Recent Clinical Techniques, Results, and Research in Wounds

Melvin A. Shiffman
Mervin Low *Editors*

Chronic Wounds, Wound Dressings and Wound Healing

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Foreword[1]

It is a great honour for me to be invited to provide a foreword for the series of six books edited by Dr. Shiffman and Dr. Low which cover a broad expanse of subjects relevant to and important in the care of patients with wounds.

Wounds have existed since the beginning of time and, until recent years, have received scant attention unless major conflicts developed which necessitated innovation in the treatment of patients with wounds. However, in recent years there has been an increasing interest in this subject as evidenced by the explosion of journals, meetings, societies and associations and initiatives that have been developed in this field.

The need for an academic underpinning of the subject of wound healing is without question. Research papers published in recent years have undoubtedly enhanced the scientific basis for wound healing. This, coupled with demographic changes in many countries around the world, has led to increasing numbers of patients developing wounds or wound healing problems. It is recognised that in the vast majority of geographies globally that the number of patients with wounds are increasing in everything other than major burns where better health and safety initiatives have been an effective preventive strategy.

This series of books attempts to deal with, not only subjects that are normally seen in wound healing text but also provides a huge amount of space to the management of wounds seen in surgical practice, both general and specialist surgery. The sections on infection are an attempt to deal with a very common but poorly managed clinical problem and one that requires urgent attention in view of the global challenge of antimicrobial stewardship. The tradition chronic wounds are also included and provide a medical as well as a nursing and paramedical focus on these subjects.

It is particularly pleasing to see books and chapters focused on specialised surgical practice as these are areas that are rarely covered in other educational products in this area. The opportunity for new therapies, measuring a range of effective and appropriate outcomes and the use of new technologies are all included.

For those of us who work in the area of wound healing, these books will unquestionably be an important reference source. For those readers who are wanting to get an insight into this common, expensive and complex problem, they will without doubt find the content of these books an important source of informed opinion and refer to the rapidly expanding evidence base that is developing in this subject area.

I would urge you to immerse yourself in these books. Read, reflect and consider how information that you have had access to can and I will change your clinical practice.

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UK

[1]P. S.

We, Melvin A. Shiffman and Mervin Low, are greatly enthralled by Keith Harding's willingness to write the Foreword for the books on Wounds. Keith Harding is the Director of TIME Institute (Translation, Innovation, Methodology and Engagement) and Head of the Wound Healing Research Unit in the School of Medicine at Cardiff University. He is Clinical Lead for Wound Healing in the Cardiff & Vale NHS Trust. In September 2013, Harding was appointed as Dean of Clinical Innovation at Cardiff University. From 2002 to 2005, he was Head of the Department of Surgery at Cardiff University. He is Editor-in-Chief of the *International Wound Journal*. Harding is a Past President of the European Tissue Repair Society. He was the first President of the European Pressure Ulcer Advisory Panel, and first Recorder of the European Wound Management Association. Harding was Chair of the International Working Group on Wound Healing in Diabetic Foot Disease in 2003. He was Chair of the Expert Working Group that produced a range of International Consensus Documents from 2004 to 2011. Professor Harding was appointed a Commander of the Order of the British Empire in the 2013 New Year Honours for services to medicine and healthcare.

Preface

We are delighted to have the book on Wounds extended into six volumes. There is so very much medical literature in journals and books that to cover the whole gamut of Wounds would be virtually impossible. We tried to include as many of the experienced practitioners in wound care as possible but many of them are too busy to spend the time committing to submitting a chapter.

The selection of topics in each of the volumes was decided by how many authors responded to each of the subjects. As usual in editing a book, many authors who agreed to submit manuscripts may finally decide that they were not available to complete the chapters. We contacted or tried to contact over 1500 authors, and the responses were mainly no response or not as good as expected.

The volumes include the following:

1. Biofilm, Pilonidal Cysts and Sinuses
2. Burns, Infections and Wound Management
3. Pressure Injury, Diabetes and Negative Pressure Wound Therapy
4. Plastic and Thoracic Surgery, Orthopedics and Ophthalmology
5. Vascular Surgery, Neurosurgery, Lower Extremity Ulcers, Antimicrobials, Wound Assessment, Care, Measurement and Repair
6. Chronic Wounds, Wound Dressings and Wound Healing

There are many expert international contributors who have worked in various aspects of wound research as well as clinical practice. We have tried to have chapters that involved humans and in vivo results and avoided as much as possible animals and in vitro results. Chapter conclusions are those of the Authors and may not be the same as those of the Editors. At times the chapter may appear cumbersome, but the authors try to show some proof of their results. Language difficulties are common when translated into English so that grammar, spelling and sometimes words have to be corrected.

Hopefully, the readers will get information that adds to their care and treatment of patients. Researchers may gain knowledge of other researchers' progress and improve on the results or can continue their work in other directions. Controversy is many times a good thing since looking in other directions to prove or disprove a result can improve knowledge. We have a long way to go to be able to treat all wounds properly and successfully in as short a time as possible.

Tustin, CA, USA — Melvin A. Shiffman
Newport Beach, CA, USA — Mervin Low

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Part I

Chronic Wounds

Impact of Host Defense Peptides on Chronic Wounds and Infections

Evan F. Haney, Daniel Pletzer, and Robert E. W. Hancock

1 Introduction

Bacterial infections are the most common cause of human infectious diseases and antibiotics, used to treat these infections, have saved millions of lives in the last century. Most antibiotics are effective against dividing bacteria, and their systemic application leads to a reduction in the bacterial burden, allowing for resolution of the infection. One of the most common indications for applying antimicrobial therapy in developed countries like the United States are skin and soft tissue infections [1]. In some cases, localized tissue damage can lead to chronic, non-healing wounds that fail to heal within 4–6 weeks and may persist for months or years, even with continuous treatment [2]. Chronic wound patients often suffer from other conditions such as diabetes or obesity [3], and this type of wound represents a growing concern in healthcare settings throughout the world. These chronic wounds include diabetic foot ulcers, pressure ulcers, and venous leg ulcers, all of which are painful and debilitating conditions that negatively impact the quality of life of affected patients. It is it estimated that up to 2% of the population will suffer from lower limb ulcerations [4], and direct hospital costs to treat skin ulcers and chronic wounds have been estimated to be as high as £5.3 billion in the United Kingdom [5] and $25 billion in the United States [3]. As the global rates of obesity [6] and diabetes [7] rise coupled with an increasing elderly population who often have comorbidities that predispose them to the development of chronic wounds [8], there exists an urgent need to develop new treatment strategies to cope with this growing health issue.

It has been proposed that the pathogenesis of chronic wounds is a result of the interplay of multiple factors: aging, damaged or reduced blood flow to the wound site, and wound colonization by bacteria coupled with an inflammatory response [9]. There is increasing evidence that the bacteria in chronic wounds occur within an organized community known as a biofilm [9, 10]. Biofilms are generally considered to be the natural phenotype of bacteria, and they are intrinsically resistant to antibiotics and to the host immune response when found in a chronic wound. Debridement can be used to remove bacterial biofilms from the wound bed [11]; however, biofilms in vivo can be exceedingly small and difficult to identify [12] which further complicates their treatment, and biofilms frequently regrow after debridement [10]. Additionally, many biofilms within wounds are often polymicrobial [13, 14] which makes it difficult to isolate

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and appropriately treat the pathogen(s) responsible for the chronic wound state.

Unfortunately, there are currently no pharmaceutical agents that specifically target the bacterial biofilms that contribute to chronic wounds. The European Society of Clinical Microbiology and Infectious Diseases released a guideline for the diagnosis and treatment of biofilm infections in 2014 [15], in which they outlined the various challenges associated with identifying and treating biofilm-associated infections. In this document, the urgent need for new anti-biofilm-specific antibiotic therapies is highlighted as is the use of anti-inflammatory approaches to reduce local tissue damage due to the host inflammatory response [15].

In this review, we highlight the emerging potential of naturally occurring host defense peptides (HDPs) and synthetic derivatives thereof as a possible treatment tool to address the growing problem of chronic wounds. HDPs have been shown to have potent immunomodulatory functions related to innate and subsequent adaptive immunity and wound repair. Indeed, several synthetic peptides have been identified that enhance wound healing in vivo, while their ability to dampen excessive inflammation (often associated with chronic wound infections) appears to be a very common property of such peptides. In addition, many reports are emerging of peptide sequences that selectively target a broad spectrum of bacteria growing within a biofilm. We propose that the sequence optimization of synthetic HDPs for an enhanced combination of wound healing properties, anti-inflammatory activity, and anti-biofilm potency could result in novel pharmaceuticals that will complement current chronic wound treatment strategies.

2 Prevalence of Biofilms in Chronic Wounds

Microorganisms can be found in any niche on earth, and they need to shelter themselves in these environments. Bacterial cells often cluster together to form communities of millions to billions of cells as an effective protection method. Within these groups, organisms are able to cross talk with each other, share genetic information, and encapsulate themselves in an extracellular polymeric matrix containing exopolysaccharides and/or proteins and DNA [16]. These dynamic communities are called biofilms and form the major lifestyle of bacterial organisms. Of increasing concern is the rise of adaptive resistance where the biofilm growth state of the microorganism leads to non-mutational high-level resistance to most antibiotics [17]. Adaptive resistance is defined as resistance that is dependent on the growth state/environmental challenges of the organism, affects susceptibility to multiple antibiotics, and reverts when the organism leaves the growth situation, e.g., upon dispersal of bacteria from biofilms [18, 19]. For example, the growth of bacteria in biofilms leads to dramatic alterations in the transcriptome, including alterations to genes influencing antibiotic susceptibility [20]. Oxygen and nutrient limitation in the deeper biofilm mass also causes bacteria to slow down their metabolism and growth rate, affecting many antibiotics like most β-lactams and fluoroquinolones that only target growing cells. This anoxic environment causes cells to switch into a more dormant, sessile, non-growing state (including so-called antibiotic-tolerant "persister" cells). Consequently, bacteria within a nondividing growth state are protected against the host immune system and various antibiotics, rendering them extremely difficult to treat compared to their planktonic counterparts [21, 22].

There is increasing evidence that bacteria grow within biofilms in chronic wounds. These biofilms can serve as foci for the emergence of systemic infections and are often the underlying cause of recalcitrant, recurring, chronic disease. Examples of biofilm-related infections include medical device-related infections, osteomyelitis, infections accompanying cystic fibrosis and other chronic lung infections, and wound infections [23]. Currently, biofilm-related infections are often treated with aggressive and intensive application of antibiotics. However, these treatments are helpful only to control biofilm-related infections, and they often fail to eradicate mature biofilms [23]. Moreover, administration of high doses of antibiotics is often impossible due to toxicity or other serious side effects such as the impact of antibiotic use on renal and hepatic function [23]. Therefore, this situation can result in a cycle of antibiotic use in patients that creates ideal conditions to select for

antibiotic resistance in a bacterial population. The Centers for Disease Control and Prevention (CDC) estimated that about two million people become infected with antibiotic-resistant bacteria in the United States each year [24]. Alarming is the fact that 23,000 [24] individuals die as a result of antibiotic-resistant infections, and this number balloons to 159,000 deaths [25] when we consider patients who die of sepsis, another serious condition where conventional antibiotics fail.

In the clinic, biofilms form on virtually all surfaces, and such contamination represents one of the biggest threats in healthcare facilities. Although this has been known for years, routine microbiological examination only assists in the diagnosis of clinical infections and does not reveal whether a microbial biofilm has been established. Additionally, non-culturable microorganisms or small-colony variants often limit detection [26]. In a study by James et al. [13], biopsy samples were collected from patients suffering from acute (16 patients) or chronic wounds (77 patients), and it was demonstrated that 60% of chronic wound and 6% of acute wound specimens demonstrated biofilm-like structures when analyzed with light and scanning electron microscopy. A recent meta-analysis of chronic wound studies reported that biofilms were found in 78.2% of chronic wounds [27], and it is now generally accepted that biofilms play an important role in chronic wounds and contribute to their inability to heal.

3 Chronic Wounds and the Healing Processes

In general, wounds can either be described as superficial (i.e., break of the epithelium), partial thickness (involving the epidermis and dermis), or full-thickness (deep cuts that reach subcutaneous fat and sometimes bone) lesions. Injuries arising from cuts, scrapes, exposure to chemicals, extreme temperatures as well as surgery, and/or disease outcomes are all possible causes for wounding. The wound healing process is a complex and dynamic process that is essential for daily survival of knocks and cuts and it is absolutely required for anyone undergoing surgical intervention. The first step after initial wounding initiates coagulation to form a blood clot in the wound bed (also known as hemostasis). The subsequent healing process can be divided into three overlapping phases: inflammation, proliferation, and maturation [28]. Since the healing process does not follow a linear order, wounds can progress both forward and backward through the different phases [29]. During the inflammation phase, blood vessels dilate to allow the entry of a variety of cells into the wound area. These include important cells of the immune system, including white blood cells such as macrophages and neutrophils, which produce a variety of enzymes, cytokines, and growth factors that are essential for the wound healing process. At this stage, the first clinical signs of healing become visible such as heat, erythema, edema, and pain. During the proliferation phase, the wound is rebuilt with the formation of new granulation tissue, the extracellular matrix reforms with collagen secreted by fibroblasts, and new blood vessels emerge (angiogenesis). Subsequently, reepithelialization occurs through the migration of keratinocytes at the surface of the wound to complete this phase. After complete wound closure, the final maturation phase remodels collagen and decreases blood vessels inside the scar tissue [28].

Chronic wounds are lesions that do not heal within a predictable amount of time and are often delayed in one (or more) of the aforementioned wound healing phases. The vast majority of chronic wounds can be categorized into three major classes: (1) leg ulcers, often associated with venous or arterial deficiencies, vasculitis, and skin malignancies; (2) pressure ulcers, which are localized skin and tissue damage as a result of constant pressure often seen in sedentary patients in hospitals and residential care homes; and (3) diabetic foot ulcers, which are a major complication of diabetes mellitus and are responsible for neuropathy and arterial damage [11, 30]. The normal wound healing processes is also often impeded by ischemia, a condition characterized by poor blood supply resulting in low oxygen levels in tissues [30].

The presence of biofilms in chronic wounds stimulates a chronic inflammatory response that attracts abundant numbers of neutrophils and macrophages to the infection site. These inflammatory cells secrete proteases to help break down injured tissue and generate reactive oxygen species (ROS)

[31]. However, this influx and retention of innate immune cells coupled with the excessive secretion of the aforementioned molecules can also damage normal and healing wound tissue [32]. Bacterial defense mechanisms, on the other hand, induce the production of biofilm matrices to protect against host defenses such as phagocytic activity, oxidative stress, and proteolytic degradation [33]. The combination of these factors contributes to a chronic inflammatory state that fails to successfully eradicate the biofilm from the wound tissue. Moreover, high densities of bacterial pathogens (and/or commensal bacterial species) inside the tissue negatively impact wound healing due to direct interactions of bacterial cells with keratinocytes and fibroblasts or through indirect modulation of the inflammatory response [9, 30].

Biofilms play a major role in bacterial infections and chronic inflammation. To clinically manage a biofilm-associated infection, necrotic and infected tissue must be physically removed (i.e., through debridement and/or vigorous cleansing) [34]. In extreme cases, limb amputation is a possibility. In fact, diabetic foot infections are the most common cause of non-traumatic amputations [35], and diabetic patients who undergo a limb amputation have high 5-year mortality rates similar to the levels seen for common cancers [36]. Unfortunately, biofilms in wound tissue are difficult to identify and often lack noticeable clinical signs. The lack of proper visualization methods to accurately identify biofilm within a wound bed makes it exceedingly difficult to remove all of the contaminated tissue. Incomplete removal of microorganisms from within the wound leads to the potential for regrowth, formation of new biofilm mass, or it could potentially promote bacterial dispersal and lead to a systemic infection. To manage such chronic, biofilm-related infections, multiple visits to a doctor are necessary to perform regular wound cleaning. In an attempt to prevent biofilm reconstitution, infections of this type are often treated with conventional antibiotics. Unfortunately, antimicrobials only prevent the growth and proliferation of planktonic bacteria and often have a minor impact on organisms still embedded in the biofilm matrix inside the wound. Another complicating factor is the polymicrobial nature of biofilms that requires patient-specific, broad-spectrum antibiotics.

The rise of antibiotic-resistant bacteria and the lack of antibiotics that efficiently work against bacterial biofilms necessitate the development of novel treatment options to help clinicians treat this type of infection in patients. In the next section, we will highlight the potential of natural host defense peptides as a potential alternative capable of fighting biofilm-related infections and promoting wound healing in chronic wound tissue.

4 Host Defense Peptides and Chronic Wounds

Host defense peptides are short (12–50 amino acids) cationic polypeptide sequences that possess antimicrobial and immunomodulatory properties and are produced by all complex life forms [37]. Larger proteins (>100 amino acid residues) are also sometimes included in the definition of HDPs including lactoferrin, calprotectin (also known as S100A8 and S100A9), psoriasin (S100A7), RNAse 7, and lysozyme [38]. Originally appreciated for their direct antibacterial activity toward microbes, they are often referred to in the literature as antimicrobial peptides (AMPs). However, subsequent studies of these molecules have revealed that these peptides exert a diverse range of immunomodulatory functions, which might indeed be their major function in the body. These include cell recruitment/chemotaxis, antiendotoxin activity, modulation of chemokine and cytokine production, angiogenesis, leukocyte activation, and wound healing properties [37, 39]. It is for this reason that we typically use the term HDP to better encapsulate the breadth of biological functions mediated by these molecules. More recently it has been shown that a distinct subset of HDP also have preferential anti-biofilm activity [40].

In humans, HDPs are produced by various cell types throughout the body. Immune cells such as neutrophils, monocytes, lymphocytes, natural killer (NK) cells, and mast cells all produce and store various HDPs [37]. The innate immune response depends on the presence of these cells to release HDPs in response to an invading pathogen and to prevent the onset of an infection. Many HDPs are also produced by the epithelial cells of healthy skin, and it is thought that the

presence of these peptides on the skin surface helps maintain homeostasis with the skin microbiota and prevents colonization and/or infection by invading microbes [41]. Examples of HDPs present in healthy human skin include RNase 7 and psoriasin [42], hBD-1 [43], dermcidin [44], and lysozyme [45]. Importantly, the expression of many HDPs is upregulated upon skin wounding, indicating that they might play an important role in the wound healing process. For instance, the human cathelicidin HDP, LL-37, is upregulated in the skin in response to inflammation [46] and in response to sterile wounding, as well as during infection by group A *Streptococcus* [47]. A more recent study found that injury of the human epidermis of the skin alone was a major inducer of a wide range of HDPs, including human β-defensin 2 (hBD-2) and hBD-3, as well as various cytokines, such as interleukin (IL)-6 and IL-8 [48].

The role of natural HDPs in wound healing has been extensively summarized in other reviews [49–51], and we will only briefly describe it here. First, the upregulation of gene expression for these molecules leads to an increase in the local concentration of HDP which, if large enough, can directly kill bacteria and prevent infection. In addition, HDPs are known to interact with various cells of the immune system as well as epidermal keratinocytes to promote the wound healing process. These activities include modulation of cytokine production, promoting cell migration and/or proliferation, and blood vessel formation [51], all of which are involved in the wound healing process described above.

There is ample evidence that the dysregulation of endogenous HDP levels contributes to impaired wound healing and chronic infections. For instance, patients with atopic dermatitis have been shown to have reduced expression levels of hBD-2 and LL-37 in inflamed skin [52], and reduced hBD-2 expression has been found in burn wounds [53], both of which may account for the increased susceptibility to bacterial infections in these patient groups. In the context of chronic wounds, LL-37 levels have been found to be low near the wound edge of chronic ulcers [54], while hBD-2 levels are insufficiently upregulated in diabetic foot or venous calf ulcers [55].

The therapeutic use of natural HDPs to treat wounds has been explored for some time. Early animal studies revealed that exogenous LL-37 promoted angiogenesis in a rabbit ischemia model through the activation of endothelial cells by the formyl peptide receptor-like 1 protein [56]. The cutaneous adenoviral delivery of a gene encoding LL-37 significantly reduced bacterial burden in rats with cutaneous burn wounds [57]. A similar adenoviral vector approach was used to deliver hBD-3 to excision wounds on Yorkshire pigs that were subsequently infected with *S. aureus*. In this case, hBD-3 expression caused a tenfold reduction in bacterial burden and significantly promoted wound closure after 4 days of growth [58]. Recently, a non-viral gene delivery method was evaluated to deliver a plasmid encoding LL-37 to wounds in vivo. Using skin-targeted electroporation, an LL-37 encoding plasmid was efficiently delivered to skin wounds in mice resulting in enhanced expression of LL-37 which promoted reepithelialization of the wounded tissue [59]. Importantly, the use of HDPs to treat chronic wounds in humans has also been shown to be safe and effective. In a randomized, first-in-man placebo-controlled clinical trial, topical treatment with synthetic LL-37 promoted healing of hard-to-heal venous leg ulcers [60], demonstrating that natural HDP supplementation may be a viable strategy to improve the clinical outcomes of chronic wounds.

5 Synthetic HDPs as Novel Wound Healing Agents

Synthetic derivatives of natural HDPs have been shown to retain many of the biological properties of this class of peptides, and in some cases, peptides with enhanced activity or reduced cytotoxicity have been identified. Most of these optimization strategies have been aimed at identifying HDPs with improved antimicrobial activity [61]. This type of study has dramatically improved our understanding of the sequence requirements of AMP sequences and has expanded the breadth of sequences that are known to possess antibacterial activity. These sequence optimization approaches have also been extended to other HDP activity types suggesting that it may be possible to optimize synthetic peptides for specific biological

applications. For instance, fragments of LL-37 have been generated that retain the antibacterial potency and chemotactic activity of the parent peptide while exhibiting reduced cytotoxicity [62]. Of importance to chronic wounds, several synthetic peptides with anti-biofilm activity have also been identified by our group [40, 63–65] and others [66], and it appears that this activity is independent of direct antibacterial activity toward planktonic cells [65]. Therefore, an optimization strategy aimed at enhancing the anti-biofilm potency of synthetic peptides could potentially address the biofilm component of a chronic wound that is not specifically addressed by conventional antibiotics alone.

Notably, the wound healing properties of natural HDPs have also been recapitulated in synthetic peptides. For instance, a frog-derived AMP, Esculentin-1a, stimulated migration of keratinocytes more efficiently than LL-37 in vitro [67]. Promotion of wound healing has also been demonstrated in vivo by IDR-1018, a synthetic derivative of the bovine HDP bactenecin, which enhanced wound healing in *S. aureus*-infected porcine wounds (Fig. 1) [68]. A recent study described the wound healing properties of DRGN-1, a Komodo dragon histone-derived peptide, in a mixed species cutaneous infection model as well as in sterile wounds [72]. Emerging evidence suggests that optimizing synthetic peptides for specific wound healing properties could generate novel peptide sequences with therapeutic potential. Nakagami et al. [73] designed a series of derivatives based on a novel angiogenic peptide sequence, AG30, to try to improve both the antibacterial and angiogenic properties. One of these derivatives, AG30/5C, in which five residues in the parent sequence were replaced with Lys or Arg residues, accelerated wound healing and angiogenesis in a diabetic mouse wound model infected with MRSA [73]. This peptide has since been further optimized to peptide SR-0379 by identifying the minimal peptide sequence required for wound healing as well as incorporating a D-Lys residue near the C-terminus to improve the proteolytic stability and reduce toxicity (Fig. 1) [70]. The sequences and wound healing activities of a number of synthetic peptides are summarized in Table 1.

The wound healing properties of HDPs do not appear to be directly related to the antibacterial properties of a given peptide sequence. For instance, HB-107 is a fragment of the insect AMP cecropin which lacks microbicidal activity but promotes wound healing in mice and enhances leukocyte migration and keratinocyte hyperplasia in wounds [75]. This observation suggests that many of the biological activities influenced by HDPs are sequence specific. Our group has demonstrated that the antibacterial properties of AMPs do not directly correlate with anti-biofilm activity. For instance, LL-37 and the synthetic peptides 1037 and IDR-1018 inhibit biofilm growth at concentrations well below their MICs against planktonic bacteria [40, 63, 65]. Moreover, IDR-1018 acts against biofilms of *Burkholderia cenocepacia*, which is completely resistant to AMP activity [65], while one of the more potent AMPs exhibited no anti-biofilm activity [63]. Furthermore, with the best anti-biofilm peptides, this activity is extremely broad in spectrum, preventing biofilm formation and destroying preformed in vitro biofilms caused by all of the major nosocomial antibiotic-resistant (so-called ESKAPE) pathogens, killing multispecies oral biofilm bacteria, and acting against in vivo biofilms [78]. Therefore, it is attractive to speculate that it may possible to screen specifically for synthetic HDPs with enhanced wound healing properties, potent anti-biofilm activities, and anti-inflammatory activity to address three of the underlying concerns for chronic wounds. Interestingly, some synthetic peptides with wound healing properties have also been shown to exert anti-biofilm activity (Table 1), demonstrating that these activity types can overlap in a single synthetic HDP.

Various in vitro screening methods are used to evaluate synthetic peptides for wound healing activity. The most common are cell proliferation assays, looking primarily at sequences that promote fibroblast and epithelial cell growth [79], and migration assays to measure movement of epithelial cells, such as keratinocytes, across a surface [80]. Both of these activities are essential during the proliferative phase of wound healing as fibroblasts produce essential components of the extracellular matrix and keratinocytes migrate to reepithelialize wounds [81]. Many of the synthetic HDPs with wound healing properties described in Table 1 were characterized with these types of assays in vitro prior to in vivo studies. Some groups are already attempting to iden-

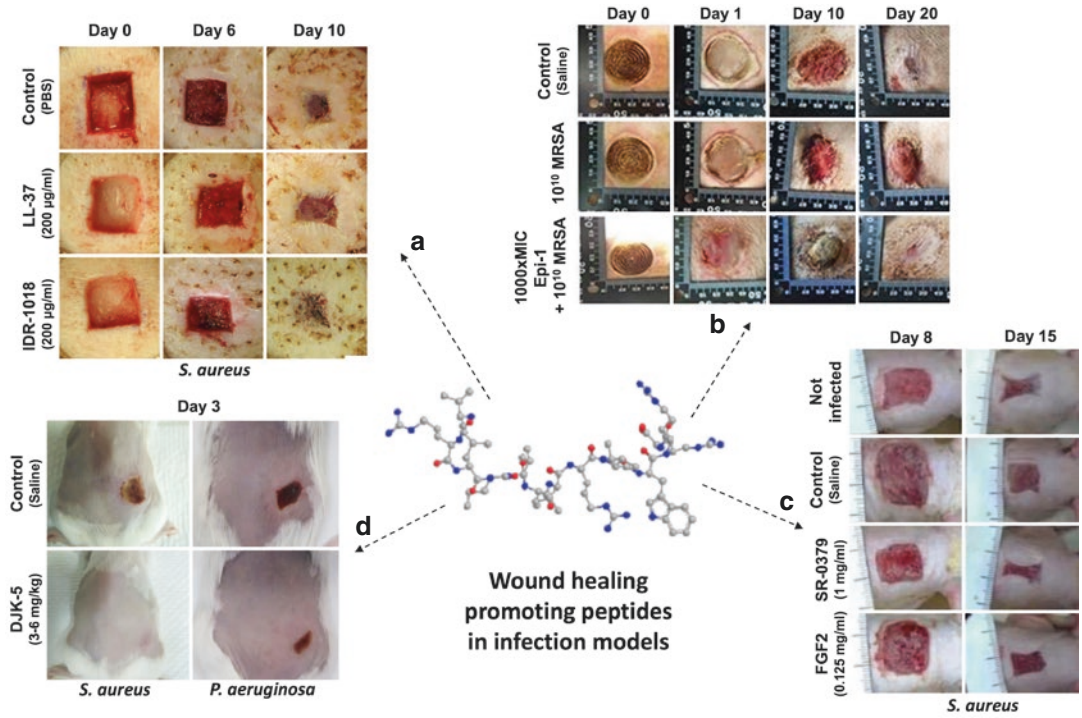

Fig. 1 Wound healing-promoting peptides in infection models. (**a**) Synthetic peptide IDR-1018 and natural peptide LL-37 compared to a PBS control in a *S. aureus*-infected porcine wound healing model. IDR-1018 demonstrated significantly accelerated wound healing when compared to LL-37 or PBS, while there was no observed change in the underlying bacterial colonization [68]. (**b**) Wound healing properties of antimicrobial peptide Epi-1 compared to a saline control and non-treated wounds in a *S. aureus* (MRSA)-infected burn wound swine model. Accelerated wound closure was observed when the infected heat-burned pig skin was treated with Epi-1 [69]. (**c**) Effects of antimicrobial peptide SR-0379 and fibroblast growth factor 2 (FGF2) in an acutely infected wound with *S. aureus* compared to saline control treatment as well as uninfected wounds. SR-0379 treatment significantly accelerated wound healing when compared to FGF2 or saline [70]. (**d**) Anti-biofilm peptide DJK-5 in comparison to a saline control in a high-density bacterial infection model in CD-1 mice which were subcutaneously injected with *S. aureus* or *P. aeruginosa*. Infections were treated with 3 mg/kg (*P. aeruginosa*) or 6 mg/kg (*S. aureus*) DJK-5. The peptide significantly reduced dermonecrotic lesions with only a minor reduction in the underlying bacterial colonization [71]

Table 1 Synthetic HDPs with demonstrated wound healing activities in vivo or in vitro and any documented anti-biofilm activity

Peptide	Sequence	Wound healing	Anti-biofilm activity
IDR-1018	VRLIVAVRIWRR-NH_2	Wound healing in pigs and mice [68]	Broad spectrum, including all ESKAPE pathogens [65]
DJK5	VQWRAIRVRVIR-NH_2 (all D-amino acids)	Reduced MRSA abscess size and bacterial burden in mice [71]	Broad spectrum, including all ESKAPE pathogens [74]
HB-107	MPKEKVFLKIEKMGRNIRN	Wound repair in mice [75]	Unknown
DRGN-1	PSKKTKPVKPKKVA	Wound healing in mice [72]	*P. aeruginosa* and *S. aureus* [72]
SR-0379	MLKLIFLHRLKRMRKRL*K*RK (*K* = D-amino acid)	Accelerates wound healing in mice [70]	Unknown
Epi-1	GFIFHIIKGLFHAGKMIHGLV-NH_2	Healed heat-burned MRSA-infected porcine skin [69]	Unknown
Tiger17	$WC_1KPKPKPRC_1H$-NH_2	Accelerated healing in mice with full-thickness skin wounds [76]	Unknown
Esculentin-1a (1-21)NH2	GIFSKLAGKKIKNLLISGLKG-NH_2	Stimulates keratinocyte migration [67]	*P. aeruginosa* [77]

tify novel wound healing peptides using these screening approaches. For example, Kosikowska et al. [82] sought to identify bifunctional AMP sequences with potent antimicrobial properties coupled with enhanced cell proliferation and migration properties. In this case, while the authors successfully identified peptides with either antimicrobial activity or wound healing potential, they unfortunately did not find a peptide that fulfilled their bifunctional objective. It is worth mentioning that this study was limited to only 15 sequences and future studies could dramatically expand the sequence space of the synthetic HDP sequences through the use of peptide arrays. We have successfully used such a strategy to simultaneously evaluate the immunomodulatory and anti-biofilm activity of hundreds of peptide sequences and generated optimized synthetic HDPs for multiple activity types [64]. As our understanding of the sequence requirements that govern the wound healing properties of HDPs improves, it should be possible to further enhance the therapeutic potential of these molecules and advance their progress to the clinic. In the next section, we will discuss various animal models that have improved our understanding of chronic wounds as well as serving an essential role in evaluating wound healing compounds in the context of a living organism.

6 Animal Models of Chronic and High-Density Bacterial Wound Infections

Molecular and cellular mechanisms underlying wound healing processes have been extensively studied in acute animal infection models. Unfortunately, most of these models fail to recapitulate the clinical features of chronic wounds and their pathology in humans. The host immune response as well as the dissimilarity of the human skin architecture to that of common lab animals (e.g., mouse, rat, rabbit) brings additional complexity limiting their ability to fully capture the clinical scenario. The implementation of a clinically relevant chronic infection model still remains very challenging, and only a handful of chronic wound models have been described (Table 2). In the following section, we describe some of the currently available chronic infection models, including a new mouse model for high-density bacterial infections developed in our lab, and we briefly discuss the strengths and limitations of these models in the context of chronic wounds.

Currently, no single animal model is able to accurately and faithfully represent the diverse etiology and heterogeneous nature of chronic wounds, and this has hampered efforts to study and understand this complex biological process. The porcine model offers the closest anatomical comparison to human skin and is widely accepted as a preclinical model for human wounds [92], but is logistically difficult to implement and associated with far greater costs than regular laboratory rodents. Benefits and limitations (such as genetic tractability, reproducibility, costs, etc.) must be taken into account when choosing an animal model, and it is important to understand how well an animal model reflects a human outcome during the development of novel therapeutic treatments. Ultimately, animal models cannot and will not replace the verification of agents and mechanisms in human wounds, but are of critical importance in providing reliable, reproducible information on the response of wounds to therapeutic treatments.

Prolonged or chronic ischemia is a major contributing factor in impaired wound healing and often leads to ulcer formation and tissue necrosis [93]. Various ischemic animal models have been described that address the issue of reduced blood

Table 2 Animal models of chronic and high-density bacterial skin wound infections

Animal model	Clinical relevance	Test animals	References
Skin/ear ischemia	Ischemic ulcers	Rabbit, guinea pig	[83, 84]
Skin flap ischemia	Ischemic ulcers	Pig, rabbit, rodents	[85, 86]
Magnet ischemia-reperfusion	Pressure ulcers	Rat, mice	[87, 88]
Diabetic wounding	Diabetic ulcers	Mice	[89, 90]
Bacterial cutaneous wound infection	Infected ulcers/abscesses	Pig, mice	[68, 71, 91]

supply and fluid drainage. In the cutaneous ischemia model using guinea pigs [83], a plastic tip is subcutaneously inserted and further ligated with a nylon strap to cause a necrotic lesion suitable for wound debridement studies. Another often used ischemia model is the rabbit ear (ulcer) model where excisional wounds on the inner aspect of the ear are produced to the depth of the auricular cartilage [84]. Since the rabbit ear dermis is firmly attached to the cartilage, this creates a full-thickness excisional wound. The advantages of this model are that many wounds can be created on one animal, lesions can be therapeutically treated, and, unlike rodent models, there is no wound contraction, which reflects granulation-type healing in humans. The model can also be extended by slowing down the healing rate through ligation of two of three supplying arteries [84].

Skin flap ischemia models are used to recreate local tissue hypoxia, which usually involves the dorsal skin. In this model, a pedicle flap of the skin is surgically removed resulting in a compromised circulatory pattern (i.e., severed blood vessels) that creates an ischemic gradient. The flapped skin shows impaired growth mechanisms often associated with tissue necrosis [85]. The model has been used to study the repair of large wound defects and allows for the investigation of wound repair and potential wound therapies. Additionally, many humanlike chronic wound characteristics such as delayed healing, increased inflammatory cytokines (such as TNF-α, interleukin-1β), and elevated proteases (e.g., metalloproteases) are observed in this model [86].

Pressure wound ulcer models use the ischemia-reperfusion model that requires surgical implantation of a metal plate under the skin followed by multiple tissue compressions using an external magnet [87]. This induces reduced blood flow, hypoxia, immune cell influx, and the release of free radicals of oxygen. Reperfusion (i.e., restoration of blood flow) of ischemic tissue is crucial for the tissue to survive, and periodic pressure application can replicate certain features of human chronic wounds where reperfusion has been restored.

Diabetes is a systemic disease that causes neuropathy and arterial damage thereby affecting various tissues and organs. Ulcers associated with diabetes can lead to medical complications, including the most severe outcome of a limb amputation [94]. Diabetes in animals can either be chemically induced, using streptozotocin (a compound that kills pancreatic β cells) [89], or by using mice deficient in leptin (a hormone made by adipose cells to regulate energy balance) or the leptin-receptor protein [95]. These mice become obese 6 weeks after birth and subsequently develop type II diabetes. Macrophages have been shown to play an important role in this model. For instance, macrophages in healthy individuals show a balanced phenotype of classical pro-inflammatory/antimicrobial (M1) cells and alternative pro-repair/anti-inflammatory phenotype (M2) cells. However, this balance is disturbed in leptin-deficient mice, and studies have shown that recruited macrophages in diabetic mice fail to polarize toward M2 phenotypes, thereby increasing M1-associated metalloprotease secretion and reducing collagen deposition [96], factors that contribute to chronic diseases. Moreover, diabetic animal models can be used to study diabetic-impaired wound healing processes.

Recently it has become apparent that bacterial colonization in wound tissue interferes with the healing process and contributes to the development of chronic wounds. Therefore, a number of animal models have been described wherein bacteria are introduced to the wound site to try to better represent the conditions of a clinical chronic wound. Our laboratory recently developed a cutaneous wound infection (abscess) model using high-density bacterial pathogens [91]. A subcutaneous injection of appropriate doses of bacteria into the dorsum of mice caused the formation of an abscess and localized necrotic tissue. This model demonstrates the significance and persistence of bacterial invaders during abscess formation and could be a valuable tool to model hard-to-treat chronic bacterial and skin infections. Furthermore, it allows the establishment of chronic wounds for several days, is technically very easy to implement, is easily adapted to study therapeutic treatment, and improves animal welfare due to the possibility of using real-time in vivo imaging techniques that drastically reduce the numbers of animals needing to be sacrificed. Critically the abscesses formed

in this model are quite resistant to high-dose intravenous antibiotic therapy although local administration of antibiotics and especially synthetic anti-biofilm peptides demonstrates efficacy [91].

The presence of biofilm-producing bacteria in wound tissue has recently received more attention [9]. Biofilms in wounds can lead to post-closure complications (such as inhibition of tissue reepithelialization) as well as recurrence of skin breaks and infections [97]. While there is still a lack of adequate in vivo models that accurately address wound biofilm infections, some promising chronic infection models have recently been established. Examples include the chinchilla otitis media model where a *P. aeruginosa* c-di-GMP overproducing mutant showed greater persistence in chinchilla ears [98], as well as the mouse polymicrobial full-thickness wound model where preformed polymicrobial biofilms, transplanted onto the top of wounds, caused an impairment in wound healing [99]. Other mouse models specifically looking at the impact of biofilms on wound healing include the diabetic murine full-thickness wound model where 2-day-old *P. aeruginosa* biofilms in punch biopsy wounds caused delayed wound healing [100] and the splinted cutaneous wound model wherein biofilm forming *Staphylococcus* in a wound prevented reepithelialization while a biofilm-deficient mutant ameliorated wound closure [97]. In addition, the rabbit ear biofilm model has been described wherein mature *S. aureus* biofilms form in wounds as confirmed by epifluorescence and scanning electron microscopy [101, 102]. The effective use of these in vivo biofilm models offers the possibility to better understand chronic wounds and develop therapeutic treatments for biofilm-related infections with the ultimate goal to clinically translate the obtained results.

7 Application of HDPs in Chronic Wound Models

The use of synthetic HDPs to supplement the wound healing process represents a novel and promising future approach toward chronic wound therapy. Selective enhancement of innate immunity with peptides, while suppressing excessive inflammation, has many advantages over direct antimicrobial compounds and has been shown to help protect against infection and inflammation in vivo [39, 103]. The following discussions will expand on some of the more recent developments and findings regarding HDPs in the context of in vivo bacterial animal models of infection and cutaneous wounds.

IDR-1018 (Table 1) is a synthetic peptide derived from the natural bovine HDP bactenecin with potent immunomodulatory and anti-biofilm properties [104]. Importantly, IDR-1018 has also demonstrated the ability to accelerate wound healing in a nondiabetic mouse splint model [68] highlighting its potential as a wound healing agent. Unfortunately, this peptide had no effect on wound healing in diabetic mice, possibly due to suppression of host immune pathways in diabetic wounds. In a cutaneous porcine infection model, where a methicillin-sensitive strain of *S. aureus* was inoculated into the dorsum of full-thickness wounds, the peptide showed superior activity by enhancing wound healing and reepithelialization, independent of antibacterial activity (Fig. 1) [68].

Epinecidin-1 (Epi-1) is a 21-amino acid antimicrobial peptide (Table 1) originally isolated from grouper fish with broad-spectrum antibacterial activity [105]. Interestingly, synthetic Epi-1 protected mice from lethal doses of MRSA administered to an excised region of the skin [106]. Recently, Huang et al. [69] demonstrated this property extended to porcine wound models revealing that treatment of heat-burned MRSA-infected wounds with Epi-1 improved healing (Fig. 1). The bacterial loads at the infection site were significantly reduced in animals treated with the peptide, and they confirmed that the peptide enhanced vascularization and extracellular collagen compound formation, as well as enhancing epithelial cell activities.

Accelerated wound healing was also demonstrated with the 20-residue AMP, SR-0379 (Table 1), in a skin ulcer model. Tomioka et al. [70] used rats that showed full-thickness defects under diabetic conditions (skin flap in streptozotocin-induced rats) as well as cyclophosphamide-

induced immunodeficient rats infected with *S. aureus*. In both animal models, treatment with SR-0379 led to significantly reduced wound areas within a week (Fig. 1). The authors explain the enhanced wound healing as being due to the beneficial effects of the peptide on angiogenesis, granulation tissue formation, and proliferation of endothelial cells and fibroblasts, as well as direct antimicrobial activity.

Bacterial infections can also lead to non-healing, recurring abscesses. Our research demonstrated that various pathogens (including *P. aeruginosa*, *S. aureus*, *A. baumannii*, etc.), when injected at high doses under the skin, were able to cause abscess formation and localized tissue necrosis in CD-1 and C57BL/6 mice. Interestingly, treatment of abscesses with the D-enantiomeric anti-biofilm peptide DJK-5 (Table 1) significantly improved visible dermonecrosis and resulted in a two- to threefold reduction in abscess size (Fig. 1) while also decreasing bacterial burden and dissemination [71, 91].

Evidently, the administration of HDPs in wound tissue to promote repair is a promising (alternative) approach in treating (chronic) wound infections. An in-depth understanding of molecular and cellular mechanisms in the future will help to deliver them into clinical trials.

8 Future Perspectives/Concerns with the Application of Peptides

In spite of their promise as alternatives to antibiotics as well as their immunomodulatory and wound healing properties, natural and synthetic HDPs have yet to fully realize their potential as pharmaceutical agents. A number of factors, such as undescribed systemic toxicities, tendency to aggregate under physiological conditions, and high production costs, might have contributed to their apparent lack of success as drug candidates [61]. Moreover, many cationic peptides tend to lose their antimicrobial activity under physiological conditions (e.g., presence of divalent ions, polyanionic glycosaminoglycan, etc.) [107], although it is unclear if this effect extends to the anti-biofilm activity of synthetic HDPs. Importantly, many of the immunomodulatory functions of HDPs are preserved under physiological salt conditions, and therefore, future optimization strategies could focus on identifying synthetic peptides under conditions that are relevant to in vivo applications.

In the context of chronic wounds, the most pressing obstacle to overcome is the susceptibility of synthetic peptides to proteolytic degradation. Proteases, particularly matrix metalloproteases, are necessary components of the natural wound healing process that are required to degrade and remodel the extracellular matrix during tissue repair [108]. Chronic wounds are characterized by increased inflammation and elevated levels of serine proteases and matrix metalloproteases [109] that can lead to over-degradation of the ECM, ultimately preventing proper healing. Another source of proteases in chronic wounds are pathogenic bacteria which produce enzymes capable of degrading peptides present at the infection site [110]. Indeed, wound fluid from diabetic foot ulcers has been shown to degrade LL-37 likely arising from a combination of bacterial- and host-derived proteases [78], highlighting the necessity to overcome this issue as the development of synthetic wound healing HDPs progresses.

Various strategies have been employed to improve protease resistance in short polypeptide sequences such as the use of D-enantiomers or other peptidomimetics. The incorporation of non-proteinogenic amino acids has been proven effective in the context of improving stability of antimicrobial peptides while retaining antibacterial potency [61]. Both the SR-079 and DJK-5 peptides described above contain D-amino acids (one residue in SR-079 whereas DJK-5 is comprised of all D-amino acids) and both demonstrated activity in vivo (Table 1, Fig. 1). This demonstrates that these types of modifications can successfully be employed in the context of synthetic HDPs with improved wound healing and/or anti-biofilm properties.

Several synthetic HDPs have progressed through various stages of clinical trials with most

of them seeking approval for topical applications [111]. One of the earliest examples of a peptide taken to the clinic is that of pexiganan (also known as MSI-78 or Locilex), a synthetic analog of the frog AMP, magainin 2 [112]. In the late 1990s, pexiganan was evaluated in two separate phase III clinical trials to evaluate the effect of topical treatment on patient with mildly infected diabetic foot ulcers [113]. Promisingly, wounds treated with pexiganan cream closed at the same rate as those patients who received oral ofloxacin treatment, and there were fewer side effects in patients who received topical pexiganan treatment [113]. However, the FDA voted against approval and controversially requested a placebo-controlled trial to establish efficacy [114]. In October of 2016, Dipexium Pharmaceuticals completed these trials, but unfortunately they reported that treatment of diabetic foot ulcers with 0.8% pexiganan cream was not superior to treatment with cream lacking the active ingredient [115]. Another example of a peptide in the drug development pipeline is that of omiganan (MX-226), a 12-amino acid analog of indolicidin with broad-spectrum antimicrobial activity [116] that initially sought approval as a treatment for catheter-associated infections. In this case, the clinical trial of MX-226 failed to meet the primary endpoint, although secondary endpoints were achieved [111]. Interestingly, development of omiganan as a therapeutic agent has not completely ceased, and Cutanea Life Sciences, Inc. is currently involved in a phase III study to evaluate the long-term safety of topical omiganan in rosacea patients [117]. Many of the synthetic HDPs that have progressed through various stages of clinical development were likely identified and optimized for their direct bactericidal activity, and they were rarely pursued as antimicrobials for indications where antibiotic therapies fail, such as for biofilm infections. It is possible that an optimal synthetic HDP for wound healing applications might lack antimicrobial activity while still proving effective as a wound closure agent in patients with chronic wounds.

Current research has shown that the combined treatment of antibiotics with HDPs demonstrates synergy against both biofilms and infections arising from dispersed bacterial cells. In this context, synthetic HDPs could form the basis for novel adjunctive therapies to treat chronic wounds and biofilm-associated infections. Thus, Reffuveille et al. [118] showed that appropriate combinations of an anti-biofilm peptide and antibiotics showed strong synergy effects against biofilms grown in flow-cell chambers. While conventional antibiotics alone were unable to decrease biofilm thickness, disrupt biofilm structure, or cause cell death, the anti-biofilm peptide IDR-1018 alone was able to trigger all of these events. When the two therapies were combined, the effect resulted in significantly reduced or even completely eradicated biofilms at low concentrations of both antibiotic and peptide. It is important to highlight that increased dispersal of bacterial cells from biofilms, as is caused by anti-biofilm peptides, represents a potential danger in clinical settings, since dispersed cells may infect other organs or cause a septic shock [119]. Fortunately, Reffuveille et al. [118] also showed that the combined treatment of low concentrations of peptide IDR-1018 with only 40 ng/mL of the fluoroquinolone ciprofloxacin eliminated biofilms and drastically reduced the numbers of live dispersed cells, most likely due to the ability of antibiotics to eliminate dispersing cells. This provides further evidence that combinations of anti-biofilm peptides with conventional antibiotics represent a powerful new strategy to treat biofilm-related infections. In addition to the increased effectiveness due to synergy, when peptides are paired with antibiotics, they often decrease the required antibiotic dose which should reduce toxic side effects and costs, as well as slow the spread of antimicrobial resistance [19]. Since biofilm-related infections are often involved in chronic diseases that are frequently untreatable with antibiotics alone, the co-administration with alternative compounds such as peptides is highly relevant.

9 Future Perspectives

Understanding the underlying mechanism operating in non-healing, chronic wounds is crucial for developing appropriate treatment strategies.

It is well established that microorganisms, and particularly biofilm-producing organisms, found in wound tissues contribute to the development of chronic wounds and prevent wound closure. Non-healing wounds should be treated with care and proper antibiotics prescribed. Unfortunately, no biofilm-active compound has reached the clinic to date, and conventional treatment strategies for wound management often fail to address the underlying biofilm component of chronic wounds. Natural HDPs play an important role during wound repair, and they are involved in the activation of cells that either enhance or promote tissue repair, or they exert direct anti-biofilm effects. Various synthetic HDP derivatives have been identified that possess enhanced wound healing and anti-biofilm activities, and these molecules represent an exciting and novel treatment option that specifically addresses the underlying causes of chronic wounds.

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Low-Level Laser Therapy (LLLT) in Wound Healing

Ravi Kumar Chittoria and Sudhanva H. Kumar

1 Introduction

Wounds are the major cause of morbidity across the world. With advances in wound care, management of wounds has evolved into a multiple modality treatment to accelerate wound healing and reduce its morbidity. Non-invasive treatment in the form of low-level lasers can act as adjuncts to management and play an important part in accelerating wound healing [1].

The acronym for light amplification by stimulated emission of radiation is LASER. It was devised by Maiman in 1960 using Einstein's research from 1917 on physical principles of simulated light emission [2, 3]. The unique properties of light produced by laser include monochromatic wavelength, coherent and collimated waves. Lasers are usually named according to the chemical/conductor responsible for producing the laser light which also determines the wavelength of the laser. FDA has classified lasers into seven classes according to their intensity and wavelength and the ability of laser beam to cause harm. Class IIIa and class IIIb lasers are used as therapeutic medical lasers and can pose a risk of retinal damage from exposure without laser protection.

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The use of low-level laser therapy (LLLT) was initiated in 1960 by Endre Mester, a Hungarian physician, and it was first utilized in the biomedical field in 1983 [4]. However, the FDA approval for LLL came only in the early 1990s. Posten et al. [5] described the properties of LLL as dose 0.01–100 J, wavelength 300–10,600 nm, pulse rate 0–5000 Hz (cycles per second) and power output 0.001–0.1 W. In contrast to ablative or cutting lasers, LLL do not produce any heat and hence are also known as cold lasers [6]. The other synonyms for LLL are phototherapy, soft lasers, biostimulation laser, therapeutic laser and laser acupuncture.

2 Types of Low-Level Lasers

The commonly used lasers for LLLT based on the substrate used are gallium aluminium arsenide (GaAlAs; 780–890 nm) (Fig. 1), helium-neon (HeNe; 632.8 nm) (Fig. 2), gallium arsenide (GaAs; 904 nm), indium-gallium-aluminium-phosphorous laser (632.8–635 nm), krypton (521, 530, 568 and 647 nm), ruby (694 nm) and argon (Ar; 488 and 514 nm). The depth of penetration is directly proportional to the length of the wavelength.

Ruby laser (694 nm) is the first laser to be discovered and used by Maiman.

Helium-neon (HeNe; 632.8 nm) is a gas laser with visible red light and shallow depth of

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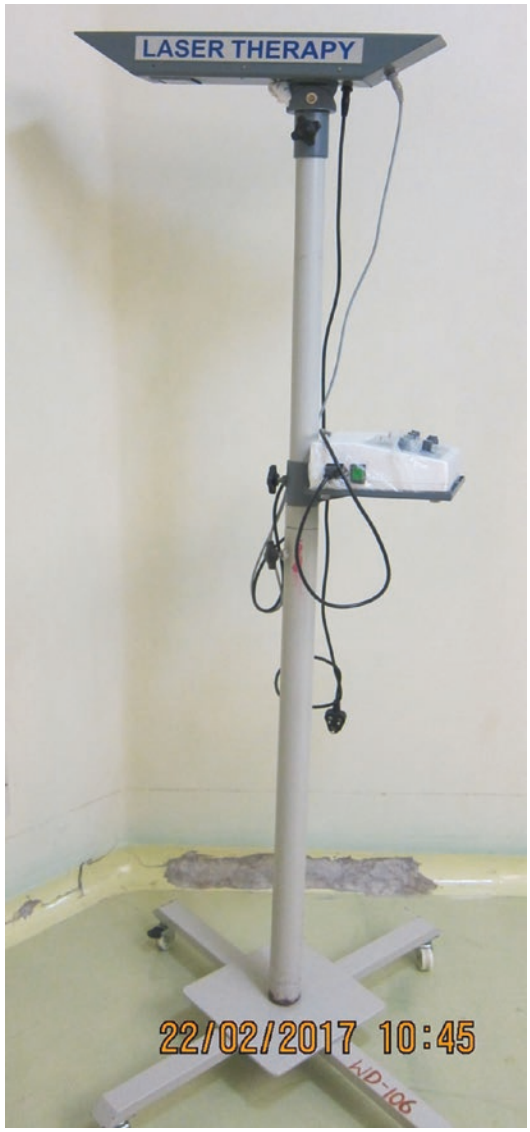

Fig. 1 Gallium aluminium arsenide low-level laser

Fig. 2 Helium-neon laser. http://used-ultrasound-equipment.net/~/dynatron/vintage-dynatron-820-helium-neon-laser-dynatronics-aperture-optical-stylus/282104748942/review

penetration. Depending on the type of tissues involved, the maximum depth of penetration achieved is 6–8 mm for a power out of 3.5 mW and 8–10 mm for 7 mW. It is used for wound healing and superficial applications like laser acupuncture.

Indium-gallium-aluminium-phosphorous laser (632.8–635 nm) is similar to HeNe lasers with visible red light, however, has more power, and is portable and inexpensive. The uses are same as for HeNe lasers.

Gallium aluminium arsenide (GaAlAs; 780–890 nm) laser is a diode laser with deeper depth of penetration and invisible light. It is one of the commonest therapeutic lasers used for the treatment of pain as it can reach deep acupuncture areas. It is inexpensive and has gallium arsenide (GaAs; 904 nm): it is the commonest therapeutic laser with the greatest depth of penetration. It is used therapeutically for treatment of pain. It is available as pulsed light or continuous wave lasers [7].

3 Mechanism of Action

LLLT is the use of nonthermal irradiance by exposing cells to low levels of red and near-infrared light. Photobiomodulation is a process by which the light produced by low-level laser either stimulates or inhibits biological processes in tissues by interacting with chromophores within the human tissue.

The power, wavelength and duration of application of laser affect the photobiological effects of LLLT [8]. The cytochromes in the mitochondria absorb the laser radiation and convert it into ATPs which are used for stimulation of cell proliferation and synthesis of proteins resulting in photobiological activation of the cell [2]. LLLT has analgesic, anti-inflammatory actions along with stimulatory effects on wound healing, tissue repair and regeneration [9]. At the cellular level, the effects of LLLT are given below [10–13]:

1. Increases cellular metabolism
2. Stimulates cell growth
3. Increase in proliferation of fibroblasts

4. Increase stimulation of keratinocytes
5. Reduces fibrous tissue formation
6. Promotes cell regeneration
7. Increase in collagen synthesis
8. Reduces oedema formation
9. Increase synthesis of growth factors
10. Decrease in inflammatory cells
11. Reduces synthesis of inflammatory mediators like substance P, bradykinin, histamine and acetylcholine production
12. Stimulates production of nitric oxide
13. Stimulates nerve regeneration and function
14. Stimulates endorphins production
15. Stimulation of angiogenesis
16. Stimulation of formation of granulation tissue
17. Improve blood microcirculation

LLLT has a biphasic dose response. Even though the beneficial effects of LLLT are well known, when given at higher dosimetric parameters, it may be ineffective or may even cause damage to the tissues [13]. It has shown to have biostimulatory effects on wound healing when given for a short duration and energy (1–4 J) [14]. Variations in the type of laser used, energy, power density, wavelength, pulse, coherence, fluence, duration of irradiation, contact or non-contact application, frequency of repetition and interval between repetitions can affect the biological effects of LLLT [13].

4 Applications

LLLT acts by photobiomodulation which is the use of photons to alter biological activity by non-thermal irradiance. In view of all the beneficial photobiological effects of LLLT, it has found a wide range of applications in medicine. It has been found to be useful in the management of acute wounds and chronic wounds (following burns, pressure sore, etc.) as adjunct therapy because of its stimulatory effects on wound healing, tissue regeneration and repair (Figs. 3 and 4). LLLT acts on all stages of wound healing by increasing fibroblasts and keratinocyte proliferation, collagen synthesis and neo-angiogenesis, improving microcirculation and stimulating granulation tissue formation, cell growth and wound contraction and anti-oedema and anti-inflammatory action. It has been used success-

Fig. 3 Low-level laser therapy being application for burns wounds

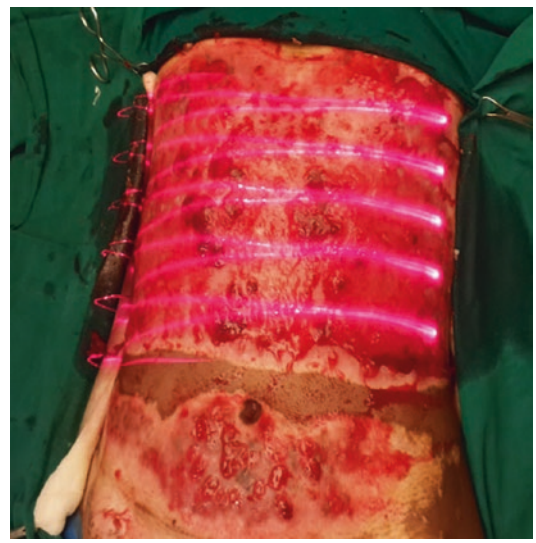

Fig. 4 Low-level laser therapy being given to postburn raw area

fully as an adjunct measure in the management of surgical wounds, non-healing ulcers, diabetic foot ulcers, pressure sores, burns wounds, venous ulcers, etc. (Figs. 5 and 6) [8, 15].

The use of LLLT in skin rejuvenation also known as photorejuvenation has been found to be beneficial for the management of fine wrinkles and photo-aged skin and reduction of acne and hypertrophic scars. It has been found to be a useful adjunct in the management of inflammatory acne, psoriasis and pigmented disorders like vitiligo [16]. It has also been found to improve hair growth and FDA approved for treatment of androgenic alopecia [17].

LLLT has been found to be effective in pain management and treatment of musculoskeletal disorders. It is used to treat acute and chronic neck pain, muscle pain, low backache, rheumatoid arthritis, osteoarthritis, fibromyalgia, tendinopathies, TMJ disorders, carpal tunnel syndrome, etc. [18–24]. It has been used in dentistry to treat chronic periodontitis and oral mucositis [25, 26].

Conclusions

LLLT is an extremely effective non-invasive tool which by its photobiomodulatory effects can promote wound healing, tissue regeneration and repair. When used within the right parameters, it can be used beneficially as an adjunct in the management of wide range of disorders ranging from wounds, skin rejuvenation and musculoskeletal disorders to alopecia.

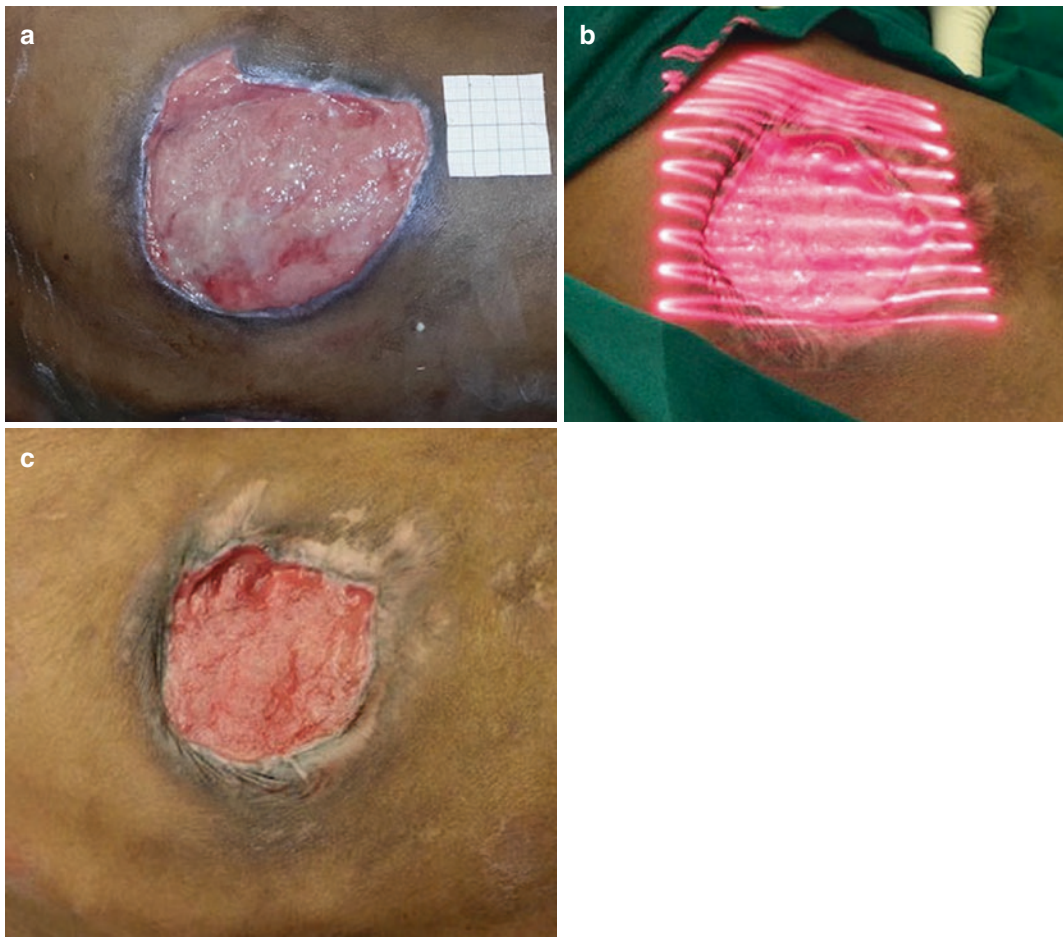

Fig. 5 (**a**) Pressure sore before LLLT. (**b**) Pressure sore during LLLT. (**c**) Pressure sore after four sessions of LLLT

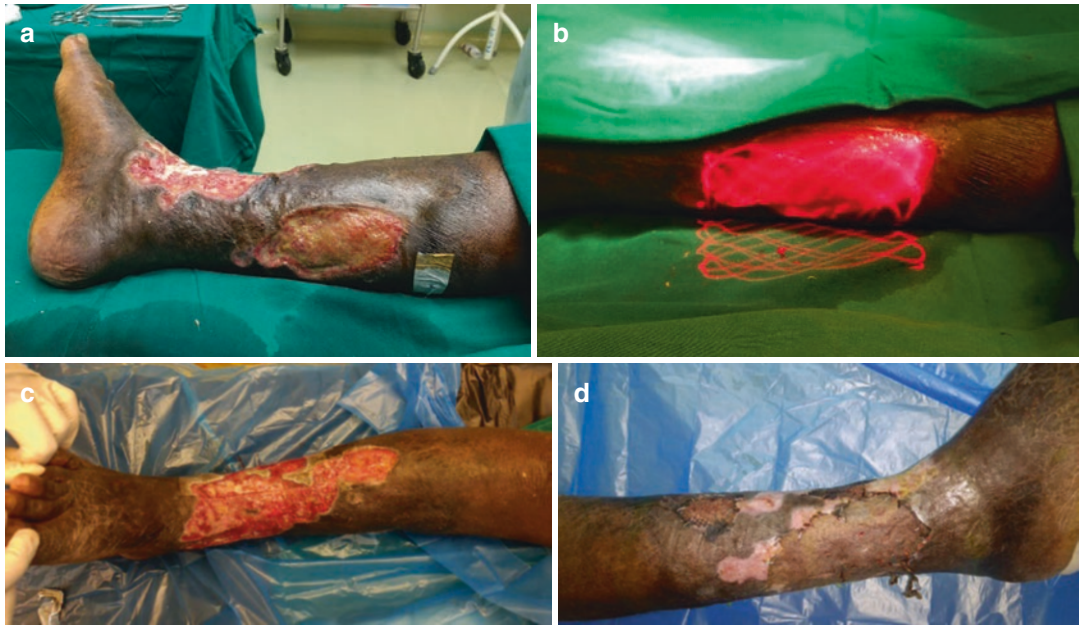

Fig. 6 (**a**) Venous ulcer before application of LLLT. (**b**) Venous ulcer during application of low-level laser therapy (LLLT). (**c**) Venous ulcer after four sessions of LLLT. (**d**) Venous ulcer after skin grafting with complete healing

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Biologic Tools for Genetic Engineering Chronic Wounds

John W. Sessions and David G. Armstrong

1 Introduction

The degenerative processes that lead to skin breakdown and possibly form into chronic wounds is nontrivial. To add to the complexity of addressing the wound itself, patients with chronic wounds frequently have complicating factors which can add to this challenging localized event. Some of these factors may include diabetes, resistant infection, immunologic suppression, retained foreign bodies, obesity, tobacco product use, excessive biomechanical stress, inadequate venous and/or arterial flow, peripheral neuropathy, etc. [1–7]. Treatment of only one of these complications (lower extremity complications of diabetes) now constitutes greater direct costs in the USA than the five most costly cancers [8, 9]. Consequently, the solutions derived to address these aberrant processes have progressively been refined to help address these complicating factors.

In practice, wound healing approaches traditionally have targeted controlling environmental aspects of the wound and neighboring tissues. Common clinical management can include revascularization surgery, mechanical compression, mechanical off-loading, sharp debridement of the wound bed, inflammation/infection control, moisture control via wound dressings, negative-pressure wound therapy, advanced biological agent application for growth stimulation and/or reepithelialization, etc. [10, 11]. These methods represent critical therapeutic elements for chronic wounds as well as provide vital areas of investigation as researchers seek to understand the fundamental behavior of wounds and how to heal them efficiently.

To build upon that common goal, genetic engineering researchers have sought to take a different approach. Instead of attempting to control the environmental aspects and heal the wound from the outside-in as is done with more traditional therapies, the genetic engineering approach is to modify the wound itself to heal the wound from the inside out. Highlighted in this chapter are biotechnologies that have been used to enhance wound healing as well as emerging technologies that can overcome previous barriers. It is intended that the reader will gain both an appreciation for previous genetic engineering work as well as understand the direction of research and intended clinical application as it relates to wound healing enhancement and genetic engineering.

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2 Traditional Viral Transduction

Viral transduction is the classical model that has been used for gene modification and generally consists of using viral machinery that has been reprogrammed to deliver a designed genetic payload. Primarily, adenovirus, adeno-associated virus, and (less commonly) *Retroviridae* family viruses (retrovirus and lentivirus) have been used in cutaneous targets [12].

Adenoviruses (ADV) are frequently used in cutaneous modification experimentation for several reasons. Unlike some viruses, ADV does not insert into the host cell's genome, which eliminates concerns for insertional mutagenesis that can occur when DNA is inserted randomly (as is the case with non-functionalized genetic loads using non-viral transfection methods) [13, 14]. This also has the added benefit of operating in the cell regardless of current cell cycle stage [15]. Furthermore, the ADV facilitates entry into the cell via receptor-mediated endocytosis, which allows for a degree of cell tropism, and transports the genetic load to the nucleus, bypassing the possible degradation that can occur during transit. These logistical benefits are significant barriers to non-viral transfection methods for gene delivery.

However, ADV do have challenges that can limit their utility. Host cell entry requires the use of the ADV capsid. Unfortunately, the ADV capsid proteins may incite inflammatory responses. In fact, it is estimated that 90% of people have formed antibodies to these capsid proteins and thereby challenge its broad use [16].

The other commonly used virus is adeno-associated virus (AAV), which is similar in function to ADV (Fig. 1). Some of the advantages of AAV include being less immunogenic than ADV [15], less likely to be deactivated by heat [17], able to target many cell types [18], and can transduce nondividing cells [15]. Unfortunately, AAV have even more modest capacity to carry engineered genes than ADV (4–5 vs. 8.5 kbp) [19]. Also, AAV viral particles require high multiplicity of infection to be effective, which can be time-consuming to generate [15].

Viral-mediated genetic modification in animal models has shown to be effective in terms of several key signaling proteins, which include vascular endothelial growth factor (VEGF), platelet-derived growth factor-B (PDGF-B), inducible nitric oxide synthase (iNOS) [20–24], as well as efficacy in ErbB3 receptor modulation [25]. While discussed here in brief, a comprehensive listing of animal experimentation with viral transduction can be found in [12]. In terms of ADV transduction, Romano Di Peppe et al. [22] reported a 3.7-fold increase in blood vessel concentration 1 week after applying a topical ADV vector that was designed to target VEGF upregulation in diabetic mice with impaired healing. In another study, a similar ADV vector targeting VEGF was delivered via microneedling to a porcine model, which did also increase VEGF levels, although not enough to induce measurable neovascularization [23]. In terms of AAV transduction and VEGF upregulation, two studies separately showed more reliable wound healing than ADV studies on the basis of being able to stimulate neovascularization, protein scaffold formation, and reepithelialization [26, 27].

One element to these early ADV studies that has been hypothesized as being a weakness in clinical application is that dysfunctional healing is not a result necessarily of there being a lack of growth factors present but rather a lack of receptors to be acted upon. Okwueze et al. [25] provides evidence for this hypothesis in terms of ADV transfection targeting the ErbB3 receptor gene, a key receptor in signaling reepithelialization. By supplying a topical EGF-like ligand topically, this work showed that significant healing maturation occurred relative to non-treated controls.

3 Emerging Biologic Tools for Genetic Modification

Viral transduction has historically experienced several limitations that has made broad application difficult [18, 19, 28–34]. However, many of these downfalls have been addressable, and workable solutions have been found, as evidenced by the animal experimentation. The one

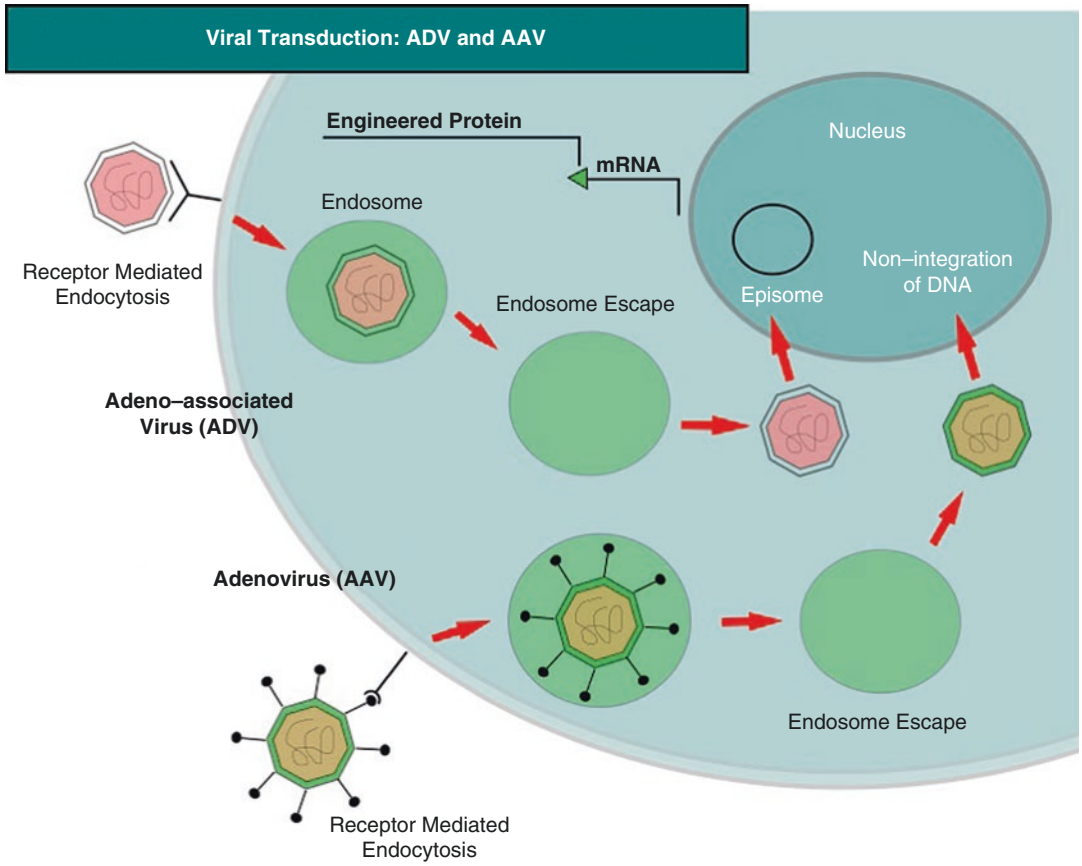

Fig. 1 Mechanism of action for ADV and AAV, two most commonly used viral transduction methods used in cutaneous experimentation. Both viruses employ receptor-mediated endocytosis for cell membrane entry. However, for nuclear membrane entry, ADV mechanism of entry is unknown, while for AAV entry occurs as the viral capsid binds to the nuclear pore complex and genetic contents are imported into the cell. It should also be noted that in rare cases, ADV-mediated transduction can have random host genome inserts

major exception to this generalized statement is the fact that viruses have a limited carrying capacity and lack mechanisms to actively promote the production of the engineered insert in a consistent manner. Consequently, with reduced capacity and precision of expression, researchers have needed to explore non-viral transfection modalities to achieve the same job of gene delivery without the same limitations. Some of these non-viral transfection methods include microseeding [35–37], particle bombardment [38, 39], liposomal reagents [40], electroporation [41–45], conjugated nanoparticles, nano-wires, microfabricated needles, and multi-electrode arrays. These non-viral transfection methods have produced a wide range of results in terms of the current context of chronic wounds. However, despite the progress that non-viral methods have made, viral delivery efficiency rates are considered to be the benchmark for all methods, reaching modification rates consistently in the 80–90% range. (Note: Although viral transduction can have high modification efficiencies, it should be recognized that some of the non-viral technologies can achieve similar rates [46].) While these technology solutions are beyond the scope of the current discussion on biologic tools used for genetic engineering, it is worth mentioning their development has been stimulated as a way to bypass historic problems faced by viral methods. For a more complete discussion on this topic, the reader is directed to [12].

Returning to viral transduction, it should be apparent to the reader that viruses can be effective at gene delivery [47–50]. However, viruses do not necessarily have the machinery to reliably implement expression of the genetic construct that they transport—thus greatly diminishing the potential for engineering biologic outcomes—that is until the recent and exciting development of clustered regularly interspaced short palindromic repeats (CRISPR) and the associated Cas9 for genetic engineering.

4 CRISPR/Cas9

The development of programmable CRISPR/Cas9 plasmids have in the last few years lead to a veritable tectonic shifting terms of the ability to reliably and precisely target and modify gene expression [51–54]. Clinically applicable projects employing CRISPR/Cas9 sweep the gamut and include treating HIV [55–57], hepatitis B [58–60], Duchenne muscular dystrophy [61–65], β-thalassemia [66], hemophilia A [67], fragile X syndrome [68], Hunter syndrome [68], Friedreich's ataxia [68], etc.

To place context to why CRISPR/Cas9 is such a pivotal element to the current state of genetic engineering, it is first important to understand why gene editing agents can struggle to genetically modify human cells. In the most basic sense, the eukaryotic cell is described as having a cell membrane which houses several organelles, each with functional contributions that allow the cell to function. Central to the cell is the nucleus which contains genomic DNA, which of course acts as the biologic coding for the cell's proteins and RNA. Because of the potentially cell-threatening effects of any alteration made to the genome, the nucleus is a highly regulated structure. In fact, the only physical passageway through the nuclear membrane comes via the nuclear pore complex. The complex consists of ~30 individual supramolecular nucleoporin structures that form an octagonal ring and act to scrutinize incoming biologic agents that seek entrance [69]. Without proper signaling elements, a biologic molecule cannot enter the nucleus (unless assisted, like in the case of ADV and AAV). Furthermore, inside the nucleus is a highly regulated histone-DNA chromosome superstructure that tightly wraps the DNA and keeps only limited portions of the genome accessible at a given time.

It is from the basis of the interaction inside the nucleus that CRISPR/Cas9 operates to facilitate gene altering functions. Simplistically described, CRISPR/Cas9 operates with two main components, the single guide RNA (sgRNA) and the Cas9 protein. The sgRNA is a short programmable RNA strand that contains a specific sequence that complimentarily matches a specific locus within the genomic DNA [70, 71]. When the sgRNA is complexed with Cas9, the complex can locate into the genomic DNA and under Cas9 action provide a variety of actions, which include transient transcriptional activation [72–74] or repression [72, 75], permanent gene insertion [76, 77] or knockout [78], inducible gene activation or repression, and genomic loci imaging/loci screening (Fig. 2). Additionally, because the sgRNA is a separate component to the Cas9 protein, several unique sgRNA sequences can be used in a single transduction/transfection event, thereby affecting multiple gene loci at a time—a process known as multiplexing [62, 79–82]. As an example of how this would be applicable in a clinical setting, in a recent study, researchers used a multiplexed CRISPR/Cas9 construct to target multiple "mutational hotspots" in Duchenne muscular dystrophy at exons 45–55. Following editing, dystrophin expression was restored to in vitro myoblasts [62].

Given the vast array of design options, the combinational use of transporting viral vectors equipped with CRISPR/Cas9 payloads capable of precision editing lends itself well for chronic wound healing applications for several reasons. First, CRISPR/Cas9 plasmids are small and relatively simple to construct, making it possible to use within the carrying capacity limits for viruses. Second, random gene insertion issues faced with some viruses are bypassed and instead can be guided to genomic sites, thus eliminating concerns for insertional mutagenesis. Third, tissue repair occurs in stages, which means at different stages there are different molecular actions that predominate and then regress as other functional

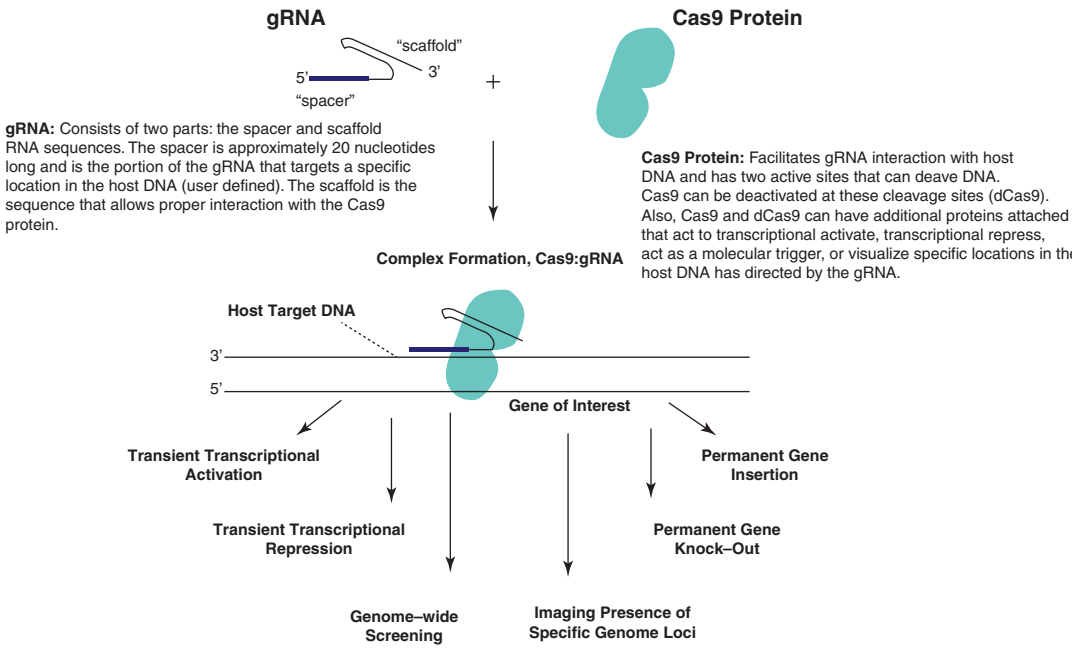

Fig. 2 The versatility of CRISPR/Cas9 for genetic engineering and genomic diagnostics

proteins act. Thus, having transiently active gene expressions that are externally controllable with molecular triggers is highly desirable—a function that has been already been demonstrated in terms of a doxycycline-regulated Cas9 [83].

5 Antibacterial CRISPR/Cas9 Applications

While there is little research in terms of CRISPR/Cas9 applied to direct wound bed healing, there has been a great deal of research activity in terms of using CRISPR/Cas9 to examine and control bacteria, particularly antibiotic-resistant bacteria, which is highly useful in infected wounds. As noted, CRISPR/Cas9 can be used to delete portions of DNA. Zheng et al. [84] demonstrated recently in *Escherichia coli* that CRISPR/Cas9 can be used to delete large portions of DNA fragments, a proof of concept that can be extended to vital bacterial components and other bacterial types. In a similar approach, several investigators have used CRISPR/Cas9 to identify antibiotic resistance genes, particularly as they behave in stress response conditions, and in some cases edit them [85–88]. Additionally, although not mentioned in great deal previously, the Cas9 protein component of the complex can be altered to be deactivated (dCas9). Albeit a CRISPR/dCas9 system lacks editing capabilities, the dCas9 protein can be linked to marker proteins, which allow the CRISPR/dCas9 complex to be used to map specific locations in a bacterial genome (in this case). This has led to many researchers being able to map antibiotic-resistant genes and identify adaptive resistance pathways with the intent to then alter the bacterial fitness to stress conditions [89–91]. This represents a critical and exciting component to chronic wound beds because of the ability to sort through heterogenous bacterial populations to identify genetic variation and antibiotic-resistant gene presence and then eliminate those pathogens deleterious to healing.

Conclusions

Chronic wounds constitute a widespread problem with complex conditions to resolve. This chapter provides contextual research in the area of biologic tools that have been and can be used to help treat them. While genetic

engineering has only begun to be explored in terms of chronic wounds, the future is promising, and with proper combinational approaches, there can be clinically viable solutions.

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Challenges and Opportunities in Drug Delivery for Wound Healing

Matthias M. Aitzetmüller, Hans-Günther Machens, and Dominik Duscher

1 Introduction

There is an ongoing shift in the distribution of the world's population toward old age, and we recently experience an increase in comorbidities like diabetes or cardiovascular insufficiency. This results in a raising number of chronic wounds which have become not only an individual medical, but also a significant economic burden, consuming 2–4% of health-care budgets worldwide [1].

Wound healing is a complex systems biology interplay depending on the timed coordination of several cell types, intra- and extracellular mechanisms, proteins, and pathways but also on external factors like infections or mechanical irritation (Fig. 1). Either defect, loss, or dominance of one factor of this convoluted interaction can cause a breakdown of the whole system resulting in chronic wounds and a loss of quality of life. A famous example for the fragility of the cellular mechanism for tissue homeostasis and repair is the connection between Vitamin C deficiency and scurvy resulting in non-healing wounds and spontaneous bleeding known since the sixteenth century [2, 3]. Mentioned first in journey books of Christopher Columbus as a result of monotone diet, the pathomechanism remained unclear until the twentieth century when it could be demonstrated that Vitamin C represents a main cofactor for collagen cross-linking and an important factor to reduce oxidative stress [4]. This example of how impactful just minimal perturbations in the underlying procuresses can be, illustrates that a highest possible understanding of all molecular and cellular players involved in wound healing is pivotal for developing treatment strategies and effective drugs.

However, no drug can be effective when its sustained and targeted delivery to the wound site cannot be assured. Specific drug delivery systems (DDSs) are key for achieving this goal. An efficacious DDS addresses the obstacles presented by the harsh wound environment and prevents the wound from mechanical, oxidative, and enzymatic stress and from bacterial contamination and provides enough oxygen while maximizing localized and sustained drug delivery to the target tissue (Fig. 2). In this chapter, we summarize the most promising recent advances in wound healing therapeutics with the corresponding delivery challenges and shed light on possible solutions for effective application.

2 Drug Delivery Routes for Wound Healing Applications

The importance of drug delivery and the challenge for translational medicine to develop effective DDS for wound healing applications are

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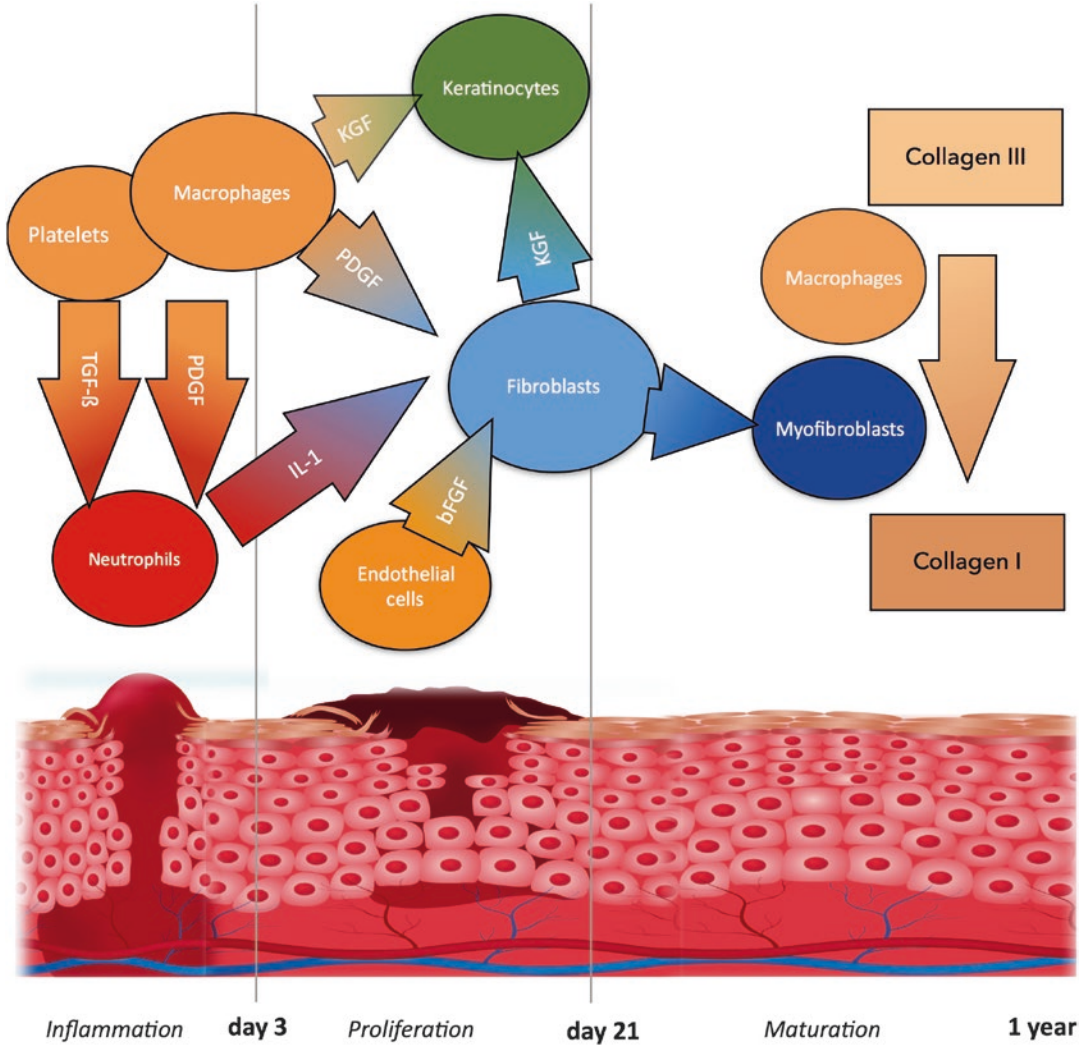

Fig. 1 Phases of adult wound healing with main affecting cells and signals. 1. Coagulation and inflammation—day 0–3. Platelets, formation of platelet plug and secretion of platelet-derived growth factor and transforming growth factor for chemotaxis of neutrophils; neutrophils, secrete interleukin 1 for the beginning of chemokine cascade for chemotaxis of inflammatory cells; macrophages, phagocyte bacteria and secrete paracrine factors for keratinocyte-based epithelialization and fibroblast activation. 2. Proliferation—day 4–21. Beginning of angiogenesis (dependent on endothelial cells), activated by vascular endothelial growth factor; formation of the extracellular matrix based on fibroblast activation, activated by basic fibroblast growth factor, interleukin 1, and platelet-derived growth factor; epithelialization, keratinocyte based as a response to keratinocyte growth factor. 3. Maturation phase—day 21–1 year. Wound contraction, transformation of fibroblasts to myofibroblast; collagen remodeling, effected by myofibroblasts and macrophages

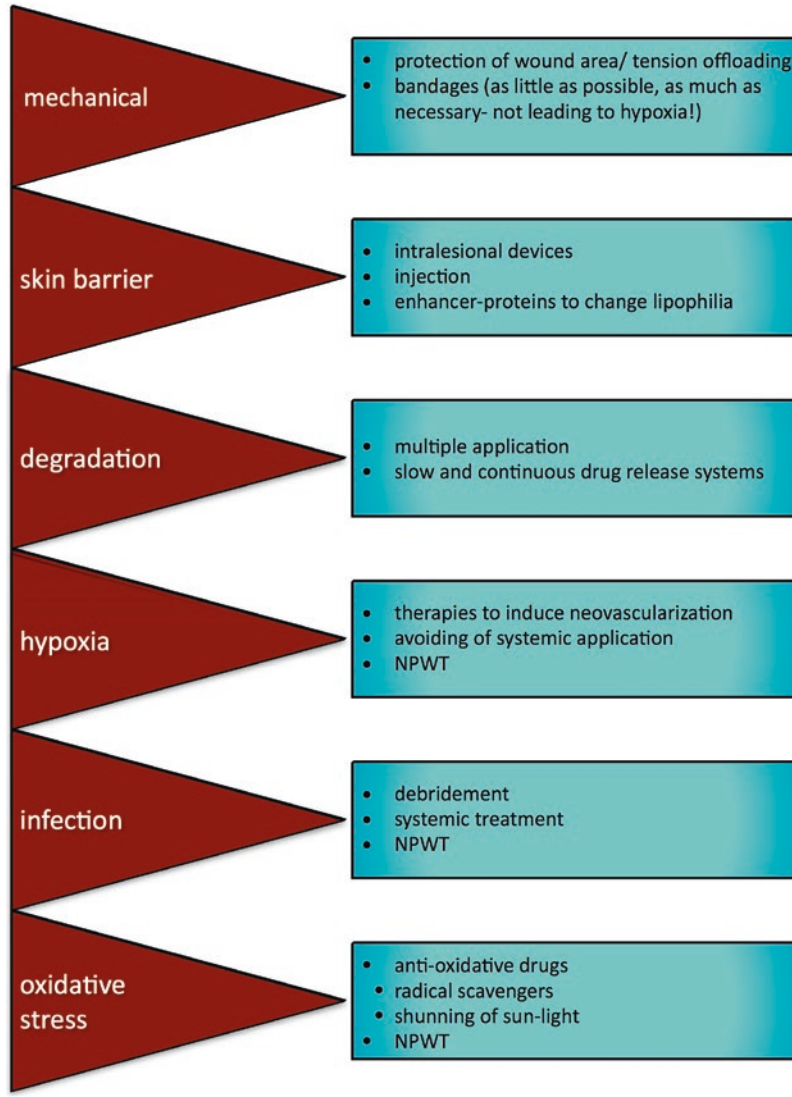

Fig. 2 Problems and preventive/solving strategies for effective drug delivery to wounds

represented by the large amount of recent studies regarding this topic (176 ongoing clinical trials registered) [5]. Different routes of drug application warrant strategies to face the obstacles represented by the systemic circulation and the harsh wound environment.

2.1 Systemic Application

Systemic drug administration is the most general form of application and common clinical practice for various substances. "Dosis facit venenum" (Solely the dose determines that a thing is not a poison) represents a rule postulated by Paracelsus already in the fifteenth century, which is still true today [6]. The limitation of dosing for orally or intravenously delivered drugs meant to act locally in a wound is essential to prevent adverse effects outweighing benefits. An additional major problem of systemic application in chronic wounds is the reduced blood circulation caused by common underlying comorbidities like diabetes, vessel diseases, tobacco abuse, or aging. Consequentially only few studies showed the benefit of systemically administrated drugs for wound healing

applications. For example, pyrvinium, a small molecule, has been shown to modulate the Wnt pathway resulting in enhanced tissue repair [7, 8]. These findings are limited by the cross-reactivity of pyrvinium and observed gastrointestinal side effects [9, 10]. Another molecule that has shown promising results as a systemically administered wound enhancer is deferoxamine (DFO), an iron-chelating agent also acting as an antioxidant. While DFO has been in clinical use for short-term systemic treatment of hemochromatosis for decades, the long-term high-dose application necessary to efficiently stimulate wound healing results inside effects as well as acute and chronic toxicity. Together with its short plasma half-life, these significant downsides limit the applicability of DFO as a systemic wound therapeutic in the clinical setting [9, 11].

Recombinant erythropoietin (rEPO), a drug-stimulating erythropoiesis, is emerging as an additive in the treatment of various chronic diseases and was shown to significantly improve outcomes of critically ill patients [12, 13]. Anemia is evident early in the courses of critical illnesses, and hemoglobin concentrations fall throughout stays in the ICU [14, 15]. A feature of anemia of critical illness is a lack of appropriate elevation of circulating erythropoietin concentrations in response to physiological stimuli [16]. Considering these mechanisms of critical illness and anemia, it is not surprising that EPO treatment for patients with severe burn wounds has shown quite promising effects on main organ function, including the kidney, liver, heart, lungs, and the central nerve system (preliminary unpublished data from our group). This novel application of a known substance might lead to a new standard in the treatment of burn wounds [17].

Given the need for high dosages and considerable side effects leads to the fact that systemic drug application currently is not part of common clinical practice, although some substances are promising to become a valuable additive for wound treatment. Systemic supplementation to optimize the nutritional state with protein and vitamins, however, increases wound healing abilities and already represents a crucial supporting therapy.

2.2 Local Application

Considering the risks for systemic toxicity and less predictable drug delivery to the target tissue, there has been a significant shift in clinical focus toward localized delivery of drugs for wound healing. Localized drug delivery permits convenient self-administration for patients while avoiding issues with gastrointestinal tract absorption and hepatic first pass metabolism, thereby improving bioavailability and maintenance of drug concentration within the therapeutic window [18]. Furthermore, local delivery enables transmission of the largest fraction of drug molecules to the target area, maximizing therapeutic potential and reducing systemic drug toxicity [19]. Despite the advantages of localized delivery, many challenges still remain, including penetration of the stratum corneum in the skin at risk for ulceration, maintaining cell survival after delivery, and the development of effective mechanisms for sustained delivery.

Growth factor and progenitor cell-based therapy research has recently centered on identifying new delivery mechanisms to overcome biological degradation and poor cell survival in the harsh wound environment. Nanoparticles, for example, have successfully been used to increase the half-life of therapeutic growth factors delivered to wounds in diabetic rats [20]. Recent advances in negative pressure wound therapy have allowed for intermittent fluid instillation, which may enable an alternative delivery method for aqueous wound therapies with the added benefit of providing local debridement during instillation [21]. Cell delivery methods such as fibrin sealant sprays or hydrogel scaffolds have been shown to improve cell retention and functional capacity at the application site both in vitro and in vivo [22, 23]. The experience with these various methods shows that an ideal delivery system is nontoxic, facilitates access of therapies to the wound site, and protects these therapies from premature degradation.

3 Recent Advances in Wound Therapeutics and Delivery Challenges

3.1 Biological Therapies

Utilized for their ability to treat both, complex acute and chronic wounds, biological therapies functionally aim to restore the body's natural repair capabilities. Creating microenvironments that encourage proliferation of both matrix depositing stromal cells and endothelial cells at the site of injury, biological therapies may facilitate the formation of a vascular network in newly forming tissue. Biologic approaches include bioactive scaffolds, growth factor-based therapies, and stem cell-based therapies.

3.2 Bioactive Scaffolds

Acellular scaffolds function by providing coverage to the wound site, establishing a matrix for resident cell infiltration, and fostering granulation tissue formation. Two clinically established acellular matrices (biologic skin equivalents, BSEs) derived from human dermis that have attempted to answer this challenge are AlloDerm (LifeCell Corp., Branchburg, NJ) and GraftJacket (Wright Medical Technology, Inc., Arlington, TX).

Alloderm is a nonliving dermal replacement composed of human cadaveric skin which has been formed by salt processing. Though initially developed for the treatment of full thickness burns, AlloDerm was quickly adapted for use in soft tissue injuries and has been shown to undergo a host-cell infiltration and neovascularization following application to the wound site. It can be used to immediately cover large wounds and reduce the need for skin grafts. However, data suggests that the take rate of split thickness skin grafts (STSGs) applied over AlloDerm is decreased when compared to STSG application alone [24].

Formed from cadaveric skin, GraftJacket is an allogeneic human tissue which uses specialized technology to maintain its basic matrix and biochemical structure after the removal of the epidermis and cellular components during processing [25]. GraftJacket has demonstrated efficacy for the treatment of diabetic chronic lower extremity ulcers in two mid-sized randomized, controlled trials (86 and 28 patients), where results revealed accelerated healing and clinically significant reductions in wound depth and volume when compared to standard wet-to-dry dressings [26]. In chronic wounds, the ECM is often dysfunctional due to the inflammatory and proteolytic environment. GraftJacket provides an intact, acellular dermal matrix that retains natural biological components, is repopulated by the patient's cells, and allows the body to initiate a normal tissue regeneration process.

Nonhuman-derived acellular biological matrices have also been used for wound care. OASIS Wound Matrix (Cook Biotech, Inc., West Lafayette, IN), derived from the submucosal layers of porcine jejunum, received FDA clearance in 2006. In separate, mid-sized randomized, controlled trials (120 and 50 patients), a significantly higher percentage of OASIS-treated chronic lower extremity wounds healed when compared to compression or moist dressing therapies [27, 28].

Despite these promising findings, a general uncertainty regarding the optimal source of tissue and processing technique forces surgeons to choose products based on familiarity, cost, and availability—rather than efficacy. The lack of randomized controlled, head-to-head trials between products, and studies often sponsored by the commercial manufactures themselves, increases the risk of potential bias. Additionally, a constant fear surrounding biological scaffolds is the risk of disease transmission and donor rejection of the graft. Because wounds vary in vascularity, the presence of infection, and amount of debris, it is essential for surgeons to prepare the wound site in order to allow these scaffolds to become successful [29]. Lastly, though BSEs hold promise, a more robust clinical comparison of host tissue must be conducted before general acceptance among surgeons can be achieved.

3.3 Growth Factor-Based Therapies

Growth factor-based therapies are vested on an understanding that specific regulatory pathways govern the host response to wound healing and are used to stimulate wound angiogenesis, matrix deposition, and reepithelialization [30]. Regranex (Ortho-McNeil, Raritan, NJ), a recombinant platelet-derived growth factor (PDGF)-based therapy, is currently the only growth factor-based biological therapy with FDA approval. Regranex accelerates the regenerative process by promoting fibroblast migration and wound reepithelialization. Randomized trials have shown that Regranex application significantly increased both the probability and time course for complete healing of leg and foot ulcers when compared to placebo gel [31]. In addition, Regranex application to pressure ulcers results in higher incidences of complete healing, as well as a significant reduction in ulcer volume compared to the application of placebo gel [32]. However, despite these promising findings, the value of Regranex for wound healing is unclear, as a recent randomized controlled trial found that the application of Regranex gel was not superior to a simple hydrogel dressing for the healing of hypertensive leg ulcers [33]. Moreover, an FDA review concluded that Regranex has the potential to increase the risk of cancer death in diabetic patientsresulting in an FDA black box warning for this product [34].

Evidence is accumulating that a mono-factor therapy like Regranex is less efficient in promoting wound closure than approaches enhancing local concentration of all growth factors involved in healing. Negative-pressure wound therapies (NWPT; vacuum-assisted closure, VAC therapy) provide an elegant way of increasing effectors of wound healing and neovascularization locally. NWPT temporarily creates relative hypoxia in the wound region resulting in significant higher levels of main regenerative factors such as VEGF, TGF β, FGF, angiopoietin 1, and BMP 2, and its application shows benefits regarding bacterial contamination [35–39]. Additionally, several studies suggest that the micro-deformation of the wound surface leads to accelerated cell migration and matrix production. By using silver-coated foams, the NWPT can be even more effective in preventing or treating bacterial contamination [40]. Rowan et al. demonstrate further that NWPT therapy does not only influence bacterial contamination by removal of microorganisms but also enhances local concentration of systemically applied antibiotics [41] making it an ideal therapeutic for contaminated wounds.

Another promising approach for multifactor therapy is to deliver a cocktail of growth factors by an injectable scaffold. Hadjipanayi et al. [42] used fibroblast-loaded collagen scaffolds and treated them under hypoxic conditions. The hypoxic stimulus led to an increased production of angiogenic growth factors which could be trapped in the surrounding matrix. Utilizing this matrix as a cell-free growth factor carrier system provided a minimally invasive method for localized delivery of growth factor mixtures, as a tool for physiological induction of spatiotemporally controlled angiogenesis. By further developing this approach, peripheral blood cells (PBCs) were identified as the ideal factor-providing candidates due to their autologous nature, ease of harvest, and ample supply [42]. Engineered PBC-derived factor mixtures could be harvested within cell-free gel and microsphere carriers. The angiogenic effectiveness of factor-loaded carriers could be demonstrated by the ability to induce endothelial cell tubule formation and directional migration in in vitro Matrigel assays and microvessel sprouting in the aortic ring assay. This approach could facilitate the controlled release of these factors both at the bedside, as an angiogenic therapy in wounds and peripheral ischemic tissue, and pre-, intra-, and postoperatively as angiogenic support for central ischemic tissue, grafts, flaps, and tissue-engineered implants.

3.4 Stem Cell Therapies

Stem cells are characterized by their capacity for self-renewal and the ability to differentiate into various tissue types via asymmetric replication. The trophic activity of these cells has led to the

development of cell-based approaches for the treatment of chronic wounds. Growth factors released from stem cells stimulate local cell proliferation and migration, increased angiogenesis, organized ECM production, and antimicrobial activity [43]. Stem cells are thought to be immuno-privileged as they are able to modulate the immune response, at least partially through the recruitment of regulatory T-cells [44]. The pro-regenerative cytokine release and unique differentiation capacity of stem cells are believed to underlay their capacity to promote healing [45]. Stem cells implanted in wounds also draw in endogenous-circulating progenitor cells, further emphasizing their role as initiators of wound repair [46, 47].

Stem cells harvested from various sources such as the bone marrow, adipose tissue, epidermis, and circulating adult blood have been utilized for wound therapy [48]. However, there are still various concerns related to the clinical application of stem cell therapeutics. To date, no study has confirmed the optimal cell source and potency (autologous vs. allogeneic; multipotent vs. pluripotent). Moreover, there is a need to promote cell survival and activity within the harsh wound environment. Biomimetic hydrogel dressings can be used to promote the survival of stem cells within a wound [45], while administering either topical or systemic EPO provides a promising approach for enhancing stem cell functionality leading to improved healing in scald wounds [49, 50].While clearly still in early phase development, stem cell therapies, used either independently or in conjunction with skin graft substitutes such as a decellularized matrix, appear to be another promising step for the treatment of non-healing wounds [48].

Looking to the future, biological products will continue to be an intriguing area of potential clinical therapy as spatially controlled drug release systems aim to minimize the quantities of drug being delivered, reduce migratory effects on surrounding tissues, and reduce overall cost [18]. Furthermore, their eventual clinical acceptance will be contingent on the development of evidence-based wound care guidelines for these biologic therapies.

3.5 Small Molecule Therapies

Though the advances in growth factor- and progenitor-based therapies hold promise for the treatment of acute and chronic wounds, they are still limited by the high costs involved, potential antigenicity, and legal and ethical issues surrounding stem cell research [51]. From a translational perspective, the application of small molecules in lieu of cells and proteins has significant advantages in terms of sterility, shelf life, and regulatory hurdles [52]. Emerging small molecule-based therapies for wound healing enhancement focus on the modulation of key signaling pathways involved in tissue repair such as the Wnt and hypoxia-inducible factor-1 (HIF-1) pathways.

Wnt proteins are highly conserved signaling molecules, which regulate embryonic development and cell fate [53]. They bind to cell surface receptors of the Frizzled (Fz) and lipoprotein receptor-related protein (LRP) family. Wnt signaling is transduced by beta-catenin, which enters the nucleus and forms a complex with the T-cell factor (TCF) transcription factor family to activate transcription of Wnt target genes [54, 55]. Wnt signaling is not only essential in development but has also been linked to mammalian cutaneous wound repair as a potential therapeutic target [56]. Thorne et al. recently identified the FDA-approved small molecule pyrvinium as a potent Wnt inhibitor [57]. Applying this molecule in an in vivo model of wound healing demonstrated that temporary inhibition of Wnt leads to increased cell proliferation, granulation tissue formation, and vascularity [8]. Furthermore, pyrvinium positively affected the engraftment and regenerative capacity of mesenchymal progenitor cells, which are promising therapeutic modalities for wound healing [7].

Another prominent pathway that can be manipulated by small molecules to enhance wound healing involves the response of tissue to hypoxia, largely regulated by the transcription factor hypoxia-inducible factor 1 (HIF-1) [58]. HIF-1 includes an α-subunit that is degraded in the presence of oxygen and iron (Fe^{2+}) by the enzyme prolyl hydroxylases (PHD) [59, 60]. Hypoxia impairs HIF-1α degradation, resulting in the expression of

a number of pro-regenerative proteins, including vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) [61, 62]. Numerous animal studies have demonstrated improvements in wound healing due to enhancement of the HIF-1 pathway [63, 64] through positive effects of VEGF on neovascularization, and SDF-1 on progenitor cell homing [65, 66]. A promising approach for the therapeutic modulation of HIF-1 signaling is the application of deferoxamine (DFO), an FDA-approved iron chelator that has been in clinical use for decades. DFO augments neovascularization and consequentially wound healing by the inhibition of HIF-1α degradation and the decrease of oxidative stress in the wound environment [67–69]. These effects synergistically promote wound healing by decreasing tissue necrosis [70, 71].

Novel small molecule treatment strategies offer tremendous opportunities in the often frustrating management of problematic wounds. However, translation into clinical practice remains challenging. Both, pyrvinium and DFO are associated with significant toxic side effects when delivered systemically, limiting dosing and duration of possible application [8, 11]. To maximize efficacy while minimizing potential side effects, localized targeted delivery directly to wound sites is essential. Controlled local drug delivery would also improve the bioavailability and enhance uptake of small molecules by maintaining drug concentration within the therapeutic window [18]. Packaging an existing FDA-approved drug into a controlled release formulation may not only improve its performance but also extend its commercial patent life. The average cost and time required to develop a new drug delivery system (DDS) (approximately $20–50 million and 3–4 years) is significantly lower than that for a new drug (approximately $500 million and over 10 years) [72]. This has led to significant growth in the US market for advanced DDSs from $75 million in 2001 to $121 billion in 2010, with the worldwide market for polymer-based controlled release system alone being estimated at $60 billion in 2010 [73]. Acknowledging the benefits of sustained local small molecule delivery, a transdermal delivery system containing DFO was recently designed [74]. This transdermal polymer patch overcomes the challenge of delivering the hydrophilic DFO molecules through the normally impermeable stratum corneum. This demonstrates that the use of a modern polymeric dressing for the delivery of an active substance to the wound site offers a promising therapeutic approach of the future.

3.6 RNAi Therapies

Unlike traditional pharmaceutical approaches, the silencing of gene function through RNAi offers selective targeting of molecules that have been difficult to regulate using growth factor- and small molecule-based therapies. RNAi is a powerful gene-silencing mechanism with enormous potential for therapeutic application [75]. Inhibiting gene expression at the posttranscriptional level, RNAi (either endogenous miRNAs (micro-RNAs) or synthetical siRNAs (small interfering RNAs)) targets specific mRNA molecules for destruction and offers an exciting therapeutic approach to wound healing (Fig. 3). A recent study illustrated the potential of this modality, demonstrating that the use of RNAi to silence Smad2 enhances wound regeneration and improves the overall wound quality [76]. Despite broad therapeutic potential, the effective delivery of RNAi to target cells in vivo remains a significant challenge due to the high rates of degradation by ubiquitous RNases, the targeting of specific tissues, and the maintenance of long-term silencing [77–79]. Developing a controlled DDS for RNAi is crucial to realize the full potential of these next generation therapeutics. Allowing for low-dose application, minimal systemic side effects, site-specific delivery, and lower costs, local delivery of RNAi offers several advantages over systemic delivery with regard to potential for clinical translation [79]. Current approaches for the delivery of siRNA in vivo include the direct injection of siRNA in saline, incorporation into liposomes, and delivery in the form of nanoparticles. However, these approaches have not demonstrated sustained RNAi activity and offer low rates of cell uptake [80]. Recent data has shown

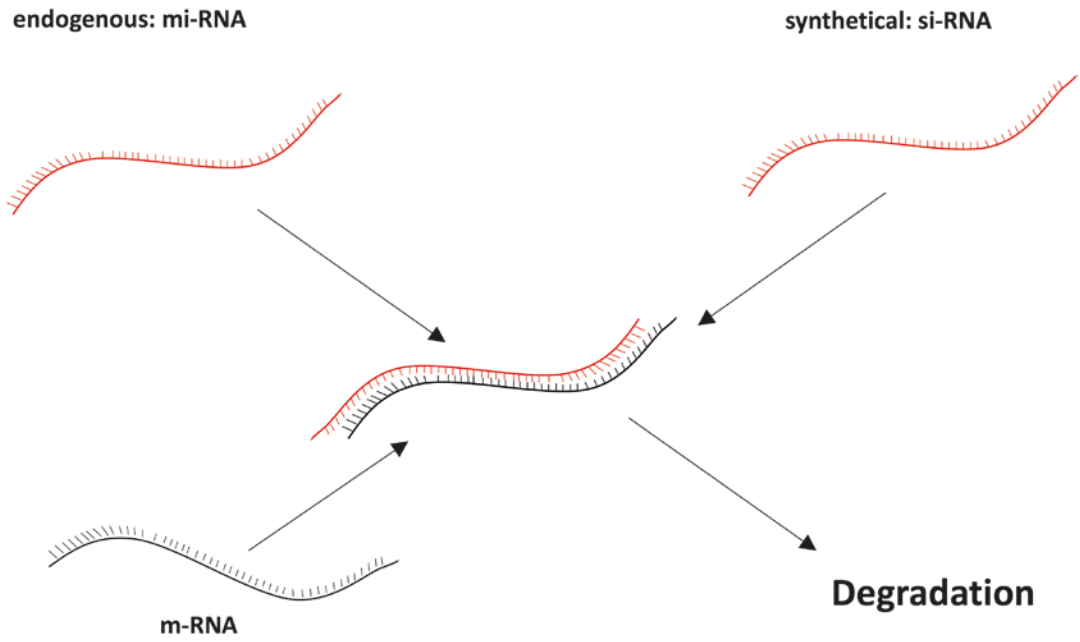

Fig. 3 Principle of RNA interference: body-own RNA (miRNA) or synthetical RNA (siRNA) binds to mRNA and leads to its degradation

that hydrogel scaffolds offer the ability to retain siRNA locally and release it directly at the sight of interest. Using a collagen-based hydrogel, the delivery of siRNA to a specific location in vivo was recorded for up to 6 days with a low fraction of siRNA being released to surrounding host tissue. These hydrogel scaffolds were applied topically and offer an exciting new platform for siRNA delivery in vivo [80].

Currently the only RNAi-based drug in clinical trials for wound healing (phase II) is RXi-109 (RXi Pharmaceuticals, Marlborough, MA) which aims to prevent the development of scarring by targeting connective tissue growth factor (CTGF) [81]. RXi-109 employs a collagen/silicone membrane bilayer BSE combined with trimethyl chitosan (TMC) and siRNA complexes to induce suppression of the transforming growth factor-β1 (TGF-β1) pathway, resulting in a functionalized matrix for scar reduction. The RNAi-BDE scaffold demonstrated high viability and suppressed TGF-β1 expression for 2 weeks. Additionally, the expression of collagen type I, collagen type III, and α-smooth muscle actin (α-SMA) was shown to be downregulated in large animals [82]. The use of RNAi-BSE parallels the structural development typically seen in normal skin repair and offers a unique delivery scaffold for the future of local RNAi delivery.

Technical difficulties restrict the development of RNAi, including stability, off-target effects, immune stimulation, and delivery problems. Researchers have attempted to surmount these barriers and improve the bioavailability and safety of RNAi-based therapeutics. However, with minimal clinical trials currently being conducted for RNAi therapeutics in wound healing, their clinical translation remains elusive. Looking ahead, as delivery methods are fine-tuned, RNAi therapeutics may develop into a drug class with the potential to exert a transformational effect on modern regenerative medicine.

4 Conclusions and Future Perspectives

The significant disability and cost to society associated with chronic wounds highlight the inadequacy of current therapeutic approaches.

The current armamentarium of therapeutic options does not fully address the impaired cellular and molecular mechanisms underlying non-healing wounds. Emerging therapeutic options have embraced the need to correct the deficits in critical signaling pathways, cellular dysfunction, and impaired neovascularization associated with chronic wounds but are largely experimental or in early phases of development. Bioactive dressings and scaffolds, growth factor- and cell-based therapies, small molecule delivery, and RNAi therapeutics all appear to be promising, but do not fully replicate the precise spatiotemporal gradients of molecules and factors during wound healing. Imperfect processing techniques and the risk of rejection continue to impair biological scaffolds, though their ability to provide volume replacement, wound cover, and a matrix for cell engraftment is encouraging. Growth factor therapies are exciting, but studies are inconclusive over their clinical benefits, potentially due to the complexity and dynamic nature of growth factor expression during the response to injury. Stem cell-based therapies are limited by the capacity of the cells to survive and function in a harsh wound environment, while small molecule-based treatments rely on efficient, targeted, and sustainable delivery systems. Finally, RNAi potentially enables the modification of downstream targets, limiting potential side effects, but is limited by its propensity for degradation by ubiquitous RNAses. A recently merging area of research is the rapid progress occurring in nanomedicine. The use of a microfluidic platform as DDS as well as controlled release applications for wound scaffolds represents a novel application for drug delivery which may hold the key to unlocking clinical wound healing therapy in the future.

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Engineered Biomaterials for Chronic Wound Healing

Matthew Davenport and Laura E. Dickinson

1 Introduction

In the United States and other developed countries, aging populations coupled with escalating rates of diabetes and obesity have significantly contributed to the increased prevalence of chronic wounds. Chronic wounds fail to progress through the systematic and reparative wound healing process and instead remain unhealed for >12 weeks [1]. Most chronic wounds can be classified into three major wound types, diabetic foot ulcers (DFUs), leg ulcers, and pressure ulcers, based on their underlying pathogenesis, i.e., diabetes mellitus, venous deficiencies, arterial perfusion, or unrelieved pressure (local tissue hypoxia) [2]. However, factors such as advanced age, poor nutrition, and immunosuppression plague the patient demographic suffering with non-healing, chronic wounds. These factors cause additional cellular and systemic stress that further contribute to wound chronicity and delay healing.

Chronic wounds are estimated to affect more than 6.5 million patients in the United States alone, and the annual healthcare burden associated with their treatment is estimated to be in excess of $25 billion [3]. Not only are chronic wounds incredibly painful for patients, significantly diminishing their quality of life, but they also require long-term treatment at high costs. Despite these high costs, reported recurrence rates for chronic ulcers remain extremely high, ranging from 23 to 40% for pressure ulcers, 24 to 57% for most chronic venous ulcers, and upward of 60% for diabetic ulcers [4]. One reason for their recurrence is because chronic wounds do not progress through the stages of normal wound healing. Even with current treatment modalities, chronic wounds are unable to regenerate tissue of complete functional integrity. The current standard of care currently focuses on compression, infection control, debridement, and selecting an appropriate dressing that maintains a moist wound healing environment. Complete wound closure is the primary clinical outcome for chronic wounds; however, successful wound closure does not necessarily correlate to regenerated tissue of a higher quality, which is desired because it is more resistant to wound dehiscence and recurrence.

2 Wound Healing

Classic wound healing is a dynamic yet well-orchestrated and highly efficient process that requires the interaction of numerous cell types, soluble mediators, and extracellular milieu to proceed linearly through the wound healing cascade: inflammation, reepithelialization, angiogenesis, granulation tissue formation, wound contraction, and tissue maturation [5, 6]. During inflammation, aggregated platelets release growth

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factors and pro-inflammatory chemokines to recruit neutrophils and macrophages to the local wound site. These inflammatory cell types phagocytose debris and bacteria and secrete mediators to stimulate the chemotaxis of cell types necessary for the proliferative phase. During the proliferative phase, fibroblasts, keratinocytes, and endothelial and smooth muscle cells migrate through the wound and proliferate to reepithelialize the denuded surface, synthesize and deposit a provisional extracellular matrix, form new blood vessels, and contract the wound size. During the final stage, the newly formed granulation tissue is remodeled by the activity of matrix metalloproteinases (MMPs) balanced with tissue inhibitors of metalloproteinases (TIMPs), which rearranges the loose, regenerated dermis and strengthens the repaired tissue [7].

Disruption of this normal wound healing cascade results in the development of non-healing chronic wounds. There is a perpetual antagonism between pro- and anti-inflammatory cytokines and an excess of oxygen free radicals and proteases, which creates a hostile microenvironment and maintains chronic wounds in a prolonged state of inflammation that is unable to progress through later phases of wound healing. Indeed, chronic wounds display a myriad of cellular and molecular abnormalities, many of which are attributed to dysregulated and dysfunctional interactions between cellular constituents and the extracellular matrix (ECM) [8].

2.1 Aberrant Microenvironment of Chronic Wounds

The wound microenvironment presents a myriad of instructive biochemical cues, cell-adhesive sites, and molecules within a structural framework of essential matrix proteins—the ECM. The ECM provides structural support and tensile strength, attachment sites for cell surface receptors, and a reservoir for signaling factors that regulate cell migration, proliferation, and angiogenesis. The ECM has a complex 3D architecture of fibrous proteins, polysaccharides, and proteoglycans that are secreted by fibroblast and epidermal cells, and it plays a significant and dynamic role in wound healing [9, 10].

Classic wound healing is a cascade of overlapping events through bidirectional interaction between various cell types and the ECM. For instance, fibroblasts synthesize and secrete collagen and ECM components, which in turn concomitantly regulates fibroblast function, such as migration, collagen synthesis, and myofibroblast differentiation [11]. Chronic wounds exhibit a host of aberrant cellular and biochemical elements that contributes to their state of persistent inflammation and significantly impairs healing. Fibroblasts from chronic wounds are phenotypically different from those in acute wounds; they are senescent and exhibit diminished migration, reduced proliferation, and decreased collagen synthesis [12]. Coupled with inhibited ECM deposition is elevated protease activity, including upregulated and amplified activity of MMPs, collagenase, elastase, and serine proteases [13, 14]. The excess of proteases degrade fibrillar collagen I to non-bioactive gelatin, cleave signaling sequences from proteins, and inactivate growth factors [15]. Fluid from chronic wounds, but not acute wounds, has been found to rapidly degrade platelet-derived growth factor (PDGF), transforming growth factor (TGF-β1), and angiogenic vascular endothelial growth factor (VEGF) [16]. The excessive degradation of the ECM, proteins, and growth factors deprives cells of attachment sites and vital signals, subsequently disrupting the progression of wound healing [17].

Keratinocytes are also dysfunctional in chronic wounds. In normal wound repair, keratinocytes migrate as a cell sheet over the granulation tissue and differentiate to reepithelialize the skin via integrin-mediated binding interactions with ECM molecules [18]. However, in chronic wounds, although keratinocytes are hyperproliferative, they are unable to migrate and close the wound [19]. This poor migratory ability is concomitantly attributed to altered integrin expression [20] and the degraded ECM components. Instead, keratinocytes at the non-healing edges of chronic wounds continuously proliferate, forming a thick, hyperkeratotic layer. Contributing to poor epithelialization is the overall excessive

inflammatory tissue microenvironment, which inhibits the migration of fibroblasts and the synthesis of new ECM, and the loss of epidermal stem cell (ESC) populations. ESC populations reside in distinct compartments or niches that regulate their self-renewal and lineage fate [21]; in response to tissue injury, the ESCs proliferate, differentiate, and migrate to reepithelialize the wounded area [22]. However, in chronic venous ulcers, it has been shown that there is a loss of SC niche signaling and subsequent deregulation and depletion of ESCs that possibly contributes to the hyperproliferative epidermis of a non-healing venous ulcer wound edge [23].

Although far from an exhaustive summary, the discussion above emphasizes the biological complexity of chronic wounds. Indeed, the impairment of the ECM in chronic wounds has long been identified as a key target for wound healing strategies. Within the last 20 years, substantial emphasis has been directed toward the development of bioengineered skin substitutes, such as living skin equivalents, acellular matrices, and polymeric scaffolds, that recapitulate multiple features of the native ECM that are so necessary in regulating the wound healing cascade [24]. Several bioengineered scaffolds that are FDA approved and commercially available will be discussed in this chapter. This chapter will also focus on emerging academic scaffolds that aim to improve on some of the shortfalls present in these available products. However, it is important to note that many of the academic products have only been tested in animal, namely, murine, models. One cannot fully understand the human wound healing response until exhaustive human clinical testing has been completed. Not all of the academic biomaterials discussed in the sections below will join the FDA-approved and CMS-reimbursed products noted below.

All of the wound healing dressings discussed in this chapter provide an ECM, whether natural or synthetic, that supports the infiltration of cells, tissue regeneration, and ultimately wound closure. These skin substitutes are designed to provide a bio-inductive and vulnerary environment by modulating the proteolytic climate and/or supplementing the wound bed with exogenous, bioactive factors that stimulate innate tissue repair mechanisms. They are precisely engineered and fine-tuned to recapitulate aspects of the wound healing milieu and target specific events in the wound healing cascade to facilitate complete skin repair with restored function and tissue integrity. However, to date, there have been limited head-to-head comparative clinical studies evaluating the performance of the plethora of advanced wound care products, which are required to guide clinical practice and payer determinations [25].

3 Bioengineered Skin Substitutes

The optimal bioengineered scaffold for skin regeneration of chronic wounds should (1) be non-immunogenic, (2) modulate proteolytic activity to reset the wound to an acute state, (3) provide a bio-resorbable scaffold that facilitates cellular migration and promotes cellular proliferation and matrix deposition, (4) recruit angiogenic and fibroblast cell types to synthesize granulation tissue, and (5) absorb and neutralize free radicals [26]. In the following sections, we will describe the various types of bioengineered skin substitutes, including those that contain natural ECM components harvested from human tissue or animal sources and synthesized, ECM-mimetic biopolymeric scaffolds. All commercially available scaffolds detailed in this chapter are listed in Table 1.

3.1 Living Skin Equivalents

Living skin equivalents comprise scaffolds, either natural or synthetic, seeded with allogenic fibroblasts and/or keratinocytes. There are several iterations of products that have been developed using this approach, which essentially provide cellular and structural components for wound healing. Apligraf® (Organogenesis, Inc.) is composed of a bovine type I collagen matrix seeded with neonatal fibroblasts to produce a neodermal layer. Human neonatal epidermal keratinocytes

Table 1 Summary of scaffolds for chronic wound healing

Product	Composition	Properties/mechanism of action	FDA
Living skin substitutes			
Apligraf®	Bovine type I collagen seeded with human neonatal fibroblasts and keratinocytes	Metabolically active cells secrete cytokines and growth factors to stimulate differentiation and proliferation	PMA (1998)
Dermagraft®	Human neonatal fibroblasts seeded on bioabsorbable polyglactin scaffold—Cryopreserved	Metabolically active fibroblasts secrete collagen, matrix proteins, growth factors, and cytokines	PMA (2001)
TheraSkin®	Cryopreserved skin allograft harvested from cadavers	Biologically active scaffold providing cellular and extracellular components Natural barrier to infection	HCT/Ps
Acellular naturally derived polymeric scaffolds			
Oasis®	Minimally processed ECM derived from porcine small intestine submucosa	Provides structural matrix and delivers growth factors to stimulate angiogenesis and cell migration	510 K (1998)
GraftJacket®	Processed (cross-linked and cryopreserved) human dermal matrix	Fenestrated acellular matrix that acts as a foundation for revascularization and cellular repopulation Reduces inflammation	HCT/Ps
DermACELL®	Decellularized human dermis allograft	Unique anionic detergent and endonuclease-based process to decellularize tissue Scaffold supports cell ingrowth	HCT/Ps
EpiFix®	Dehydrated allograft: amnion and chorion membranes derived from donated human placenta	Composed of a single layer of epithelial cells, a basement membrane, and an avascular connective tissue matrix Retains soluble biological molecules and growth factors that stimulate human dermal fibroblast proliferation and the migration of human mesenchymal stem cells	HCT/Ps
Integra™	Cross-linked bovine collagen and chondroitin 6-sulfate with a silicone membrane	Biodegradable matrix provides a scaffold for cellular invasion and capillary growth	PMA (1996) 510 K (2002)
Promogran™	Freeze-dried composite of 55% collagen and 45% oxidized regenerated cellulose	Composite matrix absorbs wound exudate to form a biodegradable gel Provides a scaffold for cellular migration	510 K (2002)
Tegagen™, Algisite™, Algi-fiber, etc.	Dressings of calcium alginate fibers	Form gelatinous mass upon contact with wound exudate Extremely absorbent (10×) Controls mild hemorrhages	510 K
Biopolymeric scaffolds			
Talymed®	Shortened fibers of N-acetyl glucosamine isolated from microalgae	Material interacts with fibroblasts and endothelial cells to stimulate cell migration	510 K (2010)
Hyalomatrix®	Non-woven pad of benzyl Ester of hyaluronic acid and a semipermeable silicone membrane	Biodegradable scaffold for cellular invasion and capillary growth. Contains a semipermeable silicone membrane to prevent water loss	510 K (2007)
Dextran	Cross-linked modified dextran and PEG diacrylate	Biodegradable matrix fills wound defect and provides a scaffold for cellular infiltration	N/A

Abbreviations: *PMA* premarket approval, *HCT/Ps* human cells, tissues, or cellular-based products

are subsequently added on top of this dermal component as a monolayer to approximate the epidermis and form a differentiated stratum corneum [27]. This results in a metabolically active bilayered skin substitute providing both a dermal and epidermal layer with living cells. Although the fibroblasts and keratinocytes in Apligraf do not persist beyond 6 weeks in patients [28], they are thought to be responsible for stimulating differentiation and proliferation via secretion of

essential cytokines and growth factors [29]. Apligraf was the first allogeneic cell-based product to be approved by the FDA in 1998 for the treatment of DFUs and venous leg ulcers. Large multicenter randomized clinical trials demonstrated a significantly higher rate of wound closure compared with conventional standard of care [30, 31].

Dermagraft® (Organogenesis, Inc.) was approved by the FDA in 2001 for the treatment of non-healing DFUs. Although it also contains neonatal dermal fibroblasts, it differs from Apligraf in that the fibroblasts are cultured onto a bio-resorbable polyglactin mesh scaffold; polyglactin is a standard suture material. The metabolically active fibroblasts proliferate within the interstices of the synthetic scaffold, secreting collagens, growth factors, cytokines, proteoglycans, and other key regulatory molecules, to create a 3D bioactive matrix, which is then cryopreserved for storage [32]. When applied to DFUs, Dermagraft significantly increased the rate of wound closure compared to the control [33].

Another biologically active human skin allograft is TheraSkin® (Soluble Systems), which is harvested within 24 h postmortem and cryogenically processed to preserve the viable fibroblasts, keratinocytes, and fully developed extracellular matrix sequestered with essential growth factors and cytokines. It has been reported that TheraSkin, which was found to be effective in the treatment of DFUs and venous stasis ulcers [34], contains a greater quantity of the key collagens (Collagen I and III) critical to wound healing compared to Apligraf and healthy tissue [35, 36]. This may be attributed to the manufacturing process of Apligraf in which a bovine collagen substrate is used to culture neonatal cells that deposit the ECM in vitro. The application of a living human dermal skin substitute delivers a smorgasbord of vital key regulatory proteins and cytokines that stimulate angiogenesis, fibroblast migration, and keratinocyte proliferation to accelerate wound healing. TheraSkin is shown to maintain key wound healing growth factors, VegF, TFG-B, and FGF-2, when compared to fresh tissue [36]. However, there is an absence of head-to-head studies that compare the clinical and cost efficacy advanced wound care products to inform clinical practice and payer determination. Indeed this stems also from the variety of chronic wound types with various etiologies—there is no single wound care product to treat and manage all wound types. Most comparative studies are either retrospective analyses or funded by the company. In a retrospective study evaluating the efficacy of EpiFix compared to Apligraf in treating DFUs, it was reported that patients treated with EpiFix required more applications compared to patients treated with Apligraf and that the median time to wound closure using Apligraf was 13.3 weeks compared to 26 weeks for EpiFix [37]. However, in a prospective study, 97% of lower extremity diabetic ulcers healed when treated with EpiFix compared to only 73% of wounds treated with Apligraf, suggesting that patients treated with EpiFix experienced a shorter time to wound closure [38]. The median graft cost was $8918 (range $1486–19,323) per healed wound for the Apligraf group and $1517 (range $434–25,710) per healed wound in the EpiFix group [38]. In a separate retrospective analysis, treatment using the bilayered living cell construct Apligraf reduced the median time to wound closure of venous leg ulcers by 44% compared to treatment using a naturally derived, acellular porcine dressing (Oasis®; to be discussed below) [37].

3.2 Naturally Derived Acellular Matrices

Acellular matrices are characterized as nonviable biomaterials. They may be animal- or human-derived, with all cells removed during manufacture, or they may be synthetic or a composite, where cells are simply not present from the outset. Natural polymers are commonly utilized in the development of acellular matrices for chronic wound treatments because of their inherent biocompatibility and bioactivity as well as their ability to mimic the structural, biomechanical, and biochemical functions of the ECM. There are cost advantages to using naturally derived ECM-based polymeric scaffolds. Using a Markov model to estimate the compara-

tive cost-effectiveness of Apligraf, Dermagraft, and an ECM-based therapy, the ECM-based therapy was economically dominant and determined to be the most cost-effective for the management of venous leg ulcers as an adjunct therapy to standard of care [39]. The expected costs for a naturally derived ECM-based scaffold (Oasis®), Apligraf, and Dermagraft were $6732, $10,638, and $11,237, for 31,29, and 17 ulcer-free weeks, respectively, suggesting that naturally derived ECM-based therapies yield potential savings compared to other cell- or tissue-derived products [39]. The most common, bioactive natural polymers utilized in the development of acellular matrices for wound healing are collagen, hyaluronic acid, chitosan, and alginate.

Collagens are the most abundant ECM macromolecule and are the main component in human skin that provides structural integrity [6]. In addition to its structural function, collagen I governs many cellular functions of fibroblasts and keratinocytes, including cell adhesion, differentiation, migration, ECM deposition, and angiogenesis [40–42]. Collagen I is also able to bind excess proteases, inflammatory cytokines, and free radicals that are rampant in the chronic wound bed [43]. The role of collagen in tissue repair and wound healing are multifactorial, which supports the extensive use of exogenous collagen-based scaffolds for chronic wound applications. Generally speaking, collagen-based scaffolds are classified as either decellularized matrices, derived from a variety of mammalian sources and anatomical locations, including porcine small intestine submucosa or urinary bladder matrix, human cadavers, and placental tissue, or they are synthesized via extraction and chemical crosslinking. There are many products that are currently used in the treatment of chronic wounds, and several of them are briefly described below as representative examples.

Oasis® Wound Matrix (Healthpoint) is a naturally occurring ECM graft (>90% collagen) derived from porcine SIS, which is a thin, approximately 0.15 mm thick translucent layer of porcine intestine that is predominately type 1 collagen. Porcine SIS possesses a porous microstructure, with pores ranging in size from 20 to 30 μm that enables oxygen diffusion and promotes cell viability [44]. Porcine SIS also retains the active forms of other biologically relevant components that provide cell and growth factor binding sites, sequester matrix-degrading enzymes, and enhance cellular infiltration into injured tissue. It is also embedded with glycosaminoglycans, proteoglycans, fibronectin, and various growth factors that imparts significant bioactivity [45–47]. In this way, the SIS not only provides a structural matrix and delivers growth factors to stimulate angiogenesis and cell migration but also regulates proteolytic activity and dampens the inhibitory effects of MMP-1, MMP-2, and MMP-9 on keratinocyte migration [48].

There are myriad acellular wound matrices available for clinical use that are processed, decellularized dermal constructs derived from donated human tissue. They are all designed to provide a scaffold for wound repair; however, each acellular dermal wound matrix differs by the way in which it is processed. For GraftJacket® (Wright Medical Technology), donated human tissue is treated to remove the epidermis and cellular components, but it retains collagen, elastin, proteoglycans, and internal matrix of the dermis, which remains intact and is chemically crosslinked to maintain the collagen architecture before cryopreservation [49]. DermACELL® (LifeNet Health) is a human tissue matrix allograft that employs a unique, proprietary MATRACELL® technology [50] that uses anionic detergents and an endonuclease to achieve >97% nucleic acid removal while retaining biomechanical strength. This allows DermACELL to be preserved at ambient temperature and offers a >3-year shelf life. Both of these products have been indicated for the treatment of non-healing ulcers and dermal wounds and have demonstrated the ability to reduce time to complete wound closure and increase healing rates compared to conventional care [51–53]. In these processed acellular dermal matrices, the removal of the cellular components reduces the risk of rejection and the critical dermal proteins that remain minimize inflammation and facilitate cell infiltration and tissue revascularization. In contrast to xeno-

geneic ECM allografts, minimally manipulated human tissue products are classified as human cell, tissues, and cellular- and tissue-based products (HCT/Ps) by the FDA. As a result, there are fewer restrictions on the applications for which these devices can be used; they are viewed as tissue transplants, and manufacturers are only required to follow manipulation guidelines to ensure materials are free from transmissible pathogens (Table 2).

The use of placental membranes for wound healing has been reported for over 100 years, which can be attributed to its collagen-rich ECM presenting biologically active components, such as developmental cytokines and elevated concentrations of regenerative growth factors [54]. Placental membranes contain a plethora of multifunctional growth factors, including, but not limited to, epidermal growth factor, basic fibroblast growth factor, PDGF, VEGF, TGF-β1, and keratinocyte growth factor, as well as MMPs and TIMPs [55–57] that support critical cell behavior and wound healing events. Also expressed in placental membranes are immunosuppressive factors and antibacterial peptides that contribute to the reduced risk of rejection of placental membranes [58, 59].Large amounts of the ECM glycosaminoglycan hyaluronan (HA) are also present in placental membranes, which has been shown to function as a free radical scavenger to remove reactive oxygen species [60, 61]. However, different processing methods impact the composition and functionality of these materials [62]. There are more than 25 commercially available placental membrane products, yet most contain no viable cells because they are either dehydrated or are cryopreserved devitalized or decellularized tissue. One such product, EpiFix® (MiMedx), is a dehydrated human tissue allograft comprising laminated amnion and chorion membranes derived from donated human placenta. During processing, the amnion and chorion tissue

Table 2 Brief overview of US FDA pathways for wound healing products (medical devices)

	PHS 361: HCT/Ps	510 K	PMA
Device classification/ risk	Low	Class II/moderate	Class III/high
Review standard	No premarket review Not required to demonstrate safety or effectiveness	Substantial equivalence in safety and effectiveness to a legally marketed predicate device	Approval requires that the safety and effectiveness of the device be established with valid scientific evidence, i.e., high-quality clinical data
Requirements	Minimally manipulated Intended for homologous use No systemic effect/not dependent on metabolic activity of cells Manufacturers follow good tissue practice to prevent the introduction, transmission, and spread of communicable diseases	Non-clinical laboratory studies for safety (performed under GLP conditions) *Clinical investigations not typically required* *Quality systems in place prior to interstate commerce* *Manufacturing not reviewed preapproval*	Non-clinical laboratory studies for safety (performed under GLP conditions) Clinical investigations (such as performed under an investigational device exemption) Detailed quality systems in place Preapproval of manufacturing facility with inspection
Regulatory burden	Low	Medium	High
Wound healing products	TheraSkin® GraftJacket® DermACELL® EpiFix®	Oasis® Integra™ Promogran™ Tegagen™, Algisite™ Algi-fiber Talymed® Hyalomatrix®	Apligraf® Dermagraft® Integra™*

Abbreviations: *PMA* premarket approval, *HCT/Ps* human cells, tissues, or cellular-based products

layers are isolated from the placenta and washed. The two layers are then laminated to form the graft, which is subsequently dehydrated and sterilized. EpiFix contains a single layer of epithelial cells, a basement membrane, and an avascular connective tissue matrix. After processing, EpiFix retains soluble biological molecules and growth factors that stimulate human dermal fibroblast proliferation and the migration of human mesenchymal stem cells [63]. When evaluated in the treatment of DFUs and venous leg ulcers, EpiFix promoted complete epithelialization and reduced the wound size in patients compared to standard treatment [64].

Integra™ (Integra Life Sciences) and Promogran™ (Systagenix Wound Management) are two wound healing products synthesized using extracted and polymerized collagen. Integra is a bilayer composite matrix of cross-linked collagen type I from bovine sources and a glycosaminoglycan (chondroitin 6-sulfate) isolated from shark skin. It has a semipermeable silicone membrane that functions as a temporary epidermal layer by controlling water vapor loss and providing structural integrity. The bilayer matrix recruits dermal fibroblasts to the wound, which then synthesize and secrete new ECM to the wound bed to facilitate healing [65]. Although initially indicated for third-degree burns via an FDA premarket approval (PMA; to be discussed below), a 300 subject clinical trial demonstrated that the non-healing DFUs treated with Integra had a more rapid time to complete wound closure and increased rate of wound closure compared to standard of care treatment [66]. Promogran is a combination matrix composed of 55% bovine type I collagen and 45% oxidized regenerated cellulose (ORC) that is freeze dried and formed into a 3 mm thick sheet that is applied directly to the wound bed. Upon application, the composite matrix absorbs wound exudate to form a biodegradable gel that enhances fibroblast migration and proliferation [67]. The composite matrix binds and stabilizes growth factors and physically sequesters and inactivates excessive MMPs while providing a scaffold for cellular migration [68]. Clinical studies demonstrate a significant reduction in the concentration and activity of proteases in the wound exudate of DFUs treated with Promogran and a greater reduction in wound size [69].

Other natural polymers used as wound dressings are alginate and chitosan. Alginate is a polysaccharide with homopolymeric blocks of (1-4)-linked β-D-mannuronic and α-L-guluronic residues that is isolated from the cell walls of a variety of species of brown seaweed. Alginate exhibits unique gelation properties and ionically cross-links in the presence of divalent ions to form a biocompatible 3D polymeric cross-linked scaffold for tissue engineering applications [70]. Alginate wound dressings are used in wound management because they provide a moist environment, are highly absorbent, and function as a hemostat. When an alginate dressing comes into contact with wound exudate, there is an ion exchange between the calcium ions in the mannuronic and gluronic groups of the alginate dressing and the sodium ions in blood or exudate [71]. As sufficient calcium ions are replaced by sodium ions, the alginate fibers swell, partially dissolve, and form a gel that maintains a moist environment for autolytic debridement and reduces pain during dressing changes [72]. Alginate dressings have also been shown to minimize microbial bioburden and sequester proteinases [73]. There are numerous alginate-based wound dressings approved for use in managing variety of wound types in which exudate is present, such as chronic wounds, including Tegagen™ (3 M), Algisite™ (Smith & Nephew), and Algi-Fiber (CoreLeader Biotech) to name a few [74, 75] .

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine that is predominately used as a hemostat but is currently being evaluated as a wound dressing for chronic wounds because of its ability to modulate the wound environment [76, 77]. Chitosan is prepared by deacetylating chitin, the principle component in the exoskeleton of crustaceans, via enzymatic or alkaline hydrolysis before being processed into various fibrous, scaffold, and hydrogel biomaterials [78]. Chitosan contributes to wound healing by stimulating the rapid mobilization, adhesion, and aggregation of platelets

and red blood cells to the wound site to facilitate rapid clotting [79, 80]. Post hemostasis, chitosan also accelerates granulation tissue and matrix formation [81, 82]. Lysozymes gradually depolymerize chitosan via hydrolysis to release N-acetyl-D-glucosamine, which stimulates fibroblast proliferation and collagen deposition and remodeling [83]. Chitosan has also been shown to stimulate inflammatory cells migration [84]. As a wound dressing biomaterial, chitosan exhibits several unique advantages, including nontoxicity, physiological inertness, antibacterial properties, biocompatibility, and an affinity to proteins [85]. Chitosan's antimicrobial properties are attributed to the presence of primary amine groups that confer an overall cationic charge, which destabilizes and permeabilizes microbial membranes [86].

3.3 Biosynthetic Acellular Matrices (Polymeric)

Acellular synthetic matrices offer several advantages over naturally derived polymeric and cellular-based scaffolds, including longer shelf life, cost efficacy, and limited risk of rejection. In a retrospective study, the number of applications needed to treat (NNT) was used to model the comparative clinical and cost efficacy of currently available advanced wound care matrices as adjuncts to compression therapy for the treatment of venous leg ulcers. It was found that fewer applications of an acellular biosynthetic scaffold was required to achieve closure compared to a human skin equivalent (Apligraf) and biologically derived polymeric scaffold (Oasis) at a significantly lower cost. The incremental costs per additional successfully treated patient were $1600 for the acellular biosynthetic scaffold (Talymed®), $3150 for Oasis, and $29,952 for Apligraf [87].

To design an efficacious biosynthetic polymeric scaffold that achieves wound closure and skin regeneration, several parameters and criteria need to be considered in addition to those listed in the above section. Scaffolds that are chemically synthetized or modified not only need to be easily manufacturable but also biocompatible, biodegradable, and nontoxic while exhibiting optimal biomechanical properties, including ideal porosity and morphology to modulate the transport cells, metabolites, and signaling molecules. Most importantly, cells must be able to appropriately respond to and infiltrate the scaffold to facilitate degradation and support a regenerative healing process [88]. Therefore, the fundamental design strategy in developing biosynthetic scaffolds is to recapitulate structural and molecular aspects of the ECM using tunable polymeric materials that simulate the elasticity and porosity of dermis. Polysaccharides possess reactive functional groups that can be modified to form nontoxic and bioactive wound healing biomaterials with optimized and tailorable characteristics, such as pore size and degradation rate, which elicit the appropriate biological response and stimulate tissue regeneration. To date, there are few FDA cleared, commercially available biosynthetic scaffolds indicated for the use in managing chronic wounds. The FDA cleared two scaffolds, both of which are polysaccharide-based.

The US FDA predominately regulates wound healing products as medical devices based on their composition and device classification, which depends on the intended use of the device. Devices that present relatively low risk are generally categorized as Class I or Class II devices, and higher risk devices are Class III. Minimally manipulated human-derived products, such as placental membrane-derived products, are regulated as human cells, tissues, and cellular- and tissue-based products (HCT/Ps) and only require manufacturers to follow good tissue practices and manipulation guidelines. Class III human-/animal-derived products, such as cellular wound matrices, are approved through a premarket approval (PMA). Devices that present relatively low or moderate risk (Class II), such as animal-derived and synthetic products, require the manufacturer to seek 510(k) clearance, which is generally granted when submitted information establishes that a new device is "substantially equivalent" to an already approved and legally marketed "predicate" device in terms of technological characteristics, such as design, mode of

action and composition, and performance. Many biosynthetic scaffolds are cleared through the 510(k) pathway (Table 2). In the European Union, there are directives that outline requirements under which a medical device could be marketed across all EU member states after earning a Conformité Européenne (CE) mark in any one member country. These directives similarly categorize devices into four classes (I, IIa, IIb, and III) on the basis of associated risks. Approval and CE marks for medical devices are directly managed by designated Notified Bodies and are subject to performance and reliability testing. Approval is generally granted if the device successfully performs as intended in a manner in which the benefits outweigh expected risks. The specific requirements for premarketing clinical studies are vague, and the guidelines for the nature of these studies are not binding on manufacturers or Notified Bodies [89].

Talymed® (Marine Polymer Technologies) is a biodegradable, wafer-thin wound matrix that was cleared in 2010 for the management of full- and partial-thickness wounds, including chronic wounds. Talymed is a bioactive scaffold composed of shortened fibers of poly-N-acetyl glucosamine derived from diatom algae. Native poly-N-acetyl glucosamine fibers are shortened to ~4–7 μm using gamma radiation, which retains the unique 3D polymeric structure and enables the nanofibers to form a thin, biodegradable scaffold membrane [90]. Preclinical animal studies demonstrated that a nanofibrous scaffold composed of shortened poly-N-acetyl glucosamine fibers initiated wound healing through material-facilitated interactions with fibroblast and endothelial cells that stimulated reepithelization via increased keratinocyte migration, granulation tissue formation, cell proliferation, and vascularization [90]. The shortened fibers of poly-N-acetyl glucosamine become completely integrated into the wound bed and upregulate the integrin-dependent Ets1 transcription factor, which regulates genes involved in cell migration, proliferation, and survival. The shortened fibers of poly-N-acetyl glucosamine stimulate endothelial cells and the increased secretion of several cytokines and growth factors, including IL-1 and VEGF, that are imperative for proper wound healing [91]. In a pilot study, 86% of patients with venous leg ulcers that were treated with Talymed biweekly achieved complete wound healing within 5 months compared to patients only receiving standard of care (45%) [92].

Hyaluronan or hyaluronic acid (HA) is a linear glycosaminoglycan composed of alternating units of D-glucuronic acid and D-*N*-acetyl-D-glucosamine that is ubiquitously distributed within the ECM and specifically in connective tissue. HA is a well-established co-regulator for gene expression, proliferation, motility, adhesion, signaling, and morphogenesis [93]. In wound healing, HA plays a key role in modulating inflammation, stimulating cell migration, and promoting angiogenesis through interactions with two cellular receptors: RHAMM and CD44 [94]. However, the role of HA in tissue repair is largely dependent on molecular size [95]. High-molecular-weight HA exhibits anti-inflammatory, immunosuppressive, and anti-angiogenic effects by inhibiting EC proliferation, migration, and capillary formation, whereas short-chain, low-molecular-weight degradation products of HA, namely, oligosaccharides of 3–10 disaccharide units, are potent pro-inflammatory molecules that induce angiogenesis by stimulating EC proliferation, migration, and angiogenic sprouting [96]. In vivo, native HA is subject to rapid enzymatic degradation by hyaluronidases and, in wounded tissue, further fragmentation by free radicals [97]. Fortunately, HA is amenable to chemical modifications due to the presence of carboxyl and hydroxyl groups on its repeating disaccharide units. The functional groups allow HA-based biomaterials to be tailored to retard and control degradation for tissue regeneration and wound healing applications. Synthetic HA derivatives have been chemically modified by esterification of the carboxylic group of glucuronic acid with benzyl groups [98]. This modification imparts higher resistance to hyaluronidase enzymatic activity and degradation. Hyalomatrix® (Anika Therapeutics) is a bilayered wound device composed of a wound contact layer containing fibers of esterified HA and an outer semipermeable silicone membrane that acts as a barrier to prevent

vapor loss and reduce bacterial colonization. Hyalomatrix acts as a regenerative matrix by providing HA in the form of a 3D scaffold. The scaffold enables rapid fibroblast and endothelial cell infiltration and modulates ECM deposition [99, 100]. In slow-healing wounds, as the matrix degrades, a high concentration of HA is locally released to the wound site that stimulates a regenerative response. When evaluated in wounds of different etiologies, including vascular, DFUs, traumatic wounds, and pressure ulcers, 83% of Hyalomatrix-treated wounds achieved some degree of reepithelialization (≥10%) within 16 days [101].

4 Recent Developments in Bioengineered Skin Substitutes

In addition to products currently in clinical use, biomaterial-based materials are constantly being developed for wound healing applications. These novel, engineered biomaterials aim to remedy current deficiencies in current acellular and cellular products. A tissue engineer has three levers by which to manipulate a wound dressing, known as the tissue engineering triad. The tissue engineering triad is comprised of novel scaffold materials, cellular loading, and/or soluble molecules (i.e., growth factors) [102, 103]. This chapter will briefly explore wound healing products that have yet to clear the FDA but utilize novel materials, bioactive deliverables, and cellular loading. The scaffolds overviewed below highlight the innovation and design necessary to address market deficiencies, and the unique strategies are listed in Table 3.

4.1 Novel Materials

Researchers have tested a variety of biocompatible materials for wound healing applications, ranging from natural polymers (chitosan, alginates, dextran, fibrin, collagen, etc.) to synthetic polymers (PLGA, PEG, PLLA, etc.) [104–106], but currently only a small number (i.e., Talymed,

Table 3 Scaffold development strategies for chronic wound healing

Approach	Mechanisms	Shortfalls	Examples
Biophysical cues			
Pore size	Regulates native cell invasion; controls structure for granulation tissue and neo-ECM; exudate absorption	Difficult to balance proper absorption with sufficient scaffold mechanics	Nanocellulose exudate control; Dextran UV cross-linking
Topography	Provides attachment sites for cells and growth factors; Directs alignment and orientation of ECM	Temporary; difficult to ensure uniformity	Electrospun microfibers aligned axially
Biochemical cues			
Material composition	Specific chemical residues elicit cellular response; binding sites for cell and GF immobilization; anti-inflammatory	Natural polymers unmodifiable; certain residues locally toxic in high concentrations	RGD peptides, MAP's cell-adhesive backbone
Small molecule release	Mediates wound healing phases and cellular response; spurs ECM deposition	High local concentrations inhibit desired response; bioactivity fades over controlled release time frame	PDGF, VEGF, and FGF2 cDNA for protein encoding
Cellular loading	Provides key growth factor and protein eluting cells to barren wound microenvironment	Short shelf life; require tightly controlled microenvironments; not patient specific	MSCs, fibroblasts
Bioactive sensing	Adaptable to the wound environment; directly targets common chronic wound deficiencies	Specialized to unique patients and wound types	pH sensing; hypoxia response

Abbreviations: *PDGF* platelet-derived growth factor, *VEGF* vascular endothelial growth factor, *FGF2* fibroblast growth factor 2, *MSCs* mesenchymal stem cells, *MAP* microporous annealed particles

Hyalomatrix) of these products have been cleared through the FDA. Despite lacking cellular loading or embedded components, many biomaterials are developed to mimic the extracellular matrix and recruit surrounding cells to heal wounds in situ. Biodegradable scaffolds must maintain a delicate balance that provides an environment for cell recruitment and structure to the wound bed void while simultaneously imparting the necessary mechanical properties for granulation and healthy tissue formation.

New evidence shows that biophysical cues provided by biomaterial wound dressings, irrespective of growth factor or cellular loading, are regulating native cell-matrix interactions [107]. Advances in scaffold formation, namely, micropatterning, allow material manipulation at the nano- and microscale and permit the study of the subsequent cellular interactions. The nanotopography of biomaterials is shown to affect dermal fibroblast migration [108], keratinocyte motility [109], and skin cell adhesion and reepithelialization [108–110]. Porous biomaterials provide a better scaffold for wound healing than solid biomaterials, especially in the stimulation of native tissue integration and cell infiltration [111]. A key study by Marshal et al. [112] tested scaffold pore sizes ranging from 10 to 160 μm and found that optimal wound healing occurs at a pore size of 35 μm regardless of material composition. A 35 μm pore supported cell infiltration and triggered an inflammatory response dominated by M2 reparative macrophages [113]. Microfabrication technologies allow wounds healing scaffolds to mimic ECM of the wound bed more accurately. Two fabrication techniques that create specific topographical alignments of biomaterials include electrospinning and microfluidics. Electrospinning aligns arrays of nanofibers uniaxially to promote cellular migration both in vivo and in vitro [114, 115]. Electrospinning creates ideal topographies for cell and biomolecular adhesion and release [116]. Microfluidics can produce biomaterials with well-defined pore structures [117] that recruit vascular cell lines and improve neovascularization by creating nonhomogeneous channels within a larger biomaterial dressing [118].

One such material-based strategy utilizes wood nanocellulose to control exudate in a wound bed. Wood nanocellulose is a biodegradable, renewable material with liquid absorption characteristics [119]. Nanocellulose is a biodegradable and renewable natural resource that is produced from wood-based pulp fibers and can be used to form cellulosic nanofibrils (CNFs) [120]. Nanocellulose is a non-cytotoxic, functional biomedical material for tissue regeneration. Nanocellulose dressings are predominately comprised of fibrillated films of varying nanocellulose grades and cross-linked with a polyethylene glycol (PEG) linker. To satisfy FDA requirements, ultrapure CNFs are isolated from *P. radiate* kraft pulp fibers with low-endotoxin levels for use in nanocellulose-based wound dressings [121]. The grade is based on the homogeneity of the nanofibril structure with varying widths and morphology [122].

Nanocellulose-based dressings are structurally very similar to alginate-based dressings; however, nanocellulose fibers have a water holding capacity of more than 70 times its own dry weight compared to 10–20 times for alginates [123]. Therefore, these films are targeted specifically for wounds with large quantities of wound exudate. The water absorption of the dressing is entirely dependent on the grade of nanocellulose, and it can vary from 1500 to 7000% [122]. As the degree of fibrillation increases, the swelling decreases because the ability for the exudate to penetrate the highly compacted fibrils is reduced [124]. Initial testing of the nanocellulose dressing was conducted in water; however, PBS is considered a more accurate representation of wound exudate because of its ionic makeup. In PBS, the liquid absorption of the nanocellulose dressing remained between 400 and 600% because the dissolved electrolytes ionically interacted with the individual fibrils [122, 125].

Despite fibril density having a minimal effect on swelling, the fibril density influences tensile properties, which, in part, impact in vivo performance. The ideal dressing provides a rigid matrix that fills the wound bed but is flexible enough to maintain contact with irregular surfaces [121]. The nanocellulose dressings have a rigid, tightly

aggregated structure in the dry form (<7 MPa) but possess a flexible structure when in PBS (10–14% maximum strain) [119, 122, 125]. Additionally, the addition of PEG to the nanocellulose mesh stabilizes the internal structure, which allows the material to reach equilibrium after 24 h [126]. Human tissue has an elastic modulus between 0.1 and10 MPa [127], and the cellulose-PEG dressings recapitulate that range with modulus of 13–48 MPa in PBS [122].The nanocellulose-based dressing highlights the advantageous tunability of biomaterial-derived dressings compared to cell-based or decellularized tissue dressings. Solely by controlling the grade of nanocellulose and the uniaxial fibril orientation, the rate of exudate absorption and in vivo flexibility can be tuned during material fabrication. For example, CNF films in the range of 0.2–1 MPa have been shown to promote the adhesion and proliferation of fibroblasts in vivo [128].

Microfluidic have been used to generate a scaffold comprised of microporous annealed particles (MAP) that function as chemically and physically tunable building blocks. MAPs are generated using water-in-oil emulsion [129–131] through a microfluidic channel. The flow rates of both the aqueous precursor and the oil are carefully controlled to produce homogenous MAP populations ranging from 15 μm for low flow to 100 μm for high flow [132]. The resulting MAPs contain a PEG-vinyl sulfone backbone with cell-adhesive peptides and activatable peptides for cross-linking [133–135]. MAPs are injected directly into the wound bed to provide the necessary interconnected network and promote cell migration. The injectable material forms to the contours of the wound bed and mimics the ECM to allow rapid cell migration, provide cellular adhesion cues, and resorb post-healing [132]. The building blocks are cured into a stabilized scaffold in vivo in the presence of FXIIIA, a ubiquitous blood clotting factor [134–136].

Injectable, MAP-based scaffolds are physically tuned by controlling the MAP block size during fabrication and range from 15 to 100 μm. The resulting scaffold pores ranged from 10 to 35 μm. By altering the PEG weight percentages and building block size, the modulus of the scaffold ranged from 10 Pa to 1 kPa. In vitro, it was shown that cells incorporated into the scaffold did not create networks in annealed MAP scaffolds that lacked the inherent chemical and mechanical properties, namely, porosity and stiffness [137]. The MAP-based scaffold had improved wound healing (39% closure by day seven) compared to the control (7%). The treated wounds also exhibited improved reepithelization, greater cell infiltration, enhanced hair follicle and sebaceous gland regeneration, and increased vascular network formation [132]. MAP scaffolds demonstrated how altering physical and chemical properties at the micron scale can directly impact macroscopic material properties and in vivo response.

Among the natural polymers, dextran is a hydrophilic, nontoxic polysaccharide composed of linear α-1,6-linked D-glucopyranose residues with a low fraction of branches extending from α-1, 2-, α-1, 3-, and α-1, 4-linked side chains. Dextran is synthesized by bacteria, *Leuconostoc mesenteroides* is naturally resistant to protein adsorption and cell adhesion, and modification of its polymer backbone allows the development of biomaterials with specific properties. Dextran is also highly water soluble and easily functionalized through its reactive hydroxyl groups. For instance, modifying dextran polymers with polymerizable vinyl groups creates functionalized dextran macromers that present available C=C groups for cross-linking. These modified dextran macromers are then combined with PEG diacrylate and photopolymerized to produce a hybrid cross-linked scaffold [138]. The biosynthetic scaffold technology was developed in Dr. Gerecht's laboratory at Johns Hopkins University. The physical properties of the biosynthetic dextran scaffold can be tuned to facilitate cell infiltration and scaffold degradation by modifying the degree of substitution of cross-linking groups and ratio of polymeric components, modified dextran, and PEG diacrylate [139]. The degree of substitution, or the number of functionalized hydroxyl groups on the dextran anhydroglucose units, and dextran content combinatorially affect the cross-linking density and, therefore, the porosity, elasticity, and degradation of the scaffold. Tissue ingrowth and regeneration is largely

dependent on these physical properties. A reduced degree of substitution of cross-linking groups affects degradation and generates a scaffold with a more porous architecture (~10 μm) that facilitates cell infiltration and migration as well as the diffusion of oxygen and nutrients. Increased dextran content generates a less rigid scaffold but retains structural integrity to enable handling and interface with the wound bed. When applied to third-degree burns in murine and porcine models, the dextran scaffold is quickly penetrated and degraded by early inflammatory cells, promoting the infiltration of necessary cells to reepithelialize the wound and facilitate skin regeneration. Third-degree burns were selected to evaluate the wound healing potential of the dextran scaffold because in preliminary studies, the dextran scaffold demonstrated rapid vascularization when implanted subcutaneously [139]. Thermal injuries display increased capillary permeability and thrombosis, so wound healing outcomes are dependent on neovascularization [140]. In mice, complete epithelial repair with mature epithelial morphologies was observed, including hair follicles and sebaceous glands, after application of the dextran scaffold (Fig. 1a) [141]. Accelerated wound closure was also observed in a porcine model after treatment with the dextran scaffold, in which a thick reticulated neoepithelium was regenerated (Fig. 1b) [142]. This is particularly exciting, because the ability to regenerate skin with functional epidermal appendages, such as hair follicles and sebaceous and sweat glands, has long been and still is a major clinical objective and challenge, particularly in the healing of chronic wounds in which obtaining wound closure is the primary objective.

4.2 Controlled-Release Deliverables

Inherent properties in biomaterials stimulate the natural regenerative wound healing response. However, in some instances, a secondary factor must be incorporated in the biomaterial to increase the wound healing response. Loaded components can include specific cell types, growth factors, or other biochemical molecules that elicit specific reactions. Unlike biophysical cues (i.e., stiffness, fiber alignment, etc.), biochemical cues interact directly with the chemical pathways that are integral to cell behavior and tissue morphogenesis [103, 143].

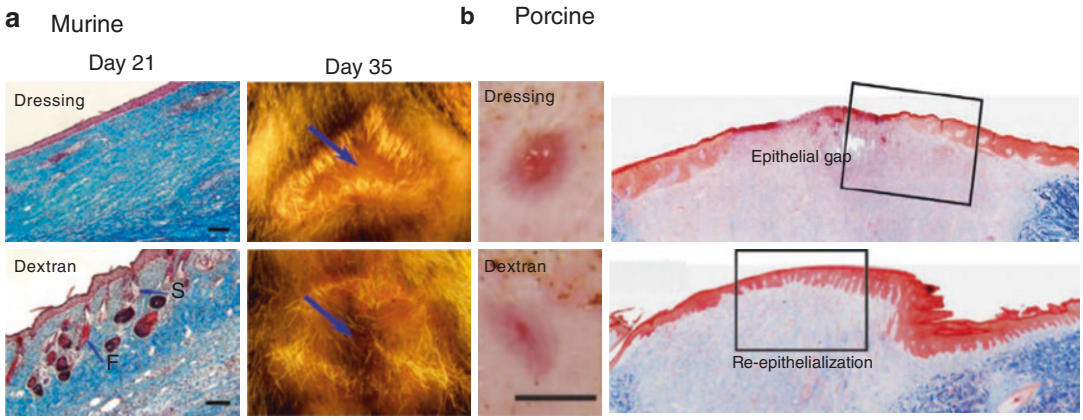

Fig. 1 Dextran scaffold facilitates wound healing in murine and porcine burn models. (**a**) Complete healing observed in mice by day 21 with mature epithelial structures, including hair follicles (F) and sebaceous glands (S) in the dermal layer (left panel; scale bar = 100 μm). By day 35, new hair growth was observed in the center of the dextran scaffold-treated wounds. (**b**) Wound closure was observed by day 14 in a porcine model, as shown by macroscopic and immunohistological images. Wounds treated with dressing only had an epithelial gap, whereas dextran-treated wounds were completely reepithelialized with a thick reticulated epithelium Scale bar = 1 cm. Modified from [141] and [142]. Copyright (2015), with permission from Elsevier

Growth factors are ubiquitously added to topical dressings targeted for chronic wounds healing. Growth factors are known regulators of cellular behavior, and their deficiencies are well quantified in chronic wounds. Dressings, both biomaterial-based and naturally derived, deliver growth factors to the wound bed. Human tissue- and animal-based scaffolds are limited in their effective growth factor delivery due to either processing methods and/or natural limits in the original tissue. Burst release strategies, such as used in Regranex [144], directly deliver growth factors topically. However, these strategies have seen limited success because a surplus of localized growth factors can negatively impact wound healing in the same manner as a low concentration, i.e., hypotension and nephrotoxicity [145]. Effective biomaterials immobilize growth factors (i.e., VEGF, TGF-β1, bFGF) in large concentrations without dampening their bioactivity [146–148]. Ideally, scaffold or peptide degradation releases a controlled concentration of growth factors to the surrounding tissue. The lower dosage spurs wound healing without the negative side effects of direct application.

Fibroblast growth factor 2 (FGF2) was loaded in a self-assembling, cross-linked PEG homodimer polymer by Decker et al. [149]. At low concentrations, FGF2 is known to improve vascularization and granulation tissue formation. The presence of FGF2 tends to be limited in wounds, especially diabetic and pressure ulcers, making it beneficial to create a biomaterial that provides additional FGF2 to the wound site [150, 151]. FGF2 is minimally active unless it is receptor bound and in a tetrameric homodimer, which is its active state [152]. The controlled release of FGF2 is used as a strategy for other ischemic diseases, including heart repair and nerve regeneration [153–156]. Topical FGF2 has been shown to have minimal effectiveness [157], but modifying FGF2 and delivering the FGF2 *superagonist* homodimer in a controlled manner via a polymeric dressing have been shown to sustain the appropriate concentration of active FGF2 to improve tissue formation and vascular density [149]. The homodimer is regulated by free, exposed cysteines (Cys-78 and Cys-96) [158] and the tether length of each dimer. Ideally, the most bioactive tether is 70 Å and is attained using a PEG-based scaffold [152]. PEG molecular weights of 2, 6, and 20 kDa were tested, and the shorter molecular weight (2 kDa) produced the optimal 70 Å tether length.

The self-assembled PEG-FGF2 scaffold increased the metabolic activity (20–30% improvement) of fibroblasts and endothelial cells in vitro compared to uncross-linked FGF2 and cross-linked FGF2 in higher PEG molecular weights. A scratch assay showed a 131% increase in cellular migration for those treated with PEG-FGF2. A co-culture of fibroblasts and HUVECs was treated with the self-assembled scaffold and formed more complex CD31+ cellular networks. Lastly, the scaffold has been tested in a diabetic murine model, and the scaffold promoted increased vascularization and granulation by day ten [149].

Altering the molecular weight of the PEG component allowed the researchers to control the growth factor release, concentration, and bioactivity in the wound bed. This was especially critical for FGF2-PEG complex because FGF2 is only active at low concentrations and specific orientations [157].

Lord et al. developed a modified chitosan scaffold with the controlled release of VEGF189, an ubiquitous angiogenic and collagen-promoting growth factor [159, 160]. VEGF189 binds almost entirely to the ECM compared to other, highly water soluble and shorter VEGF isoforms, such as VEGF165. Heparan sulfate proteoglycans, namely, perlecan, readily binds VEGF189 to the ECM [161], which maintains a much more effective local concentration of VEGF at the wound site compared to the highly water soluble and shorter isoforms [162, 163]. Studies show that perlecan interacts directly with a variety of ECM proteins and inflammatory cells and deficiencies in perlecan or VEGF provide an environment for delayed wound healing and impaired neovascularization [164, 165]. A scaffold that delivers VEGF189 to the wound sites requires a sufficient concentration of perlecan for ECM binding and VEGF189 immobilization.

Also under the umbrella of controlled release strategies, the elution of nonviral vectors (e.g.,

cDNA and RNA) encoding peptides [166] and growth factors [157] is also used in chronic would healing applications. The delivery of cDNA in ischemia studies has been shown to provide encoded target proteins without increasing the therapeutic concentration to unsafe levels either systemically or locally. This is also applicable to wound tissue regeneration [167, 168].

Lord et al. [169] employ a combination of the aforementioned strategies in a chitosan scaffold with pore sizes ranging from 0.1 to 0.15 mm. The chitosan scaffold disintegrated 14 days after implantation in a murine mode and allowed minimal cell infiltration. A mammalian plasmid (pBI-CMV1-Kan) was generated to encode the N-terminal region of both human perlecan and VEGF189. Specific base pair modifications were made to ensure proper translation without inefficiencies or recombinations. The cDNA was loaded at a rate of 90 ng DNA/mm^3. Using a rat wound model, the cDNA-loaded scaffold increased perlecan and VEGF189 expression at day 14 compared to the non-encoding cDNA-loaded control. The test scaffold also improved ECM protein formation, including collagen IV and laminin. There was no significance in macroscopic wound closure rates, but the cDNA-loaded scaffold improved blood vessel density and maturity [169].

Because chronic wounds are largely prone to infection, antibodies and silver nanoparticles are useful additives that reduce the rate of infection. Turner et al. [170] showed that antibodies, namely, flightless I (Flii) neutralizing antibodies (FnAbs), loaded into a silicon nanoparticles-based hydrogel helps to treat chronic wounds. Flii is a well-characterized protein that affects cell mobility, contraction, adhesion, and proliferation [171]. However, unlike VEGF, Flii binds to the cytoskeleton and hinders the deposition of ECM during wound healing. Flii is overexpressed in the wound bed and leads to reduced fibroblast proliferation and reepithelialization [171]. Wound models with low Flii concentrations have vastly improved wound closure and vascularization [171–173]. Therefore, Flii levels can be mediated through the controlled delivery of FnAbs to the wound bed using scaffold-based delivery. Porous silicone (pSi) is one material that can be used for the extended release of FnAbs and other bioactive small molecules. PSi is comprised of individual silicone nanoparticles that are combined to form a biocompatible crystalline structure with inherent biocompatibility and a high-surface area. PSi has a tunable pore size that ranges from the nanometer to the micrometer scale [174]. Fluorescently labeled FnAbs have been loaded into pSi scaffolds and have obtained 86% release after 7 days while maintaining their bioactivity for 14 days [170]. Murine diabetic wounds treated with pSi-FnAbs had significantly improved wound closure compared to wounds treated with unloaded silicone (30% larger).

4.3 Cellular Loading

Biomaterials laden with growth factors and other small bioactive molecules are not always enough to stimulate the necessary wound healing response. In some cases, the scaffold must be loaded with active cells, which is especially true for cellular deficient chronic wounds. Many of the current wound healing products consist of decellularized or harvested body tissue. Engineered biomaterials provide the malleability of design by combining a variety of materials with a known or anticipated response. The addition of cells to a well-formed biomaterial, with or without growth factors, allows for the flexibility to select the most desirable properties of readily available wound products for combination in a new bioengineered scaffold. Cellular loading into a biomaterial targets specific cell types that provide advantageous signals or factors to the native cells. Beneficial cell types range from stem cells (e.g., MSCs) that aid in wound closure and neovascularization [175] to more mature cell types (e.g., fibroblasts) that deposit key ECM components [176–178].

One common cellular loading strategy is to incorporate mesenchymal stem cells (MSCs) into an anti-inflammatory hydrogel [179]. Chronic wounds are characterized by their constant state of inflammation. Therefore, an anti-inflammatory hydrogel not only reduces local inflammation but also limits the body's ability to provide the cells and cell factors integral to the wound repair cas-

cade. By adding cells to an anti-inflammatory hydrogel, the necessary cells and cell factors directly transferred to the wound bed, which limits pervasive wound chronicity [180, 181].

Chen et al. fabricated a 3D biodegradable hydrogel consisting of a cross-linker and n-isopropylacrylamide (NIPAM). The specific cross-linker with an RGD-like motif was chosen for its function in cell attachment and MSC differentiation [182]. Bone MSCs are known to regulate the wound bed by excreting specific paracrine growth factors (TGF-β1, FGF) [183], and BMSCs are able to differentiate into keratinocytes, fibroblasts, and endothelial cell, which increase vascularization, granulation tissue, and reepithelialization [184–187]. BMSCs are specifically effective in treating diabetic ulcers, where poor circulation fails to provide the necessary growth factors and cells for effective and timely wound healing [175]. Despite this positive effect, a profusion of BMSCs directly to the wound bed can overpopulate the wound bed with inflammatory cytokines and contribute to overall wound chronicity. A strategy that slowly releases BSMCs and their factors to the wound bed could prevent this undesirable inflammatory response [179, 180].

The NIPAM-BMSC hydrogel allows BMSCs to adhere, proliferate, and differentiate in the wound bed while secreting bFGF and TGF-β1 at significant levels by day one and day three. The increased concentration of growth factors in the wound bed improved wound healing in a diabetic murine model. The experimental hydrogel significantly increased α-SMA expression at the wound site at days five and seven, which has been demonstrated to support wound contraction and ECM maturation compared to the control. The BSMC-laden hydrogel increased keratinocyte proliferation and granulation tissue, including neocollagen, while simultaneously reducing wound bed inflammation. M1 macrophages, which are indicative of chronic inflammation, were significantly increased in the control groups compared to the BSMC-laden hydrogel [179]. This mediation of the inflammatory response is the key to shifting a chronic wound from a perpetually open wound to one with an influx of necessary wound healing components [188]. Interestingly, BMSCs maintain bioactivity and their effectiveness in hypoxic environments, which is characteristic of most chronic wounds. Hypoxia inhibits ECM deposition in mature fibroblasts [176–178], but BMSC growth factor (TGF-β1) and ECM deposition (α-SMA) remain constant independent of oxygen concentration [189]. Hence, BMSCs have become an increasingly popular strategy for biomaterials targeting chronic wound healing [190, 191].

Conclusions

Chronic wounds are characterized by an extremely complex pathophysiology arising from varied etiologies and combined comorbidities including diabetes, immunosuppression, vascular deficiencies, and increased bacterial load that disrupt healing. These wounds suffer from severe molecular and cellular deficiencies and are, unfortunately, heterogeneous across the patient population. Because of the heterogeneity and lack of clinical evidence demonstrating significantly greater performance of specific products, there is currently no single wound dressing or scaffold that is exclusively used for the treatment and healing of all chronic wound types. The treatment paradigm for chronic wounds must shift toward precision medicine strategies that provide personalized therapy based on individual patient need. The development of novel polymers that mimic the ECM and can be modified through material design to incorporate therapeutics, growth factors, antimicrobials, or cells ushers in a new era of customized platform technologies that deliver bioactive components for the treatment of chronic wounds. As chronic wound healing is multifactorial, biopolymeric scaffolds will be designed based on specific patient need to alter the wound bed and provide the optimal wound healing microenvironment. This may include delivering bioactive VEGF to stimulate vascularization, releasing antimicrobials to control infection and/or supplying protease inhibitors to mitigate proteolytic activity and stimulate regenerative wound healing.

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Chronic Wounds of the Upper Extremity and Their Management

Antonio Rampazzo, Bahar Bassiri Gharb, Cemile Nurdan Ozturk, and Raffi Gurunluoglu

1 Epidemiology and Clinical Manifestations

Chronic wounds of the soft tissues of the upper extremities present uniquely complex challenges for the medical community.

1.1 Ischemic Wounds

These injuries are caused by different etiologies; therefore, a correct diagnosis is essential for the treatment and to decrease the recurrence [1]. The most common cause is ischemia, which occurs much less frequently than in the lower limb. It has been estimated that only 5% of patients with limb ischemia have symptomatic involvement of the upper extremity. Ischemic digital ulceration or gangrene may be caused by either large or small artery disease. Upper extremity ischemia has most commonly been associated with diabetes (Fig. 1), connective tissue disorders, embolic disease, or steal phenomena associated with access creation for renal failure patients [2].

As the number of patients requiring hemodialysis continues to grow, complications relating to vascular access become more frequent. Hand ischemia resulting from an arterial steal syndrome can be severely debilitating and can be seen in up to 5% of patients with upper extremity arteriovenous fistulas [3]. Symptoms may include ischemic rest pain, development of neurological deficits, digital ulceration, and finger gangrene (Fig. 2). The overall incidence of steal syndrome in most series is around 3% [4]. Patients who developed ischemic symptoms are more likely to be female and to have diabetes. The ischemic symptoms seen after brachiocephalic fistula creation are for the most part limited to rest pain and tissue loss and developed predominantly after fistula maturation and late vein dilatation [5].

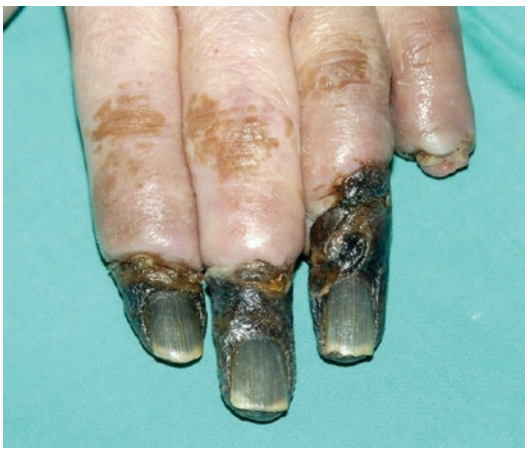

Fig. 1 Distal tip necrosis of multiple fingers in a patient with type 1 diabetes with small vessel disease. The fingers are more frequently affected than the thumb because the thumb is vascularized both volarly and dorsally

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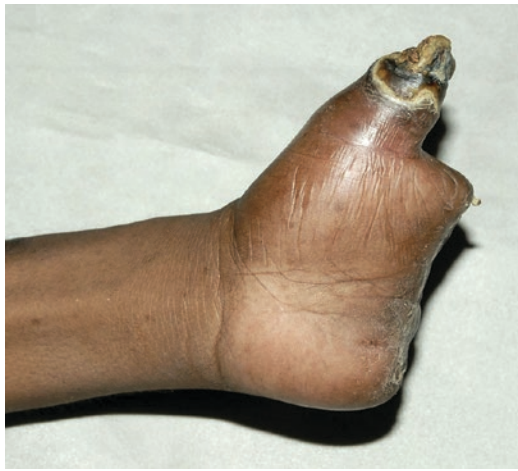

Fig. 2 This patient with renal failure developed arterial steal syndrome after creation of arteriovenous fistula for hemodialysis. He developed necrosis of the fingers and eventually the thumb although the fistula was revised. The necrotic fingers were amputated

True atherosclerotic disease of the upper extremity is infrequent. The relative infrequency of upper extremity ischemia and confusion about some entities causing this problem present challenges in diagnosis and management [2]. Connective tissue disorders can cause arteritis, which is an acute or chronic inflammatory change in the walls of small, medium, and large arteries. These conditions are uncommon too but should be kept in mind when more common causes of vascular insufficiency are not detected [6]. It is also important to distinguish the vascular etiology from factitious lesions.

1.2 Self-Inflicted Injury

Ulceration and local skin infection are frequently the presenting complaint in patients with self-inflicted injuries. These are often in areas visible to others and sometimes have a bizarre appearance, being linear or of some geometric outline with a clear demarcation from normal skin. The constant tendency toward healing requires the patient to produce new injuries, so that lesions of varying age may be present simultaneously. Almost every physician will encounter examples of factitious illness (Figs. 3 and 4) [7].

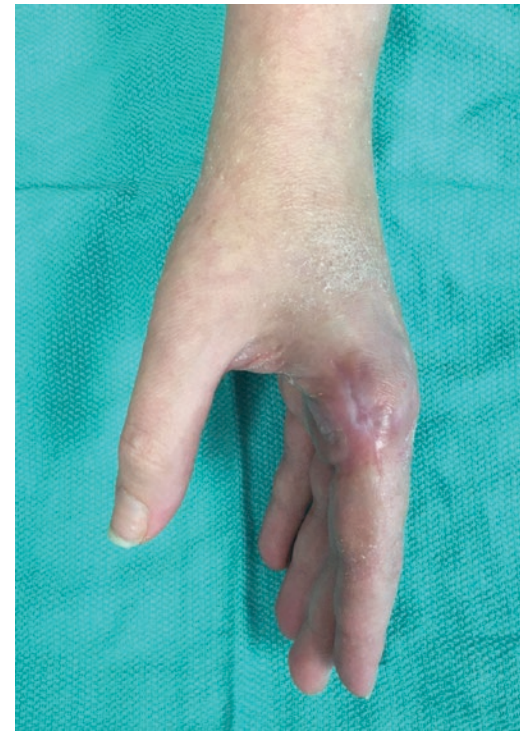

Fig. 3 Healed wound of the second metacarpophalangeal joint. The patient reported a trauma, and the wound could not heal for several months. Cultures were positive for Gram-negative bacteria. The patient was moving from hospital to hospital for assessment and treatment

1.3 Compression Neuropathy

Also severe carpal tunnel compression can rarely cause chronic wounds of the affected fingers. Carpal tunnel syndrome (CTS) is the most common entrapment neuropathy, and it usually presents with a classic triad of symptoms, including nocturnal pain, hypoesthesia, and thenar atrophy [8]. In some cases, clinical presentation may be atypical, and, in the late stages, a variety of skin manifestations such as erythema, edema, blistering, sclerodactyly, cutaneous atrophy and cyanosis, bullous lesions, ulcerations, as well as nail alterations have been described [9–12]. These manifestations usually involve the index and middle fingers and rarely the thumb. In the advanced stages, fingertip necrosis has also been reported [13], although the more severe "ulcero-mutilating" CTS, described in 1979 by Bouvier et al. [14] and associated with gangrene and acro-

Fig. 4 The wound was washed in the operating room and then casted for 4 weeks. The area healed uneventfully indicating some suspicious manipulation of the wound by the patient that had impaired the healing

osteolysis, is rare. Ulcerations and acro-osteolysis are more frequently unilateral [13, 14].

1.4 Wounds Related to IV Drug Abuse

In the current epidemic of drug addiction, injection injuries of the hands and arms causing chronic ulcers are seen with increasing frequency. The common practice of diluting (or "cutting") pure heroin to quinine or other caustic agents is the cause of widespread necrosis of cutaneous blood vessels and of adjacent connective tissue at the area of injection. Quinine has also a more specific effect on the lymphatic system causing

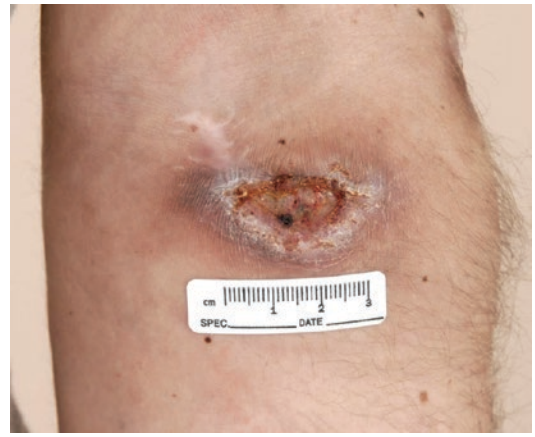

Fig. 5 Punched-out appearance of the wound at the level of the antecubital fossa. The patient was a well-known IV heroin abuser

destruction and long-term lymphedema. "Puffy hand" is the common term used to describe these hands [4, 15]. The ulcers present as a deep punched-out defect with a granulating base. There is often concomitant infection. Healing is slow, often taking several weeks or months in the best case scenario (Fig. 5) [16].

1.5 Infectious Etiology

Chronic ulcerative infections can also be caused by atypical mycobacteria, tuberculosis, and fungi. Recognition of these infections can be difficult, and they are often mistakenly treated as gout, rheumatoid arthritis, or low-grade pyogenic infections. The most common atypical mycobacterium seen in hand infections is *Mycobacterium marinum* [17]. Infection usually follows penetration by aquatic equipment, colonized marine life, or contact with contaminated water. The clinical presentation can either be verrucal skin lesions, subcutaneous granulomas, or deep infections [18].

1.6 Osteomyelitis

Osteomyelitis could potentially complicate any of the above described chronic wounds or being the reason why the wound does not heal after

treatment. Osteomyelitis of the hand is relatively uncommon, representing 1–6% of all hand infections and only 10% of all cases of osteomyelitis [19, 20]. Approximately 70% of cases involve a single bone, most commonly the distal phalanx (38%) followed by the proximal phalanx and metacarpal [21].

2 Diagnosis

When patients present with a chronic wound, it becomes imperative to establish the primary cause of the ulceration [22]. This is why a complete history and physical exam including extensive review of systems, family history, and current and relevant past medications are imperative for diagnosis and management [23]. A complete examination of the upper extremity should be performed. The skin should be inspected from the fingertips up to the neck to reveal areas of discoloration or trophic lesions. The location and the characteristics of the ulcers should be recorded. The Jamar dynamometer and the Touch-Test Two-Point Discriminator should be used to evaluate the strength and sensation. Palpation of the upper extremity can reveal increased fibrosis, edema, or masses. The nerve trunks should be examined for compression by percutaneous percussion to reveal Tinel's sign and compression tests from the wrist up to the brachial plexus. Electromyography and nerve conduction studies should be ordered if there is any clinical suspect of nerve entrapment.

The peripheral pulses should be examined (including formal assessment and by Doppler ultrasound scan if there is any concern) [24] to exclude proximal vessel disease that may require surgical intervention. Timing the return of flow in Allen's test as recommended by Gelberman [25] adds a quantitative element to identifying arterial occlusion at the wrist. The handheld Doppler is invaluable for investigating the blood flow through the radial and ulnar arteries at the wrist and the superficial palmar arterial arch in the hand, as well as assessing distal arterial patency by digital Allen's test. Noninvasive vascular tests, including segmental arterial pressure measurements, standardized as DBI (digital-brachial index), and pulse volume recordings that measure actual arterial inflow and egress via volume changes in the digits, are helpful for initial vascular assessment and postoperative follow-up [26].

Computerized tomography angiogram (CTA) and magnetic resonance angiogram (MRA) provide more detailed visualization of the vessels. These both have the advantage of not requiring intra-arterial puncture, and both techniques visualize the lumen and the vessel wall. MRA avoids the use of ionizing radiation but takes longer to perform. If surgery is contemplated, then conventional (X-ray) angiography may be indicated although this is invasive requiring intra-arterial puncture. Contrast arteriography has been and continues to be the gold standard for visualizing the upper limb arterial system. This modality can provide adequate detail to plan distal hand and finger revascularization procedures. The key point is for discussion to occur between rheumatologist, vascular surgeon, and vascular radiologist, to inform the best approach for each patient [6].

Nail-fold capillaroscopy is a key investigation in the diagnosis of systemic scleroderma and is useful in patients presenting with critical digital ischemia without a preexisting diagnosis. Immunological testing can also aid the diagnosis of the underlying connective tissue disease and may be useful in patients in whom an inflammatory component to their tissue ischemia may be represented by high titers of dsDNA (systemic lupus erythematosus), anti-neutrophil cytoplasmic antibodies (vasculitis), cryoglobulins (cryoglobulinemia), or low complement C3 and C4 (SLE and cryoglobulinemia). Immunoglobulins and electrophoresis (to look for a paraproteinemia) and urine dipsticks (to look for blood and/or protein, which might indicate systemic vasculitis) should be requested in all patients.

Embolic disease should also be considered in the context of digital ischemia, including assessment for cardiac arrhythmia with ECG and echocardiogram and, much more rarely (depending on the clinical context), a central septic source of emboli. Thromboembolism more commonly affects the lower than upper limbs. Thrombophilic states should be investigated with appropriate

tests including coagulation studies, lupus anticoagulant, anticardiolipin antibodies, and anti-β2 glycoprotein. Less common conditions that should be kept in mind when patients present with vascular insufficiency are polyarteritis nodosa and various blood dyscrasias, including polycythemia vera and vasculitis secondary to medications and hypersensitivity vasculitis. Treatment of many of these conditions requires rheumatology consultation [27]. If infection is suspected, full blood count; inflammatory markers; cultures of urine, blood, and wounds; and indwelling catheters may aid the diagnosis.

Diagnosis of osteomyelitis relies on a triad of clinical examination findings, microbiological confirmation of infection, and radiological changes [28]. Intraoperative appearance can also be helpful. Local symptoms and signs often include pain and focal tenderness, erythema, swelling, and functional impairment and may mimic a soft tissue infection. Associated nonresponse to antibiotic treatment for an apparent skin or soft tissue infection should prompt the clinician to consider underlying osteomyelitis [29]. Patients are often systemically well in the early stages. The presence of systemic signs often suggests a severe infection that requires emergent management and should prompt the clinician to look for other remote sources of infection, especially in young patients [29]; blood cultures should be performed in such patients prior to antibiotic administration. In later stages, a sinus may be present as the sequestrum drains, and osteomyelitis must be excluded in nonhealing wounds. Markers of inflammation such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and white cell count are thought to contribute little to the diagnosis of osteomyelitis, but they may have a role in monitoring the effect of treatment [20, 21, 30–32]. Plain radiographs may show signs suggestive of osteomyelitis, such as osteolysis, osteopenia, osteosclerosis, and periosteal reaction, but these usually take in excess of 1 week to appear and may be difficult to interpret [20, 28, 32]. Computerized tomography and magnetic resonance imaging (MRI) scans are said to have poor specificity with respect to osteomyelitis. Indeed MRI can be oversensitive, such as in cases of noninfective bone edema or in overestimating the extent of medullary infection in the acute phase [28, 31]. However, they do have a role to play: computerized tomography in assessing the extent of bony involvement and the identification of sequestra in known bone infections and MRI in detecting sinus tracts, to differentiate between bone and soft tissue infection, and in estimating the margins of debridement required [28]. More recently, newer imaging techniques have been employed, such as leukocyte scintigraphy, bone scintigraphy, and positron-emission tomography; not all of these modalities are as widely available as MRI, and their exact diagnostic role remains to be fully determined. It seems that until there is more conclusive evidence on the newer methods of imaging for osteomyelitis, the most pragmatic approach currently is, following a plain radiograph, to perform an MRI scan as the first choice of imaging in suspected cases of osteomyelitis.

3 Treatment

Medical optimization of signs and symptoms of chronic hand ischemia is critical both for patients with autoimmune etiologies and for patients with atherosclerosis. Patients who smoke (an intense vasoconstrictor) should be supported in their efforts to stop smoking [33, 34]. Oral vasodilator therapies include calcium channel blockers, angiotensin II receptor antagonists, alpha-blockers, and phosphodiesterase type 5 inhibitors, which are being used increasingly in severe Raynaud's phenomenon [35]. Other treatment options utilized by some clinicians (despite the lack of an evidence base to support these interventions) include statin therapy, antiplatelet therapy, and full systemic anticoagulation [36]. Despite the lack of clinical trial evidence for antiplatelet therapy in systemic scleroderma-related digital vasculopathy, most clinicians prescribe this in patients with severe digital ischemia, given that platelet activation is well recognized in systemic scleroderma [37, 38]. Recently, interest has also been expressed in fat grafting [39]. These interventions however should ideally be sub-

jected to the scientific rigor of a randomized clinical trial.

Medical therapies can reduce pain and promote wound healing, and newer minimally invasive therapies such as botulinum toxin injections show promise [40]. The first report on using botulinum toxin for chronic wounds in the American literature in 2007 provided 6- to 30-month follow-up on 11 patients. These patients received 100 units of botulinum toxin A per hand. All patients demonstrated pain relief in 24–48 h. In 9 of the 11 patients, soft tissue ulcerations healed. Some of the patients required repeat injections 3–8 months after the initial injection [41]. In 2009, Neumeister et al. [42] studied 19 patients with Raynaud's phenomenon. Patients were provided either 50 or 100 units of botulinum toxin A per hand. Of these patients, 63% require more than one injection, 84% reported pain reduction, and most of them reported that this occurred immediately after injection. The effects of the botulinum toxin A appeared to last longer than 12 months. One hundred percent of the digital ulcers healed within 60 days. Based on these data and considering the low morbidity of this procedure, botulinum toxin can be considered a valid option in patients with chronic ischemia without evidence of occlusion.

If an inflammatory etiology such as vasculitis is strongly suspected instead, then treatment with corticosteroids may be warranted. However, this approach requires caution, especially as high-dose steroids are a risk factor for renal crisis in patients with systemic sclerosis [43, 44]. Digital lesions are often infected (including with underlying bone infection) [45] and steroids may exacerbate this.

Results of imaging studies allow for categorization of patients with digital ischemia who could have either no identifiable vascular lesions (nonocclusive, spasm), discrete interruptions of patency with reconstitution of flow, or occlusive lesions that do not have reconstructible targets.

Based on vascular anatomy and occlusive lesions, three different surgical techniques are available. Patients with autoimmune chronic ischemia may be found to have a completely patent arterial tree with spasm that is severe enough to cause ischemia. Sympathectomy, first described by Flatt in 1980 [46], involves stripping the adventitia from radial, ulnar, and common and proper digital vessels to decrease the sympathetic input thought to cause pathologic vasoconstriction. A review of 17 original sympathectomy articles reveals that the vast majority (88.7%) of patients obtain pain relief and improvement of ischemic ulcers.

For patients with discrete arterial lesions and satisfactory distal targets, interposition bypass with venous conduit is appropriate. The majority of the bypass patients (77%) had ischemia secondary to atherosclerotic disease. A review of 11 original bypass articles revealed that all patients who underwent bypass (n = 76) obtain pain improvement, and a vast majority (92.7%) of those with reported preoperative ischemic ulcers experience wound healing. Other than amputation of the gangrenous digits, no further digit amputation was reported.

Patients with pain and digital necrosis who are found to have non-reconstructible occlusive disease may be candidates for venous arterialization. First described by Carrell in 1902 [47], this technique reverses venous flow in the distal hand of affected patients. This is typically accomplished by an end-to-side vein graft from the radial or ulnar artery to a dorsal hand vein. A review of five articles revealed that all patients with reported preoperative pain (n = 12) experienced improvement. In the small cohort of patients with preoperative digital ischemic ulcerations (n = 4), 75% reported wound improvement. Postoperative hand edema was the most frequent complication of venous arterialization but resolved by 3 months [48]. Although a statistical comparison cannot be calculated, the rates of patency in the arterialization group (60.0%) are much lower than in the bypass group (86.8%).

In patients with chronic end-stage renal disease and digital ischemia instead, fistula ligation remained the treatment of choice for symptomatic arterial steal. However, this approach mandated the abandonment of functioning access sites in patients who often will require many years of dialysis. A number of procedures includ-

ing anastomotic banding, small segment graft interposition, and inflow relocation both proximally and distally have been used to abolish steal symptoms and maintain graft patency [5]. Furthermore, peripheral nerve decompressions, in patients with or without chronic end-stage renal disease, usually result in total or partial remission of cutaneous lesions in most cases of peripheral nerve entrapment even if there is incomplete improvement of severe sensory deficit, as in long-standing cases of CTS [49].

If digital necrosis has developed, notwithstanding medical optimization and conservative treatment, prior surgical intervention (debridement and/or amputation) is usually required, although the necrotic digital tissue may spontaneously auto-amputate [27]. Finger amputations are typically performed as distal as possible to preserve maximum finger length in patients with connective tissue diseases; however, a much more aggressive approach should be used in chronic wounds with possible contamination or underlying osteomyelitis (Fig. 6).

Infected and necrotic tissue has to be excised with "clear" margins; no potentially contaminated or devitalized tissue is left behind. Great care has to be taken to preserve all functional structures, such as vessels, nerves, and tendons. Meticulous debridement is followed by copious irrigation to reduce the bacterial count in the wound. If any doubts remain about the viability of the wound edges or the degree of contamination, a "second look" should be scheduled. The second stage of the treatment is reconstruction of the defect, if necessary, with local or regional flap (Figs. 7 and 8) [50, 51] .Microsurgical tissue transfer is a valid option when these defects require flaps containing muscle to fill the dead space or flaps of subcutaneous tissue and skin [52].

If a chronic wound is complicated by osteomyelitis, surgical management undoubtedly plays a major role: specimens for microbiological culture should be obtained, and the infected and necrotic tissue should be debrided [29, 31]. Organisms isolated from superficial wound samples have poor correlation with the causative organisms of osteomyelitis [29]. If possible, multiple deep tissue specimens (rather than swabs) should be obtained for microbiology assessment before antibiotics are commenced. After a thorough washout and debridement to reduce the bacterial load, it may be necessary to leave the wound open to allow drainage and delay soft tissue closure until the wound is clean, viable, and not exuding pus [28]. In the immediate postoperative period, splinting of the

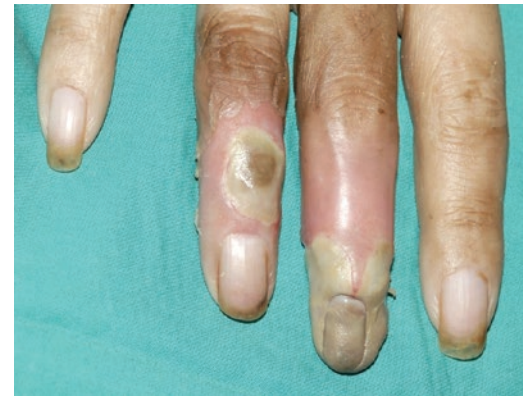

Fig. 6 This patient developed calciphylaxis and necrosis of the distal part of the fingers. The areas were debrided, but eventually she underwent amputation of the long and ring finger at the distal interphalangeal joints because the joints were exposed

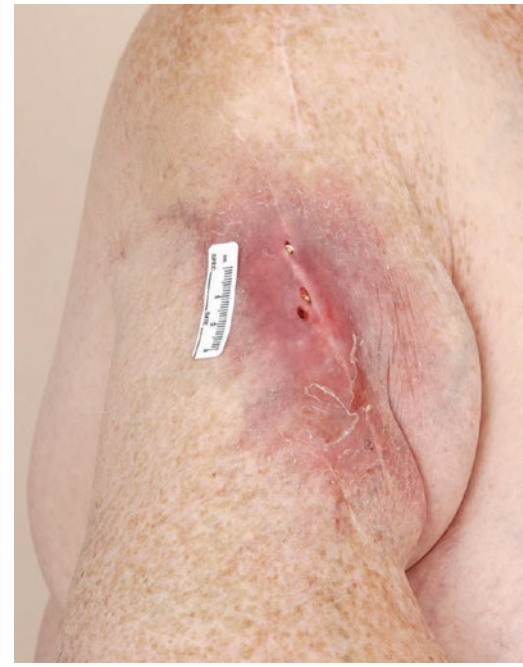

Fig. 7 Chronic wound of the arm after excision of sarcoma and irradiation

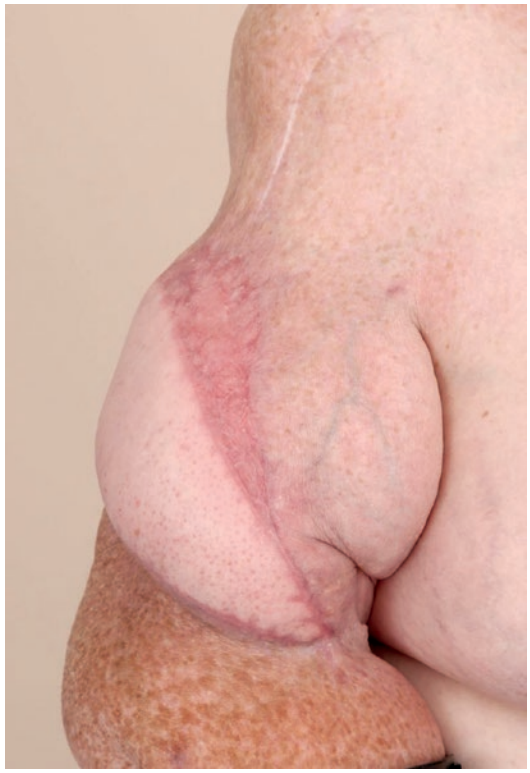

Fig. 8 There were extensive infected ectopic ossification and scar tissue which was radically removed. The area was then reconstructed with a pedicled latissimus dorsi flap and skin graft. The area healed uneventfully with stable reconstruction at 6 months after the surgery

hand for 24–48 h may provide some pain relief. However, it is important to allow mobilization of the rest of the hand soon after this period to minimize stiffness. Although thorough debridement is paramount, a decision on debridement versus amputation of a whole bone (and potentially the digit) may vary depending on the bone affected. This may particularly be the case in osteomyelitis of the metacarpal bones when their role, in provision of support of the digits as well as span of the hand, is different to that of the distal phalanges. In cases of osteomyelitis of the distal phalanx, amputation may be more appropriate to allow healing and satisfactory return of function, at the expense of loss of length of a finger. Debridement of metacarpal tissue may aim to preserve any non-affected bone, in particular the articular surfaces, to optimize the chance of maintaining the length and integrity of the hand and hence optimize function.

A balance must be sought between patient factors (medical comorbidities, expectations, lifestyle needs) and realistic outcomes of often complex reconstruction. Bony reconstruction and permanent stabilization should be performed in the absence of infection. It may be possible to perform this as a delayed primary procedure if the surrounding tissues are clean and well vascularized [20, 21, 29]. In the interim, antibiotic-impregnated polymethyl methacrylate cement can be used, either fashioned into beads (which are thought to release more antibiotic due to their increased surface area) or a tubular spacer (providing potential mechanical stability) [20, 29, 53]. As well as delivering antibiotics locally, it acts as a physical block preventing fibrous tissue invasion and over a period of 6–8 weeks induces a surrounding membrane. This membrane is thought to revascularize cancellous bone graft and stimulate bone regeneration through the secretion of angiogenic and osteoinductive factors [54]. The size of the defect may dictate the method of bone reconstruction used. Cancellous bone alone can be used in smaller defects (<1.5 cm), or corticocancellous bone grafts can provide the structural stability required in larger defects (>1.5 cm). If flap coverage is required, then a composite flap containing a vascularized osseous component has the advantage of bringing in a viable cancellous autograft with cortical stability that will be more resistant to infection, provide stability, and also integrate more rapidly with less risk of resorption [28, 29].

Regarding the ideal antimicrobial agent that should be used, and the most appropriate length of treatment, there is controversy. A recent review recommended intravenous antibiotics for osteomyelitis of the hand for 6–8 weeks [55]. Involvement of the local microbiologist or infectious diseases physician should always be sought; the importance of a multidisciplinary approach to the management of bone and joint infections has increasingly been recognized in the recent years. Ideally, empirical antibiotic treatment should only be initiated after deep tissue has been obtained for culture. Thereafter, empirical therapy should be guided by the likely causative organism and in general should cover both Gram-positive and

Gram-negative bacteria [31]. The increasing incidence of β-lactam-resistant strains of bacteria, in particular increasingly resistant Gram-negatives, should be considered. If there is concern about methicillin-resistant *S. aureus*, antibiotics such as vancomycin, teicoplanin, linezolid, or daptomycin should also be considered until culture results are available [28, 31].*Mycobacterium marinum* is naturally multidrug-resistant species, and treatment is based primarily on the personal experience and preference of individual investigators, without the benefit of large studies. In superficial cutaneous infections, minocycline, clarithromycin, doxycycline, and trimethoprim-sulfamethoxazole as monotherapy are effective treatment options, but drug resistance varies, and thereby combination therapy usually of two drugs may be required. Ciprofloxacin has shown considerable effectiveness. In cases of severe infections, including those with a sporotrichoid distribution pattern, a combination of rifampicin and ethambutol seems to be the recommended regimen. The use of isoniazid, streptomycin, and pyrazinamide as empirical treatment options should be avoided. Surgical treatment is not usually recommended and must be cautiously applied. Cryotherapy, X-ray therapy, electrodessication, photodynamic therapy, and local hyperthermic therapy have been reported as effective therapeutic alternatives. *Mycobacterium marinum* infection should always be included in the differential diagnosis of all cases with poor-healing wounds in upper extremities and a history of exposure to aquariums [56].

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Marjolin's Ulcer

Moises Menendez and Christopher Menendez

1 Introduction

Marjolin's ulcer is a very rare and aggressive cutaneous malignant transformation observed in chronic wounds and scars of long duration. It was named after French surgeon, Jean-Nicolas Marjolin, who first described the condition in 1828 [1]. But it was Dupuytren in 1839 [2] who first noted it was a malignant condition, and in 1923 the term was later coined by Da Costa (1903) [3] to describe the malignant transformation over burn injuries exemplified by two cases of carcinomatous change in chronic varicose ulcers of the leg. The most common histological tumor type found is squamous cell carcinoma, but other malignancies have been described [4]. The transformation from ulcer to malignant disease is typically slow, and the pathophysiology is poorly understood. Identification of risks factors and a high index of suspicion are the key to early diagnosis and treatment. Chronic wounds should be regularly monitored for evidence of malignant transformation. As with most malignant tumors, early treatment results in the best prognosis [4].

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The majority of the tumors are located in wounds of the upper and lower extremities. Most of the wounds were caused by burns as well as trauma [5–7], although malignant transformation has been reported in pressure ulcers, hidradenitis suppurativa, chronic fissures, lupus vulgaris, vaccine scars, radiotherapy wounds, and sinuses of osteomyelitis [8–10].

2 Discussion

Marjolin's ulcers (MU) are the malignant transformations of chronic wounds and scars [5, 7, 8, 10]. Malignant transformation, as demonstrated in these cases, has been recognized since the nineteenth century. In 1828, Jean-Nicolas Marjolin first described an indolent ulcer arising in a burn scar [5, 6]. Usually, the ulcers are squamous cell carcinomas that occur at sites of previous burns, scars, sinuses, pressure ulcers, trauma, or sites of osteomyelitis, but other less common malignancies, such as basal cell carcinomas or melanomas, have been reported [10].

Although Marjolin initially described malignant transformation of a chronic scar from a burn wound, the term Marjolin's ulcer has been used interchangeably for malignant transformation of any chronic wound, including pressure ulcers, osteomyelitis, venous stasis ulcers, urethral fistulas, hidradenitis suppurativa, anal fistulas, and other traumatic wounds. It most frequently occurs in patients of low socioeconomic status, with

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limited access to health services, as a result of burns and other neglected injuries [11].

The biologic behavior of Marjolin's ulcers is thought to be more aggressive than the other varieties of skin cancers. Most of these post-burn scar lesions may be immature/mature, atrophic/hypertrophic, keloids, and stable/unstable, which may turn malignant [12]. The various etiologic mechanisms for these tumors include release of local toxins following injury, induction of dormant neoplastic cells, and activation of injury-induced preneoplastic cells by a cocarcinogen [13].

In a review of 264 burn cases, Copcu [14] found 31 cases of Marjolin's ulcer and 14 cases of nonmalignant ulceration at previous burn sites. In the same study, 18 Marjolin's ulcers were located on the extremities, 6 on the scalp, 6 on the trunk, and 1 on the nose. There are several other common dermatologic conditions that occasionally have been linked to the development of squamous cell carcinoma. These include genital lichen sclerosus et atrophicus, oral lichen planus, erythema ab igne, and burn scars. Of these conditions, burn scars are the most likely to become malignant.

Bozkurt et al. [15] reviewed the literature and presented 16 cases of Marjolin's ulcer and concluded that the malignancy aroused in chronic wounds and in areas where the integrity of the skin was compromised because of long-term irritation at skin zones especially at the extremities, and most of the cases were admitted to healthcare facilities had a malignant transformation that occurred 20–30 years after the initial injury which happened to be burn injuries in all the cases. In 75% of those cases, the skin lesions were ulcerated, and in the 25% of the cases, the tumors were exophytic and fragile, with irregular margins. It is interesting that in 81% of the cases, the burn defects were not originally repaired or skin grafted and were left for secondary healing [13, 15]. Marjolin's ulcers frequently occurs in the unstable scar of a full thickness burn, which has not been skin grafted. The major risk factors for the development of post-burn MU include healing of full thickness skin burns by secondary intention, nonhealing burn wounds, and fragile scars that ulcerate and are easily traumatized. The post-burned scars are typically less resistant to injuries and heal poorly especially in body areas such as the joints [16].

The literature also showed the latent period between the first trauma or burn to the skin and the appearance of the malignant ulcer. Many authors suggested that there are two forms of presentation: the acute form in which the cancer occurs within 1 year from date of the triggering injury and the chronic type after 1 year [6, 9]. It is thought that there is an inverse relationship between latency period and the patient's age at the time of the burn injury, with older patients having a shorter latency period [17]. Latency until malignant transformation takes on average three decades or more. However, this period may vary from 10 to 70 years [18].

The reviewed literature indicates the various presentations of patients with this malignant ulcer to medical facilities depending on the global locations and access to medical facilities. Marjolin's ulcers most frequently occur in patients of low socioeconomic status, with limited access to health services, and as a result of burns and other neglected injuries. Most of the cases reported are from developing countries, where patients tend to have late presentations [11].

Chalya et al. [19] reported 56 cases of Marjolin's ulcers and concluded that this condition is not uncommon in Africa and commonly occurs in burn scars that were not skin grafted and left to heal secondarily. Most of those patients presented late when the disease was already in advance stages, and most of the patients were relatively young and advised that health education was imperative to discourage patient from presenting late to a hospital when the disease was too far advanced. By the same conclusion, Asuquo et al. [9] also indicated that the majority of patients in the developing countries presented with advance lesions that precluded curative surgery, and the mortality rate was significantly high in patients with advanced disease, metastasis, and local recurrence. In the authors' setting, traditional healers enjoy the patronage of the inhabitants because of strong sociocultural beliefs, and majority of times the patients seek the physicians too late [9].

3 Clinical Presentation

Most of the patients with chronic ulcers who present or are admitted to a wound care clinic or center have been referred by the primary care providers, and occasionally they are self-referred or got advice from friends or relatives.

In our wound center, we try to allocate the chronic ulcers to a well-standard protocol which permit the proper diagnosis and management. Most of the chronic ulcers are categorized as venous, arterial, diabetic, pressure, traumatic, surgical, and others or miscellaneous. Certain chronic ulcers do not match the traditional categories because of location, duration, lack of improvement, persistent infection, and other factors; therefore, these ulcers are more prone to fall in the category of "difficult chronic ulcers," and therefore they need proper identification for their management. These ulcers should be biopsied as a general rule to rule out malignancies or vasculitides or pyoderma gangrenosum.

The diagnosis of Marjolin's ulcer is based on the suggestive findings in the patient's history, the detailed examination of the ulcer and its draining nodal basin, and the histology of the lesion. The classic triad of nodule formation, induration, and ulceration at the post-burned scars should prompt a biopsy to confirm the diagnosis. Other clinical signs suggestive of MU include everted or rolled margins, exophytic granulation tissue formation, increasing size, bleeding and regional lymphadenopathy [16]. In general Marjolin's ulcers present either as nonhealing chronic ulcers or exophytic scar lesions [13]. At times they present on a previously exposed or partially exposed bone from chronic osteomyelitis as in our first patient (Fig. 1). More rarely the malignant transformation occurs on a previously treated venous ulcer or pressure ulcer [6].

Our experience was rather limited due to the fact that Marjolin's ulcer is a very rare disease [10], but we saw our first patient with this condition associated with an ulceration of the lower extremity and chronic osteomyelitis caused by a severe burn injury decades before (Fig. 1). Because of the involvement of the tibia, the patient underwent a BK amputation with good results. Likewise, our second patient a 72-year-old white female was referred for a chronic wound located in an old scar on the lateral aspect of the chest, below the left breast. The patient suffered an extensive burn on the chest when she was 4 years of age (Fig. 2). Patient underwent complete excision of the lesion and sentinel node biopsy. A skin graft was also done with good results (Fig. 3).

Several years ago we were consulted on a patient with spina bifida and large pressure ulcer with multiple ulcers compatible with hidradenitis suppurativa (Fig. 4). Multiple biopsies showed

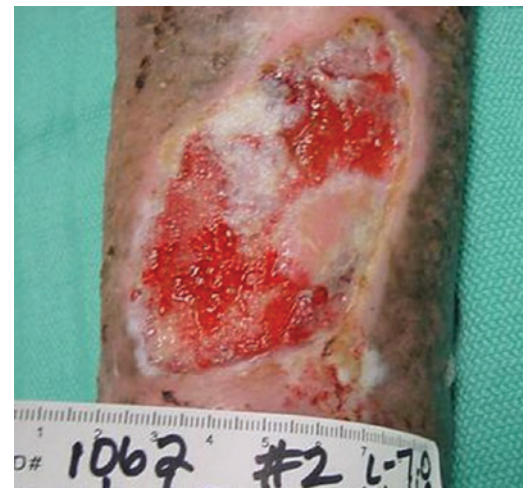

Fig. 1 The right leg had two ulcers. One of the ulcers showed the tibia was exposed partially

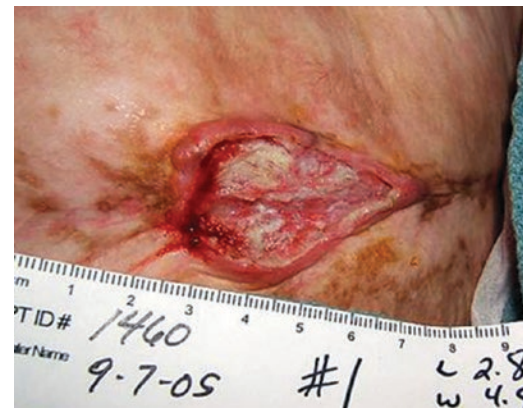

Fig. 2 Large nonhealing ulcer on left chest with elevated edges

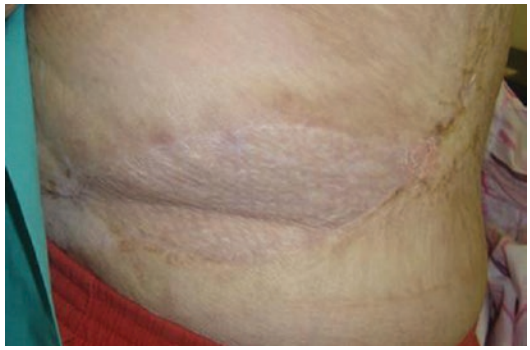

Fig. 3 Same patient after a split thickness skin graft 2 months later

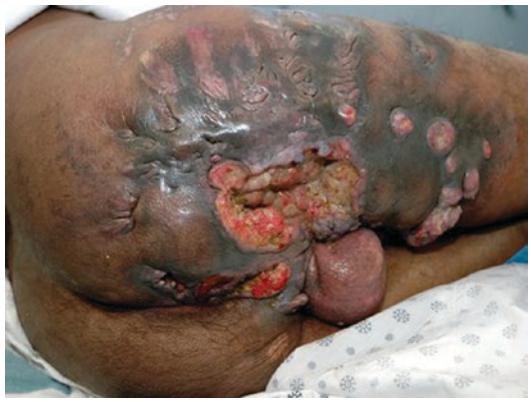

Fig. 4 This patient showed multiple ulcerative lesions compatible with Stage IV pressure ulcer right ischium, and the smaller ulcers were related to chronic hidradenitis suppurativa. Most the lesions showed invasive squamous carcinoma

squamous cell carcinoma (Fig. 5a, b) involving most the ulcerations including the large ischial ulcer. Apparently this patient had the chronic ulcers for decades and never sought medical attention until he developed fever and symptoms of hypercalcemia and sepsis. The laboratory work showed significant elevation of the calcium and WBC with leukemoid reaction and local and distant metastasis. Patient developed a paraneoplastic syndrome associated to the Marjolin's ulcer. Patient succumbed to his disease few days later.

A history of a nonhealing post-burned wound of full thickness skin loss should alert the clinician of the possibility of a Marjolin's ulcer. It is usually painless. The easy bleeding fragile areas may at times present with unprovoked bleeding, offensive discharge, or increasing pain. Superadded infection of the wound may at times be the first clinical presentation [16].

Finally, the diagnosis of Marjolin's ulcer is based on the suggesting findings in the patient's history, detailed examination of the lesion, and its draining nodal basin and regional lymphadenopathy. Once the biopsy confirms the diagnosis of Marjolin's ulcer, determination of the local extent of the lesion and staging comes to the fore.

Magnetic resonance imaging (MRI) or computed tomography (CT scan) may be performed to determine the local extent of the lesion and invasion of any underlying structures. MRI is certainly the ideal imaging tool for evaluation of the status of the soft tissues, infiltration of any underlying bone, and the involvement of adjacent neurovascular structures. Scintillography may be helpful to determine potential lymphatic node metastases as used in our second patient. Given the aggressive nature of the malignant ulcer, distant metastases are ruled out with metastatic work-up that includes the use of the CT scan, ultrasound, and MRI [16)].

4 Treatment

4.1 Surgery

Skin lesions that will not heal despite standard therapy or that their appearance look suspicious of harboring a malignant tumor should be all biopsied. Wedge biopsy is the favored method of diagnosis. Tissue specimens obtained should be taken from both the center and margin of the lesion, as the central ulcerated deposits may be necrotic. Although there is not a definitive standard protocol for the treatment of this lesion, after a confirmed biopsy of the tumor, the surgical treatment is actually straightforward: complete excision of the lesion and skin graft or musculocutaneous flap closure depending on the wound condition after the excision [5]. Amputation of the limb proximal to the lesion is also indicated like in our experience. Nodal assessment and wide surgical excision are highly

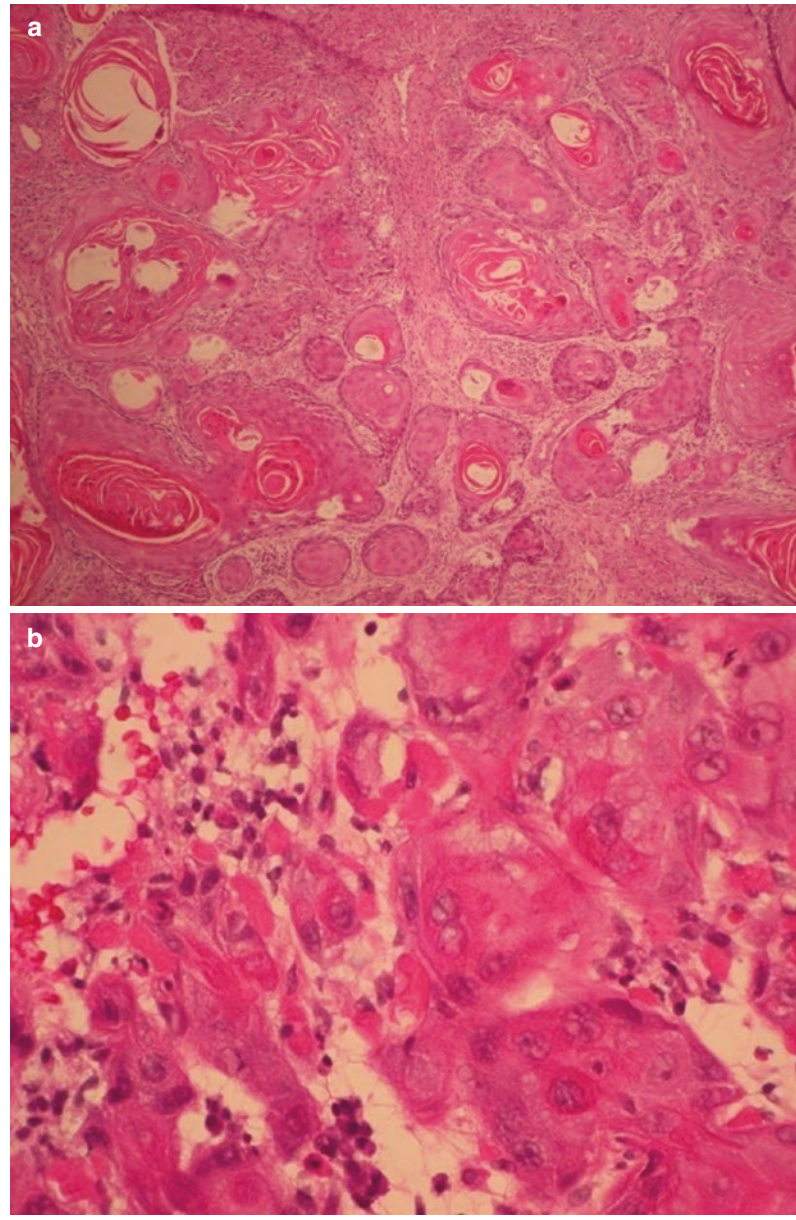

Fig. 5 (**a**) Biopsied ulcer with squamous carcinoma (low power). (**b**) Squamous cell carcinoma (high power)

recommended since Marjolin's ulcer is more aggressive than primary skin tumor. The extension of the resected-free margins varies between 1 and 4 cm depending on the location, extension, and depth of the tumor [5, 8]. Also other modalities have been used such as carbon dioxide laser, cryosurgery, and Mohs surgery [15]. Although a wide local excision and wound coverage with skin grafting or flaps is the treatment of choice [8], currently there is no universal consensus or treatment protocol regarding excision margins, lymph node dissection, or the use of neoadjuvant radiotherapy or chemotherapy. A combination of these procedures is often necessary [17].

Pekarek et al. [19] indicated that Mohs surgery, in which the surgeon serves as both surgeon and a pathologist, is now considered the gold standard for the treatment of this condition, although this technique is expensive, takes too long for final results, and only few are adequately trained to do

this procedure. He also states that the amputation is the most widely accepted treatment.

In our second patient, we did a SNB (sentinel node biopsy) of the axilla after the tumor was excised with negative results. However, the sentinel node biopsy approach may not be feasible in some cases due to extensive burn scarring [20], and its use may be debatable [19].

On the other hand, according to Eastman et al. [21], successful intraoperative lymphatic mapping/SLN biopsy was defined as the identification of blue (uptake of isosulfan blue dye) or "hot" (uptake of radiolabeled sulfur colloid as measured with a handheld gamma counter) node(s) and subsequent excision. Four of five SLN biopsies identified previously occult nodal metastasis. SLN biopsy represents a minimally invasive and accurate staging procedure for Marjolin's ulcer. Definitely if enlarged lymph nodes are encountered, node biopsy and excision is recommended.

4.2 Radiotherapy

Radiation therapy may be an important adjunctive role in managing Marjolin's ulcers. The indications for radiotherapy include (1) inoperable regional lymph node metastasis, (2) grade 3 lesions with positive lymph nodes after nodal dissection, (3) tumors with a diameter greater than 10 cm and with positive lymph nodes after regional lymph node dissection, (4) grade 3 lesions with a tumor diameter greater than 10 cm and negative lymph nodes after regional lymph dissection, and (5) lesions of the head and neck with positive lymph nodes after regional lymph node dissection [16, 17, 19, 22].

4.3 Chemotherapy

The precise role of chemotherapy is not well defined in the literature [19]. There is little support for adjuvant chemotherapy in Marjolin's ulcers, but radiation therapy has been used as palliation [17]. However, Ryan et al. [23] reported a new concept in the management of Marjolin's ulcer using topical 5-FU. They stated that recent studies suggest such cancers are in immunologically privileged sites due to the dense scar tissue. The prognosis has been shown to be much worse for tumors not having a round cell infiltrate prior to surgery, as in Marjolin's ulcers. The use of topical 5-fluorouracil

5-FU induces a round cell infiltrate. Three case reports of large Marjolin's ulcers were presented which were first treated with topical 5-FU. Radical ablative surgery was avoided in these patients with a successful outcome [23].

5 Prognosis

In the medical literature, prognosis in cases of Marjolin's ulcer estimates a 3-year survival rate of 65–75%, which increases in cases of well-differentiated carcinomas. The presence of distant metastases is obviously indicative of poorer prognosis with a 3-year survival rate of 35–50% [18]. The prognosis of Marjolin's ulcer is related to the local extent of disease, location, histological types, and degree of differentiation, patient immune status, latency period, and, most importantly, presence of lymph node metastases [11]. In conclusion, the squamous cell carcinoma of the Marjolin's ulcer seems to have a worse prognosis than other squamous cell carcinomas, and it requires aggressive treatment.

Conclusions

Chronic ulcers are at constant risk of developing squamous cell carcinoma. Fortunately,

Marjolin's ulcer is considered rare, but when it occurs, it is more aggressive than other squamous cell carcinomas appearing de novo. Also, since this condition is rare, the general practitioner should refer patients with long-standing chronic ulcers or lesions that do not heal to a wound center or to proper specialists to avoid delay in treatments.

It is imperative to provide early and definitive wound coverage of unstable scar tissue with healthy tissue.

Biopsies of suspicious ulcers and re-biopsies of the lesions should be a standard of care in institutions caring for chronic wounds. With early diagnoses and proper management and surgical treatment, the prognosis of this malignancy could be improved.

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Radiation Wounds and Their Management: Reconstructive Options

Cemile Nurdan Ozturk, Antonio Rampazzo, Joe Scharpf, and Raffi Gurunluoglu

1 Introduction

The most common cause of radiation injury is an adverse effect of therapeutic irradiation which is used to treat cancer and a few noncancerous diseases by utilizing high-energy particles or waves. Treatment is delivered via external beam radiation or by brachytherapy within the patient's body, although more rare, radiation-related wounds could also stem from occupational and environmental exposures as a result of physical contact with radioactive material.

The common pathway of injury, regardless of the source of radiation, is a structural damage to the DNA, which could lead to both acute and chronic tissue effects. DNA damage can induce apoptosis or aberrant replication and causes endarteritis, microthrombi, inflammation, and fibroblast dysfunction [1–4]. Microvascular injury leads to ineffective delivery of oxygen and nutrients. As a result of cellular changes and hypoxia, irradiated tissues heal slow and have a decreased capacity to resist infection.

Early effects after radiation exposure are similar to a thermal burn and include erythema, hyperpigmentation, edema, and desquamation. These are due to a transient increase in vascularity that peaks in the second week, gradually decreasing afterward [5]. Initial phase is usually self-limiting and resolves with minimal treatment, unless there is acute high-dose radiation, which could result in tissue necrosis. Superimposed infections may also complicate the clinical course necessitating additional treatment.

Chronic or late effects could manifest from weeks to decades after radiation exposure. These include tissue fibrosis, delayed wound healing, ulcers, lymphatic and blood vessel damage (microthrombi), malignant transformation, and bone necrosis. Irreversible fibroblast dysfunction results in permanent and progressive skin damage, decreasing wound tensile strength. The late term effects are disabling and even life threatening in some cases.

2 Initial Work-Up

Biopsy of long-standing ulcers should be done to rule out malignancy that might develop in setting of chronic wounds (Marjolin ulcer). If malignancy is present, a full cancer work-up is indicated with appropriate resection. Malignancies

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arising in irradiated beds are usually more aggressive and frequent follow-up is indicated.

Radiation injury generally extends beyond the visible field, necessitating a work-up of the condition of the underlying bone. Plain radiographs, computed tomography, magnetic resonance imaging, and biopsies are utilized as needed. Assessment of the bone is particularly important in chronic wounds with draining sinus tracts, which may indicate osteoradionecrosis.

Wound healing of oncologic patients may be impaired due to immunosuppression, malnutrition, and anemia, in addition to radiation-induced damage [6].General medical assessment is critical with optimization of nutritional parameters such as albumin and prealbumin prior to reconstructive surgery.

3 Conservative Management

Skin effects of acute radiation injury are amenable to supportive treatment, including protection from trauma and infection. Topical antibiotic ointments (i.e., silver sulfadiazine) could be used to prevent infections or to help recovery of partial thickness injury. Full-thickness necrosis is unlikely to heal with secondary intention and usually requires surgical reconstruction.

Use of hyperbaric oxygen treatment in radiation wounds is controversial. Even though it appears to be an attractive modality with potential increase in oxygen supply to tissues, stimulation of angiogenesis, and collagen formation, it is utilized as a supportive measure only due to lack of evidence of its benefits [7–10]. Another indication of hyperbaric oxygen treatment is for prophylaxis, such as when dental work is needed in a radiated field.

4 Surgical Management

4.1 General Principles

Even though surgeons are usually reluctant to operate in the irradiated field, surgery may be indicated for debridement and closure of radiation wounds or extirpation of recurrent tumors. Healing of radiation wounds is usually very slow if left to secondary intention due to minimal development of granulation tissue. As such, surgical intervention is generally required. Outcome of skin graft application to a radiated wound bed is at best unpredictable, and skin grafting generally is not advised. However, there is emerging evidence that with the advances in negative wound pressure therapy, successful results can be obtained [11]. A negative pressure dressing could be applied prior to reconstruction to optimize the wound or after skin grafting to enhance healing. Majority of the reconstructions will require a vascularized flap transfer from a regional or distant site that can provide new blood supply to the wound. The choice of flap depends on depth of the injury, tissues involved, presence of exposed vital structures, and anatomic location. Tissues that are free from radiation injury are preferred for reconstruction [4, 12].

Adequate debridement is the most critical step for a successful reconstruction. Debridement should include all nonviable structures until fresh bleeding is confirmed and existing foreign materials are usually removed [4]. Failure to do this may result in recurrent problems and infections. Zone of injury typically extends beyond the visible field which should be taken into consideration during debridements [13]. Another critical point is the amount of skin contracture present. Excision of radiated skin will result in a larger than anticipated defect due to fibrotic skin envelope.

Irradiated tissues are prone to bacterial contamination and wound breakdown. These problems can be reduced by planning prophylactic regional or distant flap use, should a need to operate on irradiated tissues without any evidence of wound arise (Fig. 1).

Meticulous tissue handling is essential in the irradiated field. Special care should be taken during free tissue transfer and microsurgery since blood vessels are fragile. Excessive bleeding is possible, and surgeons should be prepared for possible transfusions in debridement of large wounds. Microvascular anastomoses are preferably performed outside the zone of radiation where healthy recipient vessels exist. Close postoperative follow-up is also of importance in order

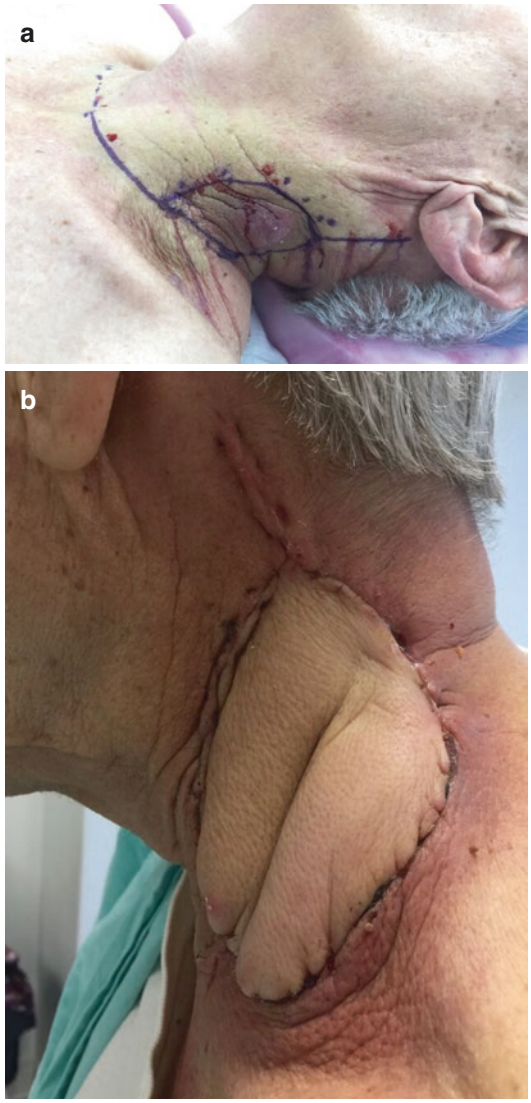


Fig. 1 (**a**) 66-year-old male patient with a history of chemoradiation for squamous cell carcinoma of the right tonsil presented with a growing left neck mass which was diagnosed as recurrent squamous cell carcinoma after fine needle aspiration. Ablation surgery and salvage radical neck dissection were performed. (**b**) A free flap was planned in advance and a left-sided radial forearm free flap was transferred to the left neck for soft tissue reconstruction

to initiate timely treatment if there are complications. Aggressive antibiotherapy and early incisional revisions could make the difference between salvage and failure.

There is emerging evidence on the role of autologous fat grafting and stem cell therapy to irradiated tissues. Adipose-derived stem cells that are transferred during fat grafting have the potential to improve healing and enhance skin quality [14]. Fat grafts are harvested via liposuction and injected into the field through small incisions, providing a minimally invasive technique.

4.2 Reconstruction According to Anatomic Site

4.2.1 Head and Neck

Cancer surgery in the head and neck region often results in defects with exposed vital structures and multiple missing layers that require complex reconstruction. Patients usually undergo induction or postoperative radiation therapy due to aggressive nature of these tumors. Bony reconstructions of maxillofacial area or calvarium offer additional reconstructive challenges as they commonly require hardware (i.e., titanium plates), which subsequently should be covered with flaps. Non-vascularized bone grafts are not advisable in the setting of radiation, as they would need a well-vascularized bed for healing.

Several local and regional soft tissue flap options exist in the head and neck region. Pectoralis flap is the workhorse due to its reliability and ease of harvest; however it is limited by bulkiness, arc of rotation, and limited extension to the oral cavity. Supraclavicular perforator flap is another good option which provides pliable, thin tissue with extensive reach [15, 16]. However, care should be taken in previously irradiated necks and with history of neck dissection as the pedicle could be damaged [17]. Temporalis muscle/fascia flap is a smaller flap which is readily usable for defects of the upper face and palate. Scalp can be reconstructed with large rotation flaps for which many designs and variations exist [18].

With the advances in microsurgical techniques, most of the larger head and neck defects, specifically irradiated areas, are now reconstructed with free tissue transfer which provides reliable long-term coverage and preservation of form and function. Fasciocutaneous flaps such as radial forearm, anterolateral thigh perforator, and scapular or parascapular are the most commonly used options. Most of these flaps could be harvested with adjacent muscle or bone as needed. Free

fibula flap is the workhorse for bony reconstruction and provides sufficient amount of bone for extensive defects, even for total mandibulectomies (Fig. 2). Other flaps (i.e., rectus abdominis, latissimus dorsi) could be indicated depending on the defect (Fig. 3). Even though there is an abundant supply of recipient vessels for microsurgery in the head and neck region, their dissection could pose difficulties due to postradiation fibrosis and adhesions. In some cases, the free flap may need to be anastomosed to the contralateral neck vessels, necessitating vein grafts for reach. Vein grafting generally increases complication rates and should be avoided if possible [19, 20].

A rare complication of radiation in the head and neck region is an infection or salivary fistula that can cause vessel rupture, which in turn could be lethal. In the setting of radiation, soft tissue healing takes longer, and neck vessels are at risk for a prolonged period of time. Sternocleidomastoid muscle transposition or pectoralis muscle flaps could be used for protection of vessels even if the skin can be primarily closed.

Osteoradionecrosis of the facial bones, specifically the mandible, could occur after radiation therapy to the oropharynx. It is defined as the presence of a nonhealing, exposed bone for 3 months in a previously radiated area [21]. Although the pathophysiology of osteoradionecrosis is evolving, there is a consensus that poor blood supply contributes to the damage [22]. Osteoclast suppression inhibiting bone turnover is also thought to play a role [9]. The necrotic bone is usually surrounded by already damaged tissues of weaker resistance, mucosa, skin, and muscle. The unhealthy bone does not allow soft tissues to heal, and the clinical picture is further complicated with fistulas, mucositis, trismus, and pain [23].

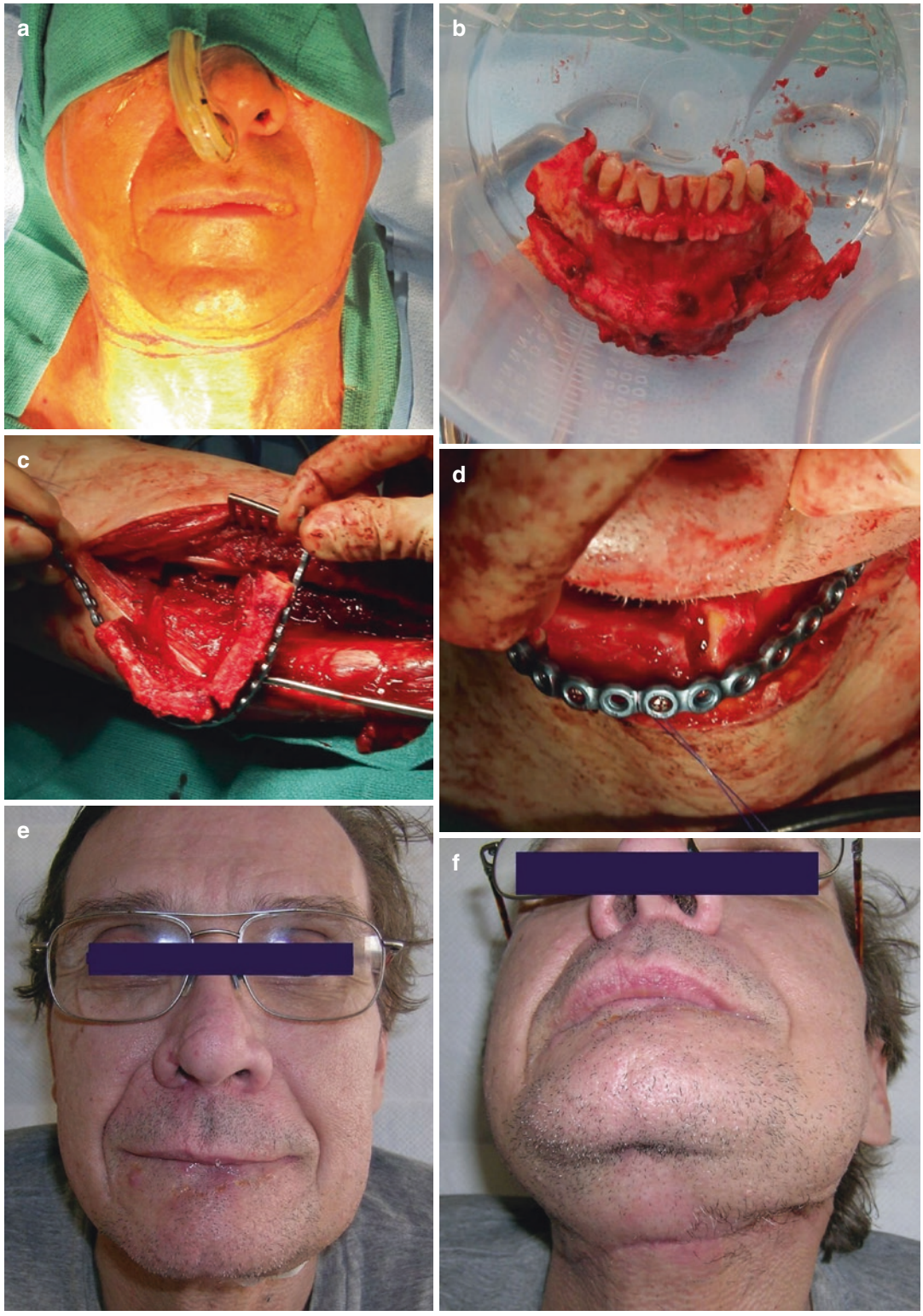
Conservative modalities such as pentoxifylline, tocopherol, clodronate, and hyperbaric oxygen may have value in treatment of early, reversible phase of osteoradionecrosis [23–25]. These medications could also be used prior to surgical intervention, to help with healing. Aggressive debridement of necrotic bone and coverage with well-vascularized tissue (free flaps) is often indicated when clinical signs of bone and tissue necrosis develop. Attempts to utilize local flaps may devascularize the surrounding tissue, further amplifying radiation-induced damage. This approach could also limit extent of resection, and if unhealthy bone is left behind, likelihood of recurrence is high [13, 23, 26]. For these reasons, distant flap reconstruction is preferred in treatment of osteoradionecrosis.

4.2.2 Trunk

Patients with previous history of radiation to the trunk, specifically chest wall, are often complicated with cardiothoracic comorbidities requiring multidisciplinary management. Chronic wounds, infections, fistula tracts tracing to sternal hardware, resection of recurrent tumors, and metastases are the most common indications for surgery. Long-standing radiation ulcers or chronic infections may need serial debridement procedures to accurately judge viable tissues.

The primary goal in the thoracic region is stabilization of skeletal structures, maintaining respiratory mechanics and protecting the vital intrathoracic organs in addition to coverage of the soft tissue defect. The chest wall is reconstructed in conjunction with thoracic surgery team. Posterior chest wall defects are usually more tolerant to skeletal resection when compared to anterior defects, yet skeletal reconstruction should be tailored according to the patient's functional status [27–29]. Skeletal reconstruction options are either synthetic mesh and hardware (i.e., polytetrafluoroethylene, methyl methacrylate, absorbable plates, titanium plates) or biologics (i.e., autologous fascia and rib grafts, acellular

Fig. 2 (**a**) A 57-year-old male patient with osteonecrosis of the central mandible secondary to radiation. Intraoperative indicates the submandibular approach for resection of the mandible. (**b**) Resection of the necrotic segment of the mandible sparing the inferior alveolar nerves bilaterally. (**c**) In situ shaping of the fibula and fixation to the reconstruction plate that is prepared according to the shape of the mandible prior to resection. (**d**) Free fibula flap was revascularized using right-sided facial vessels. (**E**, **F**) Postoperative at 3 months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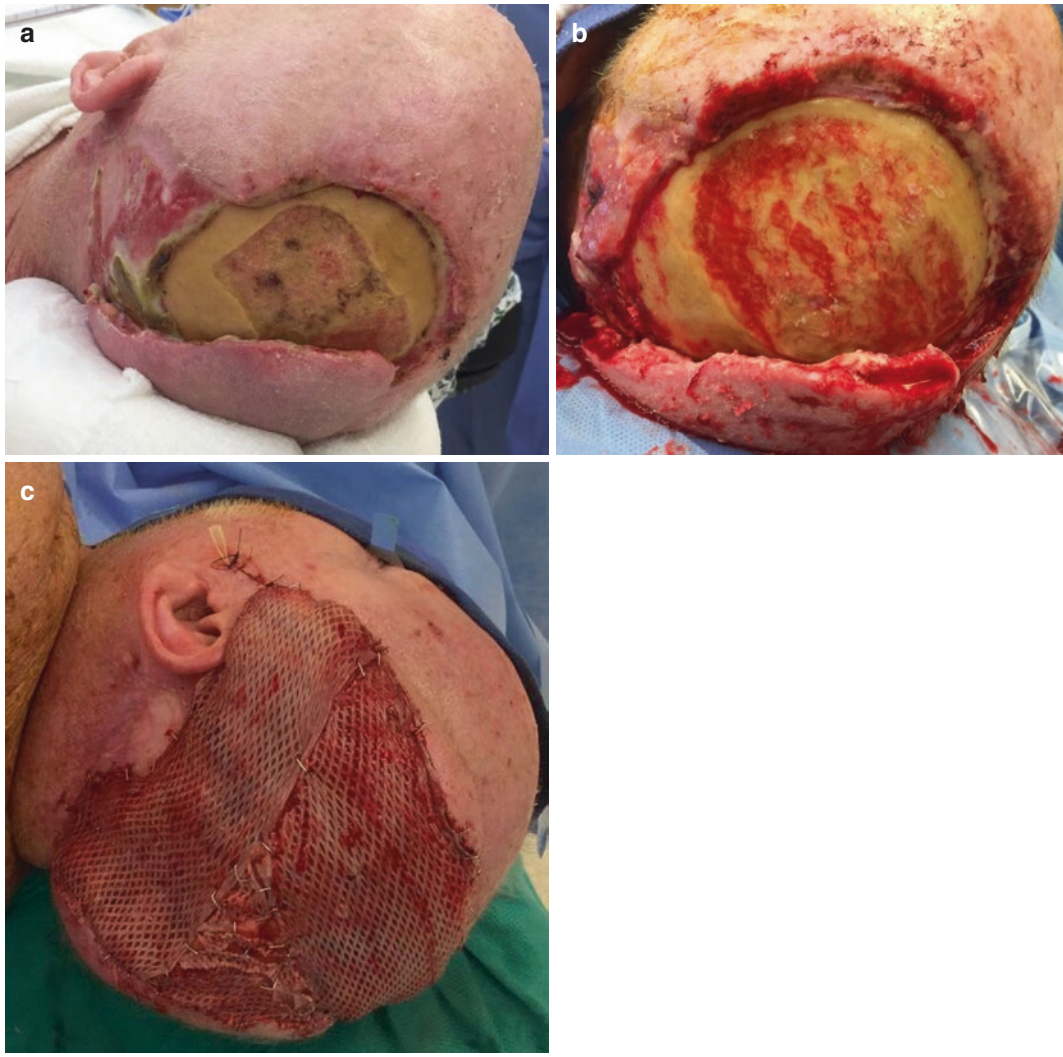

Fig. 3 (**a**) A 55-year-old male patient with a history of surgery for squamous cell carcinoma of scalp and radiation, presented with large scalp wound with exposed bone and evidence of osteoradionecrosis. He had received multiple failed attempts for wound closure using local scalp flaps and skin grafting. (**b**) Recurrent malignancy was ruled out and proper debridement of the bone and soft tissues were performed. (**c**) A free latissimus dorsi muscle flap was employed along with split skin grafts (microvascular anastomoses were done using the left-sided superficial temporal vessels)

dermal matrix). Biologic mesh is preferred when attempting to seal the thoracic cavity as utilization of synthetics in a previously radiated bed could result in recurrent infection or exposure. Once the thoracic cavity is sealed and separated, flap reconstruction is carried out. Regional flaps include pectoralis, latissimus or rectus muscle flaps, and omentum. The latter has the benefits of great vascularity, large surface area, and pliability, but has disadvantages of breach of a second body cavity and lack of support [30].

In patients with previous radiation therapy, the blood supply to a regional pedicled flap may prove to be unreliable, and augmenting the blood supply (supercharging via additional microanastomosis) should be considered [31]. Patients who require complex chest wall reconstruction are not tolerant to even minor wound complications, and all measures should be taken to optimize healing. Even though there is an abundance of regional flaps for chest wall reconstruction, in cases with multiple complications where these options have

been already utilized, free flaps may be necessary. In such cases contralateral free flaps (i.e., latissimus and rectus) and anterolateral thigh perforator flaps provide sufficient surface area and support.

The spine is the most common site of metastatic disease, requiring radiation and instrumentation to preserve neurologic function and to improve the overall quality of life. Paraspinous muscle, scapular fasciocutaneous, latissimus, and trapezius flaps have been used for coverage of defects or for prophylactic measures [32]. Reconstruction of radiation wounds in the lumbosacral region is quite challenging due to scarcity of regional options and recipient vessels [13]. The skin in this region is not elastic and has tight adhesions to the deeper layers. Gluteal artery perforator flaps are a good choice as they can be performed with minimal donor site morbidity. Field of radiation should be taken into consideration when planning these flaps, as the pedicle could have been exposed to radiation. Free flap coverage is usually needed for lumbosacral wounds that are large and irradiated.

4.2.3 Breast

Radiation-related issues in the breast usually pertain to outcomes of breast reconstruction and not to radiation wounds. Postmastectomy and post-lumpectomy radiation increases the complication rates of implant-based breast reconstruction and results in poor cosmetic outcomes [33]. Delayed surgical healing, skin necrosis, and periprosthetic infection, if severe, may require removal of implant and the surrounding capsule. The skin usually heals well with primary closure once the implant and capsule are removed. Incomplete capsulectomy or presence of unusual microorganisms (i.e., anaerobic or fungal infections) could result in continuation of healing problems. Osteoradionecrosis of ribs should also be considered if there is a chronic draining wound. In such cases, flap reconstruction is indicated, after adequate debridement.

The workhorse regional flap for breast reconstruction is the latissimus dorsi flap, which could be utilized with or without an implant, depending on contralateral breast size [34] (Fig. 4). However, since latissimus flap does not create an as large breast mound, most reconstructive surgeons utilize flaps from the abdominal region. Abdominally based flaps (deep inferior epigastric artery perforator, muscle sparing transverse rectus abdominis, transverse rectus abdominis) are almost always raised as free flaps to maximize tissue perfusion via dominant blood supply, specifically in cases of radiated breast reconstruction [35]. Pedicled transverse rectus abdominis flap (based on non-dominant blood supply) is notorious for having diminished blood supply and is more prone to healing complications and fat necrosis, which could further complicate a radiated breast reconstruction. Internal mammary artery and vein are typically selected as recipient vessels for microsurgery, which are accessed by removing a small rib cartilage. Dissection of the subcostal space could prove to be quite difficult when irradiated, and meticulous technique is necessary to avoid bleeding from these fragile but robust vessels.

Radiation-related breast wounds could also develop from elective surgery that is performed on a patient with prior lumpectomy radiation. Patients with large breasts who undergo unilateral lumpectomy commonly develop asymmetry and present requesting breast reduction after radiation. Breast reduction with extensive undermining and repositioning of tissues is not advisable after radiation [36]. Such a procedure to address asymmetry could result in nonhealing wounds that, in turn, could necessitate a salvage mastectomy. Elective surgery on a previously radiated breast should indeed be avoided or be carried out via minimally invasive techniques such as liposuction or fat grafting.

4.2.4 Extremities

Historically, amputation was the mainstay of treatment for malignancies of the extremities, specifically sarcomas, due to high recurrence rates. More attempts at limb salvage are being undertaken today due to advances in microsurgery and chemoradiation treatment [27]. Surgeons are confronted with the dilemma of salvage versus amputation, commonly in setting of radiation. Preserving limb function is the goal instead of just obliterating the defect, which sometimes means amputation could provide superior outcomes.

Extremity resections often include bone, requiring orthopedic reconstruction with prosthetics or autologous tissues. Stable coverage of prosthetic materials, bone, neurovascular

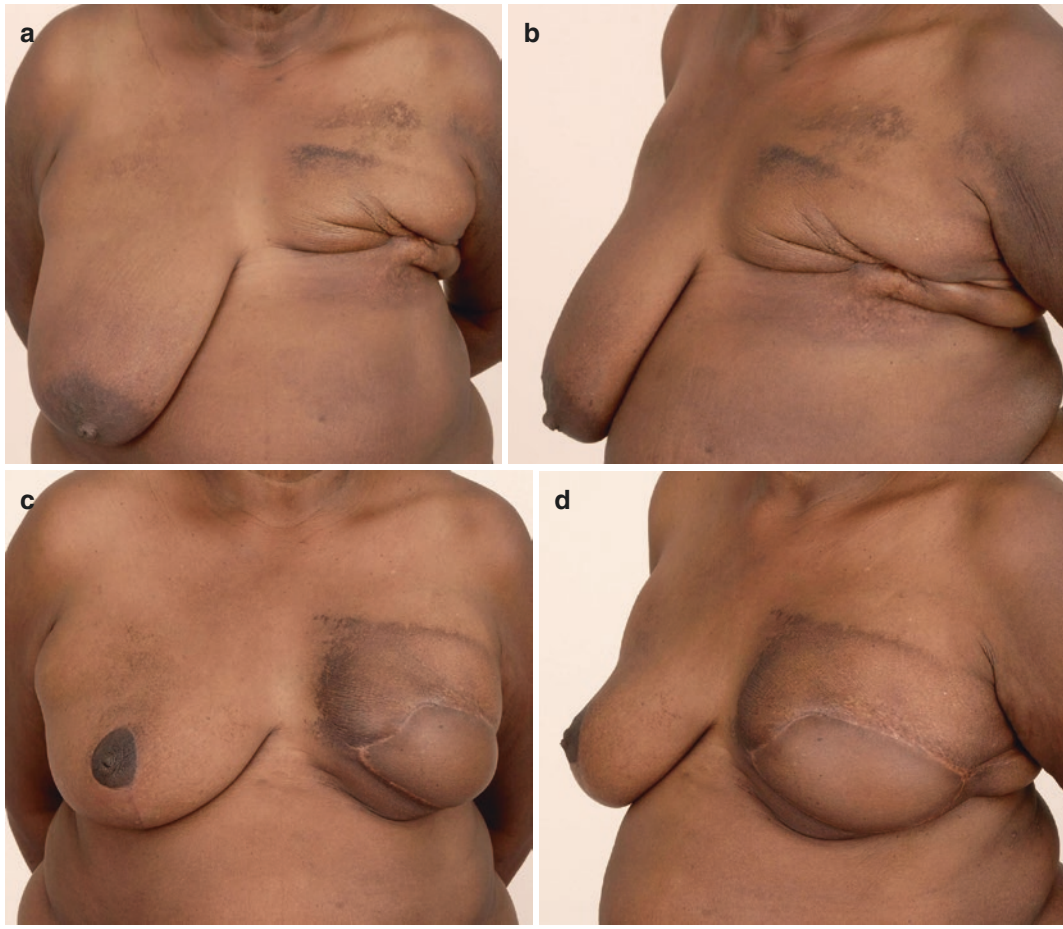

Fig. 4 (**a**, **b**) Preoperative 69-year-old female patient with a history of mastectomy and radiation presented with a long-standing left-sided breast pain and unstable scar/wound in the left breast. (**b**) Preoperative. (**c**, **d**) Postoperative at 1 year after breast reconstruction using latissimus dorsi flap and implant and right-sided breast reduction. Latissimus dorsi muscle flap provided robust blood supply to the area

bundles, and tendons is required. There is a paucity of regional flap options in the extremities, and limb salvage commonly necessitates a free flap coverage. Bringing a distant, non-radiated flap preserves available local tissues and blood supply. As mentioned earlier, it is prudent to select a flap over primary skin closure to prevent future complications in the irradiated field.

Upper extremity has unique appearance and function that are difficult to replicate with reconstruction. Functional or sensate reconstructions with tendon, nerve, or innervated muscle transfer (i.e., gracilis muscle, rectus femoris muscle) may be indicated depending on individual needs. Latissimus and rectus abdominis muscle flaps could be used to cover larger defects. Workhorse fasciocutaneous flap options are free radial forearm, anterolateral thigh, and lateral arm flaps.

Flap selection for the lower extremity depends on many different factors. In general, muscle flaps with a skin graft yield good outcomes as they atrophy in time and adhere to underlying structures better, being less mobile. However, in areas where tendon gliding is important (knee, ankle, and dorsal foot), fasciocutaneous flaps provide mobile tissue coverage [27]. The knee is particularly challenging as the reconstruction should allow a wide range of

motion. Gastrocnemius flap is a commonly used pedicled flap for lower extremity reconstruction, but its pedicle could lay in the field of radiation and its size is limited. For the foot, a muscle flap with a skin graft provides good adherence and stability. It is important to consider the eventual functional outcome of reconstruction as bulky flaps may prevent weight bearing, shoe or orthotic device wear. Outcomes of below-knee amputations are sometimes superior to flap reconstruction as they allow for a faster recovery and excellent prosthetic rehabilitation. The thigh is relatively easier to reconstruct due to the presence of more tissue bulk to work with. Flaps from regional sites, such as the abdomen, are also available (Fig. 5).

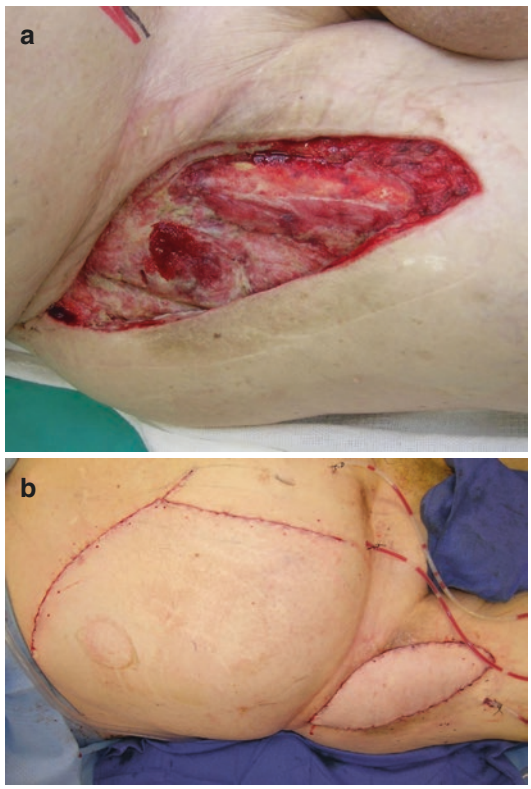

Fig. 5 (**a**) A 55-year-old male patient with a history of sarcoma resection and radiation involving the right anterior thigh presented with wound breakdown and infection. (**b**) After ensuring no recurrence and adequate debridement, pedicled rectus abdominis myocutaneous flap was utilized for soft tissue reconstruction

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Co-opting Developmental Signaling Pathways to Promote Wound Healing

Imran Khan, Sasikumar Ponnusamy, Saeed Ur Rahman, Alessio Giubellino, and Praveen R. Arany

1 Introduction

Embryogenesis involves series of well-orchestrated morphogenetic events including proliferation, contraction, and migration leading to formation of a miniature embryo which will be further maintained by the balance between cell death and replacement. Any form of external or internal injury to the tissues or organs reinitiates tissue building machinery to replace and repair the missing or damaged area. Studies involving developmental and wound healing events have highlighted significant similarities between these two phenomena that could potentially herald new insights into crucial tissues remodeling processes. This brief chapter highlights parallels between wound healing and embryonic development focusing on signaling pathways involved in key decision-making events during epithelial migration and scar formation. This information could be of immense importance for better understanding of the wound healing process and suggest novel development-inspired wound healing strategies.

2 Developmental Basis of Wound Healing

Every organism has an inherent ability to protect itself from external or internal injuries, refer to as wound healing. It functions as a primary mechanism for restoring the functional components of a living system. Wound healing occurs in four major phases, namely, clotting and coagulation, inflammation, and proliferation and matrix synthesis followed by the final maturation phase. These phases coordinate in an overlapping manner to repair damaged tissues or organs. The essential process of wound healing is seen in every living organism, but the degree and mode of healing varies widely across organisms. The ideal outcome of wound healing is the complete restoration of anatomical form (tissue-organ architecture) and physiological function termed regeneration. This regenerative process

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appears to be largely divided into two forms—epimorphic and morphallaxis where the former involves profuse proliferation of an undifferentiated "blastemal" tissue, while the latter involves direct transformation (transdifferentiation) of injured tissues or organs. As we move from lower unicellular to higher multicellular organisms, there is a dramatic shift in the nature and extent of wound healing, which is characterized by high potential of regeneration in lower organisms compared to relatively slow repair in mammals. Both the modes of healing are extremely important for the survival of organisms and probably have been chosen during the course of evolution based on the complexity of the organisms.

Wound healing has also been compared to embryonic development as both utilize planar cell polarity pathway (PCP), an evolutionarily conserve signaling system known to regulate developmental processes by actomyosin cytoskeleton formation for coordinated alignment of cell polarity across tissue planes [1]. Therefore, it has been speculated and argued that naturally occurring morphogenetic events involved in tissue movements are similar to those required for wound healing. For example, closure of epithelial holes as seen in dorsal closure of *Drosophila* embryo and ventral enclosure in *C. elegans* shows striking similarity with wound closure involving epithelial migration [2]. Tissue repair (skin) in the early fetal stage is marked by rapid reepithelialization in which actin "purse string" filaments are formed at the leading edge of the epithelial wound and hence providing coordinated cell movement for wound closure [3, 4].

Wounds formed in early gestation stage heal in a regenerative fashion leaving no scar tissues behind as it is supported by the hyaluronic acid-rich, hypoxic ECM environment [5, 6]. However, during development as blood vessels and hair follicles are formed, a transition from scarless to scar-mediated wound healing appears where healing involves hemostatic response leading to formation of fibrin clot. This is followed by the recruitment of inflammatory and fibroblast cells at the site of wound repair, along with keratinocytes from the basal layer of epidermis for reepithelialization of wounds. It is also speculated that persistent presence of myofibroblasts following normal wound healing contributes to scar formation that are notably conspicuously absent (or disappear) during embryonic scarless-wound healing [7].

3 Parallels in Signaling Pathways

The striking similarities between signaling pathways during embryonic development and wound healing emphasize both redundancies of biological systems as well as their potential for clinical therapeutics. Similes have been drawn between wound healing and development with the predominant similar biological regulatory process of the latter becoming very evident in later phases of healing, driving the tissue regenerative outcome [8]. For example, in contrast to repair in early fetal stage, repair in late fetus (as postnatal skin injury) occurs in a matured organ environment and often results in scarring. It is also fascinating to note that the stark opposite of this controlled, disciplined regulatory behavior is evident in the early phases of healing where profuse proliferation, migration, and dedifferentiation or redifferentiation of cells are evident and parallels have been drawn to a tumor microenvironment [9–11]. These persistent dysregulated changes are even more tumor-like in chronic wound scenarios [11]. These observations have led to the realization that wound healing is a key prototypical process and examining its etiopathogenesis can provide remarkable insights into both development and malignancies associated with it (Fig. 1). A major focus of these investigations has attempted to unravel the nature of the biological regulatory with various epigenetic and signal transduction approaches. Many pathways are essential for various stages of development and wound healing. This review will focus on two major pathways and their cross talk, Wnt and TGF-β, with a special reference to Hippo pathway. Among several key biological events that occur during embryonic tissue movement, epithelial migration and matrix turnover most closely resemble the normal wound healing processes.

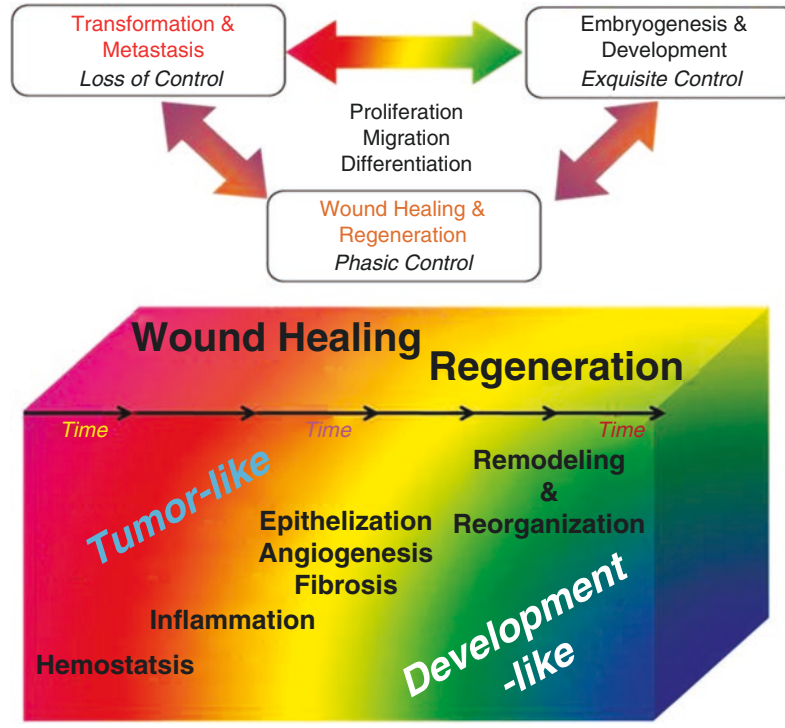

Fig. 1 Similarities and differences in three biological processes and potential insights into their overlaps that can be utilized for human clinical translation

4 Epithelial Migration

Epithelial migration during embryonic development is commonly used for filling up epithelial holes as seen in dorsal closure of *Drosophila* embryo and in *C. elegans* ventral enclosure. Dorsal closure is the process of bringing two epithelial edges together by an extraembryonic membrane composed of large flat cell (amnioserosa) to close the dorsal hole and form dorsal midline. Similarly, formation of ventral midline in *C. elegans* also involves epithelial migration wherein stretching of hypodermis takes place over the ventral surface of the embryo. One of the crucial pathways needed in both naturally occurring and wound-activated epithelial movement is JNK pathway [12]. Presence of this signaling widely imposes a tight regulation in cell divisions and epithelial movement needed for embryonic gap filling (*Drosophila* dorsal closure) or wound healing [13, 14]. Induction of JNK signaling leads to AP1 activation on the leading edges of migrating epithelial cells. This activated AP1 activity in leading edges of epithelial cells leads to induction of downstream target genes, namely, decapentaplegic and the dual-specificity phosphatase, puckered (puc). These downstream targets act as a "brake" on the activated JNK signal which is needed for dorsal closure. As epithelial migration is also needed in wound healing, studies have shown that JNK signaling is needed for wound closure as well. Tissue-specific knockout of c-Jun in the epithelium of fetal mice leads to failure of eyelid closure [15], and these lines of mice also exhibit subtle defects in wound healing [16].

Another pathway known to play a crucial role in epithelial migration is TGF-β pathway. TGF-β has been shown to promote epithelial migration in cornea and several tumor cells through activation of p38 MAPK pathway [17]. But it was observed that Smad3 knockout mice have accelerated wound healing, making TGF-β signaling an inhibitory to wound healing [18]. Similarly, TGF-β1 knockout mice showed accelerated wound repair in incisional wound due to increased reepithelialization, while transgenic mice expressing TGF-β1 (K5.TGF-$\beta1^{wt}$) in keratinocytes show delayed healing [19]. Therefore it is believed that suppression of TGF-β signaling in

cutaneous wounds may benefit wound healing despite of its known role as a key player in promoting wound healing [20]. TGF-β1 knockout mice show accelerated reepithelialization during incisional wound repair [21, 22], while TGF-β3 knockout mice show a partially fused palate (cleft palate) due to defective cell migration [23]. The difference in phenotype of different isoforms of TGF-β highlights their discrete roles or expression at specific stages during embryogenesis.

5 Scar Formation

It is known that embryo repairs its wound in absence of inflammation while inflammation is needed for adult tissue repair. But it has also been proposed to be a culprit of scarring or fibrosis. As similar pathways are used in wound healing and developmental repair, we take an insight of differential action of these signaling with an emphasis on scar formation. There are several pathways known to be involved in determining the scar phenotype, a major one being Wnt signaling. This signaling is one of the fundamental pathways driving embryonic development of multicellular organisms across all species. Wnt proteins are secreted glycoproteins which act as short- or long-range signaling molecules for inducing variety of effects including cardiac development and differentiation, stem/progenitor cell self-renewal, angiogenesis, aging, secondary body axes formation in *Xenopus* embryos, cell adhesion, and morphogenetic movements [24, 25]. Based on the mode of action, Wnt signaling activates two distinct pathways, namely, canonical or Wnt/β-catenin-dependent pathway and the noncanonical or β-catenin-independent pathway which can be further subdivided into the planar cell polarity and the Wnt/Ca^{2+} pathways. In canonical pathway, upon ligand-receptor (Fz) binding, β-catenin gets stabilized in the cytoplasm following which it gets translocated into the nucleus where it binds to the TCF-LEF transcription factors leading to gene expression [26]. In contrast, activation of noncanonical Wnt pathway leads to an increase in intracellular calcium which activates protein kinase C (PKC), phospholipase C (PLC), or Jun N-terminal kinase (JNK) that regulates planar cell polarity [27].

It is believed that both the branches of Wnt signaling may participate in the cellular decision in scarless or scar-forming wound healing outcomes. But it has been shown that postnatal wounds have increased Wnt signaling compared to fetal wounds, and this reduced Wnt signaling in prenatal wound healing could be responsible for its scarless phenotype. However, based on predominant data on scar-mediated wound healing phenotype, it is believed that noncanonical Wnt signaling mediates developmental effects, while the cross talk between canonical Wnt/β-catenin and other signaling pathways (like TGF-β signaling pathways or transcription factor HIF-1α) determines scar formation following wound healing. During embryogenesis these cross talks are less predominant, and noncanonical signaling predominates however in postnatal wound healing Wnt/β-catenin cross talk with TGF-β increases (HIF1α/PAI, etc.) leading to scar formation [28, 29].

This is also in line with the observation that Wnt signaling is needed for de novo hair follicle regeneration in wounds of adult mice, and it (Wnt3a) had a greater effect on fibroblasts from postnatal mouse skin compared to fetal fibroblasts for cell proliferation, synthesis of hyaluronic acid and type 1 collagen, and differential induction of TGF-β isoform (β1 and β3) [30, 31]. Further, recent studies show that treatment of Wnt3a ligand on fibroblast cells leads to induction of myofibroblast like phenotype by upregulating TGF-β signaling through SMAD2 pathway in canonical Wnt/β-catenin-dependent manner [32]. In light of the above observation, it can be concluded that Wnt signaling cross talk with other pathways is crucial in mediating developmentally regulated wound healing versus adult wound healing. Similarly, scar-forming wound healing in postnatal developmental stages can lead to a fibrosis-like phenotype (hypertrophic and keloid tissues), if coupled with excess activation of Wnt signaling leading to accumulation of nuclear and cytoplasmic β-catenin which could be partly TGF-β driven [33, 34].

It is also believed that absence of inflammation during embryonic healing dampens the

cytokine and growth factor profile at or around the healing area leading to less or no expression of crucial profibrogenic cytokines like TGF-β or CTGF. Similarly, if TGF-β1 is neutralized by antibody application at the site of wounded area in adult rats, wound repair shows reduced scarring, highlighting its direct involvement in scarring [35, 36].

Loss of inflammatory cytokines during embryogenesis has also been attributed to presence of miRNA which gets reduced during the adult phases and hence leading to inflammation and scar phenotype. miRNA-29b and miRNA-29c repress several ECM proteins such as TGF-β, SMADs, and β-catenin, which are known to be involved in deciding the fate of wound healing with or without scar [37, 38]. Similarly, miRNA-192 has been shown to increase collagen I α2 expression by targeting Smad-interacting protein 1 (SIP1) [39]. But the exact role of different miRNA, its expression, and its correlation with pro- and anti-fibrotic cytokines are not yet well understood and are being actively explored.

As cell proliferation and differentiation are fundamental to wound healing, recently, a new pathway, namely, Hippo pathway, has been shown to promote wound healing. It gets activated with tissue damage (changes of mechanical or biochemical environments), and a local increase of YAP/TAZ activity facilitates wound healing process [40]. Expectedly, this pathway is also needed for embryogenesis as a systematic knockout of YAP in mice is lethal because an embryo stops developing at embryonic day 8.5 (E8.5) with multiple defects in the yolk sac, vasculogenesis, chorioallantoic fusion, and body axis elongation [41].

6 Approaches That Can Invoke These Signaling Pathways for Wound Therapeutics

6.1 Biophysical Therapies

Current clinical burn wound managements are focused on preventing death, reducing pain, and scarring. Even though the conventional and current advanced treatment approaches like fluid resuscitation, removal of necrotic tissue, hyperbaric oxygen, negative pressure (vacuum) therapy, and engineering skin grafts have reduced wound-related deaths, they have several limitations such as high costs, limited efficacy, and inability to regenerate skin appendages of wound healing. The readers are referred to several excellent reviews on these biophysical modalities for more detailed information. As a major focus of our research is biophotonics therapy, the following section will highlight the use of low-dose biophotonics treatments also known as photobiomodulation (PBM) therapy (previously called low-level light/laser therapy, LLLT) [42]. In the last few decades in medicine, PBM has quite been successful in gaining major attention as a potential biophysical modality for treatment of various pathophysiological conditions, and wound healing is particularly attractive [43–45].

This field was initiated by the seminal work of Mester et al. [46], which reported nonthermal effects of lasers on mouse hair growth, and a subsequent study by the same group reported acceleration of wound healing and improvement in the post-wounding regeneration ability of muscle fibers using a 1 J/cm^2 ruby laser [47]. Commonly, PBM uses the red and near-infrared light from 620 to 1100 nm to stimulate cellular functions for numerous therapeutic benefits including regeneration of teeth [48]. Apart from wound healing, photobiomodulation has been used to treat various human diseases that result from pain, inflammation, aberrant immune responses, or lack of wound healing.

The well-known molecular mechanism of PBM therapy is absorbance of red and near-infrared (NIR) range light by photoreceptors located in the mitochondria, altering the activity of one or more endogenous enzymes and electron transport, which could initiate cell signaling pathways and alter cellular metabolism as well as proliferation [49]. PBM has been noted to modulate various biological processes in cell culture and animal models by regulating the numerous signaling pathways. Experimental evidences showed that PBM therapy could promote cell proliferation, movement and attachment, and so on. This phenomenon

of PBM has been widely applied in the treatment of various diseases including wound healing [50] and skin wound care [51]. However, the molecular mechanism associated with the stimulatory effect of PBM has not been fully understood. Research endeavors to determine novel applications of PBM therapy are continuously under progress. These PBM-activated signaling pathway can promote wound healing through beneficial effects on cells and matrix components.

6.2 PBM Therapy-Activated Signaling Pathway for Wound Healing

Evidence is accumulating that PBM therapy can improve wound healing, especially in settings where healing is impaired [52]. Meta-analyses of in vivo studies suggest that red light appears to be effective in promoting tissue repair [53, 54]. Arany et al. [55] showed the ability of low-power laser in activating the latent TGF-β1 complex in healing wounds, which suggests a key role of TGF-β1 in mediating the photobiomodulatory effects of low-power lasers in wound healing.

6.3 PBM Therapy-Activated Signaling Pathway for Collagen Production

An in vitro study on tenocytes (rat Achilles tendon cells) demonstrated that low-level laser (904 nm) stimulates cell proliferation and collagen synthesis by activating the ERK pathway through TGF-β1 production suggesting a potential basis for PBM therapy in tendon injury [56]. Ye et al. [57] showed that both extracellular signal-related kinase (ERK)1/2 and JNK/MAPK pathways that were activated by the 1064 nm laser irradiation could markedly increase collagen synthesis and inhibit collagen degradation in rat dorsal skin. The activation of ERK1/2 and JNK/MAPK seems to play a role in collagen production in the skin, induced by the 1064 nm laser. Signal pathways such as TGF-β1, ERK1/2, and JNK/MAPK seemed to play a role in regulating skin collagen expression and resulting in wound healing by laser treatments.

6.4 PBM Therapy Activates Signaling Pathway for Cell Proliferation and Differentiation

Lim et al. [58] showed that, under hypoxic/ischemic conditions, irradiation with 635 nm reduced intracellular ROS production leading to alleviation of VEGFR-1 suppression, enhanced VEGF expression, and ERK MAPK activation. Overall, the activation of ERK MAPK signaling molecules accelerated angiogenesis and may prove to be a useful alternative tool in wound healing. Similarly, Feng et al. [59] demonstrated that low-power laser irradiation 632.8 nm activates ERK/Sp1 pathway and promote VEGF expression and vascular endothelial cell proliferation. These findings highlight the important roles of ERK/Sp1 pathway in angiogenesis and provide a potential strategy to develop the therapeutic potential.

6.5 PBM Therapy for Regulating Wound Healing, Muscle Injury, and Vascular Regeneration

A study showed that PBM therapy with 632.8 nm promotes cell proliferation through PI3K/AKT activation. As PI3K/AKT signaling pathway is known to be involved in cell proliferation, induction of this pathway by low-dose light treatment could promote epithelial wound healing and regeneration [60]. PBM therapy has also been reported to promote the release of a variety of cytokines and growth factors that can contribute to wound healing [61] and to enhance blood flow via nitric oxide production [49]. Ga-Al-As diode laser (810 nm) irradiations increase the neurite PC12 cell proliferation via upregulating p38 signaling pathway [62], which could help to heal in neural healing as well. More detailed studies are needed to accurately predict the precise molecular pathways activated by PBM therapy in individual cell types that would provide a sound

molecular rationale for specific clinical wound healing scenarios.

7 Biomaterial Approaches for Wound Management

Regeneration of cutaneous wounds with large defect caused by burns or trauma remains a major challenge and costs remarkable healthcare expenditures. Therefore, there is an increasing demand for the ideal wound dressing that can accelerate wound healing. Ancient works from the Egyptians include references to the usage of grease, honey, and vegetable fiber for improving wound healing [63]. Biomaterials have been critically important in the wound care industry specifically for nanoparticle encapsulation, cell encapsulation, and wound dressings. Biomaterials can offer several advantages over conventional processes for wound dressing. One of the most favorable biomaterial scaffolds for wound dressing is nanofiber matrix (Fig. 2) and is the most reported material that has an important role in tissue regeneration. Nanofibrous scaffolds have been developed by several methods under the hypothesis that synthetic nanofiber matrix would mimic a morphological function of collagen fibrils and create a more favorable niche for the cells [64] that can physically simulate an extracellular matrix (ECM) and functioning as a network for cell attachment, migration, proliferation, and differentiation [65, 66] With its microporous structure and huge surface area, the nanofiber matrix rapidly initiate signaling pathway and attract cells, which can induce significant extracellular matrix components, such as collagen, angiogenic factors, and growth factors, to repair the damaged tissue [67, 68]. The most suitable biomaterials for wound dressing should be biocompatible and induce the growth of keratinocyte and fibroblast. Previously, novel functional wound dressing has been prepared by combining electrospun poly (Ɛ-caprolactone) (PCL) nanofiber matrix with chitosan-based nitric oxide-releasing biomaterials (CS-NO). The study showed that PCL/CS-NO dressings enhanced wound healing process through accelerating reepithelialization and improved the organization of regenerated tissues [69]. However, polymeric nanofibrous scaffold is among the most encouraging biomaterials for native ECM analogs that induce cell adhesion, migration, and proliferation and could be a promising solution for wound healing [68, 70]. Chitin nanofibers (CNFs) hydrogel has also been utilized as a directive cue to induce bone marrow mesenchymal stem cells (BMSCs) differentiation for enhancing cutaneous wound regeneration in the absence of cell differentiating factors [71].

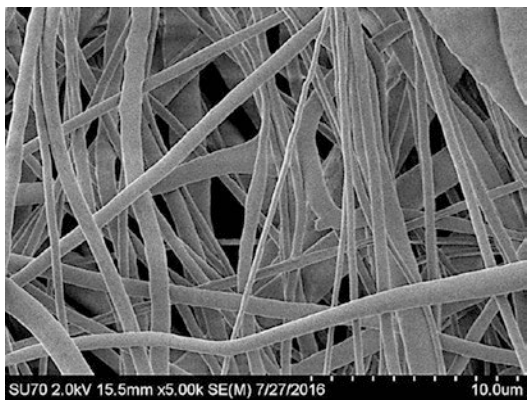

Fig. 2 Scanning electron micrograph of electrospun PCL nanofiber matrix at high magnification. SEM images reveal that the architecture of this scaffold effectively mimics the spatial features of ECM

Wound healing is a complex dynamic process that requires cellular interactions between various cell types, including keratinocytes, fibroblasts, myofibroblasts, endothelial cells, smooth muscle cells, and immune cells. These cellular interactions are mediated by several factors such as second messengers, growth factors, blood components, and hormones. Several growth factors that are released at the site of wound are considered to be necessary for wound healing. These factors include keratinocyte growth factor (KGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF). The clinical use of growth factors to enhance wound healing is currently being investigated. In the past decades, growth factors were tested as relevant treatments

to improve wound healing. However, the clinical trials of these growth factor therapies have met with only limited success. The FDA-approved clinical growth factor treatment for chronic wounds is recombinant platelet-derived growth factor-BB (PDGF-BB) (Becaplermin). Other growth factors, including epidermal growth factor (EGF), and fibroblast growth factor (FGF-2) have shown limited improvement in clinical trials [72–74]. Most of the growth factors studied have been delivered to the site of injury by direct application. The main disadvantage of direct application is that the growth factors have a short half-life and remain for limited time at the site of treatment. Therefore, microencapsulation of growth factors is a useful method for controlled and sustained delivery system. Microencapsulation method has become a popular approach to use active biomolecules, because slow and controlled release of the encapsulated factors prevents the side effects. The popularity of microencapsulation, such as synthesizing microspheres (Fig. 3), is a major medical advance for various wound healing applications. Due to its great usefulness, the polymers are the most used materials in the encapsulation of active biomolecules in microspheres. Biomaterial scaffold for wound dressings is ideal candidate for loading antibiotics or growth factors due to their release kinetics and tunable properties. Silver sulfadiazine encapsulated chitosan microspheres incorporated in polyethylene glycol (PEG) fibrin gels showed strong antimicrobial activity against *Pseudomonas aeruginosa* and *S. aureus* [75]. Polyethylene oxide (PEO) polymer composite films loaded with carrageenan [76] and PEO composite films loaded with streptomycin [77] have been used for enhancing wound healing. Ciprofloxacin has been incorporated into electrospun polyurethane and dextran composite nanofiber and suggested an efficient nanofiber-based wound dressing [78]. Many other antibiotic agents have also been used into various synthetic, natural, or composite wound dressings. Tissue microenvironment has an excessive amount of chemical cues. Therefore, delivering of one bioactive agent may not be enough to get achievement in all the issues of impaired wound healing. In addition, temporal control in release of growth factors in correlation with different phases of wound healing would likely be an essential aspect for accelerating wound healing in complex organ.

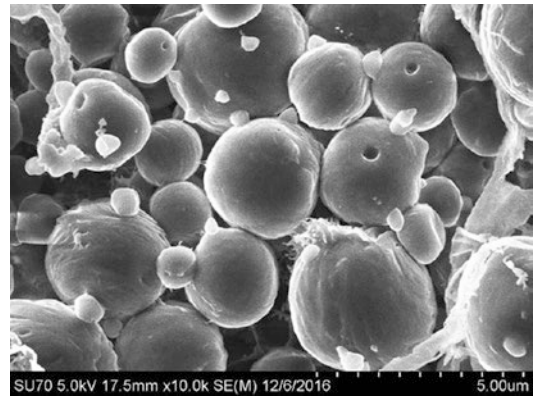

Fig. 3 Scanning electron micrograph of PCL microspheres encapsulating biomolecules of dimensionally wide range

8 Clinical Emphasis

Interestingly, all the signaling pathways discussed in this chapter are also involved in the pathophysiology of a number of ailments that are the etiologic culprit and/or are contributing to the development of chronic wounds. The Wnt/beta-catenin signaling pathway is an important modulator of the pancreatic secretion of insulin by beta cells in the islets of Langerhans and in the regulation of metabolism [79, 80]. Mutations in this pathway are responsible for many disorders, including diabetes mellitus type II and different types of cancer [25]. Activation of Wnt signaling leads to translocation of beta-catenin to the nucleus where it binds to transcription factor like TCF-4, which has an important role in type II diabetes [81], as also Wnt5b [82]. A role for the protein WISP2 (WNT1 inducible signaling pathway protein 2) in diabetes and obesity has also being recently suggested but it is still poorly understood [83].

Also TGF-beta signaling, which has pleiotropic effects, has important functions on pancreatic beta cells and diabetes. It is well known that inhi-

bition of TGF-beta can result in induction of beta cells proliferation [84]. Also TGF-beta has being recognized as a key player in the development of diabetic nephropathy [85, 86]. Moreover the TGF-beta/Smad 3 signaling pathway is crucial in the development of obesity and hepatic steatosis, besides diabetes [87]. Overall these illustrate the important role of these pathways in the pathogenesis of several comorbidities of chronic nonhealing wounds.

Conclusions

Across animal kingdom an efficient wound closure includes clotting and reepithelization to repair the damaged area. As discussed above several of the wound healing phases bear a striking similarity with embryonic developmental stages leading us to conclude that the genes or pathways involved in wound healing and development are conserved through the phylogeny. This chapter highlights the complexity and interplay of various signaling pathways that can determine eventual clinical wound healing outcomes.

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Segmentation and Management of Chronic Wound Images: A Computer-Based Approach

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1 Introduction

Computer-aided measurement of the size and characteristics of chronic wounds is a novel approach to standardizing the accuracy of chronic wound assessment. A chronic wound, as defined by Centers for Medicare and Medicaid Services, is a wound that has not healed in 30 days. An estimated 6.5 million patients in the United States are affected by chronic wounds, and it is claimed that an excess of US$25 billion is spent annually on treatment of chronic wounds. The burden is growing rapidly due to increasing health-care costs, an aging population, and a sharp rise in the incidence of diabetes and obesity worldwide [1]. As such, there is a need for a timely and accurate method to document the size and evolving nature of chronic wounds in both the inpatient and outpatient settings. Such an application can potentially reduce clinicians' workload considerably, make the treatment and care more consistent and accurate, increase the quality of documentation in the medical record, and enable clinicians to achieve quality benchmarks for wound care as determined by the Center for Medicare and Medicaid Services.

The current state-of-the-art approach in measuring wound size using digital images, known as digital planimetry, requires the clinician to identify wound borders and wound tissue type within the image. This is a time-intensive process and is a barrier to achieving clinical quality benchmarks. Our group is developing image analysis tools that will enable the computer to perform this analysis rather than requiring user input. Developing an accurate method of measuring wound size and tissue characteristics serially over time will yield clinically meaningful information in relation to the progression or improvement of the wound. The focus of the work reported in this paper is the segmentation of the wounds.

A wound exhibits a complex structure and may contain many types of tissue such as granulation, slough, eschar, epithelialization, bone, tendon, and blood vessels, each with different

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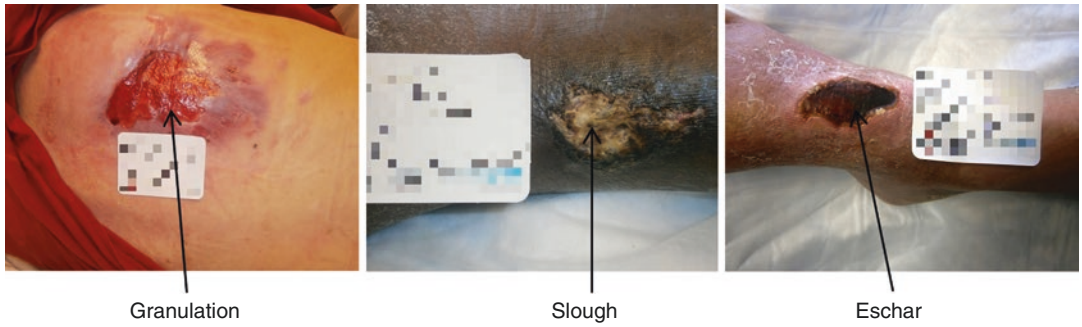

Fig. 1 Most commonly seen tissues in wounds: granulation, slough, and eschar

color and texture characteristics. In this paper, we propose a novel probability map that measures the likelihood of wound pixels belonging to granulation, slough, or eschar (Fig. 1), which can then be segmented using any standard segmentation techniques. In this work, we focus on the granulation, slough, and eschar tissues as these are the three most commonly seen tissues in wounds. A preliminary version of this work has been reported in [2]. This paper extended the previous work significantly with an extensive literature review; more elaborate explanation of the proposed method, employing two segmentation techniques to show that the probability map is adaptable to many different techniques; comparison with other existing method; comprehensive analyses on inter-reader variability between clinicians; a much bigger dataset used for performance evaluation (which was divided into three sets for analysis purpose); as well as more elaborate discussions on the results.

The paper is organized as follows: Sect. 2 presents the review of the literature on wound image analysis. In Sect. 3, we present our proposed probability map approach to wound segmentation and integrate it with two different segmentation techniques. Sections 4 and 5 discuss the experimental setup, results, and discussion. Finally, Sect. 6 concludes the paper and describes future work.

2 Literature Review

Although wound segmentation from photographic images has been the subject of several studies, most of the work in this area deals with images that are either acquired under controlled imaging conditions [3, 4], confined to wound region only [4–7], or narrowed to specific types of wounds [7–9]. Because these restrictions are mostly impractical for clinical conditions, there is a need to develop image segmentation methods that will work with images acquired in regular clinical conditions.

Table 1 summarizes current works in wound segmentation and monitoring as well as existing software tools. Wannous et al. [3] compared the mean shift, JSEG, and CSC techniques in segmenting 25 wound images, before extracting color and textural features to classify the tissues into granulation, slough, and necrosis using an SVM classifier. The wound images were taken with respect to a specific protocol integrating several points of views for each single wound, which includes using a ring flash with specific control and placing a calibrated Macbeth color checker pattern near the wounds. They reported that both segmentation and classification work better on granulation than slough and necrosis. Hettiarachchi et al. [4] attempted wound segmentation and measurement in a mobile setting. The segmentation is based on active contour models which identifies the wound border irrespective of coloration and shape. The active contour process was modified by changing the energy calculation to minimize points sticking together including preprocessing techniques to reduce errors from artifacts and lighting conditions. Although the accuracy was reported to be 90%, the method is rather sensitive to camera distance, angle, and lighting conditions.

In the work by Veredas et al. [5], a hybrid approach based on neural networks and

Table 1 Summary of current works on wound segmentation, monitoring, and software tools

Papers	Addressing	Segmentation/classification methodology	Image types	Number of images	Segmentation/classification accuracy
Wannous et al. [3]	Wound segmentation	Mean shift, JSEG, CSC, and SVM	With background, controlled conditions	25	73.3–80.2% (granulation), 56.4–69.8% (slough), 64.9–70.7% (necrosis)
Hettiarachchi et al. [4]	Wound segmentation	Active contour	Wound region only, controlled conditions	20	90.0%
Veredas et al. [5]	Wound segmentation and tissue characterization	Mean shift and region growing, neural networks, and Bayesianclassifiers	Wound region only	113 divided into 10 testing sets	78.7% sensitivity 94.7% specificity 91.5% accuracy
Hani et al. [6]	Granulation detection and segmentation	ICA and k-means	Wound region only	30	88.2% sensitivity 98.8% specificity
Perez et al. [7]	Wound segmentation and analysis	RGBSI analysis, user need to provide samples of wound and background for each image	Wound region only, leg ulcers only	Not mentioned	Visual observations only
Wantanajittikul et al. [8]	Burn image segmentation and characterization	FCM and morphology, texture analysis, and SVM	With background, but burn cases only	5	72.0–98.0% (segmentation) 75.0% (classification)
Song and Sacan [9]	Wound segmentation	Neural networks, K-means clustering, edge detection, thresholding, and region growing	Foot ulcers only	78 training, 14 testing	71.4% (MLP) 85.7% (RBF)
Kolesnik and Fexa [10–12]	Wound segmentation	SVM, texture, and deformable snake	With some background	50 training, 23 testing	Error rate of 6.6% (color), 22.2% (texture), 5.8% (hybrid)
Cukjati et al. [13]	Wound healing rate measurement	Not applicable	Not applicable	Not applicable	Not applicable
Bums et al. [14]	Software to study healing rate for a given patient population	Not applicable	Foot ulcer only	Not applicable	Not applicable
Loizou et al. [15]	Wound healing monitoring	Snake (segmentation), texture feature (healing)	Wound region only, foot wounds only	40 images from 10 cases	Not available
PictZar [16]	Software for wound analysis	Manual drawing and calibration	With background	Not available	Not available
Filko et al. [17]	Software for wound analysis and healing monitoring	Not applicable	Cropped or hand-drawn region of wound images	6 images from 1 case	Not applicable
Weber et al. [18]	Hardware and software to capture wound mapping	Using electrode to obtain wound mapping and characteristics	Not applicable	Not applicable	Not applicable

Bayesian classifiers is proposed in the design of a computational system for tissue identification and labeling in wound images. Mean shift and region-growing strategy are implemented for region segmentation. The neural network and Bayesian classifiers are then used to categorize the tissue based on color and texture features extracted from the segmented regions, with 78.7% sensitivity, 94.7% specificity, and 91.5% accuracy reported. Hani et al. [6] presented an approach based on utilizing hemoglobin content in chronic ulcers as an image marker to detect the growth of granulation tissue. Independent component analysis is employed to extract gray level hemoglobin images from red-green-blue (RGB) color images of chronic ulcers. Data-clustering techniques are then implemented to classify and segment detected regions of granulation tissue from the extracted hemoglobin images. About 88.2% sensitivity and 98.8% specificity were reported on a database of 30 images.

Perez et al. [7] proposed a method for the segmentation and analysis of leg ulcer tissues in color images. The segmentation is obtained through analysis of the red, green, blue saturation and intensity channels of the image. The algorithm, however, requires the user to provide samples of the wound and the background before the segmentation can be carried out. Wantanajittikul et al. [8] employs the Cr-transformation, Luv-transformation, and fuzzy c-means clustering technique to separate the burn wound area from healthy skin before applying mathematical morphology to reduce segmentation errors. To identify the degree of the burns, h-transformation and texture analysis are used to extract feature vectors for SVM classification. Positive predictive value and sensitivity between 72.0 and 98.0% were reported in segmenting burn areas in five images, with 75.0% classification accuracy.

Song and Sacan [9] proposed a system capable of automatic image segmentation and wound region identification. Several commonly used segmentation methods (k-means clustering, edge detection, thresholding, and region growing) are utilized to obtain a collection of candidate wound regions. Multilayer perceptron (MLP) and radial basis function (RBF) are then applied with supervised learning in the prediction procedure for the wound identification. Experiments on 92 images from 14 patients (78 training, 14 testing) showed that both MLP and RBF have decent efficiency, with their own advantages and disadvantages. Kolesnik and Fexa [10–12] used color and textural features from 3D color histogram, local binary pattern, and local contrast variation with the support vector machine (SVM) classifier to segment 23 wound images based on 50 manually segmented training images. The SVM-generated wound boundary is further refined using deformable snake adjustment. Although this study does not have the aforementioned restrictions (i.e., acquired under controlled imaging conditions, confined to wound region only, or narrowed to specific types of wounds), results were reported on a relatively small set of images. An average error rate of 6.6%, 22.2%, and 5.8% were reported for the color, texture, and hybrid features, respectively.

In addition to wound segmentation, wound healing and monitoring have been the subject of several studies on wound image analysis. Cukjati et al. [13] presented their findings on how the wound healing rate should be defined to enable appropriate description of wound healing dynamics. They suggested that wound area measurements should be transformed to percentage of initial wound area and fitted to a delayed exponential model. In the suggested model, the wound healing rate is described by the slope of the curve is fitted to the normalized wound area measurements over time after initialization delay. Loizou et al. [14] established a standardized and objective technique to assess the progress of wound healing in a foot. They concluded that while none of the geometrical features (area, perimeter, x- and y-coordinate) show significant changes between visits, several texture features (mean, contrast, entropy, SSV, sum variance, sum average) do, indicating these features might provide a better wound healing rate indication. Finally, Burns et al. [15] evaluated several methods for quantitative wound assessment on diabetic foot ulcers, namely, wound volume, wound area, and wound coloration.

There are also quite a few software tools for wound analysis and monitoring currently available. All the software, however, has yet to incorporate automated or semiautomated wound detection or segmentation so that the clinician's initial involvement can be minimized. For example, PictZar digital planimetry software [16] is a commercial software for wound analysis which provides measurements such as length, width, surface area, circumference, and estimated volume to the users. The software, however, does not incorporate automated or semiautomated wound detection; instead it requires user drawings and calibration for the above measurements to be computed. Filko et al. [17] developed WITA, a color image processing software application that has the capability to analyze digital wound images, and based on learned tissue samples, the program classifies the tissue and monitors wound healing. The wound tissue types are divided into black necrotic eschar, yellow fibrin or slough, red granulation tissue, and unclassified parts of the image, although no evaluation against the known ground truth was presented for the image analysis part of the software. To obtain wound dimensions, users must mark the distance on the photograph that is equivalent to 1 cm (or 1 in.). A different approach to wound monitoring software and hardware was proposed by Weber et al. [18]. They developed a new "wound mapping" device, which is based on electrical impedance spectroscopy and involves the multi-frequency characterization of the electrical properties of wound tissue under an electrode array. This approach, however, requires major changes to the daily clinical routine in wound care.

3 Wound Segmentation Based on a Probability Map

The wound images used in our experiments are provided by the Comprehensive Wound Center of the Ohio State University Wexner Medical Center, with Institutional Review Board (IRB) approval. The center is one of the largest wound centers in the United States, and the wound images captured in the center come from different camera manufacturers, setting, and capture conditions: different medical center employees (not professional photographers) capturing the images in routine clinical work using different cameras. This simulates the variation that we expect to see in other medical centers in terms of patient variability as well as variation due to image capture. Unlike the wound images used in the literature [3–18], these images present additional challenges. As discussed in the previous section, many previous works in this field are typically carried out in regions that contain the wound only; thus, they do not have to deal with the issue of complicated background, especially those red, yellow, and black objects, interfering with the segmentation process (Fig. 2). In order to simplify the task at this stage, the algorithm requires the user to mark a single point (i.e., a single click) inside the wound to start the segmentation process.

Our proposed method consists of several stages as shown in Fig. 3. The first step is the red-yellow-black-white (RYKW) probability map computation in a modified HSV (hue-saturation-value) color space (Sect. 3.1). Once the probability map is established, the next step is the segmentation of the boundaries of the wound in the area (Sect. 3.2). We present the results of two different segmentation approaches: region-growing segmentation and optimal thresholding. Because the distance between the camera and the wound is not recorded, this information needs to be extracted by the content in the image. We developed a novel approach, which analyzes the image to detect patient labels, typically attached near the wound, and uses the size of the label to calibrate the wound size measurements (Sect. 3.2.3).

3.1 Probability Map Computation

Granulation, slough, and eschar tissues generally correspond to red (R), yellow (Y), and black (K) tissues, respectively, in the wound area (Fig. 1). Due to the fact that the subsequent stage requires the detection of white label cards, as well as to

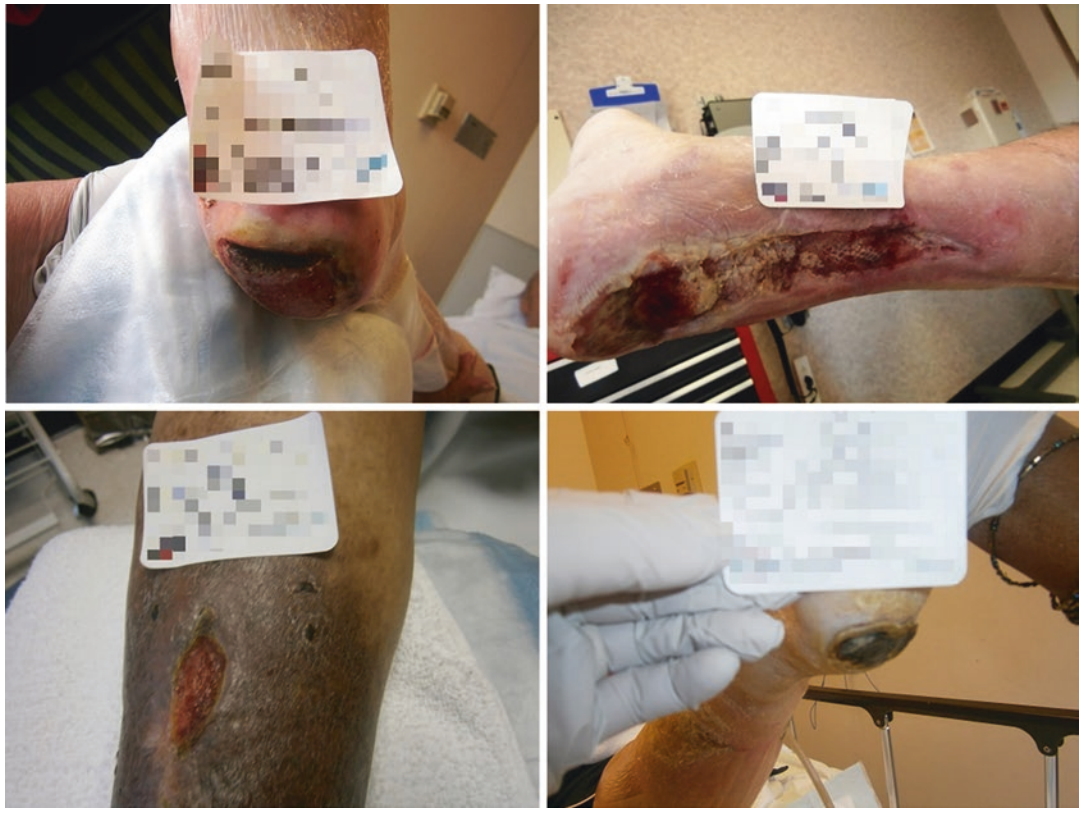

Fig. 2 Example of wound images with complicated backgrounds used in the experiment

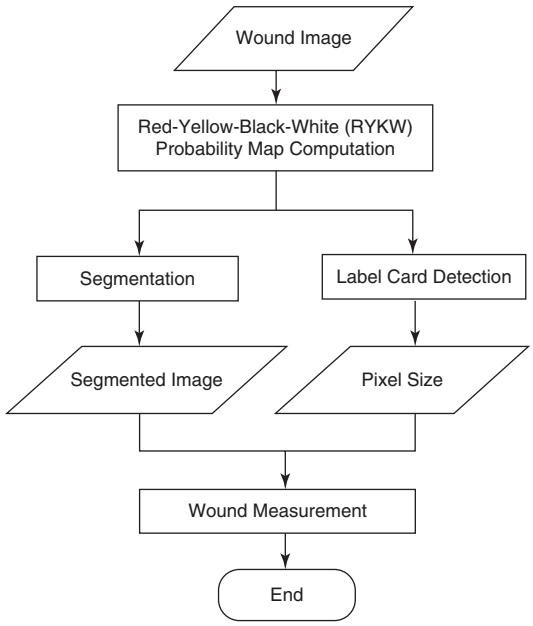

Fig. 3 Flowchart of the proposed method

avoid any white pixels in the image being wrongly classified as yellow, a fourth color, white (W), is included in the probability map computation, resulting in a four-dimensional (4D) RYKW map. Given a wound image, our method computes the probability of each pixel in the image belonging to one of these colors. The probability is computed based on the distance of the image pixels to the red, yellow, black, and white colors in a modified HSV color space. The HSV color space was chosen because it can be modified to maximize the distances between the four colors of interest (Eqs. 1 and 2. (refer to Eqs. 2 and 3)).

Consider an image I, probability matrix P, and color set $C_k = \{\mathrm{R}, \mathrm{Y}, \mathrm{K}, \mathrm{W}\}$ where $k = 1, 2, 3, 4$ represents the four colors R, Y, K, and W, respectively. For a particular pixel x within I, the probability p of the pixel belonging to a color C_k (i.e., one of red, yellow, black, or white) is computed through the following equation:

$$p_k(x) = \frac{1}{\left(\frac{d(C_k, x)}{d(R, x)}\right)^2 + \left(\frac{d(C_k, x)}{d(Y, x)}\right)^2 + \left(\frac{d(C_k, x)}{d(K, x)}\right)^2 + \left(\frac{d(C_k, x)}{d(W, x)}\right)^2}, \quad (1)$$

where $d(C_k, x)$ is the distance (see Eqs. 4–7) between the value of pixel x and the particular color C_k. In other words, the probability is inversely proportional to the relative distance between the pixel and the color of interest. The above equation is applied to all pixels for all four colors, producing a 4D probability map, P, with the sum of the probability at any one pixel equal to 1. The probability definition used here is similar to that of the fuzzy c-means clustering method without the fuzzifier parameter [19]. From the image point of view, the 4D matrix P can be viewed as a stack of four single matrices P_k, each showing the probability of the wound image pixels belonging to the four different colors. From the pixel point of view, the matrix P can be viewed as a collection of many vectors p, each showing the probability of individual pixels belonging to the four colors of interest.

One of the challenges in wound segmentation is to differentiate between regions with similar hue characteristics: e.g., dark red (granulation) versus black (eschar) regions, as well as light yellow (slough) versus white (epibole, skin, etc.) regions. Figure 4 shows an example of a dark red granulation tissue whose value channel, V, values range between 0.2 and 0.4. Taking $V = 0.5$ as the threshold, the tissue would have been misclassified as being closer to black rather than red (where 0 refers to pure black, and 1 refers to pure red). This, combined with the close proximity between red and yellow colors, makes segmentation of the three tissue types complicated, regardless of the color model used (RGB, HSV, CIE L × a × b ×, etc.). In this work, we developed a modified HSV color model to improve the accuracy of the probability map by scaling the saturation (S) and value (V) components according to Eqs. 2 and 3, respectively, to obtain S_{mod} and V_{mod}:

$$S_{\text{mod}} = \frac{\log(\alpha \times S + 1)}{\log(\alpha + 1)} \quad (2)$$

$$V_{\text{mod}} = \frac{\log(\alpha \times V + 1)}{\log(\alpha + 1)} \quad (3)$$

where S_{mod} and V_{mod} are the modified saturation and modified value, respectively, and α is a constant. In our work, we have chosen $\alpha = 8$ so that the first quarter of the original scale (dark or light regions) will be stretched to half the modified scale, while the remaining three quarters of the

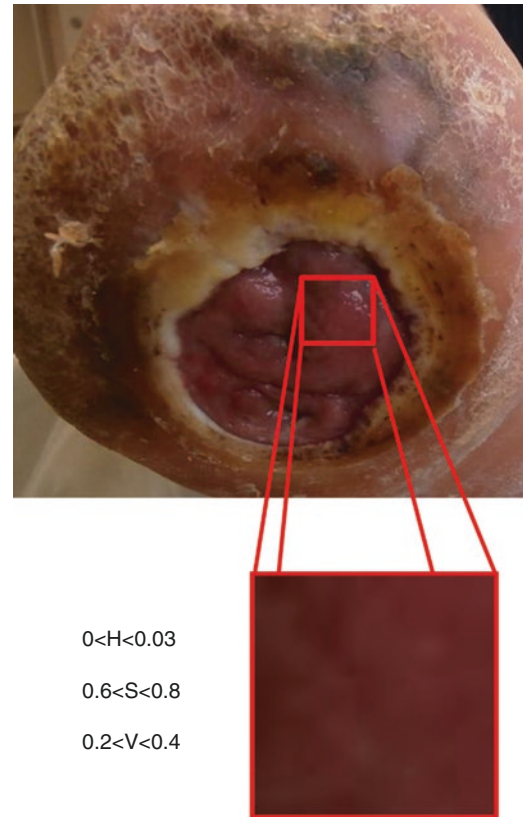

Fig. 4 Illustration of red granulation tissue mistaken as black eschar tissue

original scale (red or yellow regions) will be compressed to occupy the remaining half of the modified scale (Fig. 5). Furthermore, the Hue (H) component is also shifted by 30° to maximize the distance between red and yellow.

Figure 5 shows the transformation of S and V using Eqs. 2 and 3 and shows the transformation of the black-red, black-yellow, white-red, white-yellow, and red-yellow color transition from the standard HSV to the modified HSV color model. It can be observed that the modified HSV model better reflects the color distances between the four colors of interest. Under the standard HSV, dark red and dark yellow are closer to black; similarly light red and light yellow are closer to white. This would negatively affect the accuracy of the color probability map. The proposed modified HSV model is thus better suited to computing the probability of pixels belonging to any one of the four colors (see Sect. 5.2 and Table 14 for comparison between the modified and original HSV for region growing).

Due to the uneven color distribution of the HSV or modified HSV color models (e.g., dark colors occupied almost half of the entire color space), the calculation of distance, $d(C_k,x)$, between a particular pixel, x, and the colors is defined differently for the four colors. The distance of a pixel to black is based solely on V_{mod}, while the distance to white is based on V_{mod} and S_{mod}. The distances to red and yellow on the other hand make use of all V_{mod}, S_{mod}, and H_{mod}. For a particular pixel x, the proposed distance equations are summarized below:

$$d(R,\ x)=\sqrt{\left(H_{\text{mod}}(x)-H_{\text{mod}}(R)\right)^2+\left(S_{\text{mod}}(x)-S_{\text{mod}}(R)\right)^2+\left(V_{\text{mod}}(x)-V_{\text{mod}}(R)\right)^2} \tag{4}$$

$$d(Y,\ x)=\sqrt{\left(H_{\text{mod}}(x)-H_{\text{mod}}(Y)\right)^2+\left(S_{\text{mod}}(x)-S_{\text{mod}}(Y)\right)^2+\left(V_{\text{mod}}(x)-V_{\text{mod}}(Y)\right)^2} \tag{5}$$

$$d(K,\ x)=V_{\text{mod}}(x)-V_{\text{mod}}(K) \tag{6}$$

$$d(W,\ x)=\sqrt{\left(V_{\text{mod}}(x)-V_{\text{mod}}(W)\right)^2+\left(S_{\text{mod}}(x)-S_{\text{mod}}(W)\right)^2} \tag{7}$$

where the following values are defined:

$$V_{\text{mod}}(K)=0\quad V_{\text{mod}}(W)=1\quad S_{\text{mod}}(W)=0$$

$$H_{\text{mod}}(R)=11/12\quad S_{\text{mod}}(R)=1\quad V_{\text{mod}}(R)=1$$

$$H_{\text{mod}}(Y)=1/12\quad S_{\text{mod}}(Y)=1\quad V_{\text{mod}}(Y)=1$$

3.2 Segmentation

While there are many possible segmentation methods for use in medical applications, e.g., [20–25], we based our segmentation on two well-known and rather basic techniques, namely, region-growing segmentation and optimal thresholding. We will demonstrate that even with these two simple segmentation algorithms, when coupled with our proposed probability map approach, we are able to provide reliable segmentation of wounds. While the proposed approach works with the selection of an initial seed point by a clinician, the RYKW map has the potential to improve the segmentation into fully automated segmentation. This can be achieved by first identifying all potential wound regions throughout the entire image based on color information, before carrying out advanced image analysis to filter the false-positive regions, leaving only true wound regions as the segmented output.

3.2.1 Region Growing

Region growing [26] is a pixel-based image segmentation algorithm that examines neighboring

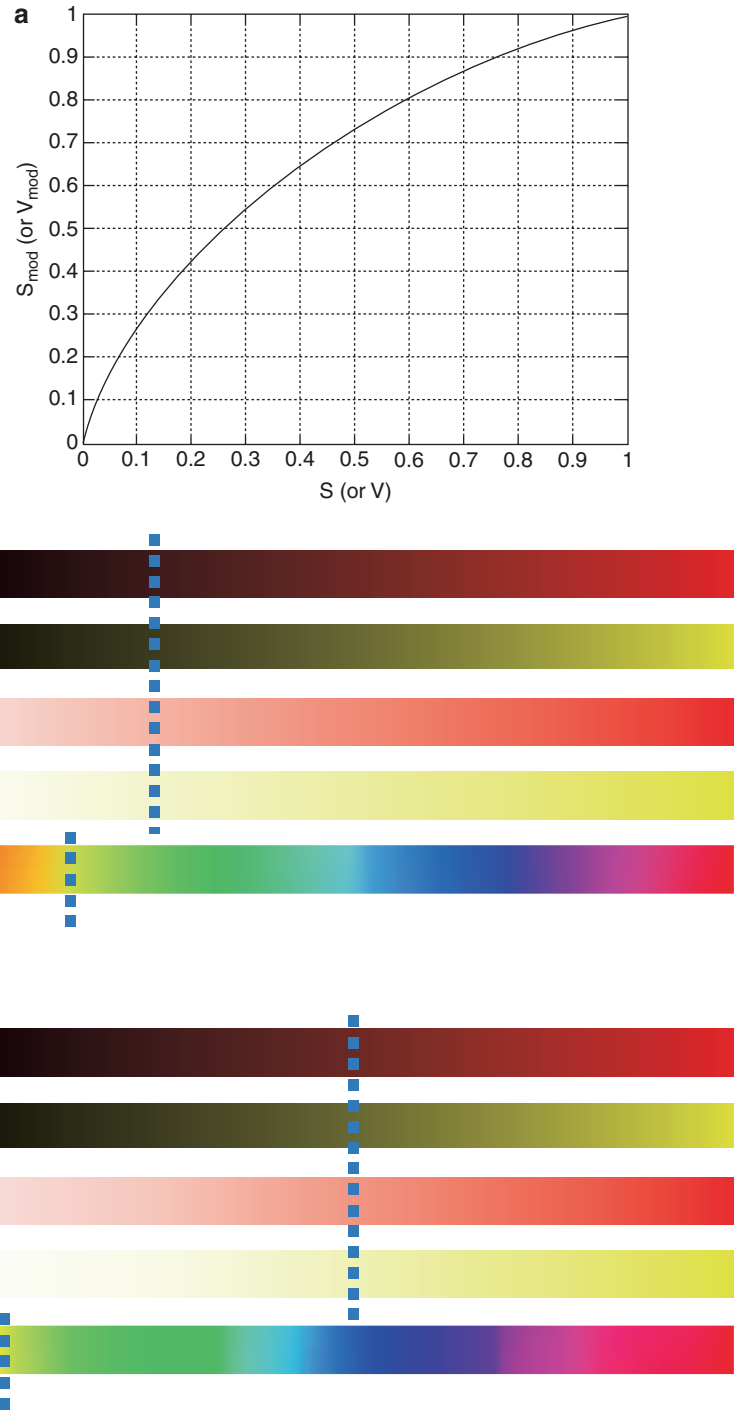

Fig. 5 (**a**) Transformation of S and V to S_{mod} and V_{mod}, based on Eqs. 2 and 3 ($\alpha = 8$), and color transitions for (**b**) standard HSV and (**c**) modified HSV color models. The dashed lines show how the H, S, and V values are shifted after the transformation

pixels of initial seed points and determines whether neighbors of the pixel should be added to the region. In our proposed method, the initial seed points are to be provided by the clinician. The regions are grown from the initial seed point's probability vector to adjacent points

based on the 4D probability map P (Eq. 1). A neighbor is added to the region if the distance between that pixel's probability vector and the mean probability vector of the region (i.e., the mean probability of each R, Y, K, and W channel over the current segmented region) is less than a certain threshold value, *t*. The process continues until either all the neighbor's distances are above the threshold or all the pixels have been processed.

To ensure the region-growing process does not stop prematurely, a mechanism is included to search for pixels with a similar probability map within a certain radius, r, from the region boundary, and the process continues. Morphological closing and filling operations are then applied during post-processing to remove noise and soften the edges. From experiments, suitable values for the threshold and radius are $t = 0.1$ and $r = 5$ pixels, respectively. Note that the proposed algorithm only segments the granulation, slough, and/or eschar regions and ignores the rest of the image as clinicians are only interested in the wounds. While region growing is generally considered as computationally expensive operation, the probability map really helps to speed up the process by providing a valuable color discriminator between the four colors of interest.

3.2.2 Optimal Thresholding

Our "optimal thresholding approach" segments the image by thresholding the difference matrix of the probability map, P, while taking into account the pixel's tissue type and strength of its probability. While there are many available thresholding methods such as Otsu thresholding that can be used to segment the probability map, these methods are rather "hard" thresholding methods; if single wounds are inadvertently separated to two or more smaller wounds (which can happen very frequently due to illumination, etc.), the segmentation can be considered to fail since the calculated accuracy (refer Sect. 4) will be very low.

The key idea behind our approach is first to identify all pixels whose color characteristics are similar to those of the seed pixel, before iteratively refining the segmentation boundary. The refinement is by simple thresholding of the difference matrix, Q, which is a matrix of the difference between the two highest probabilities for each pixel, and provides a second degree of tissue membership probability:

$$Q = P_{\max 1} - P_{\max 2} \tag{8}$$

where $P_{\max 1} = \max(P)$ and $P_{\max 2} = \max(P)\big|_{P \neq P_{\max 1}}$.

Given the seed point pixel and its probability vector, its highest probability tissue class is identified, and pixels with the following properties are considered for segmentation:

Property 1:	Pixels with the same tissue class as their highest probability. Value of Q ranges from 0 to the maximum value in Q, φ
Property 2:	Pixels with the same tissue class as their second highest probability and in which their difference with the highest probability is below a certain threshold, τ. Value of Q ranges from 0 to $-\tau$

In the strictest sense, only pixels with Property 1 should be included in the segmented region; however, due to the complicated nature of the wound tissue, pixels with Property 2 are also included to minimize false negative. The region of interest (ROI) at this point is defined as the region in Q whose pixels satisfy either Property 1 or Property 2, with values ranging between the φ and $-\tau$.

The next step is to iteratively threshold the ROI, starting from φ. At each step the mean of the segmented ROI where the seed point is located is calculated. Theoretically the mean will decrease as the threshold value decreases toward $-\tau$. The optimal threshold is defined as the threshold value where the mean values become "stable" without any sudden decreases or increases. The segmented wound region can then be obtained by thresholding the ROI with the optimal threshold value. As in the region growing, morphological closing and filling operations are then applied during post-processing to obtain the final segmentation. Experimentally, the suitable values for the threshold, τ, and step-size decrement, step, are $\tau = 0.1$ and step = 0.01, respectively. The whole process is summarized as pseudo-code in Table 2.

Table 2 Pseudo-code of the optimal thresholding-based segmentation approach

Input: 4D probability map, P
Output: Segmented wound region, I_{seg}
Procedure:
1. Compute probability difference matrix, Q
2. Based on probability map of seed pixel, identify ROI
3. Setφ = max (Q)
4. Set $\tau = 0.1$
5. Set step = *0.01*
6. Set $th = \varphi$
7. While $th > -\tau$
seg = ROI > th
segmean = mean(seg)
$th = th -$ step
end
8. Identify optimal threshold, th_{opt} based on segmean
9. I_{seg} = ROI > th_{opt}
10. Perform morphological operations on I_{seg}

3.2.3 Label Card Detection and Wound Measurement

Since the distance between the camera and the wound is not recorded, the absolute values for wound measurements—necessary for clinical reporting—cannot be recorded. To solve this problem, we have developed a technique to automatically scale the wound size. As in most medical centers, each of the wound images taken at the Wexner Medical Center contains a white label card, which we automatically detected and used as a reference to compute the actual pixel size in the image. The white label card has a standard size of 4.5 cm by 6.5 cm. With successful detection of the card and its size with respect to the image, we can easily calculate the pixel measurements in cm per pixel unit.

To detect the card, first, the white regions are identified from the same RYKW map computed in the previous step (Sect. 3.1). Then, the detected white regions are filtered based on their area, rectangularity (actual area over minimum bounding rectangle area), and convexity measure (actual area over convex hull area) to identify potential rectangular regions for the white card. The rectangularity and convexity measure helps in eliminating irregular shape region, while the area relative to the image size helps in eliminating false rectangular regions. The length and width of the identified label card are then used to calibrate the pixel size. With the pixel size available, measuring the wound size is straightforward. Currently, the proposed algorithm outputs three measurements: area, length (major diameter), and width (minor diameter) of the segmented wound.

4 Experimental Setup

This study was done with the institutional review board (IRB) approval. In our experiments, we used a total of 80 images, whose ground truth was provided by at least two clinicians. The images are of 768 × 1024 pixels in resolution, stored in JPEG format. They were captured by the clinicians following normal clinical practice and under non-controlled conditions, i.e., no measures were taken to control the illumination, the background, or the wound to background ratio, resulting in a very challenging set of images. To capture the ground truth, an in-house software tool was developed. Using this tool, clinicians cannot only draw the boundaries of the wound but also its three tissue components: granulation, slough, and eschar tissues. Again using this tool, the user can input the estimates (as a percentage) for tissue components that are already an integral part of wound documentation. The clinicians first manually drew the wound boundaries for each image independently. Based on the drawn boundaries, the clinicians were then asked to estimate the percentage of granulation, slough, and eschar tissues before proceeding to draw the boundaries for each tissue type. The tool is capable of handling as many number of wound or tissue regions as possible; hence, the clinicians were asked to provide as detail a drawing as possible. Depending on the complexity of the image, clinicians spent between 30 s to 3 min to annotate a single image.

The images were divided into three sets as shown in Table 3. Set 1, consisting of ten images, were annotated with the consensus of three clinicians and used as a training set to ensure that all three clinicians have the same understanding in

Table 3 Categorization of images

Sets	Number of images	Number of ground truth
Set 1	10	1 (consensus from 3 clinicians)
Set 2	15	3 (from 3 clinicians)
Set 3	55	2 (from 2 clinicians)

defining the different tissue types as well as their boundaries. Set 2, with 15 images, were annotated by all three clinicians separately, producing three separate ground truth files for each image. Finally Set 3, with 55 images, were annotated by two clinicians independently, resulting in two separate ground truth files. The wound and tissue boundaries from the ground truth files of Sets 2 and 3 are compared to evaluate the level of agreement between the clinicians. Tissue component percentage estimation by the clinicians was also compared to the actual tissue percentage from the drawings to evaluate the accuracy of the clinicians' estimation.

The inter-reader variability is measured using the agreement measure in Eq. 9:

$$\text{Agreement} = \frac{D_1 \cap D_2}{D_1 \cup D_2} \times 100 \tag{9}$$

where D_1 and D_2 refer to the region annotated by the first, second, or third clinician, respectively. Due to the high degree of inter-reader variability (to be discussed in the Sect. 5), it is difficult to obtain one common ground truth for Sets 2 and 3. Hence, to evaluate the accuracy of computer segmentation, the resulting segmentation is compared to each of the different ground truths. In other words, the segmentation results are compared to each clinician's manual drawings, thereby indicating with which proposed algorithm the clinicians tend to agree more.

The same measurement in Eq. 9 is used to determine the accuracy of the computer-segmented regions against the ground truth:

$$\text{Accuracy} = \frac{\text{GT} \cap \text{CS}}{\text{GT} \cup \text{CS}} \times 100 \tag{10}$$

where GT refers to the boundaries drawn by any one of the clinicians, and CS refers to the computer-segmented region.

5 Experimental Results and Discussion

We first present the inter-reader variability between clinicians on the wound boundaries, tissue characterization, as well as tissue percentage estimation in Sect. 5.1. The proceeding subsection will then report the results of the computer segmentation against all the ground truth discussed in Sect. 5.2.

5.1 Inter-Reader Variability Between Clinicians

Three clinicians independently drew the boundaries of the wounds in Set 2 as well as estimated the percentages of tissue types. In this section, this data will be used to evaluate inter-reader variability. Table 4 shows the statistics of wound boundary agreement between the clinicians for the images in Set 2. Since there are three clinicians involved, four sets of comparison are carried out. As can be observed from Table 4, the mean agreement between any two clinicians varies between 80.3 and 84.3%. The mean drops to 74.3% when all three clinicians' readings are compared, indicating that it is more difficult to reach an agreement when more clinicians are involved (the trend for the median agreement follows a similar trend). Note that the minimum agreement goes as low as 40.7%, which suggests that some of the wounds are quite complicated and thus their boundaries are relatively difficult to define.

Table 5 shows the statistics for images in Set 3. Clearly, with more images, the mean and median agreement between clinicians 2 and 3 (clinician 1 is not involved in evaluating Set 3) drops rather sharply, from 80.3% to around 67.4% in mean agreement and from 83.3 to 70.8% in median. The standard deviation also almost doubles, while the minimum agreement can be as low as 24.4%. This suggests that with increased number of images to annotate, some of which contain relatively complicated wounds, the agreement between the clinicians plummets. This is another reason why we will be comparing

the computer segmentation with the ground truth from individual clinicians instead of a combined ground.

To gauge intra-reader variability, we have also asked two of the clinicians to redraw the wound boundary for a subset of cases (ten images) after a month from their initial reading. The intra-reader variability is summarized in Table 6. As in the inter-reader variability (Table 5), the difference between two consecutive readings is relatively high, with average self-agreement of 80.4 and 84.5% for the two clinicians.

While the average agreement between the clinicians at the wound level may still acceptable, their agreement at the tissue level is much lower. Tables 7 and 8 show the tissue characterization agreement between the clinicians for Sets 2 and 3, respectively. It can be seen that the mean and median agreement are all below 60% with standard deviation of mostly more than 30%. There were many instances where the clinicians do not agree on the particular tissue types within the wound, especially when it comes to differentiating granulation and slough, or between slough and eschar, and even granulation and epithelium. This is the reason for minimum agreement (all the values in the "Min" column in Tables 7 and 8) to be 0%. In other words, there are always situations where one clinician will identify a particular region within the wound, with which the other clinician will not agree. For example, Fig. 6 shows two examples of images with the lowest agreement between two clinicians. While the clinicians show quite decent agreement when it comes to granulation, their agreement for slough

Table 4 Wound agreement for Set 2 images (percentage accuracy in Eq. 9)

Agreement between	Mean	Min	Max	Med	Std Dev
Clinicians 1, 2, and 3	74.3	40.7	88.3	76.3	12.5
Clinicians 1 and 2	84.3	69.7	94.4	86.1	7.4
Clinicians 1 and 3	81.5	41.4	92.3	87.4	13.0
Clinicians 2 and 3	80.3	55.0	92.7	83.2	10.5

Table 5 Wound agreement for Set 3 images (percentage accuracy measure in Eq. 9)

Agreement between	Mean	Min	Max	Med	Std Dev
Clinicians 2 and 3	67.4	24.5	94.5	70.8	19.5

Table 6 Intra-reader variability for wound agreement

Agreement between	Mean	Min	Max	Med	Std Dev
Clinicians 2	84.5	66.0	97.3	87.2	10.5
Clinicians 3	80.4	58.0	97.3	84.8	14.7

Table 7 Tissue agreement for Set 2 images

Tissue types	Agreement between clinicians	Mean	Min	Max	Med	Std Dev	# of Img
Granul	1, 2, and 3	42.9	0.0	86.2	42.4	31.6	19
	1 and 2	59.6	0.0	94.0	71.9	30.1	19
	1 and 3	50.9	0.0	89.2	54.2	35.0	19
	2 and 3	52.6	0.0	88.5	60.3	31.8	18
Slough	1, 2, and 3	17.8	0.0	63.1	0.2	24.3	15
	1 and 2	31.3	0.0	74.2	27.0	31.7	13
	1 and 3	29.1	0.0	72.7	17.7	31.2	14
	2 and 3	38.4	0.0	84.7	44.8	33.4	15
Eschar	1, 2, and 3	24.5	0.0	85.8	0.0	37.7	9
	1 and 2	37.4	0.0	90.5	0.0	46.7	7
	1 and 3	26.5	0.0	90.8	0.0	40.8	9
	2 and 3	48.5	0.0	91.4	55.4	34.4	8

Table 8 Tissue agreement for Set 3 images

Tissue types	Agreement between clinicians	Mean	Min	Max	Med	Std Dev	# of Img
Granul	2 and 3	42.7	0.0	93.9	51.1	34.6	65
Slough	2 and 3	15.9	0.0	90.1	0.0	27.3	42
Eschar	2 and 3	25.0	0.0	92.3	0.0	34.6	30

Fig. 6 Two examples of very low agreement between two clinicians

Table 9 Tissue percentage estimation

Sets	Clinicians	Mean	Min	Max	Med	Std Dev	# of Img
Set 2	1	23.3	7.6	51.7	19.4	12.8	14
	2	22.8	1.2	73.2	19.3	20.4	18
	3	19.5	0.7	48.4	16.4	14.8	16
Set 3	2	28.8	0.2	160.0	18.6	31.0	46
	3	25.4	0.1	132.7	16.3	27.4	53

and eschar tissues is very low. Again, as in determining agreement on wound boundaries, the more the number of clinicians involved (3 vs. 2), the lower the agreement. Similarly, the more the images (Set 3 vs. Set 2), the lower the overall agreement is.

The last comparison we made regarding the clinicians ground truth is on the accuracy of their tissue percentage estimation. During annotation, once they completed drawing the overall wound boundaries for an image, the clinicians were asked to estimate the percentage of granulation, slough, and eschar tissues within the wound boundaries. They were then required to draw the tissue boundaries within the wound, and these "actual" percentages were compared to their earlier estimates. Wounds with only one tissue type (e.g., granulation only) were excluded as for these images they were not required to estimate (automatically set to 100%). Table 9 shows the percentage differences for the three clinicians for Set 2 and Set 3. The values are computed as the absolute difference between all three tissue types (hence some differences exceed 100%). As an example, a computer-calculated percentage of 60% granulation, 20% slough, and 20% eschar against clinician's estimation of 80% granulation, 10% slough, and 10% eschar will give an error rate of 40%: 20% error from the granulation and 10% error each from the slough and eschar. It can be seen that the mean differences between the three clinicians are almost the same, which are around 20% for Set 2 and around 25% for Set 3. This suggests that even the most experienced clinicians are having trouble estimating the tissue percentages, which is an important piece of information required in wound documentation.

The results presented in this section show that wound segmentation and characterization are complicated processes, where even the most experienced clinicians have different opinions regarding wound boundaries and the type of tissues involved. The next section will discuss the results of the computer segmentation, and we will demonstrate that the proposed segmentation algorithm based on a probability map can be as good as the clinicians' consensus ground truth.

5.2 Segmentation and Measurement Accuracy

We carried out both qualitative and quantitative evaluations of the algorithm performance, and these results will be presented in the next two subsections.

5.2.1 Qualitative Evaluation

First, the performance of the segmentation algorithm was evaluated qualitatively. Figure 7 shows four examples of the results obtained using both segmentation methods. For the first case, (Fig. 7(a), granulation), the accuracy is 91.3% and 77.7% compared to the ground truth by clinicians 2 and 3, respectively, using the optimal thresholding and 83.6 and 71.2% using the region-growing segmentation. The discrepancies between the results against the different ground truths are caused by the rather big difference in the wound boundaries created by the two clinicians. For the second case, (Fig. 7(b), granulation), the accuracies for both segmentation methods against both clinicians' ground truths are all more than 90%.

For the third example, the accuracies are all more than 80% except for the optimal thresholding result against clinician 3, which is around 75%. Finally for case 4, the accuracies for the optimal thresholding are recorded as 58.6 and 86.3%, while the region-growing scores were 39.4 and 62.2%. As in case 1, the two clinicians differed in defining the wound boundary, where one of them included some parts of healed tissues as well, lowering the accuracy percentages for both methods. The optimal thresholding

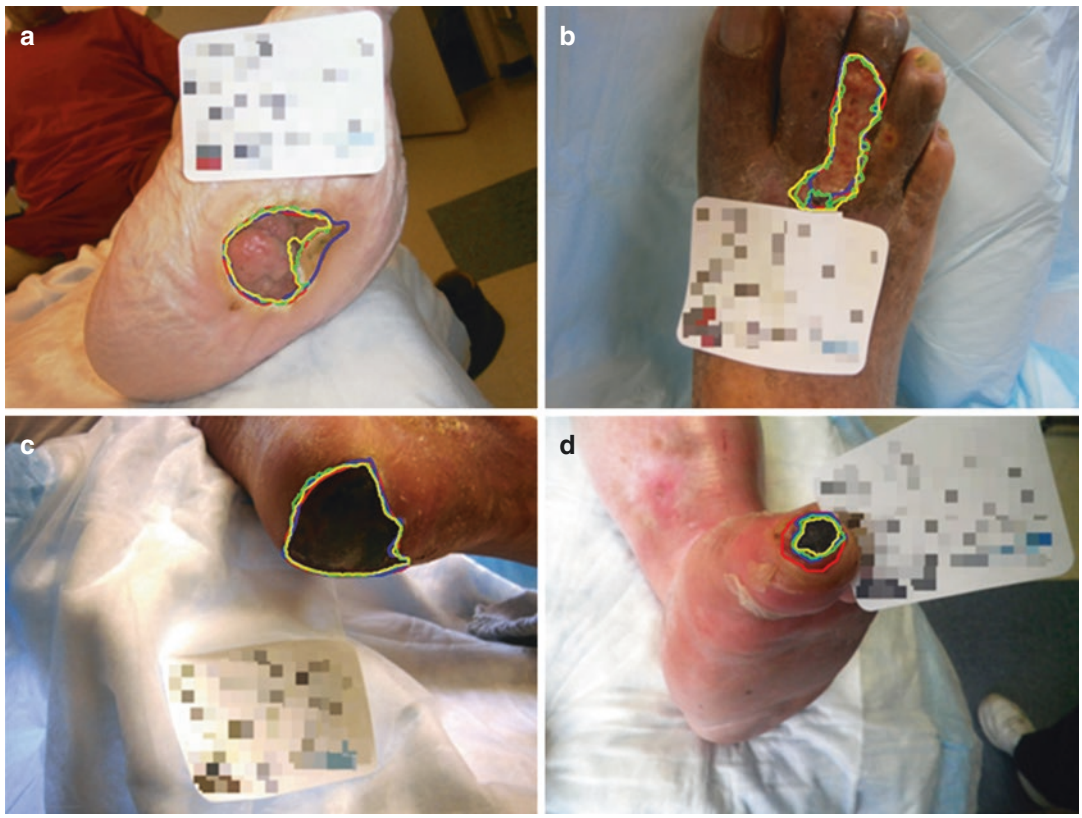

Fig. 7 Selected segmentation results. For each case, the red and blue markings show the wound boundaries drawn by clinicians 2 and 3, respectively, while the green and yellow markings show the segmentation obtained by the optimal thresholding and region-growing approaches, respectively.

method agrees well with clinician 3 with 86.3% accuracy, although the region-growing approach seems to have missed some boundary pixels. The small size of the wound also contributes to further lower the accuracy of this particular wound image, due to the "unforgiving" measurement metric used. Nevertheless, the four examples demonstrate that despite the complex nature of the wound boundary, the proposed algorithm is able to segment the wounds rather accurately.

5.2.2 Quantitative Evaluation

Tables 10 and 11 present the overall segmentation accuracy using optimal thresholding and region-growing approach, respectively. Each table presents the results according to the different image sets as well as different clinicians' ground truths. It is observed that using optimal thresholding segmentation on the probability map provides slightly better overall results compared to using region growing. However, these differences diminish as the size of the dataset increases (i.e., Set 1 → Set 3), and the average accuracies become almost identical (74.0 vs. 74.2%). This trend is also true for individual clinician's agreements with the algorithm for different methods. Optimal thresholding is also more consistent than region growing as can be deduced by the lower standard deviation for all image sets. The overall average accuracy of 75% is very promising considering the level of agreement between the clinicians varies from 65 to 85%.

For performance comparison, we also run Sefexa image segmentation tool [27], which was developed based on the work by Kolesnik and Fexa [10–12], on the same sets of images, and the results are summarized in Table 12. Their method, like ours and unlike the other works discussed in Sect. 2, is not limited to images captured under controlled environment, not confined to the wound region, or designed for specific wound types only. Furthermore, besides supervised automatic mode, their method can also work on semiautomatic mode by requiring the user to provide samples of pixels belonging to wound and non-wound regions. These two factors make Sefexa, which is based on color and texture features, the most appropriate benchmark for our proposed method. Comparing the readings in Tables 10, 11 and 12, both of our approaches outperform the Sefexa approach, which only records 68.8% average accuracy. Based on the standard deviation readings (10.5% for optimal

Table 10 Average segmentation (%) for optimal thresholding

Sets	Consensus	Clinician 1	Clinician 2	Clinician 3	Average	Std. Dev.
Set 1	78.6	NA	NA	NA	78.6	8.0
Set 2	NA	79.6	77.4	73.5	76.8	9.8
Set 3	NA	NA	74.8	73.2	74.0	10.8
Overall	78.6	79.6	75.4	73.3	75.1	10.5

Table 11 Average segmentation (%) for region growing

Sets	Consensus	Clinician 1	Clinician 2	Clinician 3	Average	Std. Dev.
Set 1	70.8	NA	NA	NA	70.8	14.3
Set 2	NA	77.1	75.7	73.6	75.4	10.8
Set 3	NA	NA	74.3	74.0	74.2	12.0
Overall	70.8	77.1	74.6	73.9	74.0	13.1

Table 12 Average segmentation (%) for SEFEXA segmentation tool

Sets	Consensus	Clinician 1	Clinician 2	Clinician 3	Average	Std. Dev.
Set 1	65.1	NA	NA	NA	65.1	22.8
Set 2	NA	78.9	78.3	80.4	79.2	10.4
Set 3	NA	NA	66.7	66.7	66.7	17.3
Overall	65.1	78.9	69.2	69.6	68.8	17.0

thresholding, 13.1% for region growing, and 17.0% for Sefexa overall), we can also deduce that our approach is more consistent. This is expected as Kolesnik and Fexa's approach depends heavily on the pixel samples to start the segmentation. While our approach requires the user to provide only an initial seed (i.e., a single click on an image), which is more convenient for the clinicians, the other method requires two sets of samples.

Table 13 shows the segmentation accuracy according to the different tissue types. Both approaches work best in segmenting granulation and eschar tissues, with lower accuracy for slough tissue. This is not surprising given the better delineated boundaries of granulation and eschar tissues. Slough tissues appear more sporadic and also may be easily confused with other tissue types. This finding also agrees with the one reported by Wannous et al. [3]. Table 14 compares the segmentation accuracies of the region-growing approach between the proposed modified HSV color space and the original HSV color space. Clearly, without modifying the HSV color space, the segmentation performance decreases considerably, highlighting the importance of our proposed modification. Without the modification, each of the overall wound segmentation as well as the granulation, slough, and eschar tissue segmentation recorded a drop in accuracy between 5 and 15%. As expected, the granulation tissue segmentation benefits the most from our modified color space because better threshold is used to distinguish dark red (granulation) and black (eschar) tissues.

Table 13 Average segmentation (%) according to tissue types

Tissues	Optimal threshold	Region growing
Granulation	76.2	75.3
Slough	63.3	63.9
Eschar	75.1	71.5

Table 14 Performance comparison (%) between modified and original HSV for region growing

Tissues	Modified HSV	Original HSV
Overall	74.0	62.9
Granulation	75.3	58.9
Slough	63.9	57.2
Eschar	71.5	66.8

Optimal thresholding has much lower computational complexity compared to the region-growing method. Region growing processes all the wound pixels; hence, the larger the image or the wound, the longer time is needed to complete processing all the pixels of interest. On average, to segment an image of size 768 × 1024 on 2.3GHz Intel® Core™ i7 processor, optimal thresholding needed less than a second, while region growing required up to 5 s, depending on the wound size. Another issue to be considered when using the region-growing approach for segmentation is the repeatability, i.e., the method should provide consistent segmentation results for different initial seeds. This is particularly even more challenging in our case as wound images tend to have "glossy" pixels within the granulation or slough area due to their wet nature. The optimal thresholding segmentation does not suffer from this problem and thus is relatively more stable. Nevertheless, the proposed probability map approach, together with the mechanism to prevent premature stopping, is able to address this issue rather well.

6 Conclusions and Future Work

We have developed a method for the segmentation of wound images into granulation, slough, and eschar regions and automatically carry out the measurements necessary for wound documentation. We propose the red-yellow-black-white (RYKW) probability map as the platform for the region-growing process in segmenting the three regions as well as the white label cards. Experiments were conducted on 80 wound images provided by the Ohio State University Wexner Medical Center. These images exhibited challenging characteristics with different types of wounds at different stages, typically pictured in a clinical setting with complicated backgrounds, some of which with similar characteristics to the color palette of the wounds or surrounding healthy skin. The analysis presented from the inter- and intra-reader variability experiment

suggests that wound segmentation and characterization are a complicated process, where even the most experienced clinicians have different opinions regarding wound boundaries and the type of tissues involved.

Using the optimal thresholding approach, the proposed method achieves an overall accuracy of 75.1%, which is very promising considering that the average agreement between the clinicians is between 67.4 and 84.3%. The wound area, length, and width measurements also give a promising accuracy of 75.0%, 87.0%, and 85.0%, respectively. We have also demonstrated that the probability map approach, computed through a modified HSV color model, is a very promising method for use with many segmentation techniques to reliably segment wound images. Based on two simple segmentation methods, optimal thresholding and region growing, the overall accuracy of around 75.1% has been observed. This suggests that the proposed RYKW map manages to identify the wound and its different tissues rather well, on par with the experts. Utilizing the RYKW map with a more advanced segmentation method can only further improve the accuracy of the segmentation and is currently being worked on in our lab. The proposed method was also evaluated against other existing technique, and experiment on the same sets of images shows much better performance for our proposed method.

We believe the proposed system will help wound experts immensely in the future. This early success could pave the way for a computer-assisted wound analysis software where the computer can segment the wounds reliably (with confirmation from the clinician) and provide a more accurate tissue characterization (as opposed to current clinicians' estimates), with possible extension into wound healing monitoring as well. With the tedious tasks of drawing the wound boundaries and populating the basic information on tissue characterization carried out by the computer, the clinicians will have more time in exercising their expertise in actual clinical work, thus achieving quality benchmarks for wound care as determined by the Center for Medicare and Medicaid Services.

It should be noted that the quality of the segmentation results as well as the resulting measurements depend on the quality of the input images. Unlike most of the previous work in this area, our work aimed at developing a solution that will work with actual, clinically captured images (all the images in this study were captured during routine clinical work, and the personnel who captured them were not aware of software development). However, there is still the expectation that the images capture the wound in a reasonable manner; for example, if only a tiny portion of the wound is visible in the image, obviously, the segmentation will fail to properly capture the wound or its tissue components. Admittedly, human readers will run into the same challenge if asked to evaluate such images. Similarly, if the labels are not placed reasonably well, the absolute measurements may be skewed. Although our software can recognize some of the variations in the placements of cards, it cannot recover from severely distorted placement of cards. A ruler and color scale in the label cards can be easily included, and these can be used to calibrate both size measurements and color variations, hence improving the overall accuracy. Other image acquisition issues include poor lighting and noise. While some of the images in our dataset do suffer from nonuniform lighting, noise, and/or other artifacts (e.g., blurring in the images due to shaking the camera while taking the picture) to a certain degree, the proposed method performs rather well in handling these types of images. A future study needs to analyze the effect of such variations on the overall performance in a controlled manner.

The proposed algorithm has some limitations in segmenting and characterizing wounds on dark skins, especially when trying to identify eschar tissues or dark granulation tissues. In some rare instances, the color of Caucasian skins tends to be very red in appearance (in which the probability of red will be very high); hence segmenting fresh granulation tissues may not work on these images. We are exploring the possibility of incorporating edge and depth analysis into the current algorithm in order to address these

problems, which could also potentially measure undermining wounds. In addition, work is currently under way to include images from other medical centers as well and to further improve the segmentation accuracy by applying other segmentation techniques on the probability map. Automatic detection of the wounds, which would eliminate the need for the seed pixel by the user, is also under consideration. The proposed RYKW map is conveniently suited to achieve this by first identifying potential wound region throughout the entire image based on color information, before carrying out advanced analysis to filter the false-positive regions. Finally, the ultimate goal of the wound software is not only to be able to characterize the wound at a single time but also at multiple time periods. By comparing the wound characteristics from the first visit to the second and subsequent visits, as well as taking into account the demographic information of the patient (age group, gender, ethnicity) and the type of ulcers (diabetic, venous, pressure), the healing rate can be estimated. This would be a significant breakthrough in wound healing management.

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Extracellular Matrix and Other Factors that Impact on Cutaneous Scarring

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1 Introduction

The repair process of wounded skin involves an intricate orchestration between resident and immigrant cells and their local epidermal and dermal microenvironments. This wound repair, also known as incomplete regeneration, refers to tissue adaption after injury with scar tissue, with some consequent loss in morphology and function. Although scar formation allows for the rapid sealing of an injured area, it can frequently prove problematic—they are aesthetically unpleasing, more prone to UV radiation, lack sweat and hair glands, and cause issues with strictures and contractions. Scarring is a morphologic as opposed to a biochemical problem, with normal molecular composition but an abnormal structural organization of the extracellular matrix (ECM). ECM constituents are essential components of the wound repair phenomenon. They not only create a provisional matrix that supports each stage of the healing process but also regulate cellular functions, mediate the cell-cell and cell-matrix interactions, and serve as a reservoir and modulator of cytokines and growth factors [1]. The nature of the ECM impacts skin cells both through specific signaling/attachment domains and via physical aspects such as stiffness and elasticity, which contribute to controlling the outcome of healing—one of repair or regeneration. Complete regeneration refers to an exact replacement of the damaged tissue, such that both morphology and function are restored [2]. While regeneration is mostly limited to invertebrates and lower vertebrates such as salamanders [3], scarless cutaneous healing has also been reported in the fetuses of mammals such as rats, mice, pigs, monkeys, and even humans [4, 5]. Many studies have shown that fetal and adult wounds heal via different mechanisms [6–8]. Early- to mid-gestation fetal healing is characterized by rapid reepithelialization, a lack of inflammation, and restoration of normal tissue architecture. In contrast, adult and late-gestational mammalian skin repairs with an inflammatory and fibrotic response that leads to scar formation [8]. The ECM of fetal skin differs from that of adult skin [6, 8]. Although collagen type I is the principal component of the ECM in both fetal and adult skin, fetal skin contains a greater ratio of collagen type III to I [9] with higher amounts of glycosaminoglycans (GAGs), hyaluronic acid (HA), and chondroitin sulfate [8, 10, 11].

In recognizing the phenotypic differences between wound repair and regeneration, it becomes therefore important to explore the distinction between these two physiologies at cellular and molecular levels (Table 1). An understanding of scarless fetal regeneration may one day enable its recapitulation in adult biology.

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Table 1 Differences in wound repair (incomplete regeneration) and complete regeneration through the four stages of healing

	Repair	Regeneration
Hemostasis	Platelets aggregate to form a clot Platelets produce large amounts of PDGF, TGF-β1, TNF-α, IL-1, and IL-6	Decreased platelet aggregation and degranulation Platelets produce less fibrogenic PDGF, TGF-β1/TGF-β2
Inflammation	Intensive Large numbers of leukocytes present in wound	Minimal Reduced or no inflammatory infiltrates, no lymphocytes
Proliferation	Fibroblasts: • Can be converted to myofibroblasts • Higher capacity to contract • Collagen synthesis is delayed, while fibroblasts take time to proliferate and migrate	Fibroblasts: • Synthesize and deposit a mature, well-organized dermal matrix • Proliferate and migrate at a faster rate • Simultaneously proliferate and synthesize collagen
	Myofibroblasts present	No myofibroblasts
	Keratinocytes: • Reepithelialize through extension of lamellipodia followed by wound-edge keratinocyte migration • Reepithelialization proceeds at a relatively slower pace	Keratinocytes: • Keratinocytes assemble an actin cable that functions like a purse-string to close the wound • Keratinocytes migrate faster, reepithelialization proceeds rapidly
	Larger amounts of granulation tissue, excessive vascularity Wound closure is via wound contraction	Reduced formation of vascularized granulation tissue Wounds close in a purse-string fashion
Remodeling	Scar formation with loss of skin appendages Restores only 70% of the tensile strength of normal skin	Scarless wound healing Completely restores form and function identical to that of the original skin

PDGF platelet-derived growth factor, *IL* interleukin, *TGF* transforming growth factor, *TNF* tumor necrosis factor

And manipulation of the ECM and cells/cytokines within it may lead to new therapeutic strategies for improving adult wound healing.

This chapter will highlight vital components of the cutaneous ECM and describe in depth their interactions and contributions to wound healing, inflammation, and pathological scarring, along with other important players in these processes—matrix metalloproteinases (MMPs) and inhibitors, growth factors/cytokines, various resident and immigrant cells, and microRNA (miRNA). Finally these factors will be discussed in the context of scarless fetal healing.

2 ECM in Skin and Its Remodeling

2.1 Defining the ECM

The skin is the largest organ in the human body, acting as a natural barrier against the outside environment to maintain internal homeostasis. Its cellular content comprises mainly keratinocytes, fibroblasts, and endothelial cells, with the ECM making up most of the remaining volume. In a physiological context, the ECM is a dynamic structure comprised of collagens, laminins, elastin, and fibrillin/fibronectin, embedded in a viscoelastic gel of proteoglycans (PGs) and other miscellaneous glycoproteins. This composition is in a constant state of flux during development and disease, controlled by the coordinated synthesis and turnover of its constituent components. When homeostasis between matrix synthesis and degradation is lost, skin fibrosis may result.

The ECM provides several diverse and vital roles including mechanical and chemical support for the hydration; cushioning and protecting the skin against external stresses [12]; structure, organization, and orientation for cells and tissues; control of morphogenesis and cellular metabolism by acting as a template for cell migration, proliferation, apoptosis, differentiation, and adhesion; regulation of cellular activity and function via direct binding to integrins and

other cell-surface receptors [13]; and a reservoir to house and regulate the bioactivity of growth factors and cytokines [14]. Recent studies also demonstrate that ECM proteins are key components in shaping the stem cell niche to maintain stem cell homeostasis and direct lineage commitment [15]. In return, the cells remodel the ECM, allowing these events to take place.

Cutaneous ECM consists of the structural and nonstructural proteins. The former consists mainly of collagen, laminin, elastin, and fibrillin/fibronectin, which govern the skin's rigidity and elasticity and facilitate cell adhesion and migration [16]. The nonstructural proteins include the PGs (decorin, lumican, dermatopontin, and HA), which possess a high water-binding ability, with important roles in hydration, pH buffering, and force dispersion within the skin. Through these they stabilize growth factors and create a charged, dynamic, and osmotically active three-dimensional space [17]. Additionally, there are a group of ECM matricellular proteins including osteopontin, secreted protein acidic and rich in cysteine (SPARC) (also known as osteonectin), tenascin-C, fibulins, and the CCN family [16]. They do not significantly contribute to ECM mechanical structure and can be absent in healthy skin, being expressed temporarily only after skin wounding. These proteins interact with each other and the local cells which produce them, by entanglement, cross-linking, and charge-dependent interactions to form a network between cells [16, 18]. ECM proteins are discussed in more detail below.

2.2 ECM Structural Proteins

2.2.1 Collagens

Collagen is the major component of skin ECM, comprising 77% of the fat-free dry weight of the human skin [19]. Collagens are synthesized by cells, particularly fibroblasts, as procollagens containing N- and C-propeptides at each end of a triple helical domain. This synthesis requires specific posttranslational enzymes including three collagen hydroxylases, two collagen glycosyltransferases, two specific proteinases to cleave the N- and C-propeptides from the procollagen molecules, and one specific oxidase to form a complex series of cross-links [20, 21]. Through hydrophobic and electrostatic interactions, collagen monomers form a quarter-staggered arrangement, which aggregate into five-stranded fibrils and subsequently into larger fibrils. The molecular arrangement into fibrils is additionally stabilized by the formation of covalent cross-links which finally contribute to the mechanical resilience of collagen fibrils. In the dermis, the fibrils orientate to form a complex network of interlaced basket weave-like fibrils. Collagen fibrils may consist of more than one collagen type. For example, the collagen type I fibrils often contain small amounts of types III, V, and XII [22]; and the collagen type II fibrils of cartilage can contain types IX and XI. Collagen types V and XI can further form hybrid molecules [23]. Twenty-nine different types of collagens have been identified with all members displaying such a similar triple-helix structure held together by interchain hydrogen bonds [24, 25]. Collagens can broadly be divided into fibrillar and nonfibrillar families.

The fibrillar collagens include highly expressed types I–III; the quantitatively minor collagen types V, XI, XXIV, and XXVII [26]; and basement membrane collagen type VI. Types I, III, and V are distributed widely in non-cartilaginous tissues, and types II and XI are found almost exclusively in the cartilage and in the eye. Fibrillar collagens are abundant in skin dermis and have enormous tensile strength [27]. Among them, types I and III are the principal collagens of both adult and fetal skin, albeit in differing ratios. Adult skin collagen content is divided into 85–90% type I, 8–11% type III, and 2–4% type V, whereas during gestation, fetal skin collagen consists of 70–75% type I, 18–21% type III, and 6–8% type V [28]. As the fetus develops, the collagen profile of the skin transitions to that of postnatal adult phenotype with lower type III to I ratio. Interestingly, this correlates with the transition from scarless regeneration to repair with scar formation [9, 29, 30]. Collagen type V is present in tissues where collagen type I is expressed. Collagen type II is an essential component of the cartilage ECM, present in the developing cartilage anlagen and

essential for endochondral bone formation. It is expressed in the epidermal-dermal junction of scalp skin at the middle stages of the human fetus and then disappears during the subsequent development and maturation [31].

Nonfibrillar collagens include those that form networks of different topologies (types IV, VIII, and X), beaded filaments (types VI, XXVI, and XXVIII), or anchor fibrils (type VII), the fibril-associated collagens with interrupted triple helices (FACIT) (types IX, XII, XIV, XVI, and XIX–XXII), and some transmembrane proteins (types XIII, XVII, XXIII, and XXV) [32]. These collagens do not form classic fibrils but instead compose reticular nets, connect cells to the basement membrane, or help in the organization of other collagen fibers. For example, collagen type IV forms the basal lamina enabling keratinocyte and fibroblast migration and adhesion to the basement membrane. Type VII secures the attachment of the epidermis to the dermis, is required for reepithelialization, supports dermal fibroblast migration, and regulates their cytokine production in the granulation tissue in wound healing [33]. Type XIV modulates fibroblast and preadipocyte growth and differentiation [34]. Type XII has been postulated to organize the ECM architecture of the skin [35].

The essential functions of various collagens have been supported by the large number of genetic disorders caused by mutations in their genes [23]. For example, collagen type III-deficient mice display very severe spontaneous skin wounds, with nonuniform diameter of their collagen fibrils [36]. Alport syndrome is an inherited disorder of collagen type IV, and the classical Ehlers-Danlos syndrome is caused by type V gene mutations indicated by skin fragility and abnormal wound healing [37]. A subset of the skin-blistering diseases, epidermolysis bullosa, is caused by a collagen type VII gene defect [38]. The skin of transgenic mice of type XII revealed a lack of matrix fiber structure in the papillary dermis [35]. The skin from collagen type XIV null mice significantly decreased maximum stress and shows a trend toward decreased modulus [39]. There are a number of human skin diseases associated with collagen mutations [40].

The presence, absence, or proportion of specific collagen types can further affect other collagens and skin function. Collagen type III-deficient mice exhibit greater myofibroblast differentiation and more pronounced wound contracture, with abnormal type I fibrillogensis in the skin, indicating that collagen type III may play a regulatory role in normal type I synthesis [36]. Similarly collagen type V has been shown to regulate type I fiber assembly, with mice deficient in type V having abnormally large collagen fibrils [41]. Both collagen types III and V modulate the expression and assembly of fibronectin in the ECM of defective Ehlers-Danlos syndrome fibroblasts [42]. A higher ratio of type III to I correlates with the observation of scarless fetal wound healing [9, 29, 30, 43], whereas a low ratio is associated with human keloids and hypertrophic scars (HTS) [44–46].

The small leucine-rich repeat PGs (SLRP), decorin, lumican, keratocan, and fibromodulin, are known to associate with collagen fibrils. For example, targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility [47]; the absence of lumican results in a significant proportion of abnormally thick collagen fibrils in the skin [48], suggestive of a role in the regulation of fibrillogenesis.

A summary of collagens and other structural ECM proteins can be found in Table 2.

2.2.2 Laminins

Laminins are high molecular weight (~400–900 kDa) cell adhesion molecules that comprise a family of glycoproteins found predominantly in basement membranes of the epithelium and endothelium, and surrounding muscle, Schwann, and fat cells [49]. They are the first ECM glycoprote in that appear in the developing early embryo. Laminins constitute the association of three genetically different polypeptides, α, β, and γ chains, the heterotrimeric subunits intersecting to form a cross-like structure. Laminins interact with collagen type IV, integrins, dystroglycans, and other components of the basal membrane matrix and the underlying interstitial stroma. They contribute to the overall ECM

Table 2 Main structural ECM proteins

		Source	Location	Function	Disorders associated with mutations
Collagen (70–80% dry skin)					
Fibrillar	I (85–90%)	Fibroblasts	Bone, skin, tendons, ligaments, cornea	Principle collagen maintaining skin structure and strength	Ehlers-Danlos syndrome, osteogenesis imperfecta
	II	Fibroblasts	Cartilage, intervertebrate disk, notochord, vitreous humor in the eye	Principle collagen in cartilage	Chondrodysplasias
	III (8–11%)	Fibroblasts	Skin, blood vessels	Principle collagen maintaining skin structure and strength	Ehlers-Danlos syndrome, Dupuytren's contracture, KO mice exhibit severe spontaneous wounds, and nonuniform collagen fibril diameters
	V (2–4%)	Fibroblasts	Fibril assembly with type I Basement membrane	Stabilizes the epidermal-dermal junction	Ehlers-Danlos syndrome, thickened skin
	XI	Fibroblasts	Fibril assembly with type II		Stickler syndrome, Marshall syndrome
Nonfribrillar	IV (2–4%)	Fibroblasts	Basal lamina	Forms a sheet-like network that enables keratinocyte and fibroblast migration and adhesion to the basement membrane	Alport's syndrome, Goodpasture's syndrome
	VI (<1%)	Fibroblasts	Papillary dermis	Resists tensile stress Maintains barrier function	Atopic dermatitis, trichothiodystrophy
	VII (<1%)	Fibroblasts	Basement membrane	Anchors fibrils to stabilize the lower part of the basement membrane to the underlying dermis	Epidermolysis bullosa dystrophica
FACIT	XII	Fibroblasts	Tendons, ligaments	Organizes collagen architecture	Lack of dermal matrix fiber structure
	XIV (<1%)	Fibroblasts	Dermal hair follicles	Modulates cell-matrix adhesion	Decreased stretch
	XVI (<1%)	Fibroblasts	Papillary dermis	Anchors fibrils to the basement membrane	Epidermolysis bullosa
	XVII (<1%)	Fibroblasts	Basal keratinocytes	Maintains adherence of the epidermis to the basement membrane	Epidermolysis bullosa, bullous pemphigoid
Laminins		Fibroblasts endothelial cells	Basal lamina	Binds to cell membrane and other ECM molecules to influence cell adhesion, migration, differentiation, and tissue phenotype	Congenital muscular dystrophy, junctional epidermolysis bullosa, nephrotic syndrome

(continued)

Table 2 (continued)

	Source	Location	Function	Disorders associated with mutations
Elastin (3–4% of the dry skin)	Fibroblasts, keratinocytes, smooth muscle cells	Skin, arteries, lungs, ligaments, cartilage, bladder	Provide elastic recoil and resilience	Supravalvular aortic stenosis, autosomal dominant cutis ataxia, Marfan syndrome, emphysema, KO mice incompatible with life
Fibronectins	Fibroblasts, keratinocytes, endothelial cells, leukocytes	Plasma, skin	Forms a hemostatic clot that serves as a provisional matrix in wound healing, binds cells to the ECM	KO mice incompatible with life

FACIT fibril-associated collagens with interrupted triple helices, *KO* knockout

structure and influence adhesion, differentiation, migration, phenotypic stability, and survival in local cells [50].

In the skin, laminin together with collagen type IV constitutes the bulk of the epidermal basement membrane ECM [51]. Laminins form weblike structures that remain in close association with cells through interactions with cell-surface receptors and resist tensile forces in the basal lamina. They support the migration and stable adhesion of keratinocytes, play key regulatory roles in the development of skin appendages, and contribute to the pathogenesis of skin cancer [52].

2.2.3 Elastin

Elastin forms a three-dimensional network that is closely interwoven with collagen fibers—an arrangement allowing it to be the major ECM component in the skin that endows resilience, permitting long-range deformability with passive recoil [53]. In mature skin, the elastin comprises approximately 3–4% of the dry weight of tissue [54]. Elastin is assembled in short repeated three to nine amino acid sequences that form flexible and highly dynamic structures [55, 56]. Elastin is synthesized by fibroblasts, keratinocytes, and smooth muscle cells. Fibroblasts are the major cell type in the dermis and are involved in elastogenesis, a process occurring in the superficial dermis. During this process, a tropoelastin monomer is synthesized as a soluble precursor; this undergoes a post-translational modification and forms a complex with elastin-binding protein before being released on the cell surface. In the extracellular space, tropoelastin is then cross-linked by the enzyme lysyl oxidase (LOX) leading to the formation of mature fibers [57], which confers the elastic recoil and resilience properties of the skin. The reticular dermis of the skin contains thick, horizontally arranged elastic fibers, whereas the papillary dermis contains thinner perpendicular elastic fibers (elaunin fibers) that merge with the microfibrillar cascade (oxytalan fibers) and intercalates into the dermal-epidermal junction. This continuous elastic network imparts elasticity throughout the skin from the reticular and papillary dermis to the epidermis. Elastic fibers regulate activity of transforming growth factor (TGF)-βs through their association with fibrillin microfibrils; they further play a role in cell adhesion, migration, survival, and differentiation and can to an extent act as a chemotactic agent [58, 59].

Humans are unable to adequately regenerate and repair destroyed elastin fibers from severe wounds [60]. Post severe wounding, elastin expression in repaired dermis may take 4–5 years and is both functionally and spatially disorganized [61, 62]. These long-lasting structures of elastic fibers begin to assemble during mid-gestation, with little adult elastic fiber assembly. The turnover of elastin in an adult is very low with its half-life probably exceeding the lifespan of the individual [63]. Once these fibers are lost, the skin loses definition of its elasticity.

Gene mutations of elastin can be divided into two groups [64]. Loss-of-function mutations, such as premature stop mutations, large intragenic deletions, and complete gene deletion, lead

to supravalvular aortic stenosis (SVAS, OMIM 185500) and eventual cardiac failure and death [59]. A second group of elastin mutations is an autosomal dominant form of cutis laxa (ADCL, OMIM 123700) and arises from nucleotide deletion, insertion, or exon-splicing errors that produce missense sequence. Missense mutations interfere with normal assembly, metabolism, and function of elastic fibers [65, 66]. The skin is the major organ affected in ADCL [59, 67]; mutations of this type can also result in abnormalities in other organ systems [28–31]. A complete lack of elastin in the body is fatal. Elastin knockout mice die shortly after birth with subendothelial cell accumulation blocking blood flow and with markedly increased arterial stiffness [59, 64].

2.2.4 Fibronectin

Fibronectin is a widely distributed multidomain glycoprotein present in most ECMs. This dimer protein is composed of two similar polypeptide subunits of ~230–250 kDa linked by a pair of disulfide bonds [68]. There are two forms of fibronectin: plasma fibronectin, which is synthesized in a soluble form by hepatocytes into the blood plasma, and cellular or tissue fibronectin, which is produced by resident skin cells such as fibroblasts, endothelial cells, and keratinocytes [69]. Along with fibrin, soluble plasma fibronectin molecules are deposited in the acute wound to form a provisional clot; this is later replaced by fibroblast-secreted cellular fibronectin which assembles into stable, insoluble, supermolecular fibrils [69]. Fibronectin binds to cell surfaces via integrins, which link extracellular fibronectin to the intracellular actin filaments. The multidomain structure of fibronectin allows binding to both cell-surface receptors, such as integrins, and to collagen, PGs, and other focal adhesion molecules, thereby mediating the assembly of other ECM proteins including collagen types I and III, thrombospondin-1, and microfibrils, as well as playing an important role in fibrillogenesis in regard to initiation, progression, and maturation of matrix assembly [70, 71]. Fibronectin also regulates the proteolytic activation of LOX, the enzyme responsible for covalent cross-linking of collagen and elastin fibrils [72].

Mice lacking fibronectins die near embryonic day 8.5 due to severe defects in mesodermally derived tissues [73]. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia, but skin wounds heal normally in plasma fibronectin-null mice [74].

3 ECM Nonstructural Proteins

3.1 PG/Glycosaminoglycan (GAG)

PGs refer to a heterogeneous group of polyanionic macromolecules that consist of a protein core, to which a variable number of linear sulfated GAG chains are bound covalently. PGs maintain ECM hydration and allow permeability of low molecular weight solutes. By interacting with other ECM components, PGs play critical roles in organizing the matrix [17]. They affect collagen organization and fibrillogenesis by binding to specific sites on collagen and controlling its rate of degradation. PGs also participate in cell-cell interactions, in cell proliferation and migration, and in cytokine and growth factor signaling associated with wound healing. The common PGs are discussed below.

3.1.1 Decorin

Decorin is the most prevalent PG in adult human skin where it effectively binds collagen and is required for normal collagen fibrillogenesis [75, 76]. Decorin knockout murine embryonic fibroblasts have greater proliferation and increased adhesion to collagen and fibronectin than wild-type cells [77], and these knockout mice exhibit a delayed healing of both excisional and incisional full-thickness dermal wounds [78]. In addition, decorin can also act as a natural inhibitor of TGF-β1; thus it is associated with decreased fibrosis and formation of normal scars [79]. Decorin levels are further reduced keloids and HTS as compared to normal skin [80–82], with fibroblasts from post-burn HTS tissue synthesizing less decorin than normal dermal counterparts [83]. A delay in the appearance of decorin has also been observed in the normal healing of burn wounds [84].

3.1.2 Lumican

Lumican is a translucent PG that is abundant in the cornea, and is also present in the skin and other connective tissue, where it plays pivotal roles in wound healing and skin collagen fibrillogenesis [85]. Lumican secreted by fibroblasts promotes fibrocyte differentiation wound fibroblast activation and skin wound; its deficiency in mice results in corneal opacity, skin and tendon fragility, and disorders of leukocyte migration [86]. Lumican is significantly reduced in HTS tissues and HTS fibroblasts compared to normal skin or cells. TGF-β inhibits lumican expression, while lumican upregulation effectively reduces the scar area and inhibits fibroblast proliferation in vitro and alleviated HTS in a rabbit model [87]. These data suggest that lumican may play an important role in fibrotic healing.

3.1.3 Dermatopontin

Dermatopontin is a recently described SLRP that increases tensile strength and collagen fibrillogenesis [88]. It also promotes dermal fibroblast attachment and cytoplasmic spreading on fibrin matrices and accelerates fibroblast adhesion to the provisional matrix in the initial stage of wound healing [89]. Dermatopontin is decreased in HTS and systemic sclerosis skin fibroblasts [90], in keloids [91], and in chronic cutaneous wounds [92]. Dermatopontin-null mice have increased skin elasticity, a thinner dermis, and 40% lower collagen content, with nonuniform diameters and irregular contours [88]. These data suggest dermatopontin is critically involved in skin elasticity and collagen fibrillogenesis.

3.1.4 Hyaluronic Acid (HA)

HA is one of the most common GAGs and the only GAG that is exclusively non-sulfated in the skin. HA is prevalent in the basement membrane and intercellular spaces between basal keratinocytes, where it augments diffusion of water and nutrients to supply epidermal stem cells, and is the key molecule involved in maintaining skin moisture. In the extracellular space, HA causes tissues to resist compression by absorbing water to provide a turgor force, particularly notable in the ECM of load-bearing joints. In the epidermis, it forms a cross-weaved, interconnected reticular pattern, whereas in the dermis it is in a striated pattern, parallel to the flattened epidermis, and is associated with blood vessels [93, 94]. HA not only provides structure and viscosity to the ECM but is also involved in early epithelial-mesenchymal transition (EMT) in development and morphogenesis, cell signaling, wound repair and regeneration, matrix organization, and pathobiology.

Under normal conditions, HA is present in its high molecular weight (HMW-HA) form with an average size range of $1–10 \times 10^3$ kDa. In addition to its role in maintaining tissue hydration and osmotic balance, HMW-HA is associated with decreased inflammation, increased expression of collagen type III, and increased activity of anti-fibrotic TGF-β3 [93, 95]. Conversely, low molecular weight HA (LMW-HA) or HA fragments that result from degradation of intact HMW-HA have been linked with increased inflammation, greater collagen type I expression, increased proliferation of fibroblasts, and increased myofibroblast differentiation [96, 97]. HA exerts a wide range of different regulatory functions by interacting with the membrane receptor, CD44 [95], a ubiquitously expressed glycoprotein present on most mammalian cells.

HA is synthesized by three HA synthase (HAS) enzymes in mammals: HAS1 and HAS2, which produce mostly HMW-HA, and HAS3, which produces LMW-HA to intermediate MW-HA [98]. HAS-1 is highly expressed in fibroblasts, HAS-3 in keratinocytes, and HAS-2 in both cell types. The turnover of HA occurs rapidly, and the enzymatic degradation of HA results from the action of five functional hyaluronidases (Hyals) with Hyal1 and Hyal2 considered the main active enzymes in tissues. In addition, HA can be degraded by oxidation reactions, particularly reactive oxygen species and free radicals [99].

4 ECM Remodeling

The ECM is a highly dynamic structure that is continuously remodeled by resident cells during development, homeostasis, and tissue repair.

ECM remodeling comprises the production, deposition, and organization of ECM molecules, balanced by degradation and rebuilding of the existing ECM [16, 100]. Remodeling of existing ECM is mediated by LOX that cross-link collagens and elastins. The existing ECM's dynamic degradation is mainly mediated by MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [101–103]. MMPs are a family of zinc-dependent, multidomain families of proteases that tightly control ECM remodeling during many physiological and pathological processes, including tissue repair. This family comprises 23 human members that are either secreted or membrane-bound and have the combined capacity to degrade virtually all ECM components. According to their substrate specificity, MMPs can be classified into distinct subgroups: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), membrane-anchored MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and a heterogeneous group containing matrilysin (MMP-7), metallo-elastase (MMP-12), enamelysin (MMP-20), endometase (MMP-26), and epilysin (MMP-28). Collagenases mainly digest collagen types I, II, III, IV, and V, elastin, and gelatines, whereas MMP-3, MMP-7, and MMP-10 degrade PGs, fibronectin, collagen types IV and IX, and laminin [104]. See Table 3 for details about MMP substrates.

Although primarily responsible for turnover and degradation of ECM substrates, the spectrum of MMP substrates goes well beyond ECM components. MMPs can process a large number of non-ECM components including cell adhesion molecules, growth factors, cytokines, chemokines, tyrosine kinase receptors, and other MMPs [104, 105], therefore regulating tissue repair and cell differentiation and transformation. Furthermore, some MMPs may have functions that are independent of their catalytic activity [104, 105]. The vital functions of MMPs in wound healing are supported by the severe effects of various MMP mutations (Table 3).

ADAMTS are secreted zinc metalloproteases with an ancillary domain containing one or more thrombospondin-1 repeats. They are synthesized as inactive zymogens and are activated by the furin cleavage of N-terminal propeptide. This superfamily includes 19 distinct members and is collectively referred to as proteoglycanases, participating in proteolysis of the large aggregating PGs aggrecan, versican, and brevican [103, 106]. ADAMTS regulate ECM turnover and development [107]. The vital functions of ADAMTS are supported by the identification disorders caused by their mutations. For example, ADAMTS-13 mutations produce inherited thrombotic thrombocytopenic purpura characterized by widespread microvascular thrombosis involving the capillaries and arterioles [108]. ADAMTS-2 mutations create Ehlers-Danlos syndrome type VIIC with extreme skin fragility, characteristic facies, joint laxity, lax skin, umbilical hernia, and blue sclera [109].

MMP activity is regulated by four tissue inhibitors of metalloproteinases (TIMPs), namely, TIMP-1 to TIMP-4. TIMPs inhibit MMPs in a 1:1 inhibitor to enzyme ratio through interaction of the N-terminal domain of the TIMP molecule with the active site of the MMP [110]. TIMP-3 is the most significant endogenous inhibitor of ADAMTS.

In the skin, TIMP-1 is synthesized by keratinocytes, fibroblasts, smooth muscle cells, and endothelial cells and especially targets MMP-1. TIMP-2 is synthesized by fibroblasts and endothelial cells and preferentially acts as an effective inhibitor of MMP-2 and is required for the cellular mechanism of pro-MMP-2 activation [111]. TIMP-3, which inhibits the activities of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13, is expressed by macrophage-like cells of granulation tissue and by endothelial cells. Both TIMP-2 and TIMP-3 are expressed by skin epidermal keratinocytes and fibroblasts. Occasional blood vessels of acute wounds are positive for TIMP-1, TIMP-2, and TIMP-3. No TIMP-4 expression appears to be present in acute human wounds [112].

Table 3 Matrix metalloproteinases and their roles in wounds

		Substates	Source	Function	Mutations/deficiency in healing	In pathological scars
Collagenases	MMP-1	Collagen types I, II, III, V, VII, X, XI Gelatines	Keratinocytes Fibroblasts Platelets	Keratinocyte migration Collagen remodeling	Not found	Decreased in HTS; increased in keloid
	MMP-8	Collagens types I, II, III, VII, VIII, X Gelatines	Neutrophils Macrophages Fibroblasts	Mitigate scarring	Delayed wound healing, persistent inflammation	Increased in HTS; decreased in keloid
	MMP-13	Collagen types I, II, III, IV, IX, X, XIV	Fibroblasts Migrating keratinocytes	Keratinocyte migration, vascularization, myofibroblast differentiation, granulation tissue formation, wound contraction	Delay in granulation tissue growth and formation of large blood vessels	Decreased in HTS; increased in keloid
Gelatinases	MMP-2	11.14.1.1.1.1.1.1. Collagen types III, IV, V, VII, X, XI, XIV Gelatines Fibronectin	Fibroblasts Keratinocytes Endothelial cells Macrophages Platelets	Migration and invasion of fibroblasts	No affect on wound healing	Decreased in HTS; increased in keloid
	MMP-9	Collagen types I, IV, V Gelatines Laminin	Keratinocytes Neutrophils Macrophages	Migration of keratinocytes	Impaired reepithelialization, wound closure	Decreased
Stromelysins	MMP-3	PGs Collagen types I, III, IV, V, IX, X Gelatines Laminin Fibronectin	Fibroblasts Macrophages Keratinocytes	Regulates fibroblast contraction	Wounds fail to contract and heal more slowly	Increased in HTS but decreased in keloid
	MMP-10	Collagen types I, III, IV, V, IX, X Gelatines Laminin	Keratinocyte Fibroblasts Macrophages	Regulates collagenolytic activity of alternatively activated (M2) resident macrophages	Increased scar formation	Not found

Metalloelastase	MMP-12	Collagen type IV Gelatines Elastin	Macrophages Endothelial cells	Antimicrobial activity, macrophage-mediated ECM proteolysis and tissue invasion	Attenuation of dermal fibrosis	Not found
Membrane type MMP	MMP-14	Collagen types I, II, III Gelatines Laminin-5 Fibronectin Fibrin Fibrinogen	Keratinocytes	Activation of pro-MMP-2	Impaired keratinocyte migration and increased apoptosis, impaired capillary formation, fibroblasts incapable of degrading collage type I matrices	Increased in keloid
Others	MMP-19	Collagen type IV Gelatines	Keratinocyte Fibroblast Endothelial cells		Not found	Comparable to normal skin in HTS; increased in keloid

HTS hypertrophic scar, *MMP* matrix metalloprotein, *PG* proteoglycan

4.1 Wound Healing and ECM Remodeling

After injury, the skin immediately initiates a complex and progressive process that includes hemostasis, inflammation, proliferation, and remodeling—leading to the restoration of its integrity and formation of new tissue [113].

Platelet-driven hemostasis is the first event, producing a blood clot as a provisional ECM. This temporary matrix comprises of cross-linked fibrin, fibronectin, SPARC, vitronectin, and thrombospondin [114]. It importantly serves as a physical barrier that protects the wound, limits blood loss, provides a reservoir of growth factors and cytokines/chemokines, and acts as a scaffold structure for the migration of immune cells and residential skin cells such as keratinocytes and fibroblasts during the wound repair process [99, 115].

This invasion of inflammatory cells allows removal of debris, foreign particles, and bacteria and release of cytokines/chemokines, growth factors, proteinases, and reactive oxygen species—amplifying inflammation and heralding subsequent phases of healing [116]. Additionally, these proteases degrade both the boundary ECM and the fibrin clot, supporting tissue ingrowth and releasing trapped growth factors for the early events of proliferation [117]. Proteases such as MMPs also provide other functions to the wound-healing process, such as regulating inflammation [104, 105].

After 2–3 days, a temporary collagen matrix enriched in PGs, GAGs, and noncollagenous glycoproteins, particularly HA and fibronectin, replaces the fibrin clot [99, 118]. The HA molecules create a woven structure that enables the incoming cells to penetrate the wound area, while fibronectin provides a scaffolding that facilitates collagen fibrogenesis. The proliferative phase now begins as the inflammatory phase withdraws. The main foci of the proliferation phase are to cover the denuded wound surface with new granulation tissue, to restore the vascular network by neovascularization, and to reepithelialize the epidermis. During this phase, fibroblasts are recruited to the injury site, synthesizing a wide range of ECM molecules to begin restoring structure and function to the area. Within the wound, HA is initially synthesized in large amounts by the fibroblasts, and collagen deposition then takes over within its fibronectin scaffolding [99, 118]. The tensile strength of collagens provides stability to any newly created granulation tissue [117]. This provides the setting for ingrowth of new blood vessels that allows leukocytes to enter the wound site and provides nutrition and oxygen to the growing tissue [119, 120]. These new vessels (or neovascularization) are the result of both angiogenesis (sprouting of capillaries from existing blood vessels) and vasculogenesis (mobilization of bone marrow-derived endothelial progenitors) [120, 121]. At the skin surface, keratinocytes migrate and proliferate to reepithelialize the wound on top of this new matrix. Keratinocytes produce collagen type IV and laminins toward their basement membrane, which in turn anchors the cells, further driving maturation [117, 118].

Once the wound is closed, physiological wound healing enters the final remodeling phase, during which the wound gradually contracts over time to regain integrity [2, 117, 118]. This remodeling is primarily mediated by (myo)fibroblasts and is regulated through various MMPs along with their inhibitors. The process may last for 12 months or longer depending on the severity of the wound. During this phase, synthesis of ECM components is reduced, and the disorganized ECM molecules laid down during the proliferative phase are realigned and cross-linked [117]. Collagen type I is increased, cross-linked, and oriented as small parallel bundles [40]. With continued remodeling, the outgrowth of capillaries is halted, blood flow to the area is reduced, and metabolic activity declines [122]. Dermal cell numbers are reduced with some myofibroblasts and vascular cells undergoing apoptosis. For most adult mammals, including humans, the normal outcome of both cutaneous and subcutaneous wounds is a fibrotic scar with excessive synthesis and deposits of ECM [2, 117, 118]. The strength of this tissue at best remains 70–80% of its original, uninjured form, with much less flexibility.

5 Excessive Cutaneous Scar Formation

Although a scar is the normal outcome of adult wound healing, excessive scar formation can be problematic, particularly when they occur across joints where they impair mobility and flexibility; on conspicuous locations, such as the face, which can result in devastating psychological consequences; and in children.

Uncontrolled synthesis, localization, and organization of ECM molecules can lead to abnormal scar tissue formation [94]. Excessive collagen synthesis and deposition results in fibrotic tissue accumulation manifested as a HTS or keloid in humans [94]. Fibrosis is defined by the overgrowth, hardening, and scarring of various tissues and is attributed to excess deposition of ECM components, primarily the fibrillar collagens [40, 123]. Collagen type I, fibronectin, and laminin are increased in hypertrophic and keloid scars when compared with immature scars and normal skin [124]. However, decorin, dermatopontin, and HA expression are decreased, and the dermal expression and localization of fibrillin and elastin fibers are altered [40, 99, 124].

Keloids are visualized as scars that grow beyond the boundaries of the original wound and rarely regress over time. They often occur in predisposed individuals with an incidence of 6–16% in African populations [125]. In contrast, HTS generally grow within the boundaries of the original wounds and appear histologically as dermal nodules, frequently regressing over time [125]. HTS represent abnormal healing responses secondary to burn injuries, traumatic injuries, and surgical procedures. Incidence rates vary from 44% following surgical wounds to up to 91% following burn wounds, depending on the depth of the wound [125–129]. These scars have raised red appearances in the skin and can cause pain, pruritus, contractures, cosmetic disfigurement, and physical impairment with multiple vital functions at risk. Often they ultimately lead to psychological stress such as low self-esteem, job discrimination, prejudicial societal reactions, isolation, depression and other psychiatric comorbidities, and decreased quality of life [130–132]. Scar contractures following burn injury are well known to cause microstomia, nasal stenosis, and lip or eyelid ectropion if severe enough. They can lead to restriction of neck movement and permanent mouth opening. Thus, scars represent a significant source of morbidity and frequently require aggressive measures to deal with their sequelae.

Even though HTS and keloids have a profound impact on the duration and quality of life, to date, no therapy has been found to reverse or arrest the progression of skin fibrosis.

While the exact etiology of keloid and HTS formation remains unclear, the pathogenesis of these scars clearly involves local conditions such as delayed wound healing, wound depth, chronic inflammation, and the skin tension around the scars. Firstly, prolonged healing is an important contributing factor for abnormal scaring. Wounds that heal within 2 weeks normally do not develop HTS, whereas a burn wound that takes beyond 3 weeks to heal has a 70% or greater risk of developing into a HTS [126, 133, 134]. Next, superficial injuries that do not reach the reticular dermis never result in HTS or keloids, suggesting that these pathological scars are due to injury to this skin layer [127]. Interestingly, HTS and keloids may be viewed as inflammatory disorders of the skin, able to be differentiated by the degree of inflammation of the reticular dermis [127, 135]. A persistent inflammatory response often leads to poor wound healing and excess fibrosis. This is relevant to many chronic wounds which are often associated with excess inflammation and become locked in an inflammatory phase. Finally in humans, excessive scarring often occurs in locations characterized by enhanced skin stresses such as the chest and shoulder [136, 137], perhaps driven by regional variations in mechanical force [138].

5.1 Inflammation and Excessive Scar Formation

A theory that has gained momentum in recent years is that scar formation is a result of an overactive inflammatory response [2, 127, 139].

Chronic wounds, often associated with excess inflammation, have a greater risk to develop HTS when compared to normal wounds [126, 134, 140]. Adult oral wounds heal rapidly with minimal inflammation and often with minimal scar formation [141]. Fetal skin wound healing is predominantly a scarless process with the onset of scar-based healing occurring in late gestation, concomitant with an increased inflammatory response [142]. Scar-based healing however does occur in fetal skin when inflammation is provoked, suggesting that the absence of inflammation contributes to the rapid and flawless repair of such wounds [141].

In *Xenopus laevis* tadpoles, the intensity of the inflammatory response and the age-related thymic morphological changes correlates to a reduced in tail regenerative potential [143]. Similarly, young froglets exhibit an age-dependent loss of scarless regeneration associated with a maturing immune system and an altered healing response [144]. Convincing evidence that immunomodulation influences regeneration comes from studies of *X. laevis* tadpoles during the refractory period, a developmental stage where regeneration is briefly inhibited [145]. Amputation of tadpole tails evokes a delayed or prolonged expression of some immune-related genes, whereas there was no obvious or transient expression of other immune-related genes in the regeneration periods. In addition, immune suppression induced by either immunosuppressant treatment or immune cell depletion by PU.1 knockdown restored regenerative ability during the refractory period. These findings indicate that immune responses have a crucial role in determining regenerative ability in *X. laevis* tadpole tails [145]. Similarly wounds also heal rapidly and scarlessly in the PU.1-null mouse, which is genetically incapable of raising an inflammatory response as they lack macrophages and functioning neutrophils [146]. Furthermore, wounds in the spiny mouse (*Acomys* spp.) which are capable of partial skin autotomy to escape predators elicit little or no increase in inflammation with less neutrophils and virtually no macrophages present, permitting a regenerative healing response [147, 148]. This contrasts with a strong, well-characterized inflammatory response in *Mus* mice wounds, which heal with much more scarring [149].

During wound healing, fragments generated from ECM degradation can promote inflammation and collagen production and contribute to events leading to fibrosis [124]. Furthermore, skin fibrosis is also linked to the phenotype of Th2 cell response without inducing local inflammation [150], whereas the inflammatory Th1 response attenuates tissue fibrosis [151]. To date, a number of treatment modalities for skin fibrosis act by reducing inflammation [127].

5.2 ECM in Excessive Scarring

5.2.1 Collagens

Collagen type III fibers, prominent in the early wound-healing process, are replaced by stronger type I fibers as the scar matures [94]. Collagen expression, in particular type I, is higher in both hypertrophic and keloid scar tissue compared with normal skin and immature scars [94]. In keloid scar tissue, collagen fibers appear as disorganized parallel thick bundles with less cross-links compared to normal skin and immature scars, which show a basket-weave network of collagen fibers. The ratio of collagen I to III is also altered in keloid tissue (~17:1) compared to normal scars (~6:1) [152]. In human fetal skin, collagen type III comprises approximately 18–21% of the total collagen but only 8–11% in adult skin [28]. The post-burn granulation tissue of the human skin has a significantly higher ratio of collagen type V to I than that of normal skin [153]. *Acomys* has a collagen profile more similar to that of fetal wounds, with greater expression of collagen types III and V [147].

5.2.2 Laminins

Despite their important role as cell adhesion molecules in the basal lamina, relatively little is known about their involvement in the mechanisms of excessive scarring [154]. Laminin β2 expression is increased in cultured keloid fibroblasts compared with normal fibroblasts [155]. However, no differences were observed in

expression of laminin between hypertrophic and normal breast scars over a period of 12 months [156]. A scarring autoimmune bullous disease was observed in a Ugandan patient with autoantibodies to BP180, BP230, and laminin 5 [157].

5.2.3 Elastin

The level and distribution of elastin is disrupted in abnormal skin scarring. Elastin is reduced in the superficial dermis of both HTS and keloids but is interestingly increased in the deep dermis of keloids [61]. This corresponds with a global decrease in fibrillin 1 [61]. Tsuji and Sawabe revealed few, irregular, thinned elastin fibers present in both atrophic and HTS [158, 159]. These data indicate that there may be a change in the composition of microfibrils during development of excessive scarring that contributes to their distinct biomechanical properties and the decreased flexibility.

5.2.4 HA

Increased expression of HA and its receptor CD44 has classically been associated with skin fibrosis [18, 99]. Levels of HA released by scarring-associated dermal fibroblasts are significantly higher than those released by oral fibroblasts, which are associated with rapid scar-free healing [160]. Yet keloid scars contain less HA and keloid fibroblasts produce less HA than their non-fibrotic counterparts. Such conflicting actions were resolved recently when it was discovered that the size of HA alters its effects. The fetus produces considerably more anti-inflammatory HMW-HA in response to injury than the adult [161, 162]. HMW-HA promotes a fetal-like environment with elevated collagen type III and TGF-β3 expression, while pro-inflammatory LMW-HA favors scar formation with stimulation of collagen type I [97]. In normal scars and normal tissue, HA staining in the dermis is typically in a striated pattern oriented parallel to the flattened epidermis, with the most intense staining in the superficial dermis adjacent to the epidermis, decreasing in the deeper dermis [93]. Mature scar tissue has a similar HA distribution, but in a thinner layer [163]. In HTS tissue, HA occurs mainly as a narrow strip in the papillary dermis. Keloid tissue shows the least HA staining of the papillary layer, in a dense reticular pattern between the unusually thick collagen bundles [93]. In contrast, the thickened granular and spinous layer of the keloid epidermis exhibits an intense HA staining [163]. This correlates with a reduced expression and different staining pattern in fibroblasts cultured from keloid tissue compared to those from normal skin [164]. Inhibition of HA synthesis reduces TGF-β1-driven fibroblast proliferation [160] and transformation to myofibroblasts [165]. HA in the skin also regulates leukocyte influx into the wound area and their associated cytokine production, possibly through the formation of HA-enriched ECM around cells in response to pro-inflammatory agonists that attract and retain inflammatory cells [166]. Failure to remove HA fragments from the site of tissue injury contributes to the unremitting inflammation and destruction observed in tissue fibrosis [99], suggesting that HA and its regulation pathways potentially represent novel targets for anti-fibrotic therapies.

Table 4 is a summary of major ECM changes in excessive scarring.

5.3 MMPs and Excessive Scar Formation

MMP levels are generally low in intact skin but rapidly increase in response to skin injury. In a wound environment, MMPs are the major proteolytic enzymes produced and activated by essentially all cell types participating in wound repair. They contribute to wound healing by cleaving ECM components and releasing biologically active peptide fragments, growth factors, or growth factor receptors on cell surfaces [167, 168], which promote the resolution of the granulation tissue and collagen scar [40, 169, 170]. Reduced MMPs produced at the wound site and an imbalance between MMPs and their natural inhibitors, TIMPs, contribute to disturbed collagen turnover resulting in keloid and HTS development [40, 169, 170]. In acute murine wounds, MMP-3, MMP-9, MMP-10, and TIMP-1 are strongly induced within 24 h after wounding [171]. In adult human wound

Table 4 Major differences between pathological scarring and scarless healing

	Pathological scars	Scarless healing
ECM	↑ Collagen deposition, ↑ type I:III ratio, disorganized parallel thick bundles ↑ Fibronectin ↑ Laminin ↓ Elastin ↓ HA, dense reticular pattern, ↑ LMW-HA	↓ Collagen type I:III ratio, fine reticular pattern of deposition Fibronectin deposition more rapid, sustained longer ↑ HA, more rapid deposition, sustained longer, ↑HMW-HA
MMP/TIMP	↓ MMP-1, ↑ MMP-2, MMP-13 ↑ TIMP-1	↑ MMP-3, MMP-9 ↑ TIMP-1 ↑ MMP/TIMP ratio
Growth factors/ cytokines	↑ VEGF, TGF-β1/TGF-β2, CTGF, PDGF ↓ TGF-β3 ↑ IL-4, IL-6, IL-8 ↓ IL-10	↑ TGF-β3 ↓ VEGF, FGF, PDGF, TGF-β1/TGF-β2 ↓ IL-4, IL-6, IL-8 ↑ IL-10
Cells	↑ (myo)fibroblast presence and activity, Th2, fibrocytes	↓ or no (myo)fibroblasts, faster fibroblast migration, with simultaneous synthetic activity ↓ Platelet aggregation and degranulation, neutrophils, M2 macrophages, T-lymphocytes
miRNA	↑ miRNA-21, miRNA-98, miRNA-181b/c, miRNA-145, miRNA-155, miRNA-382, miRNA-1908, miRNA-4269 ↓ miRNA-9-5p, miRNA-29, miRNA-143-3p, miRNA-138, miRNA-185, miRNA-196a, miRNA-200b/c, miRNA-203, miRNA-205	

ECM extracellular matrix, *HA* hyaluronic acid, *HMW-HA* high molecular weight HA, *IL* interleukin, *LMW-HA* low molecular weight HA, *miRNA* microRNA, *MMP* matrix metalloproteinase, *TGF* transforming growth factor, *TIMP* tissue inhibitor of metalloproteinase

tissue, MMP-1 and MMP-2 are initially released to injured tissue by aggregating platelets [172]. In the inflammatory phase, neutrophil-derived MMP-8 is the predominant collagenase present in normal healing wounds. Peak levels of MMP-8 occur on day 4 and MMP-1 peak levels on day 7 in wound exudates, whereas maximal levels in tissue for both enzymes occurred on day 2 [173]. As wound healing proceeds, resident cells such as keratinocytes and fibroblasts produce large amount of MMPs. Using in situ hybridization and immunohistochemistry, MMP-1, MMP-3, and MMP-10 were found to be expressed in keratinocytes bordering both acute and chronic wounds [174]. Basal keratinocytes at the migrating epithelial front express MMP-1 and MMP-10, while MMP-3 is expressed by hyperproliferative basal keratinocytes lagging behind the migrating front [175].

Expression of TIMP-1 and TIMP-3 was found in proliferating keratinocytes in 3–5-day-old normally healing wounds. In chronic ulcers, these TIMPs were abundantly expressed by spindle-shaped, fibroblast-like cells and plump, macrophage-like stromal cells, as well as by endothelial cells, but not by the epidermal cells. In normally healing wounds, TIMP-2 protein is localized under the migrating epithelial tip and to the stromal tissue under the eschar more frequently than in chronic ulcers. Occasional staining for TIMP-4 protein has been detected in stromal cells of chronic ulcers near blood vessels [112, 174]. TIMPs are temporally and spatially tightly regulated such that an imbalance between MMPs and TIMPs may lead to delayed wound healing [112, 174].

Various MMPs are upregulated during fetal wound healing in mammals that promote scarless wound healing [161, 176]. Differential expression and the temporal changes of specific MMPs and TIMPs have been seen in scarless versus scarring fetal and adult wounds, and the relative activity of MMP and TIMP is crucial in

determining the composition of the wound matrix [170]. High expression of MMP-2, MMP-3, and MMP-9 and low levels of TIMP-1 and TIMP-2 were found in skins from human fetuses at early periods of gestation [177]. This suggests that a higher ratio of MMPs to TIMPs promotes scarless repair by favoring ECM turnover and facilitating migration of fetal cells. Specific MMPs implicated in wound healing and scarring are discussed below and are summarized in Tables 3 and 4.

5.3.1 MMP-1 and TIMP-1

In adult human skin wounds, MMP-1 is expressed by keratinocytes at the epithelial tip and by fibroblasts in the granulation tissue [178]. It is required both to initiate reepithelialization by mediating keratinocyte migration on collagen [179] and for collagen remodeling in wound bed maturation [180, 181]. MMP-1 is decreased in keloids and HTS [169, 182, 183]. Concomitantly TIMP-1, a potent inhibitor of MMP-1, is increased in HTS compared with normal skin [169, 184]. Patients with severe burns that often lead to the formation of extensive fibrotic scars have significantly higher serum TIMP-1 [185]. siRNA knockdown of TIMP-1 enhances collagen degradation in keloids [186], possibly by freeing MMP-1 produced by keloid fibroblasts. MMP-1's potential use to treat fibrotic diseases was first established when pro-MMP-1 was used to decrease liver fibrosis in rats [187]. Subsequently, upregulation of MMP-1 expression in fibroblasts revealed potent anti-fibrotic properties in vivo [188]. The anti-scarring ability of an onion extract has also been associated with MMP-1 activity in vitro and in vivo [189]. MMP-1 was also increased in association with reduced scar weight and size in an animal model where fibroblast growth factor (FGF)-2 was used to treat HTS tissue implanted into nude mice [190]. Finally in a rabbit ear fibrotic model, increased MMP-1 was again linked to suppressed collagen type I and scar reduction when wounds were treated with kynurenine [188]. Together these findings indicated that MMP-1 can break down excess collagen matrix and is unique as a therapy that reverses fibrosis.

5.3.2 Gelatinases

MMP-2 is increased in keloids and HTS compared with normal human skin [169, 191–194]. Active-to-pro-MMP-2 ratio is the highest in the keloids followed by HTS, normal skin, and atrophic scars [194]. The increased MMP-2 activity may contribute to the migration and invasion of fibroblasts into the surrounding non-keloid regions that is unique to keloids [191]. Wound healing in mice was not affected by MMP-2 deficiency [195], suggesting its suppression may be a viable target for control of fibrosis.

MMP-9 is involved in early tissue repair [193] by promoting the migration of keratinocytes [196, 197]. Reepithelialization is significantly impaired in MMP-9 knockout mice [198]. Upon completion of wound resurfacing, its expression in the epithelium spreads progressively distal to the migrating front, persisting for several weeks thereafter [198, 199]. It is only transiently expressed in acute surgical wounds but is increased in many chronic wounds and in burns [200, 201]. MMP-9 is very low or undetectable in both normal skin and abnormal scars [193], although it was found to be significantly upregulated in keloid fibroblasts [202]. Interestingly, MMP-9 was upregulated during scarless wound healing in athymic nude mice, but not scar-forming wild-type mice [203]. Umbilical cord-derived mesenchymal stem cells on scaffolds facilitate collagen degradation via upregulation of MMP-9 in rat uterine scars and promote regeneration of the endometrium, myometrium, and blood vessels in uterine scars [204].

Acomys wounds that heal scarlessly have significantly higher levels of MMP-2 and MMP-9 and lower levels of expression of TIMP-1 on day 3 and 5 wounds compared to *Mus* [147]. Incisional skin wounds of MMP-2-deficient mice heal normally [195], whereas skin wounds of MMP-9-deficient mice showed delayed wound healing associated with delayed reepithelization and disordered collagen fibrillogenesis [205]. In a laser-induced mouse model of choroidal neovascularization, a model of age-related macular degeneration, the neovascularization in MMP-2 or MMP-9 single-gene-deficient mice was only partly impaired, while it was nearly completely

prevented in MMP-2/MMP-9 double-deficient mice. More fibrin deposits were found in these double-deficient mice compared to the either single knockout or wild-type mice [206].

5.3.3 MMP-3

MMP-3 is synthesized primarily by fibroblasts, and to a lesser extent by activated macrophages and keratinocytes adjacent to sites of injury, within hours of the injury taking place [207, 208]. It has a broad range of ECM targets—primarily PGs, such as decorin, biglycan, and versican, in addition to the glycoproteins laminin and fibronectin, and denatured collagens. Excisional wounds in MMP-3-deficient mice failed to contract and healed slower than those in wild-type mice, although incisional wound healing and cellular migration and epithelialization were unaffected [209]. The defect appeared to be caused by dysfunctional organization of actin-rich stromal fibroblasts, implicating MMP-3 to be crucial in the formation of a normal multicellular actin network. Fibroblasts cultured from these knockout mice further exhibited defective contractile activity in vitro that may delay wound healing [210]. Conversely, accelerated oral wound contraction, coupled with increased contractile phenotype of oral fibroblasts, was accompanied by the production of significantly higher levels of MMP-3 compared to skin fibroblasts [211]. While MMP-3 returns to normal levels in healed dermal wounds, it remains elevated in chronic, non-healing ulcers [171], perhaps due to its association with fibroblast activity.

5.3.4 MMP-8

MMP-8 is the predominant collagenase in healing skin wounds [212]. It is mainly expressed by neutrophils where it is stored as an inactive proenzyme in intracellular granules but has also been shown to be produced by fibroblasts. Upon neutrophil activation, MMP-8 is quickly released to ensure rapid availability at inflammatory sites, where it contributes to a negative feedback on neutrophils by increasing neutrophil apoptosis [213, 214]. Consistent with this anti-inflammatory effect on neutrophils, MMP-8-deficient mice show impaired reepithelization and delayed wound healing, associated with persistent inflammation and a lag in neutrophil infiltration [213]. However, MMP-8 overexpression also resulted in impaired wound healing, associated with decreased collagen deposition [215]. MMP-8 is increased 100-fold in chronic wounds compared with acute wounds [212]. MMP-8 knockout fibroblasts produce higher TGF-β1 levels, and cell culture studies suggest that MMP-8 may play a role in regulating TGF-β1 signaling of stromal fibroblasts [216]. Overall, these data suggest that MMP-8 may function to mitigate scarring.

5.3.5 MMP-10

MMP-10 is expressed by keratinocytes at the epithelial tongue of skin wounds and macrophages in response to skin injury [208]. Overexpression of active MMP-10 in basal keratinocytes led to only a minor reduction of new matrix deposition and transiently altered the pattern of laminin-5 at the wound site [217]. While neither collagen expression nor reepithelialization was significantly altered in MMP-10 knockout mice wounds, increased collagen deposition and hence scarring were noted [218]. The excess ECM was in fact associated with reduced expression of MMPs—particularly the metallocollagenases MMP-8 and MMP-13—by alternatively activated (M2) resident macrophages [218]. These data indicate that MMP-10 shapes scar deposition indirectly by controlling MMP activity without impacting collagen production by fibroblasts/myofibroblasts.

5.3.6 MMP-12

Also known as macrophage elastase, MMP-12 is primarily produced by macrophages and endothelial cells and is a key player in tissue remodeling in pathological conditions such as chronic inflammation and fibrosis [219]. MMP-12 has intrinsic antimicrobial activity [220], and intracellular MMP-12 functions as a transcription factor controlling host responses to viral infection [221]. MMP-12-deficient macrophages are unable to penetrate reconstituted basement membranes, indicating that MMP-12 is required for macrophage-mediated ECM proteolysis and tissue invasion [222]. MMP-12-deficient mice showed attenuation of dermal fibrosis [223].

5.3.7 MMP-13

MMP-13 is not detected in normally healing human adult skin wounds but is abundantly expressed by fibroblasts in chronic cutaneous wounds [174]. This collagenase exhibits preference toward cleavage of collagen type II, normally found in cartilage. Studies with MMP-13-null mice have shown a marked delay in granulation tissue growth, organization of myofibroblasts, and formation of large blood vessels [198]. A reduction in myofibroblast number and wound contraction was also apparent in these mice. MMP-13 regulates myofibroblast differentiation via activation of TGF-β [198] and augments myofibroblast function [180]. These results provide evidence for a pivotal role for MMP-13 in regulating keratinocyte migration, vascularization, granulation tissue formation, and wound contraction [180, 198]. Human MMP-13 is expressed by fibroblasts in normal human gingival and fetal skin wounds characterized by scarless wound healing [224, 225]. Rodents do not have MMP-1 in their genome, and MMP-13, a close structural homologue of human MMP-13 [18], acts as their equivalent. MMP-9/MMP-13 double mutants exhibit delayed epithelial migration [198, 205] and have longer wound-healing delays than the single mutants [198].

5.3.8 MMP-14

MMP-14, also known as membrane-type1-MMP, was originally identified as the extracellular protease responsible for activation of pro-MMP-2. In addition, it processes cell-surface-associated molecules and regulates integrin cross talk [226]. MMP-14 is expressed by normal human epidermal keratinocytes [227]. MMP-14-deficient keratinocytes showed impaired early migration and increased apoptosis during the late phase of epithelialization [228]. Similarly, transfection of epidermal keratinocytes with an antisense MMP-14 oligonucleotide correlated to reduced migration and increased apoptosis [227]. MMP-14-deficient skin fibroblasts are incapable of degrading collagen type I matrices [229]. MMP-14 is further implicated in the degradation of the deposited fibrin matrix after vascular injury, allowing endothelial cell migration and invasion [230]. MMP-14 upregulates vascular endothelial growth factor (VEGF)-A, which also promotes blood vessel formation [231]. Inhibition of MMP-14 impaired capillary formation by endothelial cells [232].

5.4 Growth Factors/Cytokines and Excessive Scar Formation

In HTS or keloids, an elevated expression of cytokines and growth factors such as interleukin (IL)-4, VEGF, TGF-β1/TGF-β2, connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF)-α [233, 234] and reduced levels of IL-10 are often observed (see Table 4). These biological molecules can act in a positive feedback loop to further promote cell growth and proliferation of abnormal ECM produced by fibroblasts and myofibroblasts.

5.4.1 IL-10

IL-10 is a potent anti-inflammatory pleiotropic cytokine produced by a number of cell types, including T-cells, B-cells, monocytes, and anti-inflammatory M2 macrophages [235, 236]. It is abundantly present in the amniotic fluid and fetal skin, with low levels in post-gestational skin [237]. IL-10 is upregulated approximately six-fold in *Mus* day 3 and 5 wounds, with no detectable expression of IL-10 in *Acomys* [147]. Liechty et al. grafted early gestation skin from either normal or IL-10 knockout mice on syngeneic adult mice to show IL-10 was critical in the regenerative fetal phenotype of minimal inflammation and scarring [238]. Inducing overexpression of IL-10 in postnatal murine wounds reduced the inflammatory response and resulted in scarless wound repair [237]. Similarly adult wounds treated with IL-10 lentivirus show low inflammation and normal collagen deposition with scarless healing (59). IL-10 acts through pleiotropic effects. It attenuates the inflammatory response by limiting the production of pro-inflammatory cytokines IL-6, IL-8, and tumor necrosis factor (TNF)-α [236, 239] and inhibiting the migration of inflammatory cells [240]. It regulates the ECM [241], minimizing the excessive collagen deposition associated with scar

formation and maintaining elevated fetal HA and inducing postnatal HA production [241]. IL-10 knockout wounds produced more collagen organized in thick, densely packed parallel layers compared to control scars [242]. IL-10 further optimizes fibroblast migration and invasion through increased HA production and protects against the formation of α-smooth muscle actin (SMA) associated with myofibroblasts [243]. Finally, it plays a role in modulating endothelial progenitor cell (EPC) survival and function; EPC is deficient in IL-10 knockout mice, but overexpression of IL-10 increases EPC in both the wound and in circulation [244].

In addition to its potent anti-inflammatory effects, IL-10 regulates fibrogenic cytokines such as TGF-β, as a part of its varied role in the regulation of tissue remodeling [245].

5.4.2 IL-4

IL-4 is mainly secreted by Th2 cells, mast cells, eosinophils, and basophils. It was first identified as a factor promoting the growth and differentiation of B-lymphocytes [246] but later found to have profound effects on not only hematopoietic cells such as B-lymphocytes and monocytes/macrophages [247] but also non-hematopoietic cells, such as fibroblasts, where it stimulates the synthesis of ECM, especially collagens [248].

IL-4 expression is significantly downregulated in *Acomys*, with no significant change in *Mus* [147]. Wounds in IL-4 transgenic mice showed a marked enhancement in expression of inflammatory cytokines/chemokines, elevated infiltration of inflammatory cells, a significantly higher level of angiogenesis, larger amounts of granulation tissue, less expression and deposition of collagen, and finally delayed wound closure and reepithelialization as compared to wild-type mice [249]. IL-4 has also been shown to be involved in the stimulation of production of components of the ECM, especially collagens synthesized by fibroblasts [248]. Blocking IL-4 with specific antibodies significantly decreased wound healing, macrophage accumulation, and collagen downregulation and fibrosis formation [250]. Perhaps lower levels of IL-4 may prevent an overproduction of collagen associated with scarring.

5.4.3 Epidermal Growth Factor (EGF)

EGF plays a crucial role in wound healing by stimulating keratinocytes and fibroblasts to migrate and proliferate and promotes neovascularization of new dermis [251, 252]. EGF binds to EGF receptor on the cell surface, initiating a signal cascade that results in a variety of biochemical changes [253]. A number of studies have addressed the therapeutic implication of EGF for HTS. In a rat full-thickness wound model, recombinant human EGF significantly promoted wound healing and reduced cutaneous scars through suppression of inflammation, TGF-β1, and collagen expression [254]. EGF gene-transfected mesenchymal stem cells increased the proliferation, migration, and adhesion of fibroblasts, contributing to wound healing [255]. Protamine-conjugated EGF was helpful for forming the skin with a more normal appearance and texture in laser-induced HTS [256]. EGF also controls ECM equilibrium by regulating MMPs. For example, EGF induced the expression of MMP-1 in human skin fibroblasts [257]. Furthermore, EGF negatively regulates the role of TGF-β1 in inducing fibroblast-populated collagen lattice gel contraction, indicating a suppressive effect of EGF on wound contraction in HTS [258].

5.4.4 VEGF

VEGF is produced by endothelial cells, keratinocytes, fibroblasts, platelets, neutrophils, and macrophages and is vital for angiogenesis in embryogenesis and in wound healing [251]. Although restoration of angiogenesis is essential for healing wound, high VEGF levels are often associated with leaky, immature, and poorly perfused vessels [259]. Increasing evidence has indicated that angiogenesis is raised and abnormal in pathological scarring. Increasing VEGF has also been shown to increase fibrosis and scar formation in the skin [260], with HTS expressing high levels of VEGF [261, 262]. Moreover, VEGF was dynamically correlated with the progression of HTS: increased in early scars, peaked in

proliferative scars, and decreased in regressive scars [251]. VEGF levels in fetal wounds are lower in comparison to adult wounds [263]. Exogenous VEGF converted the scarless phenotype of fetal wounds to a scar-forming phenotype, and neutralization of VEGF reduced the vascularity and decreased the formation of scars in adult wounds [251, 260]. Improvement of HTS by treatment with interferon (IFN)-α2b was associated with reduction of VEGF and decreased angiogenesis in patients [264]. VEGF could also stimulate the migration of human keratinocytes and fibroblasts and macrophages—non-endothelial cell types known to play critical roles in HTS—thus revealing a non-angiogenic effect on wound closure [251]. Interestingly, recent research revealed that skin wounds healed normally despite reduced angiogenesis [265], an observation supported by the reduced angiogenesis in in fetal skin and oral mucosal wounds, which heal without scarring [265, 266]. This may be explained by an inferior inflammatory response and a much faster maturation of newly formed capillary networks [265]. VEGF inhibition and anti-angiogenic therapy therefore may be promising strategies for reducing HTS and keloids. A significant proportion of new vessels in a wound are immature and not perfused [267], and these immature vessels are more sensitive to anti-VEGF agents [268]. Hence it may be possible to develop specific VEGF inhibitors that can selectively prune the nonfunctional vessels.

5.4.5 Transforming Growth Factor (TGF)-β

TGF-β are 25 kDa homodimeric proteins with multiple biological functions affecting epithelial- and mesenchymal-derived cells [269]. As one of the most potent regulators of collagen synthesis and fibroblast migration and proliferation [251], the TGF-β family influences the differentiation of fibroblasts to myofibroblasts, the transition of keratinocytes and endothelial cells to myofibroblasts [270–272], and controls scarring [251]. After tissue injury, TGF-β levels are significantly increased, which aids in recruiting immune cells, like neutrophils and macrophages, and activating fibroblasts, which release further TGF-β. TGF-β mediates the deposition of many ECM proteins, including collagen and fibronectin, and the expression and function of CTGF [95, 273]. TGF-β promotes collagen deposition both by enhancing gene expression of various collagens and by mediating the overexpression of collagenase inhibitors [274]. The family has three isotypes: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 is a chemoattractant for neutrophils, monocytes, and fibroblasts, thus promoting scar formation in adult wounds [251, 275]. Insertion of PVA sponges containing TGF-β1 into rabbit wounds caused normally scarless wounds to heal with scar [276]. Treatment of adult rat wounds with neutralizing antibodies to TGF-β1 and TGF-β2 reduced scar formation [277]. Recently, many natural compounds have been revealed to exert their anti-scarring properties by interrupting TGF-β1 signaling, which leads to reduced production of collagen in HTS fibroblasts [251]. Furthermore, siRNA-mediated knockdown of TGF-β1 receptor significantly inhibited the expression of collagen type I in human HTS fibroblasts and reduced scarring in rabbit wounds [278]. TGF-β antagonist peptide also inhibited the fibrotic behaviors of fibroblasts derived from human HTS [279]. Topical application of TGF-β1 inhibitor P144 promoted scar maturation and improved the morphological features of human HTS implanted on nude mice [280].

TGF-β3, however, inhibits scar formation via reducing ECM synthesis and fibroblast differentiation, limiting inflammation, and promoting wound closure [251, 275]. Local administration of TGF-β3 reduced dermal scarring, resulting in regeneration of skin structure [281]. Fetal skin shows an increased expression of TGF-β2 and TGF-β3 and lower levels of TGF-β1 in the dermis [282]. Embryonic wounds that heal without scars express low levels of TGF-β1 and TGF-β2, but higher levels of TGF-β3 [283]; conversely, TGF-β1 expression is increased and TGF-β3 decreased in scarring fetal wounds. Similarly, adult wounds that scar express high levels of TGF-β1 and TGF-β2, but lower levels of TGF-β3 [284]. This suggests the ratio of TGF-β3 to TGF-β1 may determine whether tissue regenerates or forms scar. Unfortunately however, the

use of TGF-β3 failed to significantly alter scarring in a human phase III trial.

5.4.6 Platelet-Derived Growth Factor (PDGF)

PDGF is another key factor that contributes to wound healing [251, 285]. It functions as a potent chemoattractant for monocytes and fibroblasts and also stimulates fibroblast proliferation and collagen synthesis. Skin scaffolds that incorporate PDGF improve wound healing due to this increased collagen deposition [286]. However high levels of PDGF are expressed in HTS and keloids [251, 287]. In HTS, PDGF is increased in both the epidermis and dermis, with the augmented epidermal PDGF leading to increased dermal matrix formation [287]. In fetal wounds however, its expression is lower and brief compared to adult wounds, further suggesting that PDGF is a profibrotic factor at high concentrations [251, 275]. Indeed, experimentally neutralizing PDGF in adult mouse, rat, and pig wounds resulted in scarless healing [275, 284].

5.4.7 Connective Tissue Growth Factor (CTGF)

CTGF is a heparin-binding glycoprotein normally localized in the cytosol and upon secretion fulfills its functions in the extracellular space, usually in combination with other specific growth factors or through direct interaction with ECM or cell-surface molecules [288]. CTGF is dramatically enriched in virtually all fibrotic conditions and is well-known for its roles in ECM production and tissue remodeling, acting as a key molecule in HTS [251]. Its expression is upregulated in human HTS fibroblasts, where it functions as a downstream modulator of TGF-β and stimulates collagen type I synthesis [251]. The growth of these fibroblasts was effectively inhibited by botulinum toxin type A, which decreased CTGF expression [289]. Similarly two PPARγ activators attenuated TGF-β1-induced expression of CTGF and collagens at both mRNA and protein levels [290]. CTGF antisense therapy appeared to have no effect on early wound closure but significantly limited the subsequent hypertrophic scarring with a marked reduction in myofibroblast numbers in scars [291]. Taken together, CTGF may prove to be a fibrogenic master switch [251, 292]—its pivotal roles in tissue repair pointing to great potential as a novel therapeutic target for pathological scarring.

6 Cells and Excessive Scar Formation

As vital components of the skin, cells have various roles to maintain homeostasis and the overall function of the skin, including in response to injury. They exert these effects largely by producing ECM and cytokines/growth factors. See Table 1 for a summary of their actions in wound repair versus regeneration and Table 4 for major cellular changes in pathological scarring.

6.1 Fibroblasts/Myofibroblasts

Fibroblasts are the predominant cell type in the skin dermal layer and are normally classified as the major ECM-producing cells in the human body. They secrete collagen and fibronectin and produce key basement membrane compounds, including laminins and collagen types IV and VII [123, 293]. When activated by TGF-β, TNF-α, PDGF, or IL-13, fibroblasts differentiate into myofibroblasts [95, 293–295], with TGF-β1 coupled with mechanical forces from the remodeled ECM being the most powerful regulator of the myofibroblast phenotype [296, 297]. Myofibroblasts are characterized by their expression of α-SMA, enhanced contractility, and increased synthetic activity of ECM proteins including collagen and fibronectin. Recently, myofibroblasts have been distinguished from activated skin fibroblasts by the expression of AOC3 and other associated markers [298]. In addition, myofibroblasts can be generated from a variety of sources including resident mesenchymal cells, vascular smooth muscle cells, and epithelial and endothelial cells, in processes termed epithelial-mesenchymal/endothelial-mesenchymal transition (EMT/EndMT), as well as from circulating fibroblast-like fibrocytes [293, 296, 297]. Once

activated, myofibroblasts are themselves capable of releasing TGF-β and MMPs, in addition to ECM components, acting as an important regulator of ECM homeostasis [296, 297].

Myofibroblasts appear in the adult wound 1 week after wounding, peaking during the 2–3-week period and then disappearing via apoptosis with time [293]. They are responsible for the closure of wounds by generating contractile forces that bring together the edges of an open wound. They also promote the formation of a collagen-rich scar [296, 297]. Myofibroblasts can prolong the inflammatory response through secreting cytokines/chemokines and growth factors, such as TGF-β family proteins, and cytokines IL-4, IL-6, IL-8, IL-13, and TNF-α [296]. The immunological regulation of (myo)fibroblasts is a key factor in the development of fibrotic scar tissue or the ECM components of regenerating tissue [297, 299]. Normally, in the context of wound healing, myofibroblasts do not persist once the activating stimulus is attenuated and tissue homeostasis is achieved. However, a chronic repair response may be triggered by a persistent or recurrent injury, by chronic inflammation, or perhaps by a deregulated signal from an absent stimulus, which in turn retains the presence of myofibroblasts [300]. The chronic presence and continued activity of myofibroblasts typically results in the continual secretion of ECM constituents outweighing their degradation by MMPs, leading to tissue irregularity and scar development. Their persistence characterizes many fibrotic pathologies in the skin and internal organs including the liver, kidney, and lung.

There are some functional differences between fetal and adult fibroblasts. Fetal fibroblasts synthesize more collagen types III and IV than their adult counterparts [301, 302]. Collagen synthesis is delayed in the adult wound, while fibroblasts take time to proliferate and migrate [303]. In contrast, fetal fibroblasts migrate at a faster rate than adult fibroblasts, simultaneously proliferating and synthesizing collagen [303]. This is in part aided by greater expression of surface receptors for HA, which enhances fibroblast migration [263, 303, 304]. Such a delayed migration speed and collagen deposition in adult wound healing likely contributes to scar formation. Wounds induced in early gestation that heal scarlessly have virtually no myofibroblasts, while scarring late fetal and postnatal wounds have progressively more active myofibroblasts, correlating with contraction and degree of scarring [303, 305, 306]. These myofibroblasts generate contractile forces that likely alter collagen fibril orientation and contribute to scarring.

An interesting experiment showed that when human fetal skin is transplanted to a subcutaneous location on an adult athymic mouse and subsequently wounded, it heals without scar formation; yet the same fetal skin heals with scar formation when transplanted to a cutaneous location [307]. In an extension of this study, immunostaining for species-specific fibroblasts and macrophages revealed an influx of adult mouse fibroblasts and macrophages in the cutaneous human fetal graft, which healed with a scar. Contrastingly, subcutaneous human fetal grafts possessed exclusively human fetal fibroblasts in the wound environment, with no inflammatory cells, culminating in scar-free repair. Thus, the highly organized collagen deposition in scarless human fetal wound repair appears to be intrinsic to the human fetal fibroblast and occurs even in the absence of an adult-like inflammatory response [308].

6.2 Fibrocytes

Fibrocytes are circulating, bone marrow-derived mesenchymal progenitors that exhibit a (myo) fibroblast-like phenotype. As they enter the wound site, TGF-β1 triggers their transdifferentiation into myofibroblasts that express collagen type I and fibronectin and increase their production and deposition of ECM components [309, 310]. Fibrocytes also secrete TNF-α, IL-6, IL-8, IL-10, and TGF-β1 into the wound [311], promote inflammation, activate myofibroblasts from existing fibroblasts [312, 313], and trigger the surrounding endothelial or epithelial tissues to differentiate toward fibroblast-like cells through EndMT or EMT [314, 315]. Fibrocytes can be distinguished in at least 4-day-old skin wounds,

and their quantity raises with time and increasing wound age [316]. Fibrocytes have been found in both adult and pediatric burn wounds, with the number of fibrocytes correlating with wound depth [317]. They contribute to collagen production and are associated with HTS [312, 313].

6.3 Platelets

Platelets are the first "cells" to invade the wound base, aggregate, and release cytokines and growth factors such as TGF-β1, PDGF, TNF-α, IL-1, IL-6, and many others that are involved in all stages of the wound-healing process [285, 293]. The resulting clot, which is composed of platelets, cross-linked fibrin, fibronectin, vitronectin, and thrombospondin and then initiates the inflammatory response. One key difference between fetal and adult platelets is that fetal platelets produce much lower levels of PDGF, TGF-β1, and TGF-β2 and do not aggregate in a similar manner to their adult counterparts [318]. Fetal wounds have decreased platelet aggregation and degranulation, and these platelets release lower levels of inflammatory cytokines [318]. Wound repair in mice treated with antiplatelet antisera had same response as controls, suggesting platelets are not essential for normal healing to occur [319].

6.4 Neutrophils

Neutrophils appear at the wound site immediately after injury and are an essential part of the innate immune system. They are, however, a double-edged sword as they can also promote persistent inflammatory response and tissue injury [293, 320]. Neutrophil elastase can promote IL-8 expression in the surrounding cells—responsible for leukocyte recruitment, initiating EndMT and increasing the survival and proliferation of fibroblasts/myofibroblasts—leading to fibrosis [293, 321, 322]. Conjunctival neutrophils predict progressive scarring in patients with ocular mucous membrane pemphigoid [323]. In the adult wound bed, there are two distinct populations of neutrophils: pro-inflammatory and anti-inflammatory types, wherein these cells differ in size, granularity, and the expression of CD11b and Ly6G [324]. The anti-inflammatory neutrophil response is strongly associated with the secretion of the anti-inflammatory cytokine IL-10 [325].

Oral wounds that show negligible scar formation have significantly fewer neutrophils compared to the skin [326]. In the absence of commensal microbiota, germ-free mice wounds show decreased neutrophil accumulation and accelerated healing with greatly reduced scarring [327]. The blood of *Acomys* mice is very neutropenic, and their existing neutrophils produce low levels of cytokines and present a low contribution to the inflammatory response [147, 148, 263]. Correspondingly, *Acomys* wounds also showed a lower level of neutrophils and healed with less scarring than those in *Mus* [148]. Reducing or eliminating neutrophils in guinea pigs revealed that the absence of neutrophils had no impact on either wound debridement or granulation tissue formation [328], although wounds in neutrophil-depleted mice healed more rapidly [329] and PU.1 knockout mice lack functioning neutrophils yet heal with minimal scarring [148]. These data suggest that while neutrophils are effective protection against infection, they may not be essential in normal healing and indeed even contribute to a scarring phenotype.

6.5 Monocytes/Macrophages

Monocytes are the dominant type of bone marrow-derived cells invading the wound site 3–5 days after injury [293, 320], where they are converted into macrophages by TGF-β [330]. Activation of blood monocytes to macrophages induces a variety of protein products needed in the wound-healing process such as TGF-α/TGF-β, bFGF, PDGF, and VEGF, which act to amplify and eventually resolve inflammation, recruiting endothelial cells and fibroblasts to initiate proliferation [331]. Macrophages in the wound bed can roughly be categorized as classically activated (M1) and alternatively activated (M2) macrophages [332]. M1 macrophages are activated by IFN-γ and

bacterial products; they exhibit phagocytic abilities and inflammatory properties. M2 are mostly activated by IL-4 and IL-13; they suppress inflammatory reactions and adaptive immune responses and play an important role in healing wound via release of anti-inflammatory cytokines IL-10 and TGF-β [333]. Macrophages can convert from a M1 to a M2 phenotype, thereby modulating the transition of the inflammatory phase to the proliferative phase [334].

During wound repair, macrophages can play a beneficial role by regulating a wide range of processes, such as removal of dead cells, debris, and pathogens, revascularization, wound reepithelialization, ECM deposition, and wound contraction [334]. Genetically modified mice lacking macrophages demonstrate retarded and abnormal repair [335], indicating that macrophages are crucial for the developing wound. Specifically, depletion of macrophages during the inflammatory phase reduces the formation of vascularized granulation tissue, impairs epithelialization, and results in minimal scar formation. In contrast, depletion of macrophages during the proliferative phase results in severe hemorrhage in the wound tissue and prevents transition into the remodeling phase [336]. Depletion of M2 macrophages in mice surgical wounds via colony-stimulating factor-1 signaling blockade leads to persistent inflammation, with an increase in neutrophils and M1 macrophages, and attenuated collagen deposition [337]. A fluid yet finely tuned balance between the relative presence and activities of M1 and M2 macrophages is pivotal in defining the various stages of wound healing. Interestingly M2 macrophages are notably absent in non-scarring fetal wounds at early gestation [263]. Oral mucosa too has significantly fewer macrophages, in particular mannose receptor-positive M2 macrophages [326]. Moreover there are little or no macrophages in *Acomys* and PU.1 knockout mice skin wounds, respectively, both of which demonstrate minimal scarring [148].

6.6 T-Lymphocytes

T-lymphocytes infiltrate the wound bed in the late inflammatory phase of wound repair and remain in the scar for up to months after wounding, suggesting that they are involved in either the late proliferative or remodeling phase. These cells produce lymphokines, activating other inflammatory cells such as macrophages [123]. T-lymphocytes also strongly modulate fibroblastic activity, with wounds in T-cell-depleted mice exhibiting significantly impaired collagen deposition and wound-breaking strength [338]. Among all lymphocyte subpopulations, Th1 and Th2 are the most studied and contribute differently to tissue fibrosis. Th1 cells mainly produce IFN-γ, which results in the differentiation of macrophages into M1 macrophages [339]. They also produce IL-10, which acts as an anti-fibrotic. Th2 cells produce two important and profibrotic cytokines, IL-4 and IL-13 [320]. IL-4 activates M2 wound-healing macrophages, resulting in collagen production and deposition. Moreover, IL-4 stimulation of collagen synthesis in fibroblasts is twice as effective as TGF-β [248]. Th2-mediated secretion of IL-4 and IL-13 enhances fibrocyte differentiation into (myo)fibroblasts [340]. Overall the Th2 response results in the production of ECM and acts as a profibrotic.

7 miRNA and Excessive Scar Formation

miRNAs are a class of small noncoding RNAs; they can modify gene expression by binding to target messenger (m)RNA, resulting in inhibition of mRNA translation or degradation of mRNA [341]. Increasing evidence shows that the miRNAs are associated with and contribute to skin fibrotic diseases [342]. The expression of many miRNAs is altered in keloid tissue [343] and HTS [344] when compared to normal skin tissue (see Table 4). Functional annotations of differentially expressed miRNA targets revealed that these miRNAs are enriched in several signaling pathways important for wound healing and scar formation including TGF-β signaling, ECM deposition, and fibroblast proliferation and differentiation [343]. Some miRNAs have profibrotic properties and contribute to excessive scaring, while others have anti-fibrotic properties.

Anti-fibrotic miRNAs include miR-29 family [345–348], miR-9-5p [349], miR-143-3p [350], miR-138 [351], miR-185 [352], miR-196a [353], miR-200 [354], and miR-205-5p [355]. These miRNAs are normally decreased in either keloid or HTS. Mimics of these miRNAs promote skin wound healing and reduce excessive scar formation, whereas inhibition of their expression is often associated with upregulation of TGF-β signaling and accumulation of collagen types I and III, leading to excessive scaring [344–355]. The miR-29 family is one well-studied miRNAs within this group. The expression of miR-29 is lower in HTS [344], in thermal injury tissue [347], and in keloids [348], when compared with healthy control tissue. This family contains four members, 29a, 29b-1, 29b-2, and 29c, which directly target collagen type I mRNA and mediate downstream signaling pathways of TGF-β, PDGF-β, and IL-4 [345, 346]. TGF-β1 significantly decreases miR-29a expression by fibroblasts [348]. Mimics of miR-29b promoted skin wound healing and reduced excessive scar formation by inhibition of the TGF-β1/Smad/CTGF signaling pathway in a mouse scald model [347]. Another study showed that miR-29b inhibits the expression of collagen type I α1 and α-SMA and the proliferation of primary human endometrial stromal cells [356]. Pre-miR-29a transfection results in a decrease, whereas anti-miR-29a leads to an increase in collagen types I/III mRNA and protein expression by keloid fibroblasts [348]. Similarly, miR-143-3p was markedly downregulated in HTS tissues and fibroblasts. Its downregulation is associated with accumulation of collagen types I and III and upregulation of CTGF, whereas overexpression of miR-143-3p induces apoptosis and reduces collagen types I and III and α-SMA in HTS fibroblasts [350]. Overexpression of miR-9-5p significantly delays TGF-β1-dependent transformation of dermal fibroblasts and deters fibrogenesis in the mouse model of bleomycin-induced dermal fibrosis [349]. The expression of miR-205-5p is significantly lower in keloid tissue; miR-205-5p overexpression dramatically impairs human keloid fibroblast viability and inhibits cell invasion and migration by directly targeting VEGF expression and subsequently inhibiting the PI3K/Akt pathway [355]. The miR-200 family, containing miR-200a, miR-200b, and miR-200c, is preferentially expressed in the epidermis [354]. HTS expressed significantly lower levels of miR-200b [357]; miRNA-200b and miRNA-200c were downregulated in keloids [343]. TGF-β1 reduced the expression of miR-200b [357], and overexpression of miR-200b may be a potential clinical strategy for treatment of HTS [354].

Fibrotic miRNAs contain miR-21 [343, 357–359]; miR-98 [360]; miR-181a [361], miR-181b [362], and miR-181c [363]; miR-145 [364]; miR-155 [365]; and miR-1908 [366]. These miRNAs are overexpressed in keloids and HTS, where they stimulate collagen and fibronectin synthesis, TGF-β1/α-SMA signaling, and (myo)fibroblast differentiation and proliferation [343, 357, 358]. For example, miR-21 was overexpressed in keloid tissue [343, 358] and HTS [357]. Anti-miR-21 reduced the expression of collagen types I and III, fibronection, and α-SMA in HTS fibroblasts [367] and suppressed HTS growth in both a mouse model [368] and in a rabbit ear HTS model [369]. In contrast, overexpression of miR-21 promoted ECM synthesis by normal skin fibroblasts; significantly increased the migration, invasion, and sphere-forming abilities of keloid-derived keratinocytes; and enhanced EMT and cell stemness [358, 359, 370]. The expression of miR-21 was increased after stimulation with TGF-β1 [357]. miR-21 levels in the skin, serum, and hair of patients were strongly correlated with the severity of skin fibrosis [359]. Similarly, HTS and keloids contain increased levels of miR-181 [361–363]. miR-181b antagomiR increases decorin protein expression in dermal fibroblasts and reversed TGF-β1-induced decorin downregulation and myofibroblast differentiation in HTS fibroblasts [362]. Overexpression of miR-181a or miR-181 mimics enhanced keloid fibroblast proliferation, whereas miR-181a suppression triggered the opposite effects [361]. A miR-145 inhibitor in myofibroblasts strongly decreased their collagen type I α1 expression, TGF-β1 secretion, contractile force generation, and migration [364]. miR-1908 mimic transfection promoted fibroblast proliferation and expression of collagen type I,

TNF-α, IL-1α, TGF-β1, and Ski-suppressing gene Meox2. In contrast, the miR-1908 inhibitor had a completely opposite effect on cell proliferation and gene expression and significantly reduced the area, volume, and fibrosis of scars in vivo [366]. Acute downregulation of miR-155 at wound sites led to reduced skin fibrosis [365].

Cheng et al. [371] investigated the unique capacity of mid-gestational mammalian skin to heal without scar. They compared the miRNA profile between E16 (scarless) and E19 (scarring) skin and identified 11 upregulated and 6 downregulated miRNAs in the E19 skin compared with the E16 counterpart. The expression of miRNAs by human fetal keratinocytes at varying gestational ages is altered [372]. Zhao et al. [372] provided evidence for 106 novel miRNAs and the dynamic expression of miRNAs that extensively target the TGF-β pathway, which may contribute to scarless wound healing in early- to mid-gestational fetal keratinocytes and thus may be new targets for potential scar prevention and reduction therapies.

A substantial level of experimental evidence has indicated that restoring downregulated miRNA expression with synthetic miRNA mimics may be a useful strategy to overcome fibrosis [346]. These results suggest that targeting miRNAs may be a successful and novel therapeutic strategy in the treatment of fibrotic diseases that are difficult to treat with existing methods. Considering the key involvement of TGF-β signaling in skin fibrotic processes, targeting associated miRNAs may prove invaluable in halting skin fibrosis. Therapies may be designed to modify the different levels of the pathway.

8 Noninflammatory Fetal Skin and Scarless Healing

In contrast to adults, fetal integumentary wounds in humans and other mammals heal rapidly without associated scarring [6]. This property appears to be intrinsic to fetal tissues rather than the protected uterine environment, as wounds in fetal skin grafted subcutaneously into athymic mice heals without scar [307]; fetal marsupials that develop outside the uterus in a maternal pouch also heal cutaneous wounds scarlessly [373]. In contrast, wounds in adult skin grafted onto fetal hosts in utero healed with scar [307]. Fetal scarless repair is also organ-specific. At early gestation, wounds in fetal skin heal without scar; fetal wounds in the stomach, intestine, and diaphragm heal with scar formation [374].

Extensive research unraveled many differences between fetal and adult skin repair mechanisms in inflammation, ECM, and cytokine/growth factors (Table 4). Fetal dermal wound healing is characterized by regenerative healing with restoration of normal architecture, a lack of fibroplasia, and a markedly diminished cellular inflammatory response [12].

The major differences between human fetal and adult skin reside at the level of dermal ECM, including differences in collagen deposition and cross-linking patterns and the content and expression of fibronectin, PG, and HA [263]. Fetal ECM contains higher proportions of collagen type III, type III to I ratio, fibronectin, PG, and HA than adult ECM [263, 303]. In scarless fetal wounds, collagen is rapidly deposited in a fine reticular pattern identical to that in uninjured skin. In contrast, adult scarring wounds have disorganized collagen type I bundles with more collagen cross-linking [40, 263, 303]. Although collagen type I and its cross-linking are essential for adult wound healing with more strength and rigidity, increased cross-linking may impede the movement of cellular mediators required for rapid cellular regeneration in the fetus. Lovvorn et al. [375] found that increased collagen cross-linking was associated with advancing gestational age that paralleled the transition from scarless to scar-forming repair in fetal sheep wounds.

Fetal skin contains more HA than adult skin; in response to injury, HA increases more rapidly, is more sustained, and is overall greater in quantity than that of adult wounds [263, 303, 304]. In addition, fetal fibroblasts express more HA and its receptors, with fewer pro-inflammatory cytokines, such as IL-1 and TNF-α, that downregulate HA expression. The increased expression and decreased degradation of HA changes the

rheology of the matrix toward a more elastic one [263, 303, 304]. The adhesion protein, fibronectin, is synthesized and deposited more rapidly and sustained longer after wounding in early gestational fetal rabbit wounds than in adult wounds [376]. Similarly, tenascin appears more rapidly in the fetus wounds compared to the adult wounds and precedes cellular migration [377]. The rapid expression and deposition of fibronectin and tenascin stimulate early cell attachment and migration of fibroblasts, keratinocytes, and endothelial cells to the site of injury [303, 377], promoting the formation of an organized wound ECM and rapid healing that results in less scarring. Fibromodulin, a PG that can inhibit collagen fibrillogenesis, is present in abundance in the fetal ECM and decreases with maturation [378]. In contrast, decorin, assisting collagen fibrillogenesis, increases rapidly with increasing gestational age in both fetal fibroblasts and skin and is further upregulated in adult wounds [378, 379]. Elastin is present in adult dermis and is not detectable in fetal dermis. These observations give some indication of the complicated and mixed functions ECM molecules may play in determining the scarless nature of early fetal wound repair.

MMPs and TIMPs, molecules that regulate ECM turnover, are also shown to be differentially regulated as fetal skin develops. In fetal rat skin, baseline expression of MMP-1, MMP-2, and MMP-14 increased with the transition into a scarring phenotype, with a higher MMP to TIMP expression ratio [170]. Overall, all scarless wounds have a higher MMP to TIMP ratio, favoring remodeling and less accumulation of collagen during wound healing [380, 381].

The cytokine and growth factor profile of fetal healing differs significantly from adult wound healing [263]. In early fetal wounds of murine, rat, and human skin, profibrotic TGF-β1 and its receptors are low [382–384]. Conversely, the anti-fibrotic TGF-β3 is higher in these fetal wounds [277]. Exogenous TGF-β1 results in scar formation in fetal skin wounds that would otherwise heal scarlessly [276, 277, 384]. PDGF is initially present in both adult and fetal wounds, but it disappears more rapidly in the fetal wounds [385].

The lack of inflammation including immune cells and inflammatory cytokines and chemokines in mid-gestational fetal skin is a key factor underlying scarless healing [263, 275]. Human second-trimester healthy fetal skin contains significantly lower numbers of immune cells and cytokine/chemokines compared to adult skin [386]. Adult wounds have a long-lasting IL-6 and IL-8 expression, whereas they are significantly lower in fetal wounds [387]. Overall, the immune deficiency in healthy second-trimester fetal skin may result in reduced inflammation during wound healing and could underlie the scarless healing capacities of the fetal skin [386].

Wounds in normal fetal skin grafts showed minimal inflammation with normal dermal reticular collagen pattern at the site of the wound, consistent with scarless repair. In contrast, wounds in IL-10 knockout fetal skin grafts showed significant inflammation and scar formation [238, 240]. This IL-10-mediated regenerative potential is dependent on regulation of fibroblast HA metabolism. The opportunity for IL-10 to regulate the fibroblast-specific formation of a HA-rich ECM may lead to the development of innovative therapies to attenuate fibrosis in diseases where inflammation and dysregulated HA dominate [388].

Fetal and adult fibroblast collagen synthesis differ in their speed of deposition, collagen type ratios, and quantity of collagen [301, 302]. Fetal fibroblasts can heal fetal skin wounds without scar despite being perfused by adult serum and inflammatory cells in an adult environment [389]. No conversion of fetal fibroblast into contractile myofibroblasts was observed at the stage of fetal wound repair where scarless healing still occurs [305]. However myofibroblasts are found in late gestation fetal wounds, after the transition to adult healing with a scar [306]. Positivity for nestin and α-SMA was higher in neonatal fibroblasts when compared with adult fibroblasts. This correlates with the low capacity of fetal fibroblasts

to contract a collagen gel and may have implications in the differences seen between fetal and adult wound contraction [390]. Murine fetal fibroblasts also produce a robust HA-rich ECM [391]. Overall, the intrinsic ability to synthesize a mature, well-organized dermal matrix is superior in fetal fibroblasts compared to adult fibroblasts.

The physiology of fetal keratinocytes and the process of wound reepithelialization differ substantially from those in the adult [305, 392]. Adult wounds reepithelialize through extension of lamellipodia followed by wound-edge keratinocytes migrating over the wound bed, whereas fetal wound-edge keratinocytes assemble an actin cable to close the wound similar to a purse-string mechanism [305, 392]. In response to injury, fetal keratinocytes produce integrins recognizing collagen, fibronectin, laminin, and tenascin more rapidly than adult cells [263].

A mammalian model of fetal skin regeneration is the African spiny mouse *Acomys* [393]. Wounds in *Acomys* reepithelialized rapidly with much less scarring compared to similar wounds in *Mus*. These wounds contain predominately collagen type III rather than type I as in *Mus*; form very few myofibroblasts, which are abundant in wounds of *Mus*; and develop dermis and epidermis that inducted and formed new hair follicles which were absent in *Mus* [149, 393].

There is a developmentally regulated threshold for scarless healing based on gestational age and the extent of injury [263]. Scarless healing persists until roughly the middle of the third trimester of intrauterine gestation, at which point a transition to the adult, scar-forming pattern of wound repair occurs [30, 394]. This "window of regenerative healing" also depends on the extent of injury and the size of the wound. Small wounds tend to heal scarlessly, whereas larger wounds heal with a scar [395].

The discovery of the ability of fetal wounds to completely heal and regenerate without any scar formation opens the door to a whole new field of research. It therefore becomes important to understand at the cellular and molecular level the distinctions between these two physiologies, in the hopes that an understanding of fetal biology may one day enable its recapitulation in the adult.

Conclusions

ECM composition is relatively stable in healthy adult tissue, but this balance is disturbed during injury. These changes involve a stepwise remodeling of specific ECM components including the formation of fibrin clot, the turnover of the provisional ECM, the generation of biologically active ECM fragments, and the replacement by a more compact and rigid ECM enriched in collagens and other fibrous proteins and finally the formation of a mature scar. Each year, more than 100 million patients acquire scars, some of which cause considerable morbidity [396]. Excessive scarring is tremendously difficult to predict, prevent, and treat; and their management remains a challenge. Clearly, effective anti-fibrotic therapies would make a significant contribution to public health. To date, therapeutic approaches to reduce scarring have been largely unsuccessful. Although the precise cellular and molecular mechanisms that control repair processes remain unclear, components of the ECM do not passively follow events that lead to fibrosis but rather play an active role in the cellular and extracellular events in this process. Targeting specific components of the ECM and/or the enzymes that modify these components may be an effective strategy to treat fibrosis in the future [124]. Despite the daunting prospect of improving a complex, imperfect wound repair process, the promise of perfect wound healing does exist. The fetal skin represents the supreme model of tissue repair due to its ability of scar-free healing. A full understanding of the most important factors governing the scarless pattern of fetal wound repair will hopefully allow for intervention in the adult wound-healing milieu to mitigate scar formation and improve the clinical outcome of those afflicted with the morbidity of scar.

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Part II

Wound Dressings

Introduction to Wound Dressings

Melvin A. Shiffman

1 Introduction

Wounds usually heal without a problem. However, the wound may have necrosis and/or infection, or the wound may dehisce because of underlying seroma or hematoma leaving the wound open. Medical disorders that affect healing include diabetes mellitus, low HGH (human growth hormone), rheumatoid arthritis, vascular insufficiency, immunosuppression, radiation, and zinc deficiency [1]. The open wound may heal by secondary intention, but wound healing needs some dressing that will help the wound to heal faster.

Wounds need a moist environment, treatment of infection, and removal of devitalized or necrotic tissue. Unnecessary dressing changes should be avoided. For healing to take place at an optimum rate, all dressing materials used should ensure that the wound remains [2]:

1. Moist with exudate but does not get macerated ("not too wet – not too dry")
2. Free from clinical infection and excessive slough or devitalized/necrotic tissue
3. Free from toxic chemicals, particles, or fibers released from the dressing
4. At an optimum temperature for healing to take place (around 37 °C)
5. Undisturbed by frequent or unnecessary dressing changes
6. At an optimum pH value

2 Dressings

2.1 Low-Adherent Transparent Polyurethane Film Dressings

Wounds can heal by secondary intention but dressings will decrease the time for healing. The polyurethane film dressings are used for superficial wounds, such as shallow pressure sores, minor burns, cuts, and abrasions. They provide moisture vapor, permeability allowing excess exudate to evaporate, helping prevent skin maceration. It seals the wound margins and reduces risk of maceration and limits the risk for damage of newly formed tissue. It is transparent for easy inspection, conforms well to body contours, maintains a moist environment, and leaves no residue, shower proof, high biocompatibility, and anti-inflammation properties. These include:

1. Mepore® Film Transparent Polyurethane Film (Molnlycke, Mundelein, Il) [3]
2. 3M TEGADERM™ Transparent Dressing (Vitality Medical (St. Paul, MN) [4, 5]

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3. Mepitel® Film IV AM Transparent Antimicrobial Securement Dressing (*Molnlycke Health Care US LLC,* Norcross, GA) [6, 7]
4. OpSite (Smith and Nephew, London, UK) [8, 9]

2.2 Hydrocolloid Dressings

Hydrocolloid dressings have an active surface treated with a gel-forming substance consisting of pectin, carboxymethylcellulose, polymers, and other adhesives. They are an opaque, flexible, wafer that adheres to the skin. When in contact with wound exudate, the polymers absorb the fluid and swell, forming a gel which is confined within the structure of the material. New dressing only needs to be applied every 3–7 days. Dressings are impermeable to bacteria.

These dressings include:

1. Tegaderm Hydrocolloid by 3M (Vitality Medical (St. Paul, MN) [10, 11]
2. Restore Hydrocolloid Dressing (Hollister Libertyville, IL)
3. RepliCare Thin Hydrocolloid Dressing (Smith & Nephew, London, UK)
4. DuoDERM CGF Hydrocolloid Dressings (ConvaTec, Bridgewater, NJ)
5. Medihoney Hydrocolloid Dressing (Derma Sciences, Plainsboro, NJ)

2.3 Alginates

Alginate wound dressings are highly absorbent and are primarily used to manage heavily exuding wounds. Designed to protect and maintain a sterile wound environment, these dressings also help keep the wound bed moist for ideal wound healing. In addition to wounds, Alginate dressings are also used for deep burns [12] and venous, pressure, and diabetic foot ulcers. Some alginate dressings are:

1. Cutimed Alginate Dressing (Chicago, IL)
2. Derma Algicell Ag Calcium Alginate Dressing with Antimicrobial Silver (Derma Medical, London, UK)
3. Maxorb Extra Ag + CMC/Alginate Dressings (Medline Industries, Northfield, IL)

2.4 Hydrogel Dressings [13–16]

Hydrogel dressings generally come in three different forms, including:

1. Amorphous hydrogel: a free-flowing gel, distributed in tubes, foil packets, and spray bottles
2. Impregnated hydrogel: typically saturated onto a gauze pad, nonwoven sponge ropes, and/or strips
3. Sheet hydrogel: a combination of gel held together by a thin fiber mesh

Hydrogel dressings are used for dry or dehydrated wounds, partial or full-thickness skin lesions, abrasions or severe scrapes, minor burns, wounds with granulated tissue development, and radiation skin damage.

The dressings create a moist healing environment which promotes autolytic debridement, epithelialization, and wound granulation. By increasing moisture content, hydrogels have the ability to help cleanse and debride necrotic tissue from the wound surface.

Some hydrogel dressings are:

1. AquaClear (Hartmann, São Paulo, Brazil)
2. AquaSite Sheet (Derma Sciences, Scarborough, ON, Canada)
3. Derma-Gel (Medline Industries, Northfield, IL)
4. Curafil (Kendall, Walpole, MA)
5. Nu-Gel Hydrogel (Systagenix, Skipton, UK)

2.5 Hydrofera Blue

This is a safe, effective, and proven moist wound dressing. It is constructed of polyvinyl alcohol (PVA) sponge complexed with two organic pigments methylene blue and gentian violet, which provide broad-spectrum bacteriostatic protection. Hydrofera Blue is intended for use on complex wounds, pyoderma, radiation burns, chemical

burns, venous stasis ulcers, yeast infections, folliculitis, and infected wounds. It can also be used to provide a safer moist environment for optimum wound healing on non-infected wounds. Hydrofera Blue is highly absorptive and bacteriostatic, and its vacuum action draws excess fluid and exudates from the wound bed. It causes debridement of necrotic tissue. Dressings should be changed every 1–3 days.

2.6 DuoDERM [17–19]

DuoDERM occurs in four forms.

1. DuoDERM® CGF™ dressing is an adherent dressing indicated for the management of exuding wounds. It promotes granulation and facilitates autolytic debridement that removes necrotic tissue as quickly as enzymatic debridement. It hydrates dry wounds and creates a moist wound environment to prevent wound drying and to allow autolytic debridement to occur. Dressings at the right point in time during the healing of a wound. DuoDERM® dressings can be worn for up to 7 days. The dressings are conformable and can be easily and gently molded into place.
2. DuoDERM® Extra Thin dressing can be used as a primary hydrocolloid dressing for dry to lightly exuding wounds and is thin, flexible, and versatile and is designed to reduce the risk of further skin breakdown due to friction. It can be used to manage stage I and stage II pressure ulcers. The translucent backing enhances dressing placement and initial monitoring of the wound. The dressings are easy to use and mold and can be cut to shape to dress awkward areas.
3. The DuoDERM® Signal™ dressing has a visual change indicator designed to make dressing change decisions easier. It is a primary dressing for low-to-moderately exuding wounds and is used to manage stage I and stage II pressure ulcers. The thin, smooth, low-friction backing is designed to reduce shearing that can prematurely dislodge the dressing.
4. DuoDERM® Hydroactive® GelSterile Gel is a clear, preservative-free, viscous hydrogel that incorporates a unique ConvaTec hydrocolloid formulation that distinguishes it from other hydrocolloid dressings. It is indicated for the management of partial and full-thickness wounds, ideally as a filler for dry cavity wounds to provide a moist healing environment.

2.7 Regranex® (Healthpoint Biotherapeutics, Arlington, TX) [20, 21]

Regranex® is a hydrogel which contains 100 microg of platelet-derived growth factor-BB (rhPDGF-BB) (becaplermin) per gram that is used to treat certain foot/leg ulcers in people with neuropathic diabetic ulcerations, sacral decubitus ulcer, and decubitus sores by increasing granulation tissue formation and epithelialization.

Conclusions

There are many dressings available for treating wounds. Physicians should be aware of the type of wounds each type of dressing is used for and how often its application is changed [22]. Many of the dressings will heal the wounds faster than simpler dressings and certainly faster than secondary intention healing.

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Wound Dressings and Comparative Effectiveness Data

Aditya Sood, Samuel Kogan, and Mark S. Granick

1 Introduction: Fundamental Wound-Healing Physiology

Wound healing is an intricate and complex process with a multitude of interdependent components. While there are several classifications that have been proposed for wounds, wound healing in its most rudimentary form consists of four phases which work in a cascade—hemostasis, inflammation, proliferation, and remodeling [1]. Comprehension of the fundamental physiology of wound healing will allow the clinician to have a better understanding of the context of the differential efficacy of various wound-dressing products available on the market.

Immediately following injury, vasoconstriction occurs resulting in reduced blood flow to the damaged skin [2]. The extrinsic coagulation cascade is initiated, with platelet aggregation at the site of injury ultimately leading to the deposition of fibrin [3]. A clot containing fibrin, platelets, and red blood cells serves both to protect the wound from contamination and as an anchor for the subsequent migration and attachment of other leukocytes whose activity is necessary for the subsequent steps of wound healing [3, 4]. In fact, the platelets within the clot release cytokines crucial for the later stages of wound healing. Platelet-derived growth factor (PDGF) is a chemotactic signal for macrophages and fibroblasts; transforming growth factor-β (TGF-β) stimulates keratinocyte migration, as well as macrophage and fibroblast chemotaxis, and epidermal growth factor (EGF) which is necessary for keratinocyte migration and replication [5, 6].

The inflammatory phase of wound healing is predominately mediated by neutrophils, which account for approximately 50% of all cells at the site of tissue injury after 24 h [7]. Neutrophils secrete proteolytic enzymes to remove devitalized tissue and kill bacteria [8]. By the third day after injury, monocytes make up the predominant cell population in the wound bed [8]. As monocytes differentiate into macrophages, they take on an important role as antigen-presenting cells and phagocytes. Macrophages secrete many important cytokines (TGF-α, TGF-β, IGF-1, FBF, PDGF, and VEGF) and are also thought to generate nitric oxide (NO), a free radical used to kill many pathogens [9, 10].

The proliferation phase of wound healing involves angiogenesis, collagen deposition, granulation tissue formation, reepithelialization, and wound contraction. Keratinocyte migration begins soon after injury and is followed by

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keratinocyte proliferation, both of which are essential for reepithelialization, which is the process to restore the protective barrier of the skin. This then allows for basement membrane proteins to reform. During the proliferation phase, granulation tissue replaces the fibrin clot and serves as a scaffold for cell migration. Fibroblasts are the key cell of granulation tissue formation, as they secrete proteolytic enzymes to disrupt the fibrin clot and secrete type I and type III collagen, fibronectin, elastin, and proteoglycans [11]. The expanding granulation tissue requires an appropriate vascular supply to meet its metabolic requirements, and thus angiogenesis is a major component of the proliferation phase. Vascular endothelial growth factor (VEGF) is the key mediator of angiogenesis and stimulates the production of new endothelial cells and capillaries at the wound boundaries [12]. TGF-β stimulates fibroblasts to differentiate into myofibroblasts, which contain α-smooth muscle actin, which provides the contractile forces needed for wound contraction [13].

Remodeling, the final stage of wound healing, occurs approximately 3 weeks after injury. In this phase, the type III collagen is replaced by type I collagen. Matrix metalloproteinases (MMPs) degrade excess collagen, vascular cells and myofibroblasts undergo apoptosis, and granulation tissue is converted into scar [13]. Within the newly formed scar, thin collagen fibers are gradually replaced with thicker collagen fibers, resulting in drastically increased strength of the wound—from 3% of normal dermis at 1 week after injury to 80% of the strength of normal dermis 3 months after injury [10]. However, despite a continuous remodeling process for approximately 1 year, scars never regain the full strength of uninjured skin.

2 Deficiencies in Wound Healing and Chronic Wounds

Given the complexity of wound healing, it is not surprising that errors in any of the numerous components may result in failure of acute wound healing, resulting in a chronic wound. A wound is classified as chronic when wound healing is delayed by more than 3 weeks or when the wound fails to return to a functional state. Chronic wounds are remarkably different than acute wounds. Chronic wounds lack key growth factors and cytokines and thus have reduced keratinocyte and fibroblast migration. Furthermore, they display an increase in reactive oxygen species, tissue proteases, and microbial contamination.

Broadly speaking, factors that may impair wound healing are either intrinsic or extrinsic. Intrinsic factors may be thought of as the general health of the patient, as well as his or her predispositions—age, immunodeficiency, hereditary disorders of wound healing, and other chronic disease states. Extrinsic factors include malnutrition, microbial infection, hypoxic conditions, smoking, cancer, radiation, and medications [14]. Both intrinsic and extrinsic factors can compromise immune function and therefore impair the inflammatory phase of wound healing. Immunosuppressive agents impede the body's ability to carry out the inflammatory phase and thus halt progress through the latter phases of wound healing. Malnutrition may result in protein, vitamin, or mineral deficiencies, all of which drastically impair the wound-healing process.

Infection is an important consideration with respect to impaired wound healing. Wound infection occurs when the bacterial burden is greater than 10^5 microbes/g of tissue. Bacterial infections impair leukocyte activity and thus negatively alter the downstream events of proper wound healing. Biofilms almost exclusively are seen in chronic wounds and rarely display the typical signs of infection. Biofilms are notoriously challenging to treat and often rely on extensive surgical debridement of tissue for effective containment [15].

3 Principles of Optimized Wound Healing

While wound healing is remarkably complex, the healing of cutaneous wounds can be broken down to three key principles: ensuring adequate blood supply, wound hydration, and reduction of microbial invasion and subsequent infection [16]. Optimization of perfusion to the wound is gener-

ally regarded as the most crucial factor and must be prioritized before attempting advanced wound care techniques. Cessation of smoking is fundamental to ensure adequate blood flow to the wound bed. Additionally, removal of nonviable tissue through a variety of possible debridement techniques—surgical, autolytic, enzymatic, mechanical, or larval—is necessary for wound healing to progress. Medical comorbidities and nutritional status must be well managed if one is to attempt wound-healing optimization (blood glucose <200 g/dL, albumin >3.0 g/dL, prealbumin >15 mg/dL, total lymphocyte count >1500).

4 Ideal Wound Dressings

Natural products have long been explored as wound dressings, with records of ancient civilizations using honey, oils, wines, animal fats, mud, leaves, and even animal dung [17]. While most of these remedies were used in the past secondary to ease of access and without any empirical evidence of efficacy, the use of honey in wound healing has been well studied and has shown to provide some benefit.

Warm, clean, and moist environments are the ideal conditions for wounds to heal [18]. These conditions are conducive to the migration of epithelial cells, ultimately leading to wound edge contraction [19]. The optimal dressing may be selected based on the conditions of the wound. Dry or desiccated wounds require hydration; wounds producing excess exudates need to have the fluid absorbed; infected wounds require appropriate antimicrobial agents; and wounds with necrotic tissue necessitate debridement (Fig. 1). The ideal wound dressing protects the wound and adjacent skin, prevents bacterial contamination, involves minimal pain upon application and

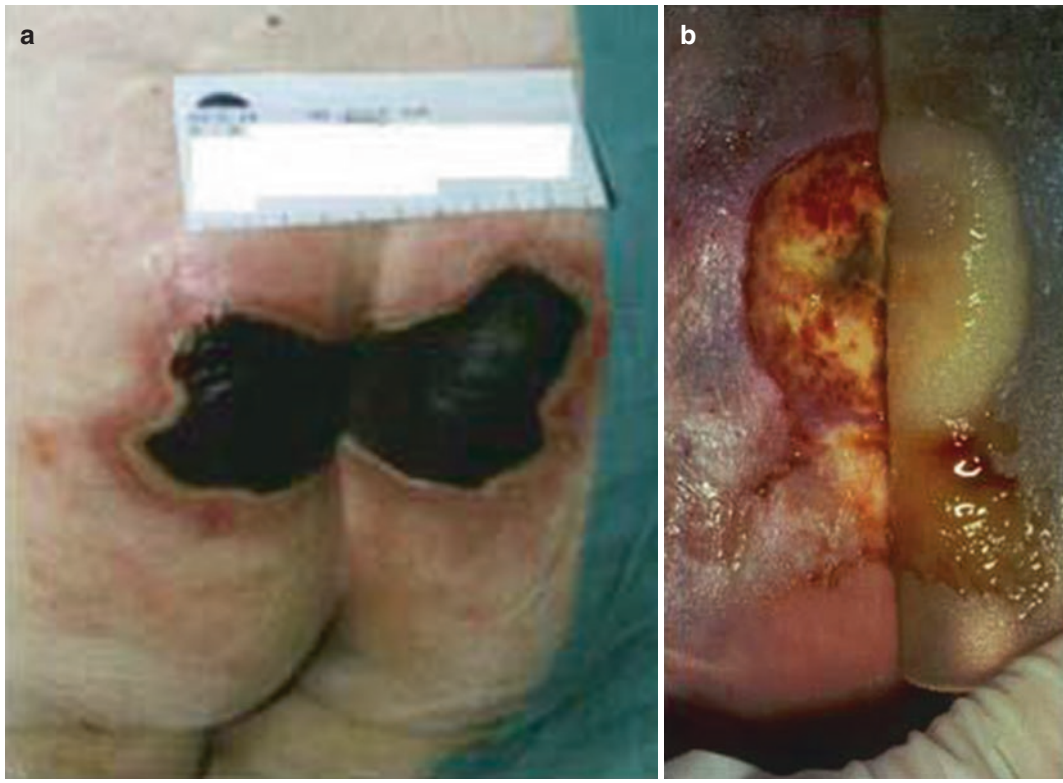

Fig. 1 (**a**) A sacral wound demonstrating moisture imbalance with a dry eschar, which delays normal wound healing and may act as a source of infection, increasing treatment time and cost. (**b**) A chronic wound of the lateral foot, with excess fluid production and signs of periwound skin maceration

Table 1 Characteristics of an ideal wound dressing

Characteristics of an ideal dressing
Creates a moist, clean and warm environment
Provides hydration to dry or desiccated wounds
Removes excess exudates
Prevents dessication and is nontraumatic
Protects periwound area
Allows for gaseous exchange
Prevents entry of microorganisms
Free of toxic or irritant particles
Does not release particles or fibers
Can conform to wound shape
Minimal pain during application and removal
Easy to use
Cost-effective

removal, does not release non-biodegradable fibers into the wound, and maintains the optimal temperature and pH (Table 1).

Given the abundance of dressings commercially available, the clinician may find multiple products that suit the needs of the wound. As such, it is also important to be cognizant of the changing wound environment and to be able to provide the most optimal dressing as the conditions of the wound change. Wound dressings may be considered as belonging to one of three broad categories. The first category of dressings facilitates autolytic debridement, in which the patient's own phagocytic cells and autolytic enzymes remove nonviable tissue [20]. The second category of wound dressings regulates the moisture of the wound. Lastly, the third category of wound dressings focuses on inhibiting bacterial growth.

5 Types of Wound Dressings

5.1 Gauze

In 1891, Johnson and Johnson began mass-producing gauze, a sterile dressing composed of cotton yarn and thread [21]. Gauze quickly became the most commonly used surgical dressing, as it is inexpensive, reliable, and highly absorbent [21]. Gauze is available in both woven and nonwoven forms, in which the latter form is composed of pressed synthetic fibers and offers increased absorbency. Gauze is a versatile dressing and is used in both infected and non-infected wounds, wounds of various sizes and shapes, and to remove exudates and prevent premature wound closure.

Despite its many utilities, it is important for clinicians to understand when gauze is an inappropriate choice for wound dressing. In particular, woven gauze may potentially cause wound trauma and/or mechanical debridement, as it requires force to remove. Additionally, woven gauze may leave behind residues, which may lead to impaired formation of granulation tissue.

Wet-to-dry dressings are the most widely used dressings in the United States. They are believed to ensure a moist wound environment, as well as function in mechanical debridement [22, 23]. However, there is tremendous controversy over these supposed benefits, as studies contest the ability of wet-to-dry dressings to keep the wound moist and their ability to remove debris and nonviable tissue [23, 24]. In particular, removal of the dried gauze may reinjure the wound, cause pain, and delay wound healing [24]. Furthermore, evaporation of the wet dressing results in cooling of the tissues, resulting in reflexive vasoconstriction, hypoxia, and impaired leukocyte activity, all contributing to impaired wound healing [24]. Additionally, removal of the dry gauze often involves some degree of pain and discomfort to the patient. When the dried gauze is removed, there is risk of cross-contamination of wounds by dispersion of bacteria into the air. As the mechanical debridement provided by wet-to-dry dressings is nonselective, healthy tissue adjacent to the wound may also be damaged.

In addition to the concerns regarding bacterial dispersion during removal of dried gauze, in vitro studies have demonstrated that bacteria readily pass through up to 64 layers of gauze and that infection rates are significantly higher in wounds using gauze compared to transparent films or hydrocolloids [25–27].

Despite these serious critiques of gauze as a wound dressing, little has been done to critically compare its efficacy against other dressings. A 2004 Cochrane Collaboration review of "dressings and topical agents for surgical wound healing by secondary intention" discovered a profound

absence of randomized controlled trials comparing differences between dressings when the primary end point was time to wound healing [28]. High-quality RCTs are necessary to accurately assess the clinical benefits and drawbacks of gauze.

5.2 Impregnated Gauze

Some of the limitations of gauze were discussed in the previous section, particularly the nonocclusive nature of gauze and its adherence to the wound as it dries. Impregnated gauze, with substances such as petroleum, iodine, bismuth, and zinc, serves to make these dressings nonadherent and moderately occlusive. Impregnated gauze dressings allow for increased retention of moisture in the wound bed and decreased desiccation/trauma during dressing changes, all of which may help promote wound healing.

Impregnated gauze is a versatile dressing that can be used both as nonadherent primary dressings and as a contact layer on granulating wound beds when used with secondary gauze dressings. They are often used both on the donor and recipient skin graft sites. They serve as an ideal dressing for burns, as their removal is without pain, unlike plain gauze dressings. As impregnated gauze dressings minimize moisture loss from wounds, localized cooling is reduced, and the negative consequences of vasoconstriction are avoided [24].

Despite their benefits over wet-to-dry gauze, impregnated gauze has some drawbacks that are important to be aware of when deciding an appropriate dressing. Bismuth is cytotoxic to inflammatory cells and may cause an exaggerated inflammatory response. For this reason, bismuth-containing dressings are not advised for patients with venous insufficiency ulcers. Iodine-impregnated dressings are also cytotoxic, but are only mildly antibacterial. They are indicated for tunneling wounds with odorous discharge, but must be frequently changed and not to be used for a duration greater than 5 days, as the cytotoxicity of iodine may cause tissue damage. Additionally, as impregnated gauze is unable to absorb exudates, they are not recommended for wounds with heavy drainage.

Despite their proposed benefits, semiocclusive dressings, such as impregnated gauze, have not been shown to reduce overall wound-healing times or costs as compared to regular gauze for surgical patients [29, 30]. While impregnated gauze dressings are changed less frequently than their dry counterparts, the higher costs of the products preclude any cost savings.

5.3 Transparent Film Dressings

Transparent film dressings are thin and flexible sheets, most often manufactured from either polyurethane or co-polyester. Given that they are transparent, they allow both the clinician and the patient to monitor wound healing, without removal of the dressing. These dressings are selectively permeable: they allow for the escape of water vapor, oxygen, and carbon dioxide, but prevent the passage of bacteria and water. By not permitting water loss, they provide a moist environment for wound healing and promote autolytic debridement. Transparent film dressings are not absorptive and thus are a poor choice for excessively exudative wounds. Furthermore, they should not be used for infected wounds, as the warm, moist, non-draining environment is conducive to bacterial growth.

Most often, transparent film dressings are used in the setting of surgical incisions, superficial wounds without exudates, skin graft donor sites, intravenous catheter sites, and areas prone to friction. In a prospective randomized study involving 80 patients undergoing elective split-thickness skin grafting, film dressings were compared to both paraffin gauze and foam dressings. This study detected no differences in healing rates at 14 days after grafting, but the film dressing group reported more comfortable use and simpler removal [31]. Earlier studies reported similar findings of reduced pain in treating skin graft donor sites, but also showed improved healing rates [32, 33]. Despite their lack of absorptive properties, film dressings are commonly used over skin graft donor sites. If exudates are seen to

accumulate under the dressing, the fluid can be released and subsequent patch coverage with another transparent film. Film dressings are also used on surgical wounds following primary closure left to heal by secondary intention. A massive case series involving 3637 surgical incisions over 8 years concluded that semiocclusive transparent film dressings were able to increase the rate of wound healing, decrease pain, cause less scarring, and promote patient mobility and hygiene when compared with traditional dressings [34].

5.4 Foam Dressings

Foam dressings are manufactured from semipermeable polyurethane and allow for the free passage of water vapor and gases, but prevent bacteria from entering the covered wound. Versions are available with a waterproof external layer. These hydrophilic dressings have absorptive properties, making them a good choice for wounds with moderate-to-heavy exudates. Foam dressings are quite versatile and can be used on granulating or slough-covered partial- and full-thickness wounds, donor sites, ostomy sites, minor burns, and diabetic ulcers. Additionally, they can be used on infected wounds, but should be changed daily [35]. On non-infected wounds, they can be left in place for 4–7 days and changed when saturated with exudates. Removal of foam dressings is painless and does not reinjure the wound. Foam dressings are not ideal for dry or eschar-covered wounds or arterial ulcers, due to their ability to further dry the wound.

A handful of studies compared different types of foam dressings for their affinity for liquids and permeability to moisture vapor. Variations in these properties make certain foam dressings more preferable for lightly exudative wounds versus heavily draining wounds. A randomized controlled trial compared the effectiveness of Allevyn (Smith and Nephew, London, United Kingdom) to Biatain (Coloplast, Humlebaek, Denmark) for the treatment of lower leg ulcers [36]. Of the 118 patients, 76% had excellent absorbency rated during dressing changes with Biatain, compared to only 7% with Allevyn. Patients treated with Biatain required fewer weekly dressing changes than those treated with Allevyn (2.14 vs. 3.34), which resulted in lower treatment cost ($10.87 vs. $18.99) for the Biatain group [36]. Unfortunately, randomized controlled trials comparing foam dressings to other treatment modalities for highly exudative wounds have not been completed.

5.5 Hydrogels

Hydrogels are complex hydrophilic organic cross-linked polymers, composed of 80–90% water. They are available in both a free-flowing amorphous of fixed flexible sheet form. Hydrogels have the ability to absorb a minimal amount of fluid by swelling, but they may also provide moisture to a dry wound, promoting autolytic debridement and thermal insulation. Hydrogels promote granulation and epithelialization of the wound bed while reducing the temperature by up to 5 °C [37, 38]. Compared to occlusive dressings, they are more permeable to gas and water, but they are a poorer bacterial barrier. Hydrogels are typically used to hydrate wound beds and facilitate debridement of necrotic wound debris.

Due to their high water concentration, they lack strong absorptive properties and are not intended for use in bleeding wounds. Often times they require a secondary dressing. The skin adjacent to the wound needs to be protected from excessive hydration, as maceration may occur. Hydrogels are often used for pressure ulcers, partial- and fullthickness wounds, and vascular ulcers. They can be used in conjunction with topical medications and antibacterial agents. Hydrogels can be left in place for up to 3 days, but require secondary dressings.

Hydrogels (Biofilm®, B.F. Goodrich, Akron, OH) were compared to hydrocolloid dressings (Duoderm®, ConvaTec, Skillman, NJ) in a 1990 RCT involving 90 patients with 129 pressure ulcers [39]. Sixty-two wounds were treated with hydrogel and 67 with hydrocolloid for a maximum of 60 days. While approximately 90% of hydrogel-treated wounds and 78% of

hydrocolloid-treated wounds improved during the treatment time, the hydrogel group had nearly double the rates of wound healing (43 vs. 24%) [39]. A 2005 prospective study compared hydrogel to gauze soaked in povidone-iodine solution in 27 spinal cord injury patients with 49 pressure ulcers [40]. Eighty four percent of patients in the hydrogel group had wound healing, while only 54% of patients in the gauze group ($p < 0.04$). The authors concluded that hydrogel dressing facilitated healing of pressure ulcers by promoting more rapid epithelialization.

5.6 Hydrocolloids

Hydrocolloids are unique two-layer dressings. The inner layer is composed of hydrophilic particles such as gelatin, pectin, carboxymethylcellulose (CMC), or some other elastomer. The inner layer is self-adhesive and does not require any external fixation. When the inner layer comes in contact with fluid such as exudate, the material swells into a gel over the wound. The gel covering ensures a moist environment for healing and also thermally insulates the wound. The outer layer is composed of polyurethane and seals the wound from bacteria, foreign debris, and shearing forces. Hydrocolloid dressings are sold in a multitude of sizes and shapes and are available in a paste, powder, or granule form. These dressings promote wound healing by providing a moist environment, preventing bacterial contamination, promoting autolytic debridement, and not requiring a secondary dressing. Hydrocolloids can be left on the wound for up to 7 days and removed once drainage is noted beneath the dressing. These dressings are regularly used for partial- and full-thickness wounds with low to moderate exudates, granular and necrotic wounds, minor burns, and pressure ulcers. They are to be avoided in the setting of infected wounds. As these dressings are self-adhesive, caution should be taken in fragile skin adjacent to the wound.

Varghese et al. [41] examined the differences in local environments of chronic wounds either under hydrocolloids or film dressings. The authors collected fluid from the wounds of nine patients who had 14 chronic full-thickness ulcers dressed with Duoderm® or Opsite® (Smith and Nephew), a transparent film dressing. They measured very low pO_2 levels beneath both dressings, despite the permeability of film dressings. Additionally, the pH of the wound fluid in the hydrocolloid group was more acidic, but the authors believe that was related to the chemical composition of the hydrocolloid product. Regardless of the reason, they suspect this increased acidity had an antibacterial effect as well as reduced the toxic effects of ammonia, a product of enzymatic digestion of material in the wound. Later studies examined the fibrinolytic activity of hydrocolloids; however, much of which has been refuted [42].

Many studies, both in animals and humans, have been conducted comparing hydrocolloids to film dressings. A study by Chvapil et al. [43] used a porcine model of split-thickness wounds to compare the effects of eight dressing regimens on the rate of epithelialization. The authors observed moderate to severe inflammatory changes in pigs treated with a variety of dressings including collagen sponge, polyethylene glycol, Duoderm®, and lanolin ointment. Compared to gauze-covered wounds (the control group), all other treatments resulted in significantly faster rates of reepithelialization. The authors suggested that the overlap between inflammatory reaction and increased reepithelialization could be explained by activation of inflammatory cells that released cytokines and growth factors, which mediated the repair process. Another porcine wound healing experiment compared Duoderm® with Clearsite® (ConMed, Utica, NY), a hydrogel sheet dressing [44]. They found that with respect to full-thickness surgical wounds, the hydrogel dressing resulted in a faster rate of closure and reepithelialization compared to the hydrocolloid product. Upon histological examination of the wounds, vacuoles and foam cells were observed in the hydrocolloid-treated group. Results from later studies suggest that wounds incorporate debris from hydrocolloid dressings, which may impair epithelial migration during acute secondary wound healing [45].

5.7 Alginates

Alginate dressings are derived from seaweeds and contain alginic acid as well as calcium and sodium salts. In addition to being highly absorbent, they are nonadherent and biodegradable. When exposed to serum in a wound, the calcium and sodium ions in the dressings form a hydrophilic gel, which functions to create a moist wound environment, absorb exudate, and prevent microbial contamination. In fact, these products are capable of absorbing up to 20 times their weight, making them a good choice for highly exudative and draining wounds, pressure/vascular ulcers, surgical incisions, wound dehiscence, tunnels, sinus tracts, skin graft donor sites, exposed tendons, and infected wounds. Alginates are believed to have some hemostatic properties, making them useful in bleeding wounds. Alginate dressings are a poor choice for dry wounds, as they do not provide hydration, but do provide absorption. They may be kept in place for up to 7 days in a clean wound, but must be changed daily in infected wounds. While most alginate dressings are manufactured as sheets, which are ideal for superficial wounds, they are also produced as ribbons and ropes, which are used for packing deep wounds and cavities [46].

A handful of reviews and studies have been published examining the efficacy of alginate dressings. The first RCT to study alginate dressings was part of a Drug Tariff reimbursement study [47]. The study consisted of 64 patients with leg ulcers treated with an alginate dressing (Sorbsan®, UDL, Rockford, IL) or paraffin gauze dressings. Individuals treated with the alginate dressing had a 31% healing rate, while those in the gauze-dressing arm had a 4% healing rate during the study period. Seventy three percent of patients in the alginate-dressing group had evidence of improved ulcer healing, as measured by a reduction in wound area compared to control. Critics of this study argued that sustained graduated compression should have been part of the treatment protocol [48, 49]. The use of alginate dressings in the setting of skin graft donor sites has been studied, with some suggesting significantly better wound healing and reduced pain compared to paraffin gauze-treated controls [50].

An RCT compared alginate, gauze moistened with a 0.05% hypochlorite solution, and a combined dressing pad (absorbent pad plus a semipermeable film dressing) in the management of 36 cases of abdominal wound dehiscence [51]. While the healing rates between the three products were not statistically significant, pain was greater ($p = 0.011$), satisfaction lower, and costs higher in the group treated with gauze moistened with a 0.05% hypochlorite solution. The authors suggested sodium hypochlorite be abandoned for surgical wounds. Another study examined the performance of the combination of alginate and film dressings, which suggested the effectiveness of this combinatorial treatment for moist chronic wounds that produce low-to-moderate levels of exudates [52].

Despite the prevalent use of alginate dressings, few studies have reported statistically significant justification of their use in any particular type of wound. Some randomized trials have yielded conflicting data. However, it has become increasingly apparent that the secondary dressing used in conjunction with the primary alginate is of tremendous importance. For heavily exudative wounds, an absorbent pad is beneficial, while a semipermeable film or foam is a preferred for light to moderately exudative wounds. Pirone et al. [53] compared alginate dressings with either polyurethane film or gauze with a hydrocolloid dressing on partial-thickness wounds in pigs. The results suggested that healing rate was related to the moisture-retaining properties of the two-part dressing system. Notably, healing rates were poorer in the alginate and gauze group, leading the authors to suggest that alginates should not be used on dry wounds or under gauze dressing. It has been suggested that there are three primary factors when considering the use of alginate dressings: (1) chemical nature of alginate, (2) amount of fiber implanted, and (3) vascularity of tissue at site of implantation [54].

5.8 Hydrofibers

Hydrofiber dressings are composed of nonwoven sodium carboxymethylcellulose (CMC) fibers,

which form a gel on contact with serum or exudates. As the fibers turn into a gel, the dressing provides a moist environment for wound healing and serves as a barrier against microbes. These dressings are an appropriate choice for heavily exudative or infected wounds. They may be left in place for up to 7 days or until saturated.

Aquacel® (ConvaTec, Princeton, NJ) is a widely used hydrofiber dressing composed of CMC fibers integrated with ionic silver. Hydrofibers are structurally similar to alginates and share many properties. Numerous studies have compared hydrofibers to alginate dressings, as both are indicated for similar wounds. Aquacel was compared to Sorbsan® in a multicenter RCT involving 132 patients with leg ulcers for 84 days [55]. Aquacel was preferred for its ease of application and removal, greater interval between dressing changes, and decreased costs. Hydrofibers were compared to paraffin gauze dressings in the treatment of split-thickness skin graft donor sites [56]. Patients treated with Aquacel reported significantly less pain and had faster rates of wound healing, with superior cosmetic results at 1 year. CMC products are a viable option for partial-thickness and small burns, but allograft skin is better suited for larger burns of mixed depth [57]. Lastly, in a theoretical cost model, Aquacel was evaluated to be more cost-effective than gauze because of reduced nursing costs associated with a lower frequency of dressing changes [58].

5.9 Hydroconductive Dressings

Hydroconductive dressings (SteadMed Medical Drawtex®) are a novel class of dressings, which were first revealed at the Symposium on Advanced Wound Care in Spring 2011. The primary layer uses capillary action to draw exudates away from the wound into the core, where it disperses into a second layer. LevaFiber™, the proprietary name of the Drawtex dressing technology, is composed of two types of absorbent, cross-action structures that facilitate the ability to move large volumes of exudates and debris through the dressing. The dressings are capable of holding 30–50× their own weight of fluid. They also are capable of moving debris away from the wound surface. The hydroconductive debridement layer helps to loosen and lift slough tissues, allowing for easy removal upon dressing change. The dressings do not shed fibers and can be left in place for up to a week.

Multiple studies have been completed looking at the activity of hydroconductive dressings. Drawtex has demonstrated in multiple RCTs to decrease wound exudate, decrease tissue bacterial levels, decrease nutrients for biofilm production, decrease MMPs, facilitate wound bed preparation, aid in burn wounds, and serve as a possible alternative to negative pressure wound therapy (NPWT).

Using an in vitro model of infected burn wounds, Oritz et al. demonstrated a significant reduction in bacterial counts in methicillin-resistant *Staphylococcus aureus* (MRSA)-containing media that had Drawtex submerged in it, with a concurrent increase in bacterial counts in the material itself [59]. Additionally, when placed in a protein-rich solution, Drawtex was able to significantly reduce the protein concentration over time, suggesting the dressing is able to absorb proteins, potentially including virulence factors, in a wound environment [59]. Ochs et al. [60] used a chronic wound model and observed that Drawtex decreased bacterial levels to $<10^2$ colony-forming units per gram and decreased MMP-1 and MMP-9 levels while simultaneously increasing the measured amount of MMP in the material itself. The authors believe this data suggests hydroconductive dressings, such as Drawtex, are capable of drawing bacteria and deleterious cytokines away from the wound bed and into the dressing.

Wolcott and Cox [61] studied the effect of using Drawtex dressing and a multilayer compression wrap in ten patients with non-healing, moderate to highly exudative venous leg ulcers in a small cohort study. Nine of the ten patients had at least 40% wound healing within the duration of the 4-week study. The authors also performed polymerase chain reaction (PCR) to quantify the bacterial burden of the wound before and after treatment. They did not detect a correlation

between the reduction of wound biofilm and wound healing. The authors concluded that the rapid removal of exudate improved wound healing, but the mechanism was not by reduction of bacteria.

5.10 Oxidized Regenerated Cellulose and Collagen

Oxidized regenerated cellulose and collagen (ORC) is a bioabsorbable topical hemostatic woven material used to control bleeding. For almost 50 years, ORC has been used in surgery and dentistry as a hemostatic agent and has been shown to reliably control capillary, venous, and small arterial bleeding. Upon contact with a bleeding tissue, ORC forms a gelatinous mass, which is absorbed within 2–7 days. In vitro studies show that ORC creates an acidic environment, suggesting a possible bacteriostatic effect [62].

A small pilot study was performed by Hofman et al. [63] examining the use of Traumacel® (Synapse Medical, Dublin, Ireland), the calcium salt of oxidized cellulose on 11 patients with 15 non-healing leg ulcers. During the course of the 12-week study, five ulcers healed and three patients reported significant pain relief. The authors concluded Traumacel was safe for use in chronic wounds and promoted wound healing in some recalcitrant ulcers. These authors also examined the effects of Traumacel P® powder on human dermal fibroblasts in vitro [64]. They observed that at 0.5 mg/mL and 1.0 mg/mL of the powder, fibroblasts were metabolically stimulated and went on to speculate that promotion of fibroblast proliferation may explain the mechanism in which Traumacel facilitated the healing of chronic leg ulcers in their first study.

ORC may be formulated in a collagen matrix. Collagen is the main structural protein of the extracellular space and is necessary for wound healing and repair. In these dual compound dressings, 55% bovine collagen is combined with 45% ORC. When the compound comes into contact with exudates, it forms a gel matrix and is also capable of binding and inactivating MMPs. In a rat model of wound healing, the combined ORC/collagen dressing resulted in statistically significant increased rates of reepithelialization compared to hydrocolloid dressings [65]. Additionally, the ORC/collagen group had decreased skin cell apoptosis, increased local growth factor concentrations, and accelerated wound healing in full-thickness excisional wounds.

5.11 Silicone Dressings

Hypertrophic and keloid scars are the result of abnormally excessive wound healing. They were typically treated with long-term application of pressure garments. In the early 1980s, it was observed that sheets of silicone gel, made from polydimethylsiloxane, could be used for these scars instead of pressure garments. Over time, silicone dressings would soften the scar tissue, allowing for a decrease in the height of the hypertrophic scar [66]. While the mechanism of action of silicone sheet dressings is not fully understood, some hypothesize that the dressing prevents water vapor loss, increasing hydration of the scar. Additionally, as fibroblast activity is reduced, less collagen is deposited, resulting in a less hypertrophic scar [67]. Silicone dressings have continued to become more widely used, as it has a non-traumatic adhesive component, which makes dressing changes less painful. Furthermore, silicone is a chemically inert dressing; thus it neither interacts with the wound nor does it have an effect on the cells responsible for wound healing.

While many studies have been published on silicone dressings, the benefits of using silicone over other dressings have been mixed. For example, De Oliveira et al. [68] compared silicone with non-silicone gel dressings in a controlled prospective trial in the treatment of keloids and hypertrophic scars. Relative to the untreated controls, individuals in both the silicone and non-silicone groups had decreased scar size and induration, suggesting that both treatments were equally efficacious in the treatment of keloids and hypertrophic scars. In another study, silicone sheets were observed to decrease the incidence of new hypertrophic scarring when applied to surgi-

cal wounds approximately 2 weeks postoperatively in high-risk populations [69].

Again, while there have been multiple studies on silicone dressings, many of them suffer from small sample size, lack of controls and comparisons to other treatment modalities, and short follow-up times. A number of reviews have been published on the use of silicone gel sheets and other treatments in the management of hypertrophic and keloid scars. Three of the reviews concluded that there was weak evidence to support some benefits associated with the use of silicone gels [70–72]. Another article suggested surgical excision and postoperative intralesional steroid injection provided a reasonable treatment outcome [73] and a separate one deemed there to be insufficient information for clinicians to make informed decisions [74].

5.12 Silver Dressings

John Woodall first described the antimicrobial properties of silver in 1617 in *The Surgeon's Mate*. Silver is a broad-spectrum antimicrobial agent with activity against bacteria, fungi, yeast, and viruses. At higher concentrations, it is also effective against MRSA and vancomycin-resistant enterococci (VRE) [75]. Due to silver's extensive activity, it can be found in a wide variety of dressings and products (Fig. 2). Silver may also aid in reducing inflammation, which promotes wound healing. To determine the optimal dosing of silver to achieve either bacteriostatic or bactericidal effects, the local wound environment must be thoroughly considered [76]. Silver has been proven to be effective against superficial microbes, but it is less potent against deeply infiltrating bacterial infections. As such, it is of far greater use in mildly infected superficial wounds, where it can decrease bacterial count.

While all silver dressings release silver upon contact with fluid, they vary greatly in the rate, duration, and peak levels of silver released. The antibacterial mechanism of action of silver is multifactorial. Once the silver cations are released, they are capable of penetrating cell walls, inactivating bacterial enzymes, and impairing DNA synthesis. Because silver affects multiple targets in the bacteria, significant resistance has yet to be documented. However, there have been some reports of isolated *Escherichia coli* and *Pseudomonas aeruginosa* resistance to silver in in vitro experiments [75]. These reports should caution practitioners to ensure maintenance of minimum inhibitory concentrations of silver in contaminated wounds.

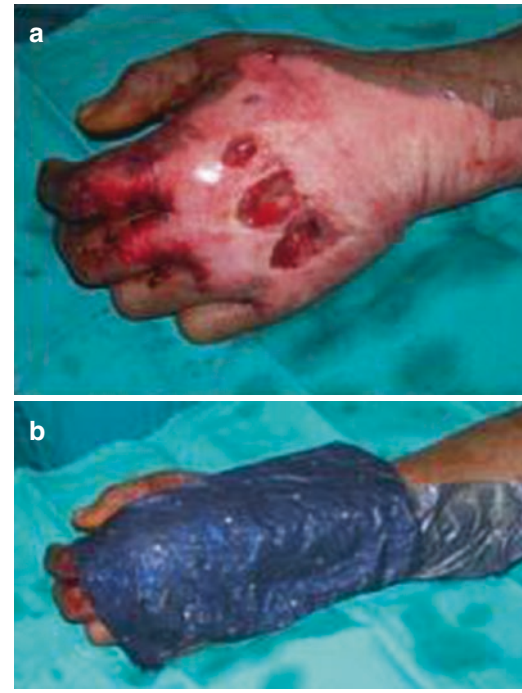

Fig. 2 (**a**) A superficial and partial-thickness burn wound to the dorsum of the hand. (**b**) After application of a silver impregnated dressing

While many in vitro models of wound fluid and silver treatment have been explored, there is a paucity of in vivo studies. The complexities and intricacies of real wounds are impossible to be fully represented in in vitro studies, and thus, it is not unjustified to have concerns about the translation of the conclusions of those studies to real-world practice. A 2007 Cochrane Collaboration review examined the effects of silver and silver dressings on wound healing in contaminated and infected wounds [77]. The authors were only able to identify three RCTs that met the inclusion criteria. The majority of the studies comparing silver dressings to other treats found no significant

difference in the rates of complete healing. Of the three included studies, one found a statistically significant reduction in relative wound size in the silver-treated group [78], another reported a statistically significant improvement in the wound-healing rate (cm^2/day) over 4 weeks [79], and the last study reported a statistically faster wound size reduction in the silver dressing group [80]. Despite all the literature on the antimicrobial properties of silver, none of the studies examined the duration of wound infection with silver treatment, and the Cochrane review concluded there was insufficient evidence to recommend the use of silver dressings or topical agents for the treatment of infected wounds. Without direct evidence from RCT trials, clinicians are left to extrapolate the efficacy of silver dressings from in vitro studies [81].

Despite a lack of quality human trial data, silver-based products are bountiful and manufactured in combination with nearly all types of dressings, including alginates, collagens, creams, foams, films, hydrofibers, hydrogels, hydrocolloids, and negative pressure sponges [82]. As mentioned earlier, silver dressings vary widely in their ability to deliver silver to the wound. To achieve an antibacterial effect, a minimum concentration between 5 and 50 ppm of silver is needed in the wound. The difficulty of accurately modeling a wound bed in the lab should not be understated, and the real-world presence of biofilms and host proteins likely affect the total delivery of silver ions to the wound bed and bacteria.

5.13 Polyhexamethylene Biguanide and Honey Dressings

Polyhexamethylene biguanide (PHMB) has historically been used as an antiseptic in contact lens cleaning solutions, wet wipes, and other products, but has recently gained the interest of wound care professionals. PHMB is available as a cleansing solution and in biocellulose dressings. PHMB is often used at a concentration of 0.3%, which is non-cytotoxic and nonirritating and carries a very low risk of sensitization [83]. It has been shown to have a broad spectrum of activity against bacteria, fungi, molds, yeast, MRSA, and VRE. Eberlein et al. [84] compared PHMB biocellulose dressings to silver dressings in 38 colonized or locally infected wounds. At the end of the 28-day study period, they observed a significantly faster reduction of critical colonization and local wound infection ($p < 0.001$) in the PHMB group. While treatment with either PHMB or silver dressings reduced bacterial burden and wound pain, the PHMB treatment was significantly faster and better with respect to eliminating the bacterial burden.

Manuka honey is produced in Australia and New Zealand from the nectar of the manuka tree. It was first described as a topical treatment for infected wounds in 1892. Since then, countless studies have explored the utility of honey products and dressings as wound-healing products. In vitro experimentation has shown Manuka honey to be effective against bacteria and fungi including *S. aureus*, *P. aeruginosa*, MRSA, and VRE [85]. There have also been reports that honey can inhibit the formation of biofilms from certain bacterial species. A recent study determined that methylglyoxal is the antibacterial component of Manuka honey [86].

It has also been suggested that honey may decrease odors from wounds by providing autolytic debridement by drawing exudates away from the wound bed. Gethin and Cowman [87] conducted a prospective, multicenter RCT to compare the desloughing efficacy and healing outcomes in venous ulcers with Manuka honey versus hydrogel. At 4 weeks into treatment, the mean reduction in slough was 67% in the honey group and 52.9% with hydrogel ($p = 0.054$), and the mean reduction in wound size was 34 and 13%, respectively ($p = 0.001$). At 12 weeks, 44% of the wounds treated with Manuka honey were healed compared to 33% in the hydrogel group ($p = 0.037$). The authors report that the Manuka honey group had higher rates of healing and a lower incidence of infection than the hydrogel group.

A retrospective study compared honey dressings to silver sulfadiazine dressings for wound

healing in burn patients [88]. The average time of healing was 18.16 days in the honey group and 32.68 days in the silver sulfadiazine group. Additionally, all wounds treated with honey became sterile by 21 days, while it took 36.5 days for the silver sulfadiazine group. From this study, the authors concluded that honey dressings resulted in shorter times to both sterility and wound healing and had better outcomes in terms of hypertrophic scars and post-burn contractures [88].

A Cochrane Systematic Review of the literature examined 19 studies, involving a total of 2554 patients [89]. The authors report that many of the studies examined were of poor quality, and thus the results of them must be interpreted with caution, with the exception of studies involving venous ulcers. They determined that in acute wounds, honey may reduce time to healing compared with conventional dressings in partial-thickness burns. The authors of the review determined there to be insufficient evidence to determine whether or not honey conferred any benefit for burns or in other types of acute or chronic wounds compared to other treatments [89].

5.14 Iodine Dressings

Iodine is an essential micronutrient that plays a key role in human metabolism, particularly with respect to the thyroid hormones T_3 and T_4. Since the initial discovery of the antimicrobial properties of iodine in 1882, iodine-based products have played important roles in the prevention of surgical site infections [90]. Iodophors are disinfectants containing iodine and a solubilizing agent such as surfactant that release free iodine when in solution. They were developed in the 1950s as an alternative to using pure iodine, because of side effects including pain and skin irritation. The most commonly used iodophors in dressings include povidone-iodine and cadexomer iodine. The povidone-iodine preparations were developed in the 1960s and are widely used as an antiseptic in the preparation of preoperative hand scrubs.

While there is substantial support for the use of povidone-iodine in wound healing, some argue against its use due to perceived issues of toxicity and systemic absorption. The controversy exists, in part, due to conflicting evidence in the literature. For example, animal models have shown some degree of iodine toxicity, whereas human trials have shown povidone-iodine can improve wound healing by reducing the bacterial burden in the wound [91, 92]. While not yet fully understood, it is believed that the antimicrobial effects are due to iodine's ability to rapidly penetrate the cell wall of microorganisms [93]. In vitro studies have demonstrated that both povidone-iodine and cadexomer iodine are effective in common bacterial wound isolates and MRSA infections.

With respect to the prevention and management of biofilms, some studies have reported that low-dose, slow-release iodine is effective in killing free-floating bacteria, and they suggest iodine is a good choice of antiseptic dressing [94, 95]. Thorn et al. [96] compared silver- and iodine-containing wound dressings with respect to their antimicrobial properties in an in vitro model of preformed mature biofilms. The authors report that both dressings were able to target the bacterial species in the biofilm, but the iodine dressing was more efficacious. Philips et al. [97] similarly reported that sustained release iodine may penetrate biofilms more effectively than either silver of PHMB. Controversy also exists regarding the cytotoxicity of iodine resulting in delayed wound healing; however, the relatively slow release of low doses of iodine can improve healing rates and is potent antimicrobial agents with a broad spectrum of activity.

Slow-release iodine dressings are indicated in a variety of wounds with either confirmed or suspected infection. These include pressure ulcers, venous leg ulcers, diabetic foot ulcers, minor burns, and superficial skin-loss injuries. Due to iodine's critical role in metabolism and thyroid function, it is imperative to carefully supervise patients with thyroid disease and iodine sensitivity, those who are pregnant or breast-feeding, and newborns. Iodine dressings should be changed when they lose their color, as that is an indicator of their antiseptic effect.

5.15 Charcoal Dressings

The primary purpose of charcoal dressings is to reduce wound odor by absorbing gases released by bacteria; thus they function as a deodorizing agents. There is an inherent difficulty in quantifying wound odor, and therefore it is much more of a subjective than objective characteristic. Anaerobic bacteria such as *Bacteroides* and *Clostridium* species are most often associated with malodorous wounds, but aerobic bacteria may also result in a foul odor. Research suggests that particular wound odors are due to specific bacterial species [98]. Leg ulcerations and fungating lesions are most commonly associated with odor production, but others can be malodorous as well.

While charcoal dressings may diminish wound odor, elimination of bacterial infection is the most effective method to curtail odor. Systemic antibiotics may be used; however, ensuring optimal concentrations in the wound bed is challenging. Topical antibiotics including metronidazole and clindamycin, honey, and sugar have been explored both to eliminate bacteria and odor [99–101]. Clinical experiences have strongly supported the ability of charcoal dressings to reduce wound odor, but objective data of pure charcoal compounds is absent from the literature.

5.16 Negative Pressure Wound Therapy

Negative pressure wound therapy (NPWT) was developed in the 1990s and quickly becomes a standard component of the armamentarium of wound-healing practitioners. NPWT involves the application of suction to the wound bed. A variety of dressings can be used in NPWT, and the result is a semiocclusive environment, which facilitates a moist wound bed. The constant negative pressure on the wound bed removes excess fluid, allowing for increased circulation and the elimination of cellular waste, reducing the risk of bacterial contamination.

NPWT is indicated for use in a wide range of wounds including acute and traumatic wounds, surgical dehiscence, pressure ulcers, diabetic, arterial and venous ulcers, and both fresh and compromised flaps (Fig. 3). It can also be used in skin grafts to increase the rate of granulation and epithelialization. In most situations involving NPWT, dressings can be changed every 2–3 days, but must be changed every 12 h in the setting of infection.

A multitude of foam products are available to suit different wound types. For example, black, sterile polyurethane foam contains large pores and is best suited for stimulating granulation tissue and wound contracture. On the other hand, white, sterile, polyvinyl alcohol foam is denser with smaller pores and is better suited for deep wounds, undermined flaps, sinus tracts, or overexposed vertebra. While white polyvinyl alcohol foam is less prone to adhering to the wound bed,

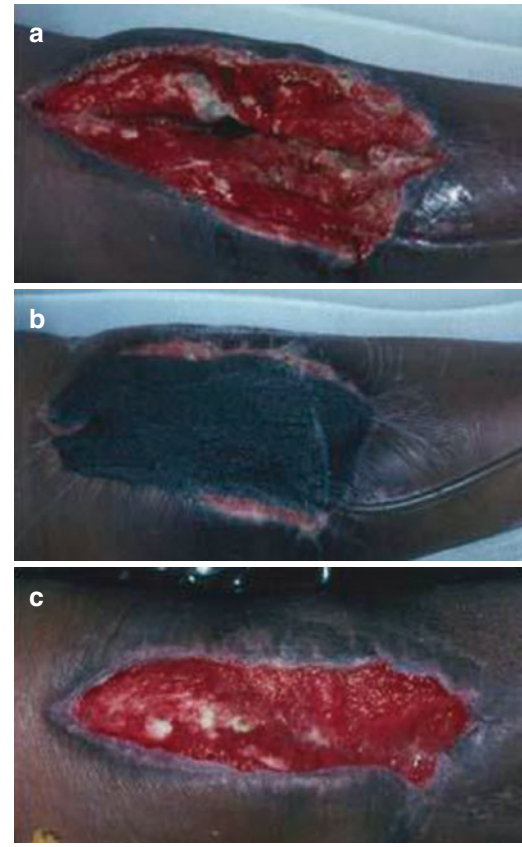

Fig. 3 (**a**) A traumatic wound of the anterior lower leg after surgical debridement. (**b**) The same wound after NPWT application. (**c**) Three weeks later, demonstrating wound contraction and a healthy wound bed

it also does not stimulate granulation tissue formation. Other wound contact dressings, including gauze and polyurethane, may be used with NPWT, but there is a stark paucity of published data comparing the efficacy of the various contact layers.

Numerous case reports, review articles, and clinical trials have been published on NPWT, with a wide range in quality. In 2008, Vikatmaa et al. [102] analyzed 14 RCTs using NPWT in a variety of wound types. The authors were only able to classify two of the studies as "high quality," while the remaining 12 were deemed to have poor internal validity. In all 14 studies, NPWT was found to be at least as effective and, in many cases, more effective than the control treatment.

Philbeck et al. [103] attempted to judge the benefits of NPWT by performing a retrospective study comparing the costs of NPWT against conventional therapies. They examined the records of 1032 Medicare patients with 1170 NPWT-treated wounds of all types. Based on their calculations, a 22.2 cm^2 wound would take 97 days and cost $14,546 to heal when using NPWT, but would take 247 days and $23,465 days to heal using more conventional therapy. The authors concluded that NPWT were associated with 61% faster healing rates and 38% lower costs when compared to saline-soaked gauze for the treatment of certain pressure ulcers.

Studies have also been conducted to determine the optimal time to initiate NPWT. Kaplan et al. [104] performed a retrospective study in which they compared the clinical and cost-benefits of initiating NPWT therapy for traumatic wounds at an early stage (days 1–2, 518 records) or later stage (days 3–4, 1000 records). Individuals in the earlier intervention group had fewer hospital inpatient days (10.6 vs. 20.6 days; $p < 0.0001$) and fewer treatment days (5.1 vs. 5.0 days; $p = 0.0498$) than those in the later intervention. Additionally, early intervention had lower total and variable costs than later intervention. The data generated by this study supports the notion that NPWT is both clinically and financially a sound option for the treatment of traumatic wounds.

While evidence does exist supporting the use of NPWT in a variety of wound types, some clinicians still hesitate to initiate therapy due to the initial costs. Larger, more powerful RCTs examining the cost-effectiveness of NPWT will be necessary to overcome this hurdle. NPWT has proven to be an effective dressing choice for large wounds that cannot be closed primarily, heavily exudative wounds, and those that have lymphatic involvement. An excellent summary of the indications and contraindications of NPWT was published in 2008 as a consensus document [105].

5.17 Amnion/Chorion Membrane Products

Human amnion/chorion membrane products have begun to be extensively explored as a multifaceted tissue regeneration and wound-healing modality. These products are derived from human placental tissue, which contains many growth factors and cytokines that are associated with wound healing [106, 107]. Historically, fresh amnion/chorion membranes were harvested from patients undergoing elective cesarean section, and they were quickly processed for use in patients. Recently, significant advances have been made in the processing and preservation of dehydrated human amnion/chorion membranes (dHACM), which allows these products to be stored for extended periods of time, and used when necessary. This has expanded the utility of dHACM to both emergent and elective procedures [107–109]. One such product, EpiFix® (MiMedx Group, Inc., Marietta, GA), has been studied both in the laboratory and in human patients. ELISA assays of EpiFix have detected and measured the levels of vascular endothelial growth factor (VEGF), platelet-derived growth factors AA and BB (PDGF-AA and PDGF-BB), transforming growth factors alpha and beta (TGF-α and TGF-β1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and granulocyte colony-stimulating factor (GCSF) [106, 107]. Prior to the advancements in the processing and preservation of dHACMs, Apligraf® (Organogenesis, Inc., Canton, MA), a bilayered bioengineered skin substitute, was brought to market in the late 1990s for the treatment of ulcers that failed standard wound care [110]. Apligraf is prepared by culturing

human foreskin-derived neonatal fibroblasts in a bovine type I collagen matrix, which is then cultured with human foreskin-derived neonatal epidermal keratinocytes. Like dHACMs, Apligraf provides cytokines, growth factors, and a matrix to promote wound healing. Apligraf is regularly used for the treatment of both chronic venous leg ulcers and diabetic foot ulcers (Fig. 4).

Within the past decade, numerous cohort studies and RCT have been performed examining the clinical utility of amnion/chorion membrane products in a broad spectrum of wound types. A prospective, randomized, controlled, multicenter study compared two dHACM products against standard of care in diabetic patients with chronic lower extremity ulcers [111]. Patients were stratified into three groups of 20 patients each to receive a weekly application of either Apligraf® (Organogenesis, Inc., Canton, MA) or EpiFix® (MiMedx Group, Inc., Marietta, GA) or standard wound care with colla-

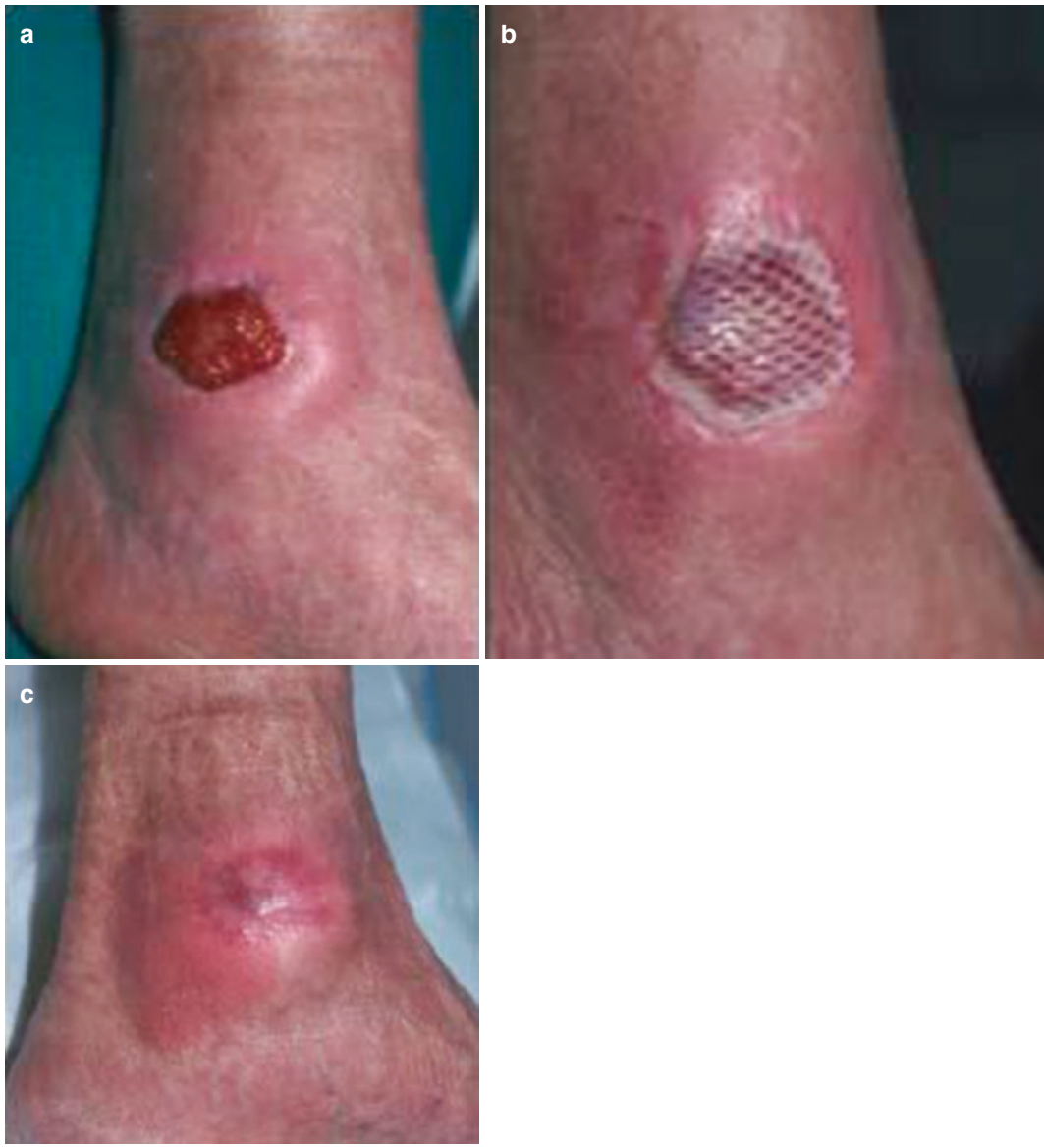

Fig. 4 (**a**). A chronic right malleolar wound retardant to conventional wound healing. (**b**) After application of Apligraf® (Organogenesis, Inc., Canton, MA) in a meshed fashion. (**c**) Fully healed wound after four repeated weekly applications

gen-alginate dressing. After 6 weeks of treatment, 95% of patients in the EpiFix group had complete wound healing, while only 45% in the Apligraf and 35% in standard of care group achieved complete wound healing. The combination of dHACM with multilayer compression therapy was studied against multilayer compression therapy alone in an RCT for venous leg ulcers [109]. Four weeks after the onset of treatment, 62% of patients in the dHACM group showed greater than 40% wound closure, while only 32% of patients in the compression therapy group did ($p = 0.005$).

Human amnion/chorion products have also been explored for their use in the closure of post-laryngectomy pharyngocutaneous fistulas [112] and vesicovaginal fistulas [113]. Both of these studies reported successful wound closure using amnion/chorion membrane products, but the sample sizes were small, and thus larger studies are necessary to draw more generalizable conclusions. These membrane products were also evaluated on graft take in split-thickness grafts of extremity burns [114]. This study selected individuals with bilateral extremity burns who required split-thickness skin grafts for wound coverage. One limb of each patient had the skin graft fixed with staples (control group), while the skin graft on the contralateral limb was simply wrapped with an amniotic membrane. Each individual patient had one limb to serve as an internal control for the amnion membrane. The graft take rate was 96.8% in the amnion group and 88.8% in the control group. The amnion group had a mean duration of graft take of 6.98 ± 1.35 days and 13.9 ± 1.66 days in the control group ($P < 0.001$) [114]. The initial cohort of studies involving amnion/chorion membrane products are very promising, but larger studies are necessary in order to fully understand the benefit of this technology over existing wound dressings.

Conclusions

Numerous wound dressings are readily available, ranging from the most ancient and rudimentary of remedies (i.e., honey) to the most modern and high-tech (i.e., dHACMs). Despite the abundance of therapeutic options, after a careful review of the literature on wound dressings and management, it is apparent that a paucity of high-quality RCTs has been performed to evaluate wound dressings, with even fewer demonstrating a clear-cut benefit of a particular dressing or treatment modality.

It is evident that the wound care field would benefit from comparative effectiveness research. Large, high-powered studies with measureable, clinically significant outcomes are needed so that clinicians can accurately determine whether an intervention may help a patient and whether the clinical and economic benefit exceeds the potential harm and cost of a particular intervention. To address these needs, the European Wound Management Association established a Patient Outcome Group to generate recommendations on clinical data and collection on wound care [115]. Until the next generation of data is generated, clinicians must continue to systematically evaluate, categorize, and treat each wound using the guiding principles of debridement, managing exudates, and preventing microbial colonization.

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Early Wound Dressing Removal

Esther Eberstark, Steven L. Warsor, and David Peleg

1 Introduction

The primary function of the skin is to serve as a protective barrier against the environment. Wound healing occurs as a complex interplay of multiple biological and cellular processes, which are codependent. Full thickness wound healing is carried out in three phases, inflammation, proliferation, and remodeling. The purpose of the inflammatory phase is to control bleeding and establish a clean wound bed. This phase lasts approximately 3 days. The proliferative phase consists of three components, angiogenesis, collagen synthesis, and epithelization, and lasts for weeks to months. The final stage of wound healing is remodeling which can continue for over 1 year [1].

Covering of a primarily sutured surgical wound with a sterile dressing is ordinarily considered to be a routine conclusion to an aseptic operation. The purposes of a surgical dressing when used to cover a wound healing by primary intention are to control any postoperative bleeding, absorb exudate if anticipated, ease pain by providing support, provide protection for newly formed tissue, and restrict the ingress of bacteria into the wound and thus prevent contamination.

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Previous research has shown that bandaged wounds heal up to 40% more rapidly than non-occluded wounds [2]. This is thought to be due, in part, to re-approximation and easier migration of epidermal cells in the moist environment created by the dressing [3]. Another mechanism for improved wound healing may be the exposure of the wound to its own fluid [4]. Acute wound fluid is rich in platelet-derived growth factor and basic fibroblast growth factor and has a balance of metalloproteases serving a matrix custodial function [5]. These interact with one another and with other cytokines to stimulate healing [6].

There are a multitude of dressings available for a primary wound, ranging from plain gauze to advanced materials like permeable films and antimicrobial dressings. Walter and colleagues [7] examined 16 controlled trials on meta-analysis and did not find one dressing type superior to another or significant differences in patient pain or scarring [8]. Modern wound care dressing selection considers factors such as the phase of healing, the volume of exudate, and the presence of necrotic tissue to determine the type of dressing that will be most supportive of wound healing [1, 2, 9].

Wound complications include infection, seroma/hematoma formation, and dehiscence. As is true for all abdominal surgery, there is very little evidence as to how long the surgical bandage needs to remain over the incision with regard to minimizing wound complications.

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Some have even questioned whether the bandage is needed at all.

2 Animal Studies

In 1962 Winter [9] published a study comparing the effect of air drying to occlusive dressings on resurfacing. His study conducted on domestic pigs showed a 30% difference between the two treatments, with occlusion providing a greater benefit.

A study conducted on swine in 1988 concluded that there is a window of time during the first 6 h in which the presence of an occlusive dressing will produce an increased rate of epithelialization [10]. Other confirmed that when a suitable dressing is applied to a wound and changed appropriately, the dressing can have a significant impact on the speed of wound healing, wound strength and function of the repaired skin, and cosmetic appearance of the resulting scar [11–13].

3 Human Studies

Historically, surgical dressing removal has been at 24–48 h postoperation, as recommended by the CDC [13]. Beyond 48 h, it is unclear whether an incision must be covered by a dressing or whether showering or bathing is detrimental to healing. Small crossover trials indicated that occlusion of the wound increases the speed of re-epithelialization, although complete healing appears to occur at about the same time when compared to uncovered wounds [11, 12, 14]. Similar results have been obtained in other studies [2, 3, 15].

The Cochrane Collaboration reviewed the medical literature up to September 2016 to assess the effect of wound dressings and the effect of alternative wound dressings in preventing surgical site infections [8]. The review included 29 trials (5718 participants) and contained 11 comparisons in total. Four randomized controlled trials investigated early (permanent removal of dressings within 48 h of surgery) versus delayed removal of dressings (permanent removal of dressings after 48 h of surgery with interim changes of dressing allowed). The authors concluded that no significant differences were reported between the two groups in terms of superficial surgical site infection (infection of the wound), superficial wound dehiscence (partial disruption of the wound that results in it reopening at the skin surface), or the number of people experiencing more serious adverse events. There were no cases of deep wound infection or complete wound dehiscence (complete disruption of wound healing, when the wound reopens completely) in the studies that reported these complications. However, the studies were not large enough to identify small differences in various complication rates. None of the studies reported on quality of life. Participants in the group that had early removal of dressings had significantly shorter hospital stays and incurred significantly lower treatment costs than those in the delayed removal of dressings group, but these results were based on very low-quality evidence from only small randomized controlled trials [16–20].

A small RCT published in 2011 of 124 patients with clean surgical wounds compared surgical wounds left without postoperative dressing to those with a surgical occlusive dressing concluded that wounds left open do not have an increased incidence of surgical site infection and wound dehiscence compared with similar types of wounds [21].

Another multicenter RCT (n = 857 participants) investigated the effect of removing the wound dressing within the first 12 h compared with keeping the wound covered for 48 h postoperatively. Participants were patients from a primary care setting who were undergoing minor skin excisions. The primary outcome was surgical site infection defined by CDC criteria. The study found no statistically significant difference between the two groups (RR 0.96, 95% CI 0.62 to 1.48) [22].

One quasi-RCT (n = 1202 participants) examined the effect of leaving a postsurgical wound uncovered after the first 24 h following surgery on the incidence of infection. The control group had the dressing removed only at the time of removal of the sutures. Participants were surgical

patients undergoing clean and clean-contaminated operations. The study found no statistically significant difference between the two groups (RR 0.97, 95% CI 0.59 to 1.60) [23].

4 Cesarean Section

The US cesarean section rate released by the National Center for Health Statistics for 2015 was 32.0%, making it the most frequent operation in women aged 18–44 years performed in the United States [24] and an important contributor to surgical site infection. Cesarean delivery continues to increase with advanced maternal age: women aged 40 and over were more than twice as likely to deliver by cesarean as were woman under 20.

Indications for cesarean delivery fall into two general categories: medically indicated or on maternal request. Approximately 70% of cesarean deliveries in the United States are primary (first) cesareans. The three most common indications for primary cesarean delivery in the United States account for almost 80% of these deliveries [25].

1. Failure to progress during labor (35%)
2. Non-reassuring fetal status (24%)
3. Fetal malpresentation (19%)

Today, cesarean section is performed with a transverse skin incision (Pfannenstiel) since it is associated with less postoperative pain, greater wound strength, and better cosmetic appearance than the vertical midline incision [26]. We rarely perform a vertical incision. We use a vertical incision when we believe it will be faster and the incision-to-delivery time is critical, as well as when we believe a transverse incision may not provide adequate exposure or may be too prone to hematoma formation. Vertical incisions generally allow faster abdominal entry, cause less bleeding and nerve injury, and can be easily extended cephalad if more space is required for access [27].

The resulting wound from a cesarean section is considered to fall into the clean-contaminated category because the surgical procedure involves an incision into the genitourinary tract with minimal spillage of contents [13]. We re-approximate the skin with intracuticular sutures or surgical staples and cover using a wound bandage.

The incidence of wound complications in the obstetric population varies in the literature, with rates ranging from 2.8 to 26.6% [1]. Post-cesarean wound infection is the cause of significant maternal morbidity, extended hospital stays, additional staff allocation, and sepsis management.

A myriad of risk factors have been reported for post-cesarean-related surgical site infections.

1. Antepartum factors
   Low socioeconomic status, limited prenatal care, obesity, tobacco use, diabetes mellitus
   Significant maternal comorbidities (American Society of Anesthesiologists class of three or more), hypertensive disorders, multiple gestations, and corticosteroid administration
2. Intrapartum factors
   Unscheduled or nonelective cesarean, length of labor, length of rupture of membranes, number of vaginal examinations, internal fetal monitors, chorioamnionitis, duration of operation, absence of antibiotic prophylaxis, management by teaching service, and wound length
3. Postpartum factors
   Subcutaneous drains, anemia, and postoperative hematoma [1, 28–47]
   A trial involving 320 patients who underwent scheduled cesarean delivery reported no detrimental effects from dressing removal at 6 vs. 24 h postsurgery. The secondary finding of this study proved that early removal of the bandage allowed woman to wash or shower sooner, which led to increased satisfaction with their postoperative care [48].

5 Current Recommendations

Guidelines for prevention of surgical site infection have been published by the CDC [13, 49]. These include pre-procedure showering within 24 h of surgery, hair removal with clippers rather

than shaving immediately before surgery, antibiotic prophylaxis, proper preparation of the skin, good surgical technique, and covering the incision site with a sterile dressing for 24–48 h. Despite these measures, a considerable percentage of women will still experience wound complications.

The conclusion of the latest update in 2013 of the National Institute for Health and Clinical Excellence (NICE) clinical guidelines addressing the prevention and treatment of SSIs stated there is no robust evidence to support the use of a dressing in the immediate postoperative period for the prevention of surgical site infection. However, it is generally accepted good clinical practice to cover the wound with an appropriate interactive dressing for a period of 24–48 h unless otherwise clinically indicated, for example, if there is excess wound leakage or hemorrhage.

The systematic review of the Cochrane group published in 2016 concluded that at present, there is insufficient evidence as to whether covering surgical wound healing by primary intention with wound dressings reduces the risk of SSI or whether any particular wound dressing is more effective than others in reducing the rates of SSI or whether any particular wound dressing is more effective than others in reducing the rates of SSI, improving scarring, pain control, patient acceptability, or ease of dressing removal.

Guidelines for managing surgical closed wounds instruct that patients keep their wounds dry and covered for 24–48 h [1]. However few published studies suggest that there is no conclusive evidence of harm from postoperative showering within 48 h of surgery in patients with closed surgical wounds [50–54].

Although data from randomized trials are limited, there seems to be inconsistency regarding the proper time to remove dressings to attain rapid epithelialization, moreover if albeit wound dressings are necessary at all.

Surgical site infections after wound incisions are an economic burden and threat to the health of patients. There is a wide array of modifiable risks, ranging from patient differences to intraoperative factors.

There is a lack of high-quality research evidence regarding whether choice of wound dressing, or indeed use of wound dressings at all, affects the rate of infections in people whose surgical wounds are healing by primary intention.

Existing research has not found that any dressing reduced infection in surgical wounds that heal by primary intention. While uncertainty remains regarding the best approach to dressing these surgical wounds, decision to use surgical wound dressings might be better based on the other properties and qualities that dressings can offer.

Using short dressing time not only reduces the number of nursing hours but also limits the need for costly dressing material. In addition, both wound observation and patients' personal hygiene are made easier.

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Electrospun Antimicrobial Wound Dressings: Novel Strategies to Fight Against Wound Infections

Liis Preem and Karin Kogermann

1 Introduction

Chronic wound infections are responsible for considerable morbidity and increased healthcare costs [1]. The scale of social and economic impact of non-healing wounds and wound infection is clear from harsh statistics. There are over 50 million patients suffering from severe wounds including 15% of diabetic foot ulcers that eventually required amputations. It is reported that in the USA, an excess of US $25 billion is spent annually on the treatment of chronic wounds [2]. The cost of managing wounds in the UK in 2012/2013 was retrospectively estimated to be £4.5–£5.1 billion [3], and in Scandinavian countries 2–4% of the total healthcare budget account for costs associated with chronic wounds [2].

Wound infection occurs when the virulence factors of wound pathogens overwhelm host resistance mechanisms resulting in invasion and division of the bacteria and local tissue damage [4]. Recently, it has been recognized that biofilm formation (i.e., complex microbial community) is one of the main problems associated with chronic wounds [5–7]and persistent infections causing delayed healing [8].

Current therapies to treat severe bacterial infection in the wound rely mostly on the systemic administration of antibiotics [9], but the related major concerns are the risk of toxicity during treatment and insufficiently low local drug levels in the wound [4, 8]. As an alternative, traditional topical pharmaceutical formulations have been used [10]. These topical formulations require frequent application and thus are ineffective in the presence of wound exudate or biofilm [1]. Importantly, persistent infections require prolonged antibiotic treatment due to the phenotypic heterogeneity of infecting bacteria [11, 12]. It has been shown that nondividing cells (persisters) often residing in biofilms are not efficiently killed by antibiotics, although they do not possess antibiotic resistance genes [11, 12]. Moreover, low drug concentration itself may initiate persister formation [13]. Therefore, local antibiotic levels in the wound during the therapy should be carefully considered.

Novel management strategies have been proposed for the removal and prevention of microbial bioburden and biofilms in the wound, and one important part is the use of topical antimicrobials in advanced dressings [8, 14, 15]. Antimicrobial wound dressings are applied when there is a risk of infection. Often these dressings are used prophylactically to prevent the wound infection development. There is an increasing interest to develop advanced antimicrobial wound dressing which is not only used to remove the microbes but also to interact with the wound and support the normal wound healing. In order to develop such dressings, several important properties need to be investigated which enable to

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understand the dressings and their potential applications for treating the wound infection and support the wound healing. In most cases, the selection of an appropriate antimicrobial agent becomes a critical factor. It is evident that biofilm plays a significant role in the development of hard-to-heal chronic wounds, and therefore novel strategies to fight against microbial biofilms are of relevance and need to be integrated into the wound dressing design and development.

Electrospinning is a promising method for the preparation of nanofibrous matrices for wound care. Electrospun nanofibers have several characteristics that favor their use in such applications including (1) ability to mimic the fibrillary structure of natural extracellular matrix (ECM), (2) the high surface area to volume ratio, (3) interconnecting porous structure with high permeability, and (4) the ability to incorporate active pharmaceutical ingredients (APIs) [16]. Appropriate nonwoven nanofibrous mats used as localized drug delivery vehicles can remarkably improve the wound healing and also reduce the systemic absorption of APIs and toxicity problems [17]. During the last decade, a wide range of APIs, including antimicrobials, have successfully been incorporated within the nanofibers, while the amount of literature has increased tenfold within last few years on the application of electrospun antimicrobial nanofiber mats as wound dressings.

In the present chapter, electrospun antimicrobial nano-/microfiber mats as wound dressings are overviewed as regard of their material and relevant physicochemical/mechanical properties and possible clinical relevance. Specific focus will be put on the wound dressings that are under the development which may have huge potential to be used as novel wound care products. Different data sources will be used including peer-reviewed literature, animal studies and reports, patents, as well as regulatory guidelines where available.

2 Wound Infection and Biofilm Formation

It is important to note that practically all wounds are contaminated (presence of nondividing bacteria), but not always an infection (dividing bacteria causing tissue damage) is present. It is even shown that some bacteria seem to aid the healing process [1]. Nowadays, a concept of wound infection continuum is recognized, implying that there are no clear-cut boarders between harmless contamination and clinically symptomatic infection presenting all classical signs of inflammation (pain, heat, swelling, redness, loss of function). An intermittent phase where no such visible symptoms exist, but bacteria already delay healing and cause subtle detrimental effects to the host, is referred to as a critical colonization. A widely held opinion is that non-healing is associated with a bacterial load of more than 10^5 bacteria per gram of tissue. Still, different wounds may be differently affected by bacteria due to differences in underlying pathology [18]. Also, the virulence of individual pathogens needs to be considered, together with synergistic interactions of some bacterial species. Due to these reasons, it is not an easy task to detect this state where healing is compromised, but no other signs are present due to multiplying microorganisms in the wound, and it might be necessary to arm oneself with antimicrobial strategies in advance of symptomatic infection. It is clear that if bacteria prevent wound healing, intervention is required. Table 1 summarizes clinical signs and symptoms of infection in acute and chronic wounds.

Table 1 Clinical signs and symptoms of infection in acute and chronic wounds

Acute wounds	Chronic wounds
Pain	Abnormal or excessive granulation tissue
Swelling	Bleeding from fragile surface at dressing change
Warmth	Increasing pain
Redness	Persistent odor
Loss of function	Bridging and pocketing of purulent material
	Delayed healing

Roberts CD, Leaper DJ, Assadian O. The role of topical antiseptic agents within antimicrobial stewardship strategies for prevention and treatment of surgical site and chronic open wound infection [19]

The diversity of microorganisms in a wound is influenced by many factors, like wound type, depth, location, quality of tissue perfusion, and host immune response. Also, microbial proliferation is facilitated by the presence of foreign

material and devitalized tissue [4]. Broadly speaking, there are two main sources of wound contaminants: environment (exogenous microorganisms) and endogenous sources (surrounding skin, gastrointestinal, oropharyngeal, genitourinary mucosae). Although environment is a source of various wound contaminants, most of them are not able to replicate in a wound. Thus, most wound colonizers are endogenous.

Most commonly, *Staphylococcus aureus* and coagulase-negative staphylococci are isolated from the wounds. Other commonly observed bacteria in wounds include *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Prevotella* spp., *Bacteroides* spp., *Peptostreptococcus* spp., *Porphyromonas* spp., etc. [1, 18]. Routine microbiological cultures struggle to provide representative images of microbiological communities in infected wounds, but novel molecular techniques have shed light on the true diversity and polymicrobial nature of those infections. The number of strict anaerobic bacteria and yeasts is thus much bigger than previously estimated [7]. Although it is not always clear if the presence of certain bacteria is clinically relevant, especially in low numbers, some pathogens are always considered significant, e.g., group A beta-hemolytic streptococci, mycobacteria, and *Clostridium perfringens* [1].

It is a matter of debate whether the colonizing microbiota changes over the course of wound progression. There appears to be a correlation that the longer the wound persists, the higher is the number of both aerobic and anaerobic bacteria. Moreover, as chronic wounds have low oxygen levels, the growth of anaerobes is especially facilitated [1].

Microorganisms tend to aggregate on surfaces in most natural and clinical environments, forming biofilms. Biofilms are complex communities of bacteria embedded within a matrix of extracellular polymeric substance attached to a surface of a substrate [20]. The significance of biofilms lies in the phenotypic differences of sessile and planktonic bacteria, which makes them highly tolerant to antibiotic treatment and immunological reactions of the host, also increasing overall resistance to hostile environmental conditions. Thus biofilm-related infections are typically characterized by slow development and persistent nature, they rarely seem to be resolved by immune defenses and respond transiently to antimicrobial therapy. All in all, the eradication of these infections is highly impeded.

3 Strategies to Fight Against Wound Infections

One of the most successful strategies for eradication of biofilms is still physical debridement, aiming to completely remove all necrotic tissue and biofilm in wound bed, leaving healthy tissue unharmed. Although debridement alone may be insufficient, it is still considered as a cornerstone for a successful therapy. In addition to debridement, topical antiseptics and, less commonly, antibiotics are used. Wound dressings without any specific antimicrobial agent are also shown to possess features that help to reduce the bioburden. Being in close proximity with the wound, dressings can prevent open spaces on the wound surface which are prone to microbiological contamination. Also, hydrophobic dressings have the capability to adsorb bacteria onto their surface and consequently remove them from the wound [21]. In addition to relieving wound from bioburden, bacterial toxins can also be adsorbed, e.g., using dressings with activated charcoal (ACTISORB™, KoCarbonAg® dressings currently on the market).

Antimicrobials can help to reduce the number of planktonic bacteria, preventing their reattachment and biofilm regeneration [8]. Antibiotics are administered either systemically or topically. Systemic administration is indicated if deep tissues are infected or infection is systemic. However, it is known that systemic antibiotics have limited efficacy against dormant cells in a biofilm – they only suppress rapidly growing cells. It has been shown that systemic antibiotics have only 25–32% efficacy in treating or preventing wound biofilm formation [22, 23]. Poor circulation in many chronic wounds further impairs reaching appropriate antibiotic concentrations. Thus, local delivery could have its benefits in order to avoid systemic actions and a risk of side

effects. Although biofilm phenotype possesses high tolerance to antibiotics, it has been shown that immature biofilm that remains after physical debridement is more susceptible to treatment [24]. Antiseptics are applied topically on the intact skin or some on open wounds, and they are a valuable tool in the fight with infection, however, they can cause some harm to human cells. Antiseptics have broad-spectrum and they often have multiple microbial targets, which is beneficial in the treatment of polymicrobial wound infections. Most common antiseptics used on wounds are silver, polyvinylpyrrolidone (PVP) iodine, octenidine, and polihexanide (polyhexanide, polyhexamethylene biguanide, PHMB). Similarly to antibiotics, antiseptics are less effective against biofilm than against planktonic cells.

There are also several specific antibiofilm agents, like lactoferrin, ethylenediaminetetraacetate (EDTA), gallium, xylitol, dispersin B, and others. They act via various mechanisms, including blocking the attachment of planktonic bacteria to a surface, binding nutrients that are vital to microbial growth, interference with metabolic processes, degrading extracellular polymeric matrix, inhibition of quorum sensing, etc. Some of these antibiofilm agents are well recognized, and some still remain to be further studied and introduced to a clinical setting. Other antibiofilm strategies include the utilization of bacteriophages – viruses that infect bacteria – due to their bactericidal effects. It is proposed that using classical antimicrobials (antibiotics, antiseptics) together with antibiofilm agents could have synergistic and superior action against wound infection [7, 8, 25].

Complicated microbiological nature of chronic wounds and elusive symptomatology of critical colonization impede making a correct diagnosis and deciding on a course of appropriate treatment. Thus, wound dressings intended for chronic wounds are expected to prevent and cure infection. Wound dressings impregnated with antimicrobials provide currently indispensable aid in controlling wound bioburden. However, the release of those antimicrobials is often uncontrolled and result in rapid increase in local concentrations, favoring local toxicity but also systemic absorption and related side effects. Advances in nanotechnology have given a novel direction in the development of efficient wound dressings, enabling to modify the carrier material properties together with drug release and other biopharmaceutical aspects. Novel fibrous polymer-based wound dressings have the capability to deliver drugs to the site of action at a controlled rate for an extended period of time, establishing localized, clinically relevant drug concentrations and hence potentially improving the therapeutic efficacy, reducing toxicity, and enhancing patient compliance [26]. Thus, antimicrobial drug-loaded nano- and microfibrous dressings have emerged as a major interest in the development of topical drug delivery systems for managing wound infections.

4 Wound Dressing Properties

Wound dressings (e.g., solid and semisolid wound dressings) can be divided into three main categories based on their main action mechanisms: passive, interactive, and bioactive. Some literature has also differentiated advanced dressings from other interactive dressings (e.g., hydrocolloids, alginates, hydrofibers, dextranomers) [27]. Table 2 shows the main types of wound dressings together with their description and the expected mechanism of action.

Passive dressings are nonocclusive (e.g., gauze, tulle) which are used to protect the wound from contamination and to cover the wound to restore its function underneath. Interactive wound dressings are semiocclusive or occlusive dressings, including products like hydrogels, hydrocolloids, hydrofibers, silicones, and foams. Interactive products are mostly transparent, permeable to water vapor and oxygen and impermeable to bacteria, thus provide protection from microorganisms and contamination. All these advanced dressings have the capacity to provide a moist wound healing environment supporting the TIME concept (tissue, infection/inflammation, moisture balance, and edge of wound) [29]. Bioactive dressings include drug delivery devices,

Table 2 Wound dressings together with their description and mechanism of action [28]

Wound dressings	Materials	Desription	Mechanism of action/application
Passive (inert) dressings	Gauze (polyester or cotton), tulle (petroleum jelly)	Nonocclusive. Cover the wound to restore its function underneath	No regulatory function, some dressings may absorb exudate. Superficial acute wounds
Interactive dressings	Semipermeable polymeric films and foams, hydrogels, hydrocolloids, hydrofibers	Semiocclusive or occlusive, highly elastic and flexible, non-absorptive and/or moderately to highly absorptive. Barrier against penetration of bacteria to the wound environment, permeable to water vapor and oxygen	Regulate wound healing by simple physicochemical means control moisture level (e.g., gel formation). Epithelializing wound, superficial wound, and shallow wound with low amount of exudates (dry wound). Burn wounds. Moderately to highly exudating wounds. Chronic wounds
Bioactive dressings	Dressings including bioactive polymers such as alginate, collagen, gelatin, chitosan, or dressings consisting antibiotics, antiseptics, growth factors, enzymes, stem cells, plant extracts, phages, etc. Skin grafts, skin substitutes	Semiocclusive or occlusive dressings, barrier against contamination, highly elastic and flexible, tunable adsorptiveness. Specific mechanism of action depends on the properties of active substance and the carrier polymer, the incorporation method of the active substance, and its release	Delivering bioactive substances that assist in wound healing or dressing is constructed from material having endogenous activity. Regulate wound healing by means of physiologically active substances. Control the moisture balance in the wound. Infected wound, burns, chronic wounds (e.g., pressure ulcers, venous ulcers, and diabetic foot ulcers)

biological dressings, and skin substitutes. Advances in complex dressing technologies have enabled the development of epidermal, dermal, and extracellular or scaffold-like replacement products such as US Food and Drug Administration (FDA)-approved Alloderm™, TransCyte™, Biobrane™, and Epicel® cultured epidermal autograft and Suprathel. These products mainly consist of allogeneic dermal cells incorporated into the scaffold. Interactive and advanced bioactive dressings are capable of modifying the physiology of the wound environment and interacting with the wound surface to optimize healing by promoting debridement, enhancing granulation and reepithelialization, and reducing the exudate levels and bacterial colonization counts. Their main advantage is that these enable to maintain an optimal environment for wound repair. However, the disadvantages of these products are their high cost and low stability. For chronic wounds, more complex dressings are usually required [30], and therefore drug-loaded bioactive dressings have been developed. These dressings are functionalized and deliver the APIs such as antimicrobials or other relevant substances (e.g., growth factors). These substances have a direct role in changing the chemical and cellular environment of the local wound, stimulating the wound healing. In Table 3 some commercially available wound dressings with antimicrobial agents are summarized.

4.1 Characteristics of Ideal Wound Dressings

After TIME concept was introduced [29] and understanding about the importance of moisture in the wound reported [31], several new developments for wound care have been proposed [32]. These include both the new procedures performed on wound bed as well as new wound care products. An ideal wound dressing should on one hand provide protection from extraneous matters (e.g., microbial contamination) and on the other hand have antimicrobial properties to fight with an occurring infection. Furthermore, an ideal wound dressing should absorb the exudates from wounds and enhance aesthetic appearance after recovery. It has been highlighted that wound should have its moisture balance, which means that dry wounds are more prone for scar formation; however highly exudating wounds have

Table 3 Some examples of antimicrobial dressings currently of the market (not an exhaustive list)

Antimicrobial agent	Brand names	Wound dressing type
Silver	AQUACEL® Ag EXTRA™	Hydrofiber dressing
	ComfortFoam™ Ag Border	Silicon foam dressing
	PROMOGRAN PRISMA™ Matrix	Freeze dried oxidized regenerated cellulose and collagen dressing
	SilvaKollagen® Gel	Collagen gel
	SILVERCEL™	Alginate dressing
	Supreme Ag Calcium Alginate with Antibacterial Silver	Alginate dressing
	3M™ Tegaderm™ Alginate Ag Silver Dressing	Alginate dressing
	Absorbent Dermanet® Ag+ Border (DagB)	Multilayered composite dressing
	ACTICOAT	Contact layer dressing
	ACTISORB™ Silver 220 Antimicrobial Binding Dressing	Contact layer dressing with activated charcoal
	ALGICELL® Ag	Alginate dressing
Chitosan	Dextrosan®	Granules
Iodophor	IodoFoam®	Foam dressing
	IODOFLEX 0.9% Cadexomer Iodine Pad	Impregnated dressing
	Inadine	PEG impregnated woven dressing
Polyhexamethylene biguanide	Curity® AMD™ Antimicrobial Gauze Sponges	Gauze sponge
	Kendall™ AMD Antimicrobial Foam Dressing	Foam dressing
	Kerlix® AMD™ Antimicrobial Super Sponge	Sponge
Chlorhexidine	IV Clear™	Transparent film dressing with silver and chlorhexidine
	SurgiClear™	Transparent film dressing with silver and chlorhexidine
	Bactigras	Paraffin gauze
Honey	MEDIHONEY® HCS	Superabsorbent gelling dressing
	TheraHoney Gel Honey Dressing	Gel

problems with maceration of the periwound area. As all wounds are contaminated, the clinical recognition of wound infection is critical. As discussed in previous paragraph, the presence of biofilm needs to be considered when designing, preparing, and choosing effective wound dressings. The use of topical antiseptics to control bioburden in wounds has taken enormous attention, since it is emphasized that the increasing antibiotic resistance of antibacterial agents may lower their relevance for the treatment of chronic wounds.

Despite the fact that there are more than 3000 different dressings currently on the market, no one and only solution exists. There is no one single dressing suitable for every kind of wound. The selection of suitable dressing is based on the clinical symptoms, and only after appropriate diagnosis and wound bed preparation [33], the most appropriate wound dressing is selected [34]. There is no superior product that heals chronic wounds like venous leg ulcers, diabetic wound, and pressure ulcers which often fail to achieve complete healing and may result in complications. Hence developing novel dressings that address the major interfering factors of normal healing process will help patients and wound care practitioners largely. For clinicians even their own classification has been proposed which helps to classify the wound dressings and helps to understand which dressings should be used based on the clinical symptoms [16]. It has been shown that polymeric fiber mats are suitable candidates to be used as novel advanced bioactive wound dressings.

5 Electrospinning Technique

Various techniques, including phase separation or self-assembly [35], can be used for the fabrication of polymer meshes/matrices as wound dressings, but electrospinning is most frequently chosen because it is a straightforward, cheap, and unique method to produce fibrous matrices. Electrospinning of micro- and nanoscale fibers is a well-documented method for preparing wound dressings [36, 37]. Setup of monoaxial electrospinning is shown on Fig. 1.

Typical needle-based electrospinning setup requires four main components: (1) polymer solution/melt in a syringe together with an appropriate needle (nozzle or spinneret), (2) syringe pump, (3) high-voltage power supply, and (4) grounded metal collector plate. In electrospinning (electrostatic fiber spinning), a polymer solution or a melt is expelled from a capillary toward a grounded metal collector plate by applying a high voltage between the capillary and the plate. A pendant drop of a solution becomes unstable under the action of the electric field, and a jet is issued from its tip. After the jet flows away from the droplet in a nearly straight line, it will bend into a complex path and other changes in shape occur, during which electrical forces stretch and thin it by very large ratios. If molecular cohesion or chain entanglement in the droplet is sufficiently high, the droplet does not breakup (electrospray) but instead continues to stretch to form fine fibers on a grounded collection target. After solvent evaporation, a birefringent nano- or microfibers are obtained [38]. One example of electrospun nanofibrous mats prepared from polycaprolactone (PCL) in different environmental conditions is shown on Fig. 2.

In addition to the needle-based setups, also various other novel electrospinning techniques have been developed. Modifications have been mostly performed on the collector and spinneret parts. Core-shell nanofibers are prepared using the same needle-based electrospinning setup with a coaxial needle instead of the common spinneret [39–41]. In order to increase the yield of

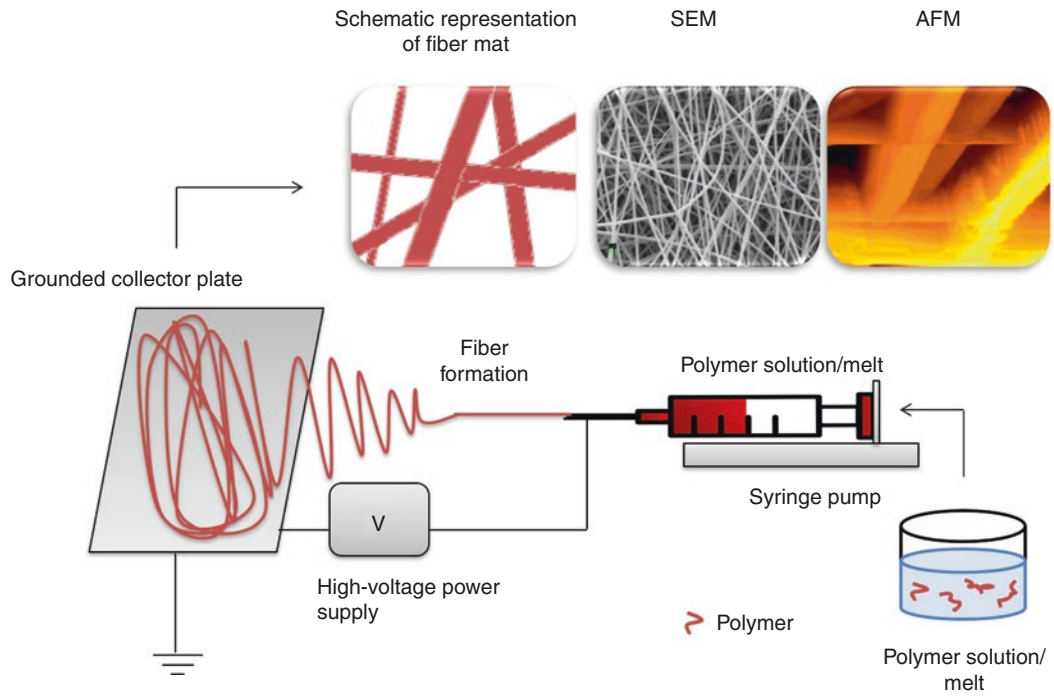

Fig. 1 Monoaxial electrospinning setup with polymer mats together with scanning electron microscopy (SEM) and atomic force microscopy (AFM) figures, respectively

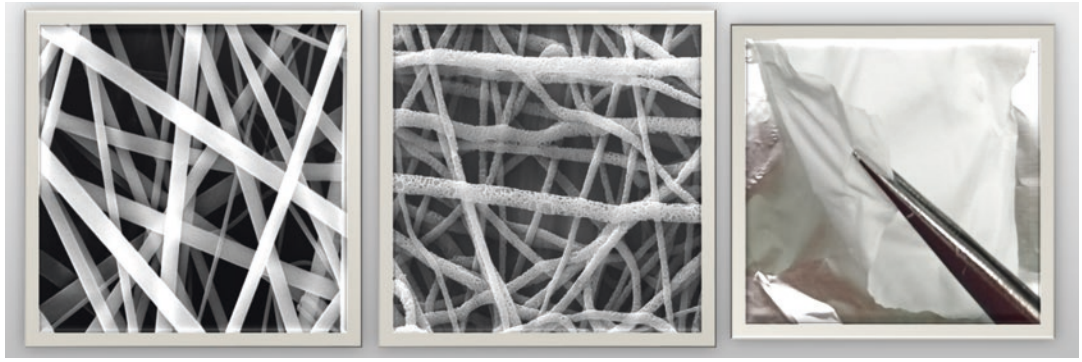

Fig. 2 Scanning electron micrographs of polycaprolactone (PCL) nanofibers (smooth) and microfibers (porous) obtained using different solvent systems and environmental conditions and representative picture of PCL nanofiber mat on aluminum folio

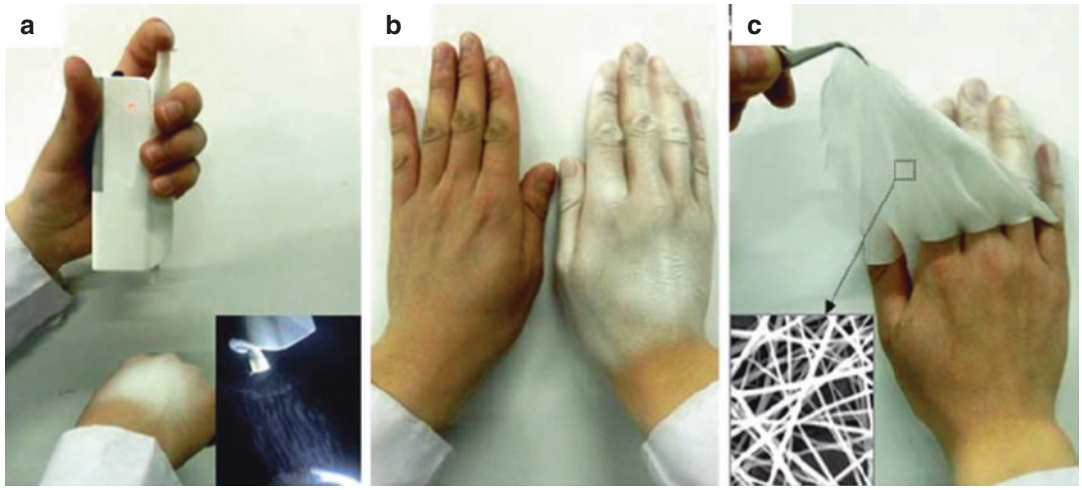

Fig. 3 (**a**) The electrospinning process of the battery-operated electrospinning apparatus (BOEA) directly on hand. (**b**) Polylactic acid (PLA) fibrous membrane was fabricated on another hand within 2 min. (**c**) The good flexibility and compactness of fiber membrane [48]. Reproduced with permission

electrospun nanofibers, multi-needle and free surface spinning as alternatives to needle-base system have been proposed [42–45]. Recent advances have also used corona electrospinning [46], ultrasound-enhanced electrospinning (USES) [47], and ball electrospinning techniques (patented SNC BEST™ technology). These advancements enable to produce nanofibers by high throughput either with a simple construction spinneret [46], use of ultrasound which provides the formation of ultrasound pulses protrusions (fountains) at the surface of spinning solution and after high voltage is applied to the polymer solution, thin jet is drawn from the fountain [47], or by rotating a ball in a polymeric solution and providing multiple liquid jets ejection from the surface, respectively. In addition to scale-up and large-scale production, to use and electrospinning technique as everyday practical tool to support the wound healing, a handheld electrospinning device operating using batteries has been developed [19, 48]. One example of a handheld electrospinning device and produced nanofiber mats has been shown on Fig. 3.

Electrospinning seems to be the only method which can be further developed for mass production of one-by-one continuous nano- to microfibers from various polymers. Although the process itself seems simple, there is a wide variety of parameters that can be changed, and all these parameters modify the structural as well as mechanical properties of the obtained fibers or fiber mats [49]. These parameters include material (e.g., polymers, solvents), processing (e.g., voltage, flow, distance, needle/free surface) and environmental parameters (e.g., temperature, humidity).

6 Electrospun Fibrous Wound Dressings

Electrospun nanofibrous dressings are ideal wound dressings with unique architectural features. The structure of the fiber mat enables to mimic the ECM and provides an ideal environment for wound healing [19]. Wound healing involves a complex interaction between epidermal and dermal cells, the ECM, controlled angiogenesis, and plasma-derived proteins – all coordinated at the molecular level by an array of cytokines and growth factors [50]. Normal wound healing consists of four main overlapping stages: hemostasis phase, inflammation phase, proliferation phase, and maturation/remodeling phase. Hence all these phases occur during normal healing of the wound, thus interactive or bioactive wound dressings mostly reveal their mode of action to one or several of those phases. It needs to be understood that all molecular, biochemical, and cellular processes are affected in chronic wounds, and these overlapping steps may be shortened/prolonged, therefore the design and development of effective wound dressing for chronic wound are much more complex. Usually normal wound healing leads to the anatomical reconstitution of the biological barrier, and an ultimate goal of wound healing is rapid recovery with minimal scarring and maximal function [51].

It is a known fact that for wounds to heal, they must have consistent and sufficient supply of oxygen, nutrients, enzymes, and cells. Once the mentioned factors are controlled and the wound bed is prepared with adequate debridement and moisture control, normal wound healing will be supported. The ideal wound dressing accelerates the healing process, prevents infection, and restores the structure and function of the skin [27]. Electrospun nanofibers are also ideal scaffolds for skin tissue engineering possessing a lot of suitable properties for skin wound healing. Nanofibrous scaffolds have been shown to act as a suitable dermal analogue to assist in skin cover and regeneration [52]. Electrospun polymer nanofibrous mats have shown to facilitate cell migration and proliferation of the wound bed [53] and provide hemostasis, gaseous exchange, and management of wound exudate [54]. These fibrous dressings reduce pain, trauma, inflammation, and scarring [55]. Polymeric electrospun nanofiber dressings provide excellent surface topography and are a non-touch, feasible, and safe method to promote wound healing with the potential to reduce wound infections. The properties that make electrospun nanofibrous systems important and "ideal" for wound healing are summarized in Table 4.

Electrospinning technique has several advantages such as easy control over fiber diameter, morphology, surface characteristics, porosity, and ease of getting fiber diameter into nano range. The structures of the nanofiber mat, for example, its small fiber diameter, high porosity, and specific surface area, are the major advantages that electrospun nanofiber systems have compared to other modern wound dressings, such as hydrocolloids, hydrogels, etc. Nontoxicity, hemostaticity, and the ability to maintain a moist wound environment are the other desired properties of an ideal dressing material. Therefore, the superior properties of electrospun nanofibers are mainly related to the electrospinning technology, which provides three-dimensional scaffolds with relevant structural properties mimicking the ECM [57]. By combining the electrospinning technology with the relevant materials for wound healing, improved wound dressings are obtained. Therefore, the design of clinically relevant wound dressing requires deep knowledge about the wound characteristics and its microenvironment,

Table 4 Relevant characteristics of electrospun nano- and/or microfiber systems to be used as "ideal" wound dressings [10, 56]

Characteristics	Advantages
Structural properties resembling ECM	Allow adequate gaseous exchange Tunable morphology (porosity, fiber diameter)
Mechanical properties	Material and processing parameter modification provides suitable mechanical characteristics
Possibility to include active substances	Wide variety of APIs and other active substances relevant for wound healing can be incorporated (antimicrobials, anti-inflammatory agents, hemostatic substances, peptides, proteins, DNA, etc.)
Modification of drug release	Depending on the carrier polymer, drug release modification is possible
Moist wound environment and thermal insulation	Creates a moist, clean, warm environment Provides hydration if dry or desiccated
Debridement of wound site	Incorporation of enzymes/ debriding substances which remove the dead skin Debridement promotes the wound healing
Moisture absorption and swelling/gel formation Bioadhesiveness	Removes excess exudates and blood, necrotic tissue Materials allow to design gelling and/ or bioadhesive systems
Impermeable to microorganisms	Protection from contamination – capable of protecting the wound from further trauma and infection
Low adherence, ease of removal	Prevents desiccation and is nontraumatic Provides protection to periwound area Minimal pain during application and removal
Biodegradability	Long shelf-life
Free of toxic or irritant particles	Nontoxic and non-allergenic Does not release particles or fibers
Comfortable and conformable	Easy to use Minimal frequency of dressing change Can conform to wound shape
Cost effectiveness	Various polymers can be electrospun

Key: *ECM* extracellular matrix

and then it is possible to develop supportive bioactive dressings that help the wound to heal. The selection of appropriate materials and their physicochemical and internal structural properties affect largely the properties of the obtained fibers. However, as an advantage, it is possible to tailor these properties by modifying the electrospinning parameters. Some of these properties will be overviewed in order to shed light on their importance to the electrospun fiber dressings.

6.1 Structure, Geometry, and Morphology of the Fibers

Structural properties of the fibers affect the wound healing properties (cell attachment, exudate absorption), mechanical properties, and drug release of the wound dressings. It has been shown that the high porosity of the nanofiber matrix allows oxygen and water permeability and nutrients exchange and also the removal of metabolic waste preventing fluid accumulation at the wound site. Furthermore, the high surface area favors cell attachment and subsequent proliferation and differentiation during tissue regeneration. The moisture under the occlusive dressing helps in healing, and it provides optimum barrier to wound exudate which may enclose cytokines and proteins supportive in injury [58].

The morphology of electrospun polymer fibers depends on the strength of the electric field, the solution viscosity (e.g., concentration), the charge density of the solution (by salt addition), and the solution feeding rate [59]; hence these properties need to be controlled and optimized during electrospinning in order to obtain the desired wound dressing properties. The fiber diameter and alignment can easily be tuned by manipulating the processing, material [60], and/or environmental parameters [61]. For example, solution conductivity and polymer concentration play an important role in controlling the fiber diameter [62]. Usually the fiber diameter increases together with the solution concentration if suitable concentrations for electrospinning are achieved. Lower relative

humidity values cause rapid solvent evaporation, resulting in thicker nanofibers, whereas higher relative humidity causes slower solvent evaporation, resulting in thinner nanofibers [61]. Nano- or microfiber mats have shown different interactions due to different fiber diameter and porosity of fiber mats [63]. Moreover, as it is known that cell functioning is largely dependent on different environmental interactions, specific interactions between the dressing and eukaryotic cells could promote cell migration, proliferation, and differentiation to enhance wound healing. In a normal situation, such cues are provided by the ECM, but dressing with similar structure and physicochemical properties could do the same. As discussed earlier, electrospun fibrous matrices do exhibit structural similarities with dermal ECM, thus they are especially attractive for wound care applications.

Cell attachment is mostly important when developing tissue scaffolds and less favorable for dressings which eventually need to be removed. However, if the dressing is biodegradable and resorbs after being in contact with the wound, cell attachment can be desirable. It has been shown that higher surface roughness leads to increased cell affinity and attachment and surface roughness relates to fiber diameter (roughness increases with increasing diameter). The combination of both micro- and nanofibers enhances cell penetration and infiltration to the matrix as it creates greater pore interconnectivity and larger pore size [64]. If synthetic polymers without specific binding sites are used for fiber production, the surface of these fibers can be modified with different functionalities to promote interactions with cells (e.g., coating with natural molecules like fibronectin, vitronectin, collagen, etc.) [64]. Another possibility is to modify the topology of the fiber surface (e.g., with photolithography) to create patterns enhancing specific cell functions, like adhesion, migration, or differentiation. Mimicking the anisotropy of tissue could also bring such results.

The electrospun nanofibers are typically collected as nonwoven mats with random orientation. For the development of scaffolds (e.g., artificial dural substitute), cell adhesion is required for supporting the tissue regeneration. Aligned fibers have better cell adhesion compared to random fibers; however, only uniaxially aligned nanofiber scaffolds can promote cell migration along one specific direction [65]. It is desired to have radially aligned fibers. Radially aligned nanofibers increase the migration rates for cells compared to random fibers and shorten the time for healing and regeneration of dura mater [66]. Radially aligned fibers can be obtained by using a collector comprised of a central point electrode and a peripheral ring electrode [66] or some other advances in electrospinning technique. Uniaxially aligned arrays of nanofibers are obtained by using an air gap collector or a mandrel rotating at a high speed. Yang et al. [60] have demonstrated that the neural stem cells elongated and their neurite outgrew along with the fiber direction for the aligned scaffolds, whereas the fiber diameter did not show any significant effect on the cell orientation. Interestingly, no effect was seen between differentiation rate and fiber alignment, whereas the effect on fiber diameter was clearly shown. The differentiation rate was higher for nanofibers compared to microfibers. For skin wounds, Xie et al. [66] for the first time developed a method and electrospun radially aligned PCL nanofibers to generate scaffolds potentially useful as dural substitutes.

Electrospun fibers may show conglutination and garlands in their structures. The first one is the process by which partially solidified jets can produce fibers that are attached at points of contact. Garlands are nanofiber networks formed when loops of an electrospinning jet conglutinate in flight [38]. Both these features will strongly affect the mechanical properties of the nonwoven fiber mats; hence strong attachments at crossing points stiffen the fiber mat. As mentioned, the morphology of the nanofiber mats can be modified by varying the process parameters and/or materials, such as type of polymer, type of solvent, and thermal treatment. A porous structure made out of nanofibers is a dynamic system where the pore size and shape can change, unlike conventional rigid porous structures [67]. High

porosity enables to obtain a high surface area of the fiber mesh. Depending on the materials as well as the material properties, the porous nanofibrous mats are able to create a moist environment around the wound area to promote healing. An increase in polymer concentration decreases the porosity and increases the mechanical properties (Young's modulus and tensile strength) of the fibers. Porous structure enables to absorb excess of wound exudate from the wound, does not build up under the wound covering, and does not cause wound desiccation.

The cross-linked fibers possess more tensile strength and high Young's modulus with appropriate flexibility [68]. Since good mechanical properties are required for electrospun fibers to be used for wound healing/tissue engineering, several studies have investigated different material and processing parameters effects on fiber mat mechanical properties. The strength and deformability of nanofiber mats influence in vitro cell migration, proliferation, and differentiation, along with cell morphology [69]. The mechanical properties of the fibers can be tuned by combining suitable materials as discussed in next paragraphs. While it is known that the crystallinity of the polymer is a great contributor to a material's stiffness and largely affects the polymer degradation behavior. Electrospun nanofibers typically display tensile strengths below 300 MPa and Young's moduli below 3 GPa, which can be mainly ascribed to the low degree of orientation and chain extension of the polymer chains along the fiber axis [70]. An increase in mechanical properties with decreasing fiber diameter has been reported for different nanofiber mats by different authors [71, 72]. However, the presence of beads (e.g., defects in the nanofiber) may totally change the mechanical behavior of the nanofiber mats [71]. In addition, fiber alignment has been reported to significantly affect their mechanical properties [73–75]. Baker et al. [75] have shown that the key mechanical properties for electrospun PCL fibers, such as viscoelasticity, yield point stress and strain, relaxation times, total and elastic tensile modulus, and energy loss with increasing strain, are dependent on sample age. Post-electrospinning treatment is also commonly used to increase the mechanical properties of electrospun nanofibers. Effect of both physical (e.g., heat treatment, stretching, and twisting) and chemical (e.g., cross-linking, surface coating) treatments have been reported [76].

6.2 Water Absorptiveness, Water Vapor Transmission, and Oxygen Permeability

The swellability and water absorptiveness are one of the main properties investigated for wound dressings as medical devices since these enable to make a decision on their suitability to be used for different wounds. Contardi et al. [77] have shown that nanofiber mats absorbed wound exudate faster than their respective transparent films. Good water vapor transmission and oxygen permeability are needed in order to support normal wound healing. Therefore, all wound dressings should be protective against contamination but porous having sufficient water vapor transmission and oxygen permeability behavior. It enables to make conclusions about the performance of the barrier. It has been highlighted that there is a need for new and smart wound dressings capable to either give signals (diagnostic purposes) or provide treatment (smart dressings) [78]. As an additional advancement, nanofiber mats can be functionalized in order to be sensitive against changes in the wound. Hence smart wound dressings can be developed. Oxygen sensors in different forms have been found applications in wound dressings. Oxygen is crucial to wound healing and used for cellular energy production by adenosine triphosphate. It acts on different levels of wound healing by inducing angiogenesis, keratinocytes differentiation, migration, reepithelialization, fibroblast proliferation, and collagen synthesis and promotes wound contraction [79]. Xue et al. [80] have developed nanofiber-based sensor systems that could be developed further for wound dressing application. Hu et al. [81] reported an electrospun hybrid nanofibrous dressing for wound healing, with a high porosity, wettability, and the ability to simultaneously decrease the H_2O_2 and increase the O_2 concentration. Polyvinyl alcohol (PVA) solution

and a suspension of hematite nanoparticles were mixed to electrospin the nanozyme-containing nanofibers. Encapsulated hematite showed a high catalase-like activity and quickly converted H_2O_2 into O_2, thus these dressings could be used for wound healing acceleration.

Wound dressings should have a good water holding capacity by absorption and swelling; in addition to high porosity and surface area, these properties are highly related with the material characteristics and will be discussed in more detail in next paragraphs. The biocompatibility of the nanofiber/microfiber scaffold is usually tested ex vivo by culturing skin fibroblasts or keratinocytes on the scaffold and monitoring the cell growth and proliferation. An animal model is used to study the biocompatibility of the scaffold in a biological system before the scaffold is introduced into patients for wound healing applications. The bioadhesiveness of electrospun nanofiber mats has been investigated using ex vivo pig skin and texture analyzer by Tamm et al. [82]. Furthermore, all these properties (e.g., mechanical, water absorptiveness, bioadhesiveness, O_2 permeability, hydrophilicity, biodegradability) can be modified by using surface modification techniques which enable to change the properties of the carrier polymers [83, 84].

6.3 Odor Control, Comfortability, Sterility, Safety, and Efficacy

Odor control and comfortability play a major role for the final use. These properties of wound dressings determine whether the product manages to ease the life of people with chronic wounds and prevent the social withdraw from the community. If the formation of the odor cannot be prevented, it may be necessary to use a wound dressing that adsorbs the volatile molecules released from the wound which are responsible for the smell. The most effective way of dealing with malodorous wounds is to prevent or eradicate the infection responsible for the odor which will be discussed in more depth in the antimicrobial wound dressing paragraphs. However, also the use of activated charcoal consisting wound dressings enables to prevent the malodor. According to authors knowledge, no wound dressing made using electrospinning of activated charcoal is on the market, but it has been shown that activated charcoal can be efficiently electrospun, and high specific surface area webs are obtained [85, 86]. While likely in the near future, these electrospun fiber mats will be tested for wound healing applications. The comfortability on the other hand plays a major role whether patients start using the product or not. This property is difficult to design and test beforehand, while in several cases the actual patient comfortability will be known during clinical trials on humans.

As an additional important feature of electrospun nanofibers/microfibers used as wound dressings for open skin wounds, their sterility needs to be confirmed. Assurance of microbiological safety is one important requisite for any biological/medical device to be used in clinical settings. Although there are literature reports that for surgical wounds also nonsterile clean dressings could be applied [87], general guidelines suggest to use sterile wound dressings. Lipp et al. [88] evaluated the effect of wound dressings on bacterial bioburden using six different wound dressings and two pathogens commonly found in biofilms, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* biofilms. It was found that bacteria can grow unchallenged within a dressing environment and that an antimicrobial dressing can limit this bacterial growth. Some wound dressing materials support the bacterial growth therefore highlighting the importance of including preservatives or antimicrobials into the wound dressings. Sterilization and desinfection methods for wound dressings are available (e.g., heat, chemical sterilants ethylene oxide or glutaraldehyde, ultraviolet or gamma irradiation, immersion into 70% ethanol solution, etc.), but their suitability for nanofiber/microfiber mats and scaffolds needs to be tested case by case [89]. Some of the main drawbacks may include toxicity (e.g., toxic metabolites; residuals in the dressing), denaturation, or degradation of the mats or structure alterations [90]. In case of drug-loaded or growth factor loaded electrospun nano-/microfiber bioactive dressings, not all sterilization methods

can be used due to the degradation of the API/protein or release of the API before application. The selection of appropriate sterilization method also highly depends on the material properties.

As with other wound dressings, also nano-/microfiber dressings should be evaluated for their characteristics and expected efficacy and safety. Current challenges are related with the development of suitable analytical techniques to prove their efficacy and safety in vitro before testing in in vivo conditions. Antimicrobial effectivity, biocompatibility, stability, and sterility testings as well as water vapor permeability and water absorptiveness make a huge part of the characterization of the antimicrobial nanofiber/microfiber dressings.

6.4 Regulatory Requirements for the Development of Wound Dressings

In general, nano-/microfiber wound dressings must be biocompatible, non-irritating, and have suitable mechanical properties for the application. There are several characteristics that nanofiber/microfiber mats need to have in order to be used as effective wound dressings as listed in Table 4. All these characteristics are usually investigated while designing and developing novel wound dressings. It is desired to have rapid hemostasis property, good antimicrobial property, good mechanical properties and water-vapor permeability, etc. It is recommended that wound dressings (including nanofiber/microfiber mats) should ensure a moist wound environment while readily absorbing wound exudate. Dressing removal should be atraumatic and minimally painful. Dressing care should be patient-centered and individualized [91]. It is evident that wound dressing needs to be biocompatible, so it does not adversely affect healing by eliciting undesirable effects to cells. International Standard ISO-10993: Biological Evaluation of Medical Devices lists the requisites to call a dressing biocompatible. FDA guidelines require that wound dressings intended to be used on compromised skin for prolonged duration (24 h to 30 days) are tested for cytotoxicity, sensitization, and irritation; in addition, acute systemic toxicity, material-mediated pyrogenicity, and subacute/subchronic toxicity are recommended endpoints for consideration [92].

At the moment wound dressings are considered as medical devices, hence there are several regulatory requirements and guidelines that need to be followed, e.g., FDA and International Standardization Organization (ISO) guidelines [92–94]. The decision about regulatory classification for a given wound management product lies with the regulatory authority of the country in which the product is to be marketed and the manufacturing site making the product comes within their jurisdiction by virtue of the intent to market the product. Currently, medical devices require an investigational device exemption for clinical evaluation in humans; they are regulated through and receive 510(k) approval through a premarket approval scheme regulated by the FDA in the USA and are approved through the CE marking process which was introduced in the early 1990s in Europe [95].

Antimicrobial dressings consisting antiseptics have been regulated as medical devices (Class II or III), although there are changes taking place while it is recognized that these antimicrobial-consisting dressings require more clinical testing and proof for their safety. Whether the wound dressings that contain drug(s) should be classified into Class II or Class III depends on their possible risks and whether these risks can be mitigated. In some cases, when the drug is included, these may be considered as drugs and should follow the guidelines accordingly. When human growth factors are included into the wound dressings, the wound dressings should follow guidelines for drugs and/or combination product guidelines. In some cases, depending on the added molecules, the dressings may be classified as biological products under the Public Health Service (PHS) Act. Both the FDA's Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER) have regulatory responsibility for therapeutic biological products, including premarket review and oversight; however, the two institutions take care of different biological products

accordingly. It is advised that sponsors contact the Office of Combination Products (OCP) to confirm the classification of any products they may wish to market in order to confirm the actual classification category for the product (drug, device, biological product, or combination product) [96].

Most of the guidelines for wound care practice state that topical antimicrobials/anti-infectives are not typically recommended for wounds that do not exhibit clinical signs of infection. Recent literature and systematic reviews (e.g., Cochrane Database of Systematic Reviews (CDSR)) on the clinical relevance of the antimicrobial wound dressings has concluded that there is a lack of appropriate randomized trials evaluating the effects of wound dressing combined with topical antimicrobials in wound care, and further good quality research is needed before definitive conclusions can be reached about the effectiveness of these topical antimicrobials (including antimicrobial dressings) products [97, 98].

6.5 Current Materials Used for Electrospun Fibrous Wound Dressings

Electrospinning has been considered quite flexible preparation technique since there are several polymers available that have good spinnable properties. The latter enables to design and prepare advanced wound dressings as well as bioactive wound dressings by modifying the materials and incorporating relevant APIs or other substances. Different polymers can be electrospun, while the materials selected for making electrospun nanofiber mats enables to tune the characteristics of the fiber mats which support the wound healing.

Electrospun nanofibers are being studied and have been developed because they hold considerable promise for realizing some advantages of nanostructured materials. Over past years the amount of publications that have developed electrospun nanofiber and/or microfiber mats has increased enormously. Several review publications have overviewed the different materials suitable to be used as wound dressings and also suitable for electrospinning [56, 99]. Here, the recent understandings of the materials suitable for electrospinning and wound healing applications are summarized based on available publications on electrospinning. When materials are varied, it needs to be kept in mind that not all polymer solutions are electrospinnable. Usually, in the currently available literature, the solvent selection is broader and does not always follow the FDA recommendations; however, these become of importance when the authors are interested to move toward the development of clinically relevant wound dressings. For clinically relevant wound dressings, FDA recommendations for safe solvents need to be followed [100].

Both natural and synthetic polymers have been electrospun in order to obtain electrospun fibrous mats as wound dressings in the current literature. As a third option, the blends of the two are used. As a standard procedure, the materials for wound dressing preparation are always selected to be nontoxic, with good mechanical properties, biodegradable and biocompatible, and are considered safe materials that are approved by the FDA/European Medicines Agency (EMA) for numerous applications in tissue engineering, wound healing, and drug delivery. In addition to these properties, the dressing materials should be hydrophilic in order to be biocompatible with the wound while this enables to remove the excess of wound exudate when required.

6.6 Natural Polymers

Natural polymers used for the preparation of electrospun wound dressings include collagen-based matrices and other natural polymers such as chitosan, fibrin, elastin, gelatin, and hyaluronic acid. Natural polymers have been widely used for biomedical application because they have better biocompatibility and low immunogenicity, compared to synthetic polymers. Furthermore, natural polymers can promote the cell attachment and proliferation. Main disadvantages of natural polymers are their weak mechanical properties, relatively

rapid degradation profile and potential for immunogenicity, batch-to-batch variation, their limited supply, high cost of production, and susceptibility to cross-contamination. The latter disadvantages may limit their clinical applications. Their hydrophilicity also promotes fast degradation; therefore, it is a challenge to use natural polymers alone for prolonged drug release applications, while cross-linking should be used which may increase the toxicity problems. Among natural polymers, alginate and chitosan are two frequently used polysaccharides for wound care applications. Another natural polymer, silk fibroin, is considered a favorable scaffold material for the incorporation and delivery of a diverse range of drugs in tissue regeneration applications, owing to its biocompatibility, relatively slow biodegradability, facile processability, and better mechanical properties. Although some of their dressing products have been commercialized, their cellular skin grafts are only in the laboratory stage, and further clinical data are needed for FDA approval or commercialization [101].

6.7 Synthetic Polymers

Synthetic polymers are usually more tunable and their production is easier and at lower costs. These can be divided into nonbiodegradable and biodegradable. As an example, polyurethane (PU) is a nonbiodegradable polymer. While, PCL, polyvinyl alcohol (PVA), poly(glycolic acid) (PGA), and poly(D,L-lactide-co-glycolide) (PLGA) are broken down during hydrolysis or enzymatic degradation. These on the other hand can be divided into hydrophilic and hydrophobic polymers. It has been shown that hydrophilic polymers show better wound healing properties; however hydrophobic polymers enable better control over drug release, and these have slower degradation rates. Hydrophilic polymers use involves also additional technological steps (e.g., cross-linking), which prolong their existence and modify the drug release from the matrix. It has been found that synthetic polymers provide more stable scaffolds and other advantages include durability, relative inexpensiveness, and tunable degradation time. However, the cell affinity toward synthetic polymers is generally poor because of their low hydrophilicity and lack of surface cell recognition sites. In terms of wound dressing materials, nonabsorbable synthetic polymers often cause serious complications. These may include induction of excessive granulation tissue formation due to their chronic stimulation of the surrounding tissues and long-term foreign body reaction [102]. Examples are shown for silicone and expanded polytetrafluoroethylene (ePTFE), specifically in nanofiber form. To tackle with this problem, Wade et al. [103] has synthesized electrospinnable macromers that degrade through peptide cross-links and demonstrated their suitable in vitro degradation and in vivo erosion profiles. These results present a novel degradation mechanism for engineered electrospun hydrogels. Shishatskaya et al. [104] have shown that electrospun nanofiber mats prepared using novel synthetic biodegradable natural polymer hydroxy derivatives of carboxylic acids (PHAs) and copolymer 3-hydroxybutyrate/4-hydroxybutyrate P(3HB/4HB) have improved characteristics compared to the respective films, and these nonwoven membranes combined with cells synthesizing growth factors facilitated wound healing, neovascularization, and regeneration and enabled complete wound healing by Day 14 postsurgery. The authors have proposed that these mats can reduce the inflammation, enhance the angiogenic properties of the skin, and facilitate its healing.

6.8 Blends of Natural and Synthetic Polymers-Composite Fibers

Natural polymers can be easily combined with synthetic ones during electrospinning. Composite polymers are made of two or more polymers including natural and synthetic components, which combine advantages of both types of polymers such as their resemblance to the ECM and desired mechanical properties and/or tunable biodegradability. It can be seen that in several cases composite fibers are used, where natural and synthetic polymers are used together. The most

tested combinations include chitosan with polyethylene oxide (PEO) or PVA or gelatin and PCL with gelatin or chitosan or polylactic acid (PLA). For example, it was found that PCL/gelatin 70:30 enhanced the nerve differentiation and proliferation much better compared to PCL nanofibrous scaffolds alone [105]. Hence composite fibers enable to obtain multifunctional wound dressings. Anjum et al. [106] have reported the development of gelatin-PCL composite fibers for wound healing and for the use as scaffolds for adult human skin-derived precursor cells (hSKPs). Since gelatin alone has poor tensile strength, it was blended with PCL to achieve more desirable handling characteristics. The study showed that the transplantation of acellular scaffolds into wounds exhibited improvement in dermal-epidermal thickness, axonal density, and collagen deposition. These results demonstrated that PCL-based nanofiber scaffolds show promise as a cell delivery system for wound healing. Chitosan inclusion into hybrid and core-shell structured nanofibers together with PLGA using co-electrospinning or coaxial electrospinning, respectively, both improved the water absorption behavior of the PLGA mats and also showed better cytocompatibility than the PLGA membrane in adhesion, viability assays, as well as morphology observation [107].

6.9 Functionalized Nano-/Microfiber Mats-Bioactive Wound Dressings

More complicated systems have included also growth factors into the electrospun fibers. These may be classified as bioactive dressings, since the materials itself support the wound healing in addition to the fibrous and porous structure. Some publications about electrospun bioactive dressings that have been shown to be effective in in vivo animal wound models in recent literature have been summarized in Table 5.

Using growth factors and their combinations in vivo has been suggested to be an advantageous treatment method to promote active wound healing. Angiogenic factors [e.g., VEGF, basic fibroblast growth factor (bFGF), endothelial growth factor (EGF), and platelet-derived growth factor (PDGF)] have been incorporated into the electrospun scaffolds, resulting in an efficient way of increasing the formation of blood vessels. The main drawback of using electrospun mats consisting growth factors is their poor stability, while clinical trials have shown that scaffolds with only one type of angiogenic factor are insufficient to induce a mature, stable, and vascular structure. Therefore, the interaction of a number of stimulating factors that act at different stages of angiogenesis is recommended. Growth factors easily lose their activity upon chemical or physical processing. Therefore, the preservation of protein activity is a prerequisite for successful growth factor delivery supporting the wound healing. One interesting study has shown that the electrospun PVA combined with carbon nanotubes (CNTs) and EGF composite bioactive dressings have a well-distributed structure and display an improved ability to release EGF at a sustained rate with the activity of the EGF released being favorable. The bioactively released EGF accelerated the growth of L929 fibroblasts; hence its application in wound dressing has been demonstrated [118]. There are publications showing that the in vivo wound healing rate on rats is increased by the use of nanofibrous wound dressings [55, 118]. The same has been shown also in porcine wound models. Lai et al. [112] have encapsulated multiple growth factors including bFGF, EGF, VEGF, and PDGF in collagen-gelatin nanoparticles loaded into collagen-hyaluronic acid nanofibers as controlled release vehicles for the facilitation of angiogenesis and dermal and epidermal layer regeneration. They have shown that the sequential release of growth factors, which is analogous to the natural physiological environment, offered precise control of the proliferation and migration of human umbilical vein endothelial cells during the vascularization process, as well as epidermal and dermal tissue regeneration in diabetic rats. bFGF and EGF directly embedded in the nanofibers exhibited an initial rapid release, whereas VEGF and PDGF-BB encapsulated in the gelatin nanoparticles showed a gradual slow release pattern.

Table 5 List of publications where electrospun nano-/microfibrous mats have been tested in animal models and their potential clinical relevance as interactive or bioactive wound dressings revealed

Polymer	Active substances	Proposed mechanisms of accelerated wound healing	Clinical application/in vivo testing	References
Dibutyrylchitin (DBC) and PCL	–	Increased granulation tissue weight and blood vessel number, better hydration, no impairment to total collagen synthesis or no excessive fibrosis	Wounds with increased exudate Rat study, wound model	[54]
Gelatin+PCL	–	Proper adhesion, morphogenesis, motility, formation of respective tissue compartments	Soft tissue defects, for example, oral mucosal for the first 4 weeks Minipig study; cell cultures	[108]
Collagen+ PCL	–	Resolution of inflammation, the accelerated migration of fibroblasts and keratinocytes, the promotion of angiogenesis	Chronic wounds In vivo diabetic rat model	[55]
PLGA+polydioxanone	–	Reduced inflammation, faster rates of wound closure with granulation tissue, as well as achieving deposition of mature collagen and vascularization	Partial or full-thickness cutaneous wounds; partial and full-thickness wounds, chronic wounds (e.g., ulcers), and severe wounds caused by trauma or surgery In vivo minipig model	[109, 110]
Collagen	Fusion protein (CBD-MSC-peptide)	Enhanced MSC adhesion and infiltration	Acute full-thickness wounds (burns) In vivo pig model	[111]
Collagen+HA+ gelatin NPs	EGF, basic FGF, VEGF + PDGF	Accelerated wound closure rate, elevated collagen deposition, and enhanced maturation of vessels	Chronic wounds In vivo diabetic rat model	[112]
Chitosan, PEO, PVA, PLGA	PDGF-BB, VEGF	Angiogenesis promotion, increased reepithelialization and controlling granulation tissue formation, quicker collagen deposition and earlier remodeling, faster full regeneration of skin	Chronic complex wounds, e.g., diabetic ulcers, all in one (antibacterial and wound healing acceleration) Full-thickness rat skin wound model	[113]
Chitosan + PCL	Nitric oxide	Pro-angiogenesis, immunomodulation, enhanced collagen synthesis due to the sustained release of nitric oxide	Chronic wound caused by the ischemia In vivo mice model	[114]
PEO	Soy protein	Enhanced reepithelialization and dermal tissue regeneration, 4 weeks after application presence of dermal appendages, (e.g., sweat glands and hair follicles)	Chronic wounds; bioactive scaffold or wound dressing In vivo pig model	[115]

Table 5 (continued)

Polymer	Active substances	Proposed mechanisms of accelerated wound healing	Clinical application/in vivo testing	References
PEO	Human albumin	Non-adhesive to cells and susceptible for degradation by macrophages	Non-healing wounds; an anti-adhesive dressing In vivo mice model, cell cultures	[116]
PVA	pNSR16	Promote wound healing and basic fibroblast growth factor expression	Wound healing application In vivo rat model	[117]
PVA (+gauze)	CNTs + EGF	Well-distributed microstructure; sustained release of growth factors	Wound healing application In vivo rat model	[118]

Keys: *bFGF* basic fibroblast growth factor; *CBD* collagen-binding domain; *CNTs* carbon nanotubes; *EGF* epidermal growth factor; *MSC* mesenchymal stem cells; *NPs* nanoparticles; *PDGF-BB* platelet-derived growth factor-BB, *PLGA* poly-lactic-coglycolic acid; *pNSR16* recombinant spider silk protein; *VEGF* vascular endothelial growth factor

Other natural polymers including chitosan, fibrin, elastin, gelatin, and hyaluronic acid have been investigated for wound healing. Although some of their dressing products have been commercialized, their cellular skin grafts are only in the laboratory stage and further clinical data are needed for FDA approval or commercialization. It is also expected that wound dressings have multiple functions, for example, in addition to the wound healing promotion, the dressing should suppress the scar formation. In addition to promoting neovascularization, also inflammatory phase needs to be taken under the control in chronic wounds. Hence, reduced pain helps patients to manage with the wound and clinical experience suggests that hypertrophic scar formation is an aberrant form of wound healing [119]. Several nonsteroidal anti-inflammatory agents (NSAIDs) have been included into the electrospun nanofibers [120, 121]. The addition of NSAIDs or their selective cyclooxygenase-2 (COX-2) inhibitors have been proposed to inhibit prostaglandin E2 production, which might exacerbate excessive scar formation, especially when used during the later proliferative phase [122].

Silk fibroin-PVA-based dressings have shown good wound healing properties via enhancing cell migration toward the wound bed. Additional functionalization of nanofibrous mats with EGF and bFGF reestablished the growth factor pool in the wound fluid, thus triggered the wound healing [123]. Similarly, the reduction in the amount of metalloproteinases (MMP) needs to be solved in chronic wounds, when the enzymatic degradation is overwhelming other relevant processes in the wound. Normally, remodeling of the ECM is dependent on the balance between matrix MMPs and tissue inhibitors of MMPs [124]. Surface functionalization of nanofibers as well as incorporation of small interfering RNA (siRNA)-loaded nanoparticles into the electrospun nanofibers have been examined to investigate the release mechanism and achieve matrix MMP-responsive release of siRNA, respectively [125, 126]. Kim et al. [126] have shown that the wound recovery rates of diabetic ulcers were significantly increased when siRNA incorporated into PCL-PEO block copolymer nanofibrous mats was administered. The authors proposed that the suicidal treatment with the MMP-2 siRNA-decorated nanofibrous mat is expected to improve the prognosis of diabetic ulcers with reduced side effects.

Several other strategies have been proposed useful for the treatment of chronic wound or wounds with eschar. For example, it is known that enzymatic debridement of necrotic tissues without harming healthy tissue is also a crucial part to promote normal healing process. Papain- and

collagenase-based dressings have been tested to digest necrotic tissue. Collagenase acts on the collagen by attacking native collagen and gentles on viable collagen by gradual breakdown of tissue, whereas papain attacks cysteine residue and associated with inflammatory response [28]. It is expected that also these bioactive dressings using electrospinning approach will be tested further. One clinically relevant electrospun medical device is already on the market – Restrata Wound Matrix [109]. This is a matrix composed of synthetic polymers, polyglactin 910 poly(lactic-co-glycolic acid) (PLGA) (10:90), and polydioxanone. Both these are approved by the FDA and currently utilized in biomedical applications such as resorbable sutures. These polymers are co-electrospun into nonwoven sheets that are roughly 0.5 mm thick. It has been shown that this wound matrix is fully resorbable. MacEwan et al. [109] have shown that the fully synthetic electrospun matrix has faster rates of wound closure characterized by granulation tissue, deposition of mature collagen, and vascularization at earlier time points than in wounds treated with a bilayered xenograft. The authors propose that this matrix could be used for partial and full-thickness wounds, chronic wounds (e.g., ulcers), and severe wounds caused by trauma or surgery.

Thorough literature search revealed thousands of research papers discussing about electrospun nanofiber development for wound healing. Moreover, a high number of patents were found during patent search using keywords "electrospun dressings." This highlights that most likely in the following few years several new electrospun wound care products are entering the market. For the treatment of skin wounds, a multilayered wound care product has been developed comprising at least one ECM layer and at least one polymer layer comprising collagen and at least one further biodegradable polymer [127]. Polymer layer could be produced by various methods, and electrospinning is one of the methods that could be used.

When designing antimicrobial electrospun polymeric nano-/microfiber scaffolds or mats, the type of polymer is of crucial importance because it affects the wettability (hydrophilic/hydrophobic properties) and degradation rates of the fiber mats (affected by its microstructure and crystallinity). These are also key factors in controlling the drug-release profile when bioactive antimicrobial dressings are under the development. In addition to the properties of polymers, other factors including the types of drugs and drug-loading techniques are equally important for designing long-term drug delivery vehicles. In the following sections, we will review these factors in detail.

7 Antimicrobial Electrospun Fibrous Wound Dressings

Usually, the polymers used for fabricating antimicrobial electrospun nanofibers possess inherent abilities which support the wound healing. Table 6 summarizes some of the main physicochemical, mechanical, and biodegradation characteristics of the polymers used for the preparation of antimicrobial electrospun fiber mats for wound healing application together with the recent references.

Most of the listed polymers in Table 6 have already been tested together with some antimicrobial agents (most common ones PCL, PLA, PLGA, PVA, and chitosan). Some polymers are known to have inherent antimicrobial properties, such as chitosan, hence enable to produce antimicrobial wound dressings. As much as there are different polymers which can be used to prepare wound dressings, the list of APIs, and even more specifically antimicrobials, is no shorter. Common antimicrobials incorporated into fiber mats are antibiotics, antiseptic agents, metal (oxide) nanoparticles, a wide variety of natural products, and other compounds.

7.1 Preparation of Antimicrobial Drug-loaded Electrospun Fibrous Dressings

There are several methods to incorporate a drug into the dressing matrix, e.g., (1) blending with the polymer solution prior electrospinning, (2) encapsulating the active ingredient into the fiber core by coaxial electrospinning or emulsion electrospinning, and (3) encapsulating the API before mixing with electrospinning solution or surface immobilization (Fig. 4).

Table 6 List of properties of natural and synthetic polymers most prevalently used for the development of electrospun antimicrobial nano-/microfibrous mats as wound dressings. Summary of recent publications

Natural polymers	Crystallinity/ physicochemical properties	Mechanical properties/ biodegradation	Composite fibers	Antimicrobial agent	References
Collagen	Semicrystalline/ hydrophilic	Weak/enzymatic	PCL, PLLC	Silver nanoparticles; graphene oxide; berberine; gentamicin	[128–131]
Fish Collagen	Semicrystalline/ hydrophilic	Weak/enzymatic		Bioactive glass	[132]
Gelatin	Hydrophilic	Weak/enzymatic	PCL	Trimethoxysilylpropyl octadecyldimethyl ammonium chloride; vancomycin; silver nanoparticles	[133–135]
SF	Liquid crystals/ hydrophilic	Strong/enzymatic	PVA	Ciprofloxacin hydrochloride monohydrate; Manuka honey; Polyethyleneimine	[136–138]
Chitosan	Semicrystalline/ hydrophilic	Weak/enzymatic	PEO, PVA, PCL, Na-alginate, PVP, sericin	Silver nanoparticles; chlorhexidine; thyme extract; phthalocyanines; clover honey; sulfadiazine; gentamicin	[128, 139–146]
HA	Semicrystalline/ hydrophilic	Weak/enzymatic	SF	Olive leaf extract	[147]
Na-alginate	Semicrystalline/ hydrophilic	Weak/enzymatic, slow hydrolytic	PVA, chitosan, PEO	Silver nanoparticles; moxifloxacin hydrochloride	[108, 148–150]
Dextran	Crystalline/hydrophilic	Weak/enzymatic	PCL, CA	Tetracyclin hydrochloride	[151]
PHBV	High crystallinity/ hydrophobic	Weak/enzymatic, slow hydrolytic	Collagen	ZnO NPs; graphene oxide	[130, 152]
PHB	Semicrystalline/ hydrophobic	Weak/ enzymatic	Pluronic F-108 (PF)	Doxycycline	[153]
Gum tragacanth (GT)	Amorphous/hydrophilic/ swellable part	Weak/ enzymatic	PCL	Curcumine	[154]

(continued)

Table 6 (continued)

Synthetic Polymers	Crystallinity/ physicochemical properties	Mechanical properties/ biodegradation	Composite fibers	Antimicrobial agent	References
PVA	Semicrystalline/ hydrophilic (gel formation)	Strong/ biodegradable: enzymatic	Pluronic F127; PAA, chitosan; PSSA-MA, SF	Silver nanoparticles; chlorhexidine; silver sulfadiazine; soursop leaves extract; clover honey; tetracycline hydrochloride; nisin; antimicrobial peptide LL-37; ZrO_2; neomycin	[123, 141, 143, 155–162]
PLA (PDLLA) (mixture of PLLA and amorphous PDLA)	Semicrystalline depending on PLLA/ PDLA ratio/hydrophobic	Strong/ biodegradable: hydrolytic	Chitosan, PEO	Cinnamaldehyde; ZnO nanoparticles;	[162–166]
PLLA	Semicrystalline/ hydrophobic	Strong/ biodegradable: hydrolytic		Nitrofurazone; polymyxin B sulphate	[163, 167]
PLGA	Semicrystalline/ hydrophobic	Strong/ biodegradable: hydrolytic		CuO nanocrystals; ciprofloxaxin; magainin II; fusidic acid;	[168–172]
PCL	Semicrystalline/ hydrophobic	Strong/ biodegradable: hydrolytic	Chitosan, gelatin, CA, dextran, GT, collagen, PEO, HA, PLA, whey protein, PNIPAM	Trimethoxysilylpropyl octadecyldimethyl ammonium chloride; tetracycline hydrochloride; silver sulfadiazine; curcumine; silver nanoparticles; chloramphenicol; gatifloxacin; levofloxacin	[56, 128, 129, 134, 151, 154, 156, 173–176]
PEO	Semicrystalline/ hydrophilic	Weak/ biodegradable: enzymatic or hydrolytic	Chitosan, PVA, PVP, Na-alginate, PCL, PLA, curdlan	Silver nanoparticles; chlorhexidine; phthalocyanines; tetracycline hydrochloride; cefazolin sodium; ZnO nanoparticles; imipenem/cilastatin	[141, 142, 144, 145, 177–179]
PU	Semicrystalline/ hydrophilic or hydrophobic depending on the structure	Strong/ nondegradable	CA, chitosan, gelatin, dextran	Ciprofloxacin hydrochloride; silver nanoparticles; mupirocin; ampicillin	[180–184]
PVP	Semicrystalline/ hydrophilic	Weak/ biodegradable: enzymatic	Chitosan, PEO, PVA, CA; PVP/VA	Silver nanoparticles, chlorhexidine; chloramphenicol; ciprofloxacin; curcumine	[77, 82, 141, 185, 186]

CA	Semicrystalline/ hydrophilic	Strong/ biodegradable: enzymatic or hydrolytic	PVP, PCL, dextran, PU	Tetracycline hydrochloride; streptomycin sulfate; curcumine	[151, 180, 186, 187], [188]
PAA	Amorphous/hydrophilic superabsorbent	Weak/ biodegradable: enzymatic	PVA	Ciprofloxacin hydrochloride; aloe vera; silver nanoparticles; doxycycline hyclate	[189–191]
PEUU	Semicrystalline/ hydrophobic	Strong/ biodegradable: hydrolytic		Tigecycline	[192]
PVDF	Crystalline/hydrophobic	Strong/ nondegradable		Enrofloxacin	[193]

Keys: *CA* cellulose acetate; *GT* gum tragacanth; *HA* hyaluronic acid; *PAA* poly(acrylic acid); *PCL* polycarpolactone; *PEO* polyethylene oxide; *PEUU* poly(caprolactone urethane urea); *PLA* polylactic acid; *PLGA* poly(D,L-lactide-co-glycolide); *PLLA* poly(L-lactic acid); *PNIPAM* poly(N-isopropylacrylamide); *PSSA-MA* poly(styrene sulfonic acid-co-maleic acid); *PU* polyurethane; *PVA* polyvinylalcohol; *PVDF* polyvinylidene fluoride; *PVP* polyvinylpyrrolidone; *PVP/VA* polyvinylpyrrolidone vinyl acetate; *SF* silk fibroin; *PLLC* poly(L-lactide-co-caprolactone); *PHB* polyhydroxybutyrate; *PHBV* poly(3-hydroxybutyrate-co-3-hydroxyvalerate); *SF* silk fibroin

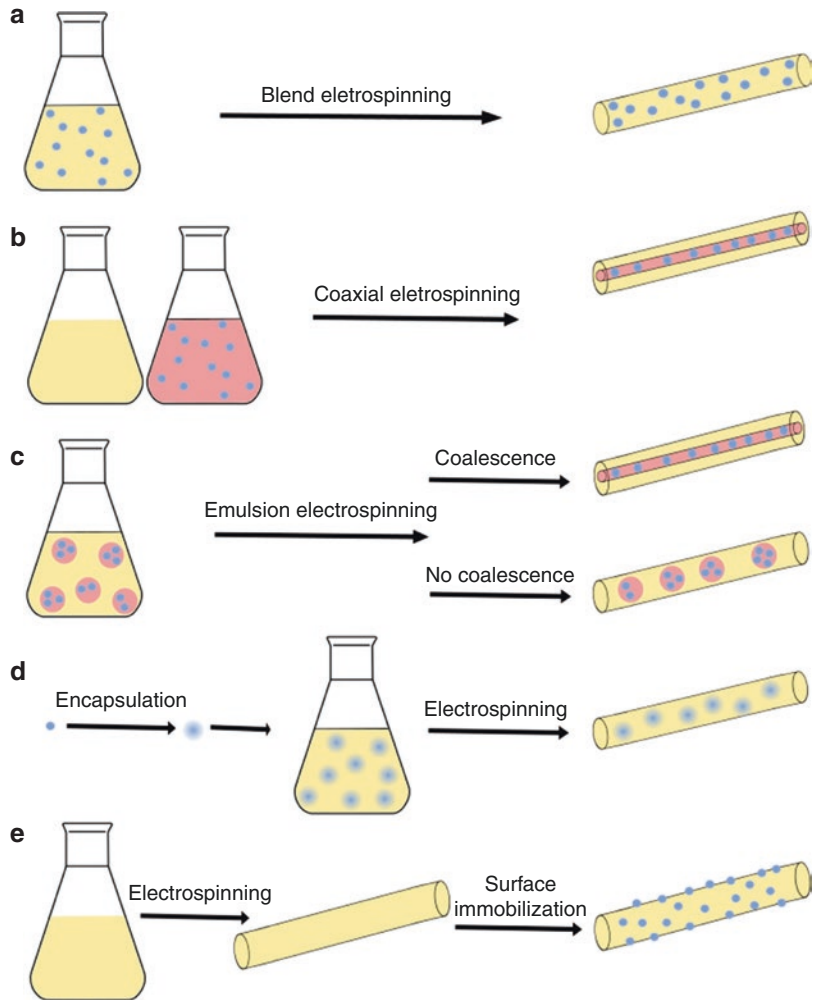

Fig. 4 Drug incorporation strategies. (**a**) Blend electrospinning, where drug(s) and polymer(s) are co-dissolved in solvents to be electrospun. (**b**) Coaxial electrospinning, where drug(s) and polymer(s) solutions are separately electrospun through two concentric nozzles. (**c**) Emulsion electrospinning, where drug(s) solutions are emulsified into immiscible polymer solutions, followed by electrospinning. (**d**) Encapsulation of drug(s) before mixing with electrospinning solution, followed by electrospinning. (**e**) Post-immobilization, where drug(s) are conjugated onto fabricated polymeric nanofiber matrices through physical or chemical interaction

The method for incorporating the drug is important as it most likely has an impact on the drug release. Suitable drug release profile is crucial for pharmaceutical products to elicit desirable outcomes. In wound therapy, we aim for prolonged drug release as this reduces the frequency of inconvenient and painful dressing changing. If antibiotics are concerned, local concentrations need to be carefully maintained at optimal levels to avoid promoting antibiotic resistance and persistence. A bimodal release profile with initial burst release following sustained release could have the greatest advantage in wound infection treatment as it helps to quickly reach effective antibiotic concentrations and at the same time provide extended activity. For this, several strategies are possible. It is commonly achieved with blend electrospinning slowly degrading hydrophobic polymers, e.g., PLA or PCL [56, 173], as the drug deposited on the fiber surface will release quickly and the drug loaded into the fibers will release in a prolonged manner due to limited water uptake [194]. Blend electrospinning is a simple one-step method to incorporate a drug into the fiber structure by dissolving polymer(s) and drug(s) in a single solution prior electrospinning. Homogenous mixture of polymer and drug in the formed fibers is usually aimed for, although if polymer and drug are not compatible and solvents are poorly chosen, the drug could be deposited on the fiber surface and thus release rapidly. This is more than likely if

hydrophobic polymers are combined with hydrophilic drugs or vice versa. Hydrophilic polymers are usually not sufficient for achieving prolonged release, thus they are either mixed with more hydrophobic polymers or processed otherwise (e.g., cross-linking) to overcome that problem [195]. From this, it is evident that it is much more difficult to prolong the release of hydrophilic drugs, as they tend to crystallize on a fiber surface if hydrophobic polymers are used, and on the other hand, more compatible hydrophilic polymers are usually not capable of sustaining the release. Another possibility is to create multilayered dressings. Different layers can be composed of the same or different polymers, whereas the drug can be added only to the inner polymer layer [196]. Coaxial electrospinning and emulsion electrospinning can help to overcome some limitations of blend electrospinning, like fast release of the drug or problems associated with incompatibilities between drug(s), polymer(s), and solvent(s). Coaxial electrospinning utilizes concentric nozzles through which two (or more) different solutions are fed, thus creating fibers with core and shell structure. In case of emulsion electrospinning, two phases are emulsified with the help of surfactants and fed through a conventional nozzle. During electrospinning the inner phase coalesces and core-shell fibers are created. It is also possible that the emulsion is conserved as droplets inside the fibers which form from external phase. Coaxial and emulsion electrospinning allow production of core-shell structured fibers where drug can be deposited only in the inner core of the fiber, thus prolonging the release compared to simple blend electrospinning. Another option to avoid rapid drug release is to encapsulate it prior electrospinning into nanoparticles, although this can also serve other purposes, like stabilizing and protecting the drug. Drug can also be added to the fibers after electrospinning either by simply impregnating them in drug solution which relies on drug adsorption onto the fibers or by surface modification via covalent bonding between the polymer and the drug. This can be laborious and the fibers need to be very stable in the solvents used to carry out the immobilization process.

7.2 Drug Release from Electrospun Fibrous Dressings

Drug release mechanisms from electrospun fibers are often rather complex as several processes are happening simultaneously. A combined effect of diffusion, polymer degradation, drug partitioning in polymers, and drug dissolution determines the release rate. These are largely determined by inherent properties of the polymers and drugs used, but other factors, like fiber diameter, drug loading, etc. also play their part in shaping the final effect. Solid-state properties of the drug and polymer need also to be considered, as the crystallinity of materials can, and most often do, change during electrospinning. As polymer crystallinity can affect water uptake and mechanical properties, it can also affect the drug release. Electrospinning often results in reduced crystallinity or even amorphous form of the drug [197]. On one hand, amorphous form of the drug has greater dissolution rate and apparent solubility, thus enhancing drug release; on the other hand, recrystallization often takes place on the fiber surface compared to amorphous drug which is more likely deposited inside the fibers [194]. Due to large surface area of the fibers, even crystalline drug can dissolve quickly if it is deposited on the fiber surface. Thus, the prevailing effect is not straightforward and several factors need to be taken into account. Still, by carefully selecting compatible polymer(s), drug(s) and solvent(s) together with appropriate processing parameters, dressings with finely adjusted and sustained release profile can be produced for advanced therapy.

7.3 Antibiotics in Novel Electrospun Fibrous Dressings

Antibiotics are drugs that act selectively on bacteria to reversibly inhibit their growth (bacteriostatic) or irreversibly kill them (bactericidal). Due to the selectivity, they are less harmful to the host compared to bacteria. Problems associated with topical antibiotics are related to allergic reactions and emergence of antibiotic resistance.

Due to these problems, current consensus is advised against routine use of topical antibiotics in the treatment of wound infection [198]. Systemic antibiotic treatment is, however, indicated if the infection is spreading.

Still, antibiotic-loaded wound dressings are extensively being developed for the management of wound infection, and it is argued that skillful use of topical antibiotics using more sophisticated delivery platforms together with general wound care principles should not be disregarded. The faith in topical antibiotics is clear from the number of scientific publications. Most commonly, broad-spectrum antibiotics are used in novel dressings due to polymicrobial nature of wound infections. Extensive work has been done in electrospinning antibiotics, like tetracyclines, vancomycin, sulfonamides, aminoglycosides, fluoroquinolones, beta-lactams, chloramphenicol, nitrofurazone, fusidic acid, polymyxin B, and mupirocin (Table 6).

Antibiotics can be broadly classified into five classes based on their mode of action: (1) cell wall synthesis inhibitors, (2) cell membrane function inhibitors, (3) protein synthesis inhibitors, (4) nucleic acid synthesis inhibitors, and (5) metabolic process inhibitors.

7.3.1 Cell Wall Synthesis Inhibitors

The bacteriostatic effect of beta-lactam antibiotics is related to the inhibition of essential enzymes (transpeptidases, carboxypeptidases) involved in the terminal stages of peptidoglycan biosynthesis. The bactericidal effect of these antibiotics is due to the activation of an autolytic system causing cell death [199]. Fazli and Shariatinia [177] incorporated cefazolin sodium-loaded fumed silica nanoparticles into chitosan/PEO matrix, thus achieving improved hydrophilicity, higher tensile strength, and sustained drug release compared to matrices with cefazolin but no nanoparticles or with nanoparticles but no cefazolin. Fazli et al. [178] also reported similar imipenem/cilastatin-loaded ZnO nanoparticles in chitosan/PEO matrix. Sundaran et al. [200] produced a wound dressing with a sandwich structure, where chitosan microbeads loaded with ampicillin were placed between PCL layers. Chitosan microbeads swell in aqueous environment and release the antibiotic while retaining the beads within the electrospun mat, thus preventing microbeads entering the wound bed but allowing sufficient amount of drug to be released.

Vancomycin is a glycopeptide antibiotic that inhibits the cell wall synthesis by binding to the C-terminal L-Lys-D-Ala-D-Ala motif present in cell wall precursor. It is active against staphylococci, streptococci, and other gram-positive bacteria. Dhand et al. [133] utilized matrix-drug interactions of gelatin and polyhydroxy antibiotics, like vancomycin, to sustain antibiotic release for more than 20 days. Liu et al. [201] used vancomycin hydrochloride to modify multiwalled carbon nanotubes later anchored to polyurethane nanofibers to produce highly antibacterial material with potential application in the preparation of wound dressings. As carbon nanotubes are also known for antibacterial activity, the material had activity against both gram-positive and gram-negative bacteria.

7.3.2 Cell Membrane Function Inhibitors

Polymyxin B as a cationic antibiotic binds to lipopolysaccharides in the outer membrane of gram-negative bacteria and disrupts the cell membrane structure [202]. Zhang et al. [167] encapsulated polymyxin B sulfate into halloysite nanotubes and mixed these with PLA and dexamethasone to produce dual-loaded wound dressing, significantly improving burn wound healing of rats and protecting those burn wounds against bacterial infection.

7.3.3 Protein Synthesis Inhibitors

Tetracyclines are a family of broad-spectrum antibiotics inhibiting protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. Zahedi et al. [56] demonstrated better performance in water uptake, water permeability, drug release rate, and antibacterial activity of electrospun nanofibers loaded with tetracycline compared to commercial wound dressing.

Chloramphenicol is a broad-spectrum antibiotic first isolated from *Streptomyces venezuelae*. By diffusing through the bacterial cell wall and reversibly binding to the bacterial 50S ribosomal subunit, it interferes with peptidyltransferase activity and prevents peptide bond formation, hence impairing bacterial protein synthesis and bacterial cell proliferation [203]. Chloramphenicol has been electrospun together with suberin fatty acids, natural antimicrobial agent, to produce potential wound dressing material [82]. Preem et al. incorporated chloramphenicol into two different dressings, showing that different hydrophobicity/hydrophilicity of the dressing resulted in different drug release rates but also interactions between wound bacteria and the dressings [173].

Primary mechanism of action of aminoglycosides is the inhibition of protein synthesis by binding to 30S ribosomal subunit at aminoacyl-tRNA acceptor site [204]. Unnithan et al. produced streptomycin sulfate-loaded polyurethane/cellulose acetate/zein nanofibrous dressings showing enhanced blood clotting and excellent platelet activation ability [180]. Monteiro et al. [139] immobilized gentamicin-loaded liposomes on chitosan nanofibrous mesh resulting in more sustained release of the drug compared to liposomes in suspension. Reshmi et al. [128] loaded gentamicin into PLLC/collagen-layered matrix and achieved prolonged bioactivity for 15 days. Nandagopal et al. [205] showed synergistic effect of gentamicin and titanium dioxide nanoparticles loaded into PCL fibers. Nitanan et al. [155] demonstrated in vivo enhanced wound healing on Wistar rats using neomycin-loaded dressings.

Mupirocin reversibly inhibits isoleucyl-transfer RNA and thereby inhibits bacterial protein and RNA synthesis [206]. Chen et al. [181] incorporated mupirocin into polyurethane fibers to treat burn wounds and demonstrated continuous release of antibiotic for 3 days. Due to unique mechanism of action, they proposed small cross-reactivity with other antibiotics.

Fusidic acid binds to elongation factor G necessary for translocation on the bacterial ribosome after peptide bond formation during protein synthesis and thereby inhibits protein synthesis [207]. Said et al. [168] have reported about developing bioburden responsive fusidic acid loaded PLGA nanofibers with enhanced drug release resulting from the activity of microbial esterases.

7.3.4 Nucleic Acid Synthesis Inhibitors

Fluoroquinolones interact with DNA gyrase and topoisomerase IV and convert them into toxic enzymes damaging the bacterial chromosome [208]. Contardi et al. [77] compared ciprofloxacin-loaded PVP nanofibers with respective films and saw that fibers were resorbed much faster than films, but at the same time, in vivo bioresorption was still a lot longer compared to in vitro degradation in PBS. Li et al. [209] observed accelerated in vivo wound healing with ciprofloxacin-loaded PNIPAAm/PLCL composite fibers compared to those loaded with only PLCL or ciprofloxacin and commercial gauze soaked with ciprofloxacin solution. Chouhan et al. [136] achieved scarless healing in rabbit wound model with PVA-fibroin dressing functionalized with EGF and ciprofloxacin hydrochloride monohydrate compared to surgical gauze and PVA treatment. He et al. [193] showed improved healing in rat wound model using enrofloxacin-loaded fibrous dressing compared to the treatment with gauze. Similarly, enhanced wound healing on rats was shown with gatifloxacin-loaded fibrous dressings [174].

7.3.5 Metabolic Process Inhibitors

Sulfonamides inhibit bacterial growth by competing with para-aminobenzoic acid at the first biosynthetic step of the folic acid pathway [210]. Mohseni et al. [156] report about potential wound dressing containing sulfadiazine with a capability of cell seeding. Munteanu et al. [140] have incorporated sulfadiazine or sulfadiazine-modified chitosan in electrospun dressings in the form of functional nanoparticles. Abbaspour et al. [211] incorporated mafenide acetate into chitosan/PVA scaffold and demonstrated prevention of bacterial penetration through the dressing, hence providing protection from a secondary infection despite of highly porous nature of the dressing.

Nitrofurazone is an antibiotic with an unknown mechanism of action, showing bactericidal activity against variety of both gram-negative and gram-positive bacteria. Zhao et al. [163] prepared a dual-layered wound dressing loaded with nitrofurazone. They demonstrated that adjusting the amount of drug in different layers allows to control the release profile and showed superior wound healing in vivo compared to commercial dressing occurring with a dressing having both initial burst release and following long-term sustained release.

7.4 Antiseptics in Novel Electrospun Fibrous Dressings

Antiseptic agents have a clear place in the prevention and management of wound infections. They have broad-spectrum activity which can kill or inhibit bacteria, fungus, protozoa, viruses, and prions. Topical antiseptics can be used instead of antibiotics to prevent emergence and dissemination of antibiotic resistance. However, some antiseptics have been associated with toxicity to granulation tissue and hence delayed wound healing [212]. Also, rare but serious allergic reactions are reported [213]. It is also important to note that resistance can also develop to antibiotic compounds and, even worse, cross-resistance between antiseptics and antibiotics has been described [214]. Still, there are several antiseptics which can have clinical value in wound infection prevention and treatment, e.g., silver, PVP-iodine, chlorhexidine, polyhexanide, octenidine hydrochloride, and quaternary ammonium compounds.

Silver is one of the most widely used antiseptics in wound care. Although used for centuries, the exact mechanism of action of silver is not entirely known. Probably the mechanism relates to interactions between silver ions and thiol groups in enzymes and proteins, but it has also been shown that silver is capable of penetrating cell membranes and attacking bacterial respiratory chains. It can also interact with DNA and thus deactivate bacterial replication [215, 216]. Silver can be incorporated into the dressing in various forms, e.g., silver metal, silver acetate, silver nitrate, silver protein, and silver sulfadiazine, the latter being a combination of silver and sulfonamide antibiotic. With the emergence of nanotechnology, silver nanoparticles are increasingly studied to be used in wound dressings. As a result of their nanoscale, nanoparticles have unique physicochemical properties compared to their larger counterparts. Antibacterial properties of silver nanoparticles are strongly influenced by their shape, size, concentration, and colloidal state. Shape is an important characteristic as it determines the effective surface area, e.g., truncated triangular silver nanoparticles have enhanced antibacterial action compared to spherical or rod-shaped particles. So does also the size matter – 10–15 nm size is proposed to have the best stability, biocompatibility, and antibacterial properties, although it has also been shown that the smaller the particles, the better the antibacterial activity due to enhanced penetration into the cell. Colloidal stability needs also to be accounted for as suspended form of silver nanoparticles has superior activity [217]. There is a wide range of different silver-loaded commercial wound dressings, but several novel dressings also utilize the virtues of silver. Hassiba et al. [141] loaded silver nanoparticles into the outer layer of multilayered wound dressing with a primary purpose of hindering secondary contamination from the environment. Dongargaonkar et al. [218] incorporated silver into gelatin-dendrimer nanofibers, allowing sustained release of silver from the dressing. Abdelgawad et al. [219] showed that wound dressings containing silver nanoparticles and chitosan have synergistic antibacterial properties. Silver is the most potent antimicrobial metal, although not the only one. Copper [220], zinc [152], and titanium [158] are all used as well for this application, and as they are considerably cheaper than silver, they can be looked as an economic alternative. Gold nanoparticles are also known for antimicrobial properties and excellent biocompatibility; thus they remain an interest in biomedical field despite of higher price. Highly stable antimicrobial coating of gold nanoparticles and lysozyme has been produced for cellulose acetate nanofibers, making it

an attractive composite material for wound dressings [221].

Iodine and iodophors are another type of widely used antiseptics. Iodine penetrates into microorganisms and attacks their proteins (thiol groups of amino acids), nucleotides, and fatty acids, causing cell death. PVP-iodine and cadexomer iodine are the most commonly used iodophors, overcoming the problems associated with irritating iodine solutions [222]. Sebe et al. [185] incorporated PVP-iodine into PVP and PVP vinyl acetate (PVP/VA) fibers produced by alternative technique to electrospinning – high speed rotary spinning that utilized centrifugal forces instead of electrostatic forces. The produced fibers had good antibacterial properties, and the time-kill profile was closely correlated with their available iodine content.

Chlorhexidine is an antiseptic belonging to a class of biguanides widely used in handwashing and oral products but also in wound care. The mechanism of action of chlorhexidine is biphasic – initially it causes membrane leakage, but as the concentration increases, it brings about coagulation of the cytosol. It is important to note that the activity of chlorhexidine is greatly reduced in the presence of organic matter, like wound debris, and the action is pH dependent [222]. Polymeric biguanides, like PHMB, act via interactions with the negatively charged bacterial cell membrane components. After binding to the membrane phospholipids, permeability of the membrane increases initially causing loss of K^+ ions and ultimately resulting in precipitation of intracellular constituents and cell death. Hassiba et al. [141] observed that molecular interactions with carrier polymer PVP reduced the antimicrobial activity of PVP-chlorhexidine-loaded fibers compared to PEO-chlorhexidine fibers, although both showed activity. Liu et al. [223] investigated wound healing properties of PHMB-loaded nanofibers on rat burn wounds and saw that the best results were present in case of cospin 4:1 PEU/CA fibers, due to high wettability and water uptake, good moisture retention, air permeability, and mechanical properties.

The mode of action of octenidine hydrochloride is also based on plasma membrane destabilization. In liquid forms, it is often administered together with phenoxyethanol which acts as a cosolvent but is also associated with strong irritability limiting the use in open wounds and mucous membranes [224]. Jiang et al. [225] incorporated octenidine together with peppermint oil and/or chlorhexidine digluconate into nanocapsules later loaded into nanofibers and demonstrated synergistic antibacterial properties of these compounds.

7.5 Antimicrobial Natural Products in Electrospun Fibrous Dressings

Many natural plant extracts possess antimicrobial, anti-inflammatory, or anti-oxidant activities, making them a promising source of novel compounds of interest in wound infection treatment [226]. Antimicrobial phytochemicals belong to various classes and so are the mechanisms of action also diverse, including attacking cell membrane or cell wall, substrate deprivation, enzyme inhibition, etc.

Many crude plant extracts have been loaded into electrospun matrices for antimicrobial activity, e.g., thyme extract [142], soursop leaves extract [157], propolis extract [227], henna leaves extract [228], chamomile extract [229], garcinia extract [230, 231], and *Tridax procumbens* extract [232].

Much interest is put into electrospinning essential oils as this allows encapsulation of volatile substances to enhance the stability and provide a suitable vector for clinical applications. The mechanism of action of essential oils is related to their hydrophobic nature, as they partition into lipid cell membrane and disrupt its structure. Some examples of antimicrobial essential oils incorporated into the electrospun matrices are derived from rosemary, oregano [187], cinnamon [233, 234], lemongrass, peppermint [233, 235], and lavender.

Curcumin, an active ingredient derived from turmeric, has many beneficial properties, including antioxidant, anti-inflammatory, and antimicrobial characteristics, making it attractive for wound

therapy. Curcumin has low in vivo bioavailability due to poor solubility in water, but it has been shown that encapsulating it into nanofibers could increase its bioavailability [236]. Mohammadi et al. [154] loaded curcumin into PCL/gum tragacanth electrospun fibers. These fibers showed high activity against methicillin-resistant *Staphylococcus aureus* and extended spectrum beta-lactamase producing bacteria; moreover, the dressing promoted healing of diabetic wounds in rats.

One of the most important natural products used in wound therapy and modern dressings is honey. The antimicrobial activity of honey is based on several mechanisms: (1) hygroscopicity, resulting in dehydration of bacteria; (2) high sugar content causing hyperosmolarity; (3) hydrogen peroxide generated by glucose oxidase in situ upon dilution; and (4) low pH (3.4–5.5). In addition to antimicrobial properties, honey is also an effective debriding agent, and it can help to control the malodor [237]. Honey-based wound care products are commercially available as topical semisolid formulations (gels, creams, ointments) and dressings. Active research still continues to develop novel honey-based dressings. Sarhan and Azzazy [143] produced electrospun chitosan nanofibers loaded with 40% honey. This is rather high concentration, but Yang et al. [137] incorporated even up to 70% of honey to silk fibroin/PEO fibers, showing that adding honey to the dressing can significantly enhance its antimicrobial properties whereas not adversely affecting biocompatibility. Moreover, in vivo wound healing in animal model was also improved.

7.6 Other Antimicrobial Strategies Used in Wound Dressings

In addition to aforementioned active ingredients, antimicrobials with novel approaches to infection treatment are also investigated. As they offer a completely different view on therapy, they can be especially beneficial if thought about problems with antibiotic resistance. Photodynamic therapy can be one such approach, as it utilizes photosensitizers together with light irradiation at specific wavelength. As a result, reactive oxygen species are created which have multiple targets within a microbial cell. Some examples of photosensitizers incorporated into wound dressings are phthalocyanides [144], tetraphenylporphyrin [238], and methylene blue [239]. The effectiveness of photodynamic therapy was demonstrated on *S. aureus*-infected wounds in immunocompromised rats [239] and even on 82 patients with chronic leg ulcers (ulcer area reduced from 12.5 to 8.1 cm^2, $P < 0.01$) in a randomized study [238]. Another approach is utilizing nitric oxide (NO) donors. NO induces bacterial cell membrane damage and DNA deamination while having numerous functions advantageous for wound healing, like modulating hemostasis, reducing inflammation, and promoting healing [240]. NO can be incorporated into polymer structure, so the polymer itself acts as an NO donor [240–242]. Worley et al. [242] on the other hand blended NO-donating dendrimers into polyurethane matrix. Nogueira et al. [243] propose another fascinating and highly effective possibility to fight with bacterial infections without the use of antibiotics by immobilizing bacteriophages in wound dressing nanostructure. Nisin, an antimicrobial peptide, has also been incorporated into wound dressings [159, 244]. Antimicrobial peptide LL-37 (AMP LL-37) addition to the silk fibroin nanofiber mat functionalized with EGF and bFGF enabled to avoid the occurrence of biofilm and bacterial colonization in the diabetic wound [123]. In addition, enzymes have been included into the fibers to obtain antimicrobial bioactive dressings [245]. Miao et al. [245] employed a cell lytic enzyme, lysostaphin (Lst), with specific bactericidal activity against *S. aureus*, to generate anti-infective wound dressings. Lst was immobilized onto biocompatible fibers generated by electrospinning homogeneous solutions of cellulose, cellulose-chitosan, and cellulose-poly(methylmethacrylate) (PMMA) from 1-ethyl-3-methylimidazolium acetate. The developed mats showed activity against *S. aureus* in an in vitro skin model with low toxicity toward keratinocytes, suggesting good biocompatibility for these materials as antimicrobial matrices in wound healing applications.

Biofilm formation in the wound is one of the most important causes of delayed healing and failing antimicrobial therapy. Thus, novel approaches to specifically target biofilm phenotype of bacteria are urgently searched. This topic

is well summarized in a review paper by Sadekuzzaman et al. [246]. It is proposed that using classical antimicrobials (antibiotics, antiseptics) together with antibiofilm agents could have synergistic and superior action against wound infection [7, 8, 25]. Thus, novel wound dressings most probably target wound infection through several mechanisms and biofilm will be one of the key targets in the future therapies.

7.7 Antimicrobial Wound Dressing Interactions with Bacterial Cells

As wound dressings are in direct contact with the wound, they become a part of this complicated environment, and dynamic interactions occurring between the wound, the dressing, and the colonizing bacteria need to be elucidated to understand how a dressing can help or deter healing. Knowledge about interactions between the dressings and bacteria is unfortunately still scarce, although the importance of these has been highlighted in the studies that do exist. The first stage of biofilm formation is adhesion of bacteria onto a surface. As discussed earlier, biofilms have increased resistance to antimicrobials and immune system, so they are difficult to eradicate. The mechanisms of bacterial attachment to design wound dressings that do not promote biofilm formation should be understood. Said et al. [247] showed that bacteria quickly colonize ultrafine fibrous PLGA dressings and cause enhanced drug release due to detrimental changes in morphology of those fibers together with decreased pH. They point out that nonmedicated or inadequately medicated bioresorbable fibrous dressings could impose a risk of wound reinfection and resistance and the need for carrying out studies in biorelevant conditions. Similarly, Preem et al. [173] have shown that biofilm can form quickly onto fibrous dressings, most notably on hydrophobic dressings. They also showed that drug release profile can have a great impact on dressing colonization, prolonged release of antibiotic being favorable to reduce biofilm formation. Abrigo et al. [248] have shown that fiber diameter can also determine how bacteria attach and spread throughout the fibrous substrates – the best support for bacterial adhesion and spreading occurring if the average fiber diameter is close to bacterial size. Surface roughness, wettability, hydrophobicity, charge, and charge density have been all been correlated to bacterial attachment and proliferation on surfaces [249]. Abrigo et al. [248] also looked into the influence of surface chemistry to bacterial attachment to fibrous meshes. They demonstrated that fibers with hydrophilic and positively charged surface induced the highest attraction of viable cells, whereas hydrophilic negatively charged fiber surface had significantly lower attraction. At the same time, they show significantly different attachment of viable bacteria on two hydrophobic meshes with different surface chemistries, emphasizing that hydrophobicity is not the only parameter affecting the attachment.

7.8 Smart Wound Dressings to Fight Against Wound Infection

There is a growing awareness that wounds need to be monitored during healing (specifically chronic wounds) to detect the physiological changes indicating healing or deterioration of the situation, often related to the absence or presence of bacterial infection. However, the frequent dressing change may disturb the wound healing; therefore, the ability to monitor the wound healing process under the dressing without any interference is desired. Smart wound dressings capable of signaling changes in the wound such as infection development provide means to start immediate treatment of the wound and avoid infection-related complications (e.g., amputations, systemic infections). As increased pH is proposed to indicate the onset of infection, several attempts have been made to develop wound dressings capable of monitoring it. Different approaches are possible, e.g., using disposable electrochemical pH-sensitive sensors [250] or using colorimetric detection with pH indicators [234]. Wound temperature is another parameter that could be used for assessing wound situation with smart dressings [251]. More elaborate

systems are able to detect more specific bacterial biomarkers, like pyocyanin (a quorum-sensing molecule produced by *Pseudomonas aeruginosa*) [252], or virulence factors secreted by pathogenic bacteria only [253]. The detection of these markers can be linked with the drug release to produce on-demand drug delivery systems which offer additional benefit of providing treatment only if infection is present, thus avoiding problems related to misuse of antimicrobial agents and resistance.

Conclusions

Electrospun polymeric micro-/nanofibers loaded with antimicrobials have gained significant popularity for use as antimicrobial wound dressings because of several advantages, such as an inherently high surface area to volume ratio, tunable fiber diameter, high porosity, and ECM-like structure. Incorporation of antimicrobial drugs using various technological approaches enables to design the wound dressings with the desired drug release kinetics relevant to fight against wound infections. Controlled delivery dressings can provide an excellent means of delivering antimicrobial drugs to wound sites in a consistent and sustained fashion over a long period of time without the need for frequent and uncomfortable dressing change. As was summarized in this chapter, the careful selection of suitable materials and electrospinning conditions/setups is of utmost importance while developing novel antimicrobial dressings. Not only the active substances, but also the polymers need to be selected appropriate for electrospinning as well as wound healing. It can be seen that these electrospun fiber mats have several characteristics important for wound healing. All different properties of nanofibers (e.g., structural, mechanical, etc.) need to be considered while developing effective bioactive wound dressings. In some cases, the appropriate selection of polymers and/or active substances enables to produce multifunctional dressings suitable to be applied also for difficult to heal wounds such as chronic wounds. The knowledge about interactions between wound bed, bacteria, and wound dressings is still an unexplored field in most parts, although pioneering studies have shed some light on those important issues, which help in designing more appropriate novel wound care products. It is expected that also novel antibiofilm agents and antibiofilm strategies together with antimicrobial substances and supportive polymers will aid to improve to the fight against wound infections and support normal wound healing. In a near future, it is expected that smart wound dressings consisting smart polymers and feedback systems enable to improve the wound healing even more. This will improve the patient compliance thus avoiding the prolonged treatment and complications of wound infection.

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Cognition and Wound Healing

Vahid Rakhshan

1 Healing and the Stress Response

Healing is a natural process initiating spontaneously to restore homeostasis and tissue integrity [1, 2]. The conventional model of physical recovery is wound healing [3–5] that can be affected by factors such as adequate nutrition, blood supply, rest, hygiene, physical health, and psychological well-being [3, 5–7]. A wound might be described as a "disruption of normal tissue structure and function" [8] which occurs usually in the skin or the mucosa [9] and can be caused by surgery, trauma, pressure, or pathologies [10, 11]. Wound healing progresses through a complex process formed of several overlapping and interwoven stages, namely, clot formation, inflammation, proliferation, and remodeling with some site-specific differences [11–22]. Almost after vasoconstriction and blood coagulation, the inflammatory stage begins. It is characterized in part by the cascade of various substances such as growth factors and cytokines (e.g., platelet-derived growth factors, interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor (TNF), transforming growth factor-β, and vascular endothelial growth factor) from damaged cells and platelets. These biomarkers attract the migration of phagocytes which debride the wound and destroy pathogens. These cells as well release substances such as matrix metalloproteinases (MMPs), cytokines, and other chemoattractants which help protect against bacteria and induce the migration of fibroblasts, epithelial/endothelial cells, and other cells necessary for the proliferative phase, i.e., revascularization and tissue regeneration [14, 15, 18, 23–27]. Cytokines and MMPs are again crucial to the last phase which may continue for weeks or months [12, 27, 28], during which cell numbers reduce and collagen fibers are remodeled, strengthening the scar tissue [11, 15, 29].

A healthy immune response plays a pivotal role in successful wound healing during the inflammatory stage and its downstream cascade [14, 15, 20, 30, 31]. However, it can be modulated by acute or chronic stresses [32] which may disrupt the inflammatory phase by affecting various healing processes directly or indirectly [15, 28, 33–38]. Stress might indirectly influence the immune system and recovery potential through deteriorating the lifestyle such as alcohol drinking, smoking, lack of exercise, obesity, etc. [37, 39]. The direct effect of stress is exerted through the stress response [32, 37, 39–42]: under normal circumstances, the parasympathetic nervous system (vagal output) is active and acts as an endogenous anti-inflammatory factor by inhibiting the secretion of inflammatory cytokines; nevertheless, under stress, this inhibition is lost [43, 44]. Upon detection of signs of an upcoming threat, cortical networks

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responsible for the cognitive component of stress as well as subcortical (limbic) networks accountable for the emotional stress component relay "danger" signals to the hypothalamus [45], which activates the sympathetic-adrenal-medullary and the hypothalamus-pituitary-adrenal (HPA) axes [46–49]. These systems release, respectively, catecholamines (such as adrenalin and neuroadrenalin) and glucocorticoids (e.g., cortisol and corticosterone) to elicit the fight-or-flight response [45, 46, 48–54]. These can disrupt the inflammatory phase via various mechanisms [15, 28, 33–37]. Cortisol is the primary stress hormone which, among other effects, can compromise the immune system, impede the rate of healing, and accelerate bone resorption [44, 48, 55–57]. Its ongoing elevation might have immunosuppressive effects on antigen presentation, antibody and cytokine secretion, proliferation or traffic of lymphocytes, and T helper (Th)-2 predominance [49, 58].

Increases in cortisol, epinephrine, and norepinephrine might deteriorate the proliferation and infiltration/activation of macrophages and neutrophils, alter the secretion of many pro-inflammatory cytokines, and shift the function or differentiation of T cells from Th1 cellular to Th2 humoral immunity [30, 36, 41, 47, 49, 58–61], resulting in the inhibition of inducible nitric oxide synthase (iNOS) release from macrophages [17, 30]. This attenuates the concentration of nitric oxide which is vital to angiogenesis, cell proliferation, and cell migration [30, 62]. Adrenaline elevation might also elevate iNOS to cytotoxic levels, which is again detrimental to wound healing [30, 36, 63]. Adrenaline can also prolong inflammation via persistent trafficking of neutrophils to the wound [61, 64].

The stress-induced humoral immunity upregulates B lymphocytes, increasing antibody production instead of contributing to wound healing; it also activates mast cells, which release histamine and aggravate inflammation [30, 58]. Stress can prolong inflammation by reducing pro-inflammatory cytokines and impairing neutrophil mRNAs [41, 61, 65–67]. The stress response can as well disrupt further stages of healing by reducing MMPs (such as MMP-2 and MMP-9) which contribute to cell migration, collagen turnover, and wound maturation [68]; this leads to less mature collagen scaffolds and delays the remodeling of the granulation tissue [30, 36]. Cytokines are crucial to healing [17], and their stress-induced imbalances might impair it [30, 69]. Among stress-induced changes, IL-1β, IL-6, IL-10, and TNF-α may be increased by stress [70, 71], while IFN-γ might be downregulated [71].

Given these alterations, psychological stresses may cause clinically relevant delays in wound healing [11, 15, 52, 60, 69, 72–75] with a medium effect size ($r = -0.42$) [11]; it can also raise morbidity and mortality of various conditions, worsen postoperative complications [11, 39, 67, 76–80], and accelerate aging through increasing telomere shortening [81–84]. On the other hand, psychological stress can speed up blood coagulation [85]. It is suggested that during stress, blood plasma moves to the extravascular space, increasing the hemoconcentration of molecules larger than 69 kiloDaltons which cannot pass through vascular pores [86–89]. Moreover, under stress, prothrombosis activity is heightened more than is fibrinolysis activity, favoring coagulation [89]. This might help limit bleeding in a potentially dangerous situation.

Psychological health is a combination of factors and states such as optimism, self-esteem, life satisfaction, and perception of social support [39]. It can affect several health indices [90–92] such as the prognosis of disease and the capacity for recovery and survival [2, 3, 5, 39, 93–104]. For instance, the immune function is associated with personal relationships [31, 105], partly because the oxytocin released during social interactions can suppress the reactivity of HPA axis to stress and reinforce the wound healing potential [72, 106, 107]. A negative mood has been linked to lower salivary levels of antibody to an orally ingested antigen as well as declined natural killer (NK) cell lysis potentials [38, 108, 109]. A conflict-inducing psychiatric session for couples has been reported to delay healing [110]. Higher activation of the right prefrontal cortex (which is associated with experiencing and expressing negative emotions [111]) might be linked to lower NK cell activity [31, 112]. Optimism and depression have been linked to better and poorer

recoveries, respectively [33, 113, 114]. Among kidney donors, positive associations were observed between delayed wound healing with higher life stress before the surgery, lower conscientiousness, and lower optimism, while faster wound healing was associated with a greater emotional stability [115].

Pain is another factor interfering with the healing response [116–119]; it is a pathogen capable of dysregulating neuroendocrine responses, weakening the immune function, increasing metabolism, changing thermoregulation, impairing wound healing, and even facilitating the progression of metastatic diseases [120–127]. Pain may as well cause suffering and stress, which again can interfere with the healing cascade in various wounds [30, 34, 41, 67, 110, 126–130]. Wound healing might be complicated by inadequate pain treatment [60, 131, 132]. For instance, a 5-week investigation of standard punch biopsy wounds in patients who had undergone gastric bypass surgery demonstrated that postsurgical pains were associated with healing delay [126].

Another emotion affecting recovery is anger, which is correlated by the hostile attitude of the person toward the others; it can be controlled, suppressed (i.e., "anger in" or boiling inside without showing it [133]), or expressed in various intensities (i.e., "anger out") [73, 133–136]. The way one interacts with anger might have psychophysiological influences [73, 137]. Anger, its expression or its suppression, might have adverse effects such as heightened glucocorticoid release, decreased immunological activity, longer postoperative recovery, more postsurgical complications, delayed wound healing, or an increased cancer risk [73, 76, 138–147]; whereas, anger control (i.e., monitoring, regulating, and controlling the temper [133]) has been linked to less severe cortisol elevations or more efficient NK cell cytotoxicity [73, 148]. Positive attitudes as well might enhance healing [149]. Symptoms of post-traumatic stress disorder (such as intrusive thoughts) might result in hyperarousal, hypervigilance, catastrophizing, fatigue, sleep disturbances, and intensified endocrine or inflammatory activities [150–159]. A systematic review [39] evaluated studies on the associations between psychological traits and healing of punch biopsy wounds [67, 130], suction blister wounds [73, 110], or oral mucosa wounds [160] in 1473 subjects (1071 patients and 402 volunteers), of whom 37 received dental surgery [161], 861 underwent cardiac surgery [76, 162–165], and 173 received general surgery [118, 128, 166–168]. Of the five studies assessing the associations between trait anxiety and wound healing [76, 128, 130, 161, 168], two identified a significant wound healing attenuation in stressed subjects [128, 130]. Of the five studies examining state anxiety [67, 76, 128, 161, 167], three reported impaired wound healing [67, 128] and postoperative complications [76]. In the four papers on anger and hostility [73, 76, 110, 162], anger control accompanied faster wound healing [73], while anger suppression or its expression were not related to clinical outcomes. Trait anger deteriorated the complications [76, 162] and wound healing [110]. State anger worsened postoperative complications [76]. An external health locus of control was associated with faster healing, while low pain expectations mitigated facial swelling [161]. Two of the studies on coping (vigilance vs. avoidance) [161, 166, 167] noticed more complications in vigilant subjects [166, 167]. Subclinical depression, distress (defined as depression, anxiety, and stress), and loneliness were researched in three articles [160, 162, 166]. Distress and subclinical depression were, respectively, associated with postoperative infections [166] and healing [160]. Perceived social support [162] or loneliness [160] did not affect healing or complications. Optimism had no influence on surgical outcomes in one study [162]; in another one, it merely ameliorated ventilation [163].

Besides the adaptive immunity, stress can also weaken the innate immunity of the skin [32, 61]. Peripheral nerve terminals can release factors like neurotrophins and neuropeptides (e.g., substance P) within the skin, which act as local stress responders and neurogenic inflammatory mediators [49, 169]. Substance P is a pro-inflammatory neuropeptide released from cutaneous peripheral nerve endings, and increased during stress

[49, 170]. It might aggravate the virulence of skin microflora by upregulating cysteine-aspartic proteases and changing the actin cytoskeleton, thus contributing to neurogenic inflammation [171]. It can also stimulate inflammatory cell and neutrophil infiltrates [172] as well as the release of different cytokines (such as IL-1, IL-6, and IL-12) from T cells and monocytes, resulting in proliferation of T cells and inflammation [173, 174]. It also induces mast cell degranulation and macrophage infiltration [170]. In addition, it is involved in the effect of corticotropin-releasing hormone on mast cell degranulation during stress [49, 175]. Nerve growth factor is a neurotrophin which can induce proliferation, migration, and differentiation of fibroblasts into myofibroblasts, which are critical in cutaneous wound healing [49, 176]. Furthermore, stress can lower the number of Langerhans cells in the epidermis [61, 177], slowing the healing and increasing the risk of wound infections [61, 178].

In view of the effects of psychological conditions on biological well-being, it seems that cognitive-behavioral interventions might be cost-effective treatments to alleviate pain, stress, anxiety, and decrease HPA/sympathetic activity; hence, they may better immune function and aid recovery and wound healing while reducing postoperative complications and hospital stay [3, 5, 96, 97, 99, 179–189]. This chapter briefly reviews some of such interventions, namely, relaxation and meditation, music therapy, expressive writing, hypnosis, and placebo.

2 The Relaxation Response

The relaxation response is a psychophysiological state opposite of the fight-or-flight response. It is characterized by the reduction of sympathetic/HPA activities and increases in the parasympathetic tone, neutralizing the excessive stress and inflammation [44, 118, 190–194]. Relaxation techniques, especially in long term, might provoke this response and might even modulate numerous genes associated with vital functions such as antigen processing and presentation, oxidative phosphorylation, or apoptosis [195]. They can downregulate genes involved in stress-related pathways and inflammatory responses while enhancing genes linked to telomere maintenance, mitochondrial function, energy metabolism, and insulin secretion [194]. They can also increase melatonin [196, 197] which regulates the circadian rhythm and improves sleep, is a potent antioxidant, inhibits tumorigenesis, and mediates regenerative functions such as bone fracture healing [44, 196–199]. Additionally, relaxation and meditation might reduce oxidative stress by increasing the activity of antioxidant systems and thus can positively affect wound healing [200–202]. A number of practices can induce this state and thus impact endocrine, autonomic, and immune functions [21, 203–205].

Muscle relaxation is the practice of eliminating residual muscular tensions, which might diminish excitatory impulses and irritability of neural centers [206]. It can be performed using different techniques mainly involving a variant of progressive muscle relaxation (PMR). PMR was introduced by Jacobson [206] as a series of tensing and releasing of 16 muscle groups. According to him, a stressed mind cannot exist in a relaxed body [206–208]. This method has been modified by others, in order to be integrated into different therapies [207–209]. It can be facilitated by cognitive processes such as guided relaxing imagery, music, nature sounds, or hypnotic imaginations. Although their exact mechanism is unknown, muscle relaxation techniques are believed to relax the mind; relieve stress, suffering, and pain; and refresh the mood perhaps by triggering the relaxation response [118, 190–193]. Exposure to nature might as well be relaxing through different mechanisms such as relief from the direct attention needed for pursuing life goals and enhancing cognitive processes via providing a rich environment (which allows the shift of attention toward the involuntary mode) [210] and the decrease of HPA activity or cortisol concentrations while elevating parasympathetic activity (i.e., the relaxation response) [84, 211–213]. Another approach to evoke the relaxation response is meditation [191–193]. Meditation is the moment-by-moment self-regulation of attention constantly focused either on a single experience—such as a mantra, breathing, or an object—for a rather long time

without being distracted (in concentration meditation) or on the present moment and whatever is being immediately experienced (in mindfulness meditation) [214–218]. Yet another way for inducing the relaxation response, reducing stress and anxiety, and improving health is practicing yoga [194, 219–221]. Yoga can exert its wound healing influence via the HPA suppression [222], pain perception [223, 224], stress control [118], and probably oxytocin release that might regulate bone mass [225] regardless of its beneficial psychophysiological impact mentioned earlier [72, 106, 107, 226], which might enhance healing of the wound and bone [118, 226, 227].

Relaxation might lessen pain and distress [190]. It might lower postoperative tension, anger, and tachycardia in cardiac patients [164]. A systematic review [185] indicated that among mind-body interventions, relaxation training might have the strongest evidence to influence the immune function. Meditation has been shown capable of increasing the antibody response to influenza vaccine [228]. Brief sessions of relaxation with guided imagery can help control state anxiety, cortisol levels on the first postoperative day, and erythema at the wound site, while accelerating postsurgical recovery [118]. Five sessions of PMR might reduce cortisol levels [229]. Exposing hospitalized patients to a view of natural setting might decrease hospital stay compared to a view of a brick wall [230]. Also walking in a forest had cortisol-reducing effects [212, 213]. Ten sessions of relaxation and guided imagery strengthened the knees and diminished anxiety or pain after knee surgery [231]. In patients suffering from chronic nonhealing foot ulcers, biofeedback-assisted relaxation (PMR + autogenic training + deep breathing) was first taught to patients and then self-administered at home (using a prerecorded tape) five times a week for 3 months; this method doubled up the rate of healing (87.5%) compared to the standard care group (43.8%) [232]. Also an extensive program of stress inoculation training (which included relaxation training among others) was noted to be effective in shortening the postsurgical recovery period and lessening pain and anxiety felt during the rehabilitation process [233]. Listening to a short stress-reduction audiotape based on mindfulness meditation during ultraviolet light therapy might promote the rate of resolution of psoriatic lesions [234]. However, some authors did not remark favorable results. For example, Gouin et al. [73] compared the effect of a 45-min relaxation program (including deep breathing, PMR, imagery, and self-hypnosis performed four times in the day of wounding) on healing of suction blisters with "reading" as control; they found no significant differences despite their adequate sample size (n = 98); they attributed this to the low baseline stress of the subjects in both groups, which could hinder the effects of stress-reduction protocols [73]. A study reported a significant decline in circulatory leukocytes at rest, in people practicing meditation compared to controls; meditation did not alter the immune response to exercise stress [235]. Furthermore, a rather large trial (n = 140) concerning the effects of a brief preoperative relaxation session with guided imagery [236] could not identify significant effects on morphine use, preoperative anxiety, postoperative pain, and patient satisfaction. The authors concluded that it might not be that simple to offer a relaxation CD to patients before surgery and expect notable results [236]. On the contrary, a much smaller experiment (n = 44) on the effects of a 28-min session of presurgical relaxation plus guided imagery demonstrated that relaxation might alleviate anxiety and immediate postoperative pain and might slightly shorten the hospital stay (though only marginally significantly) [237]. In another study, a short preoperative relaxation session reduced stress and enhanced hydroxyproline accumulation (a component of collagen and a major marker of wound healing) in the wounds of 60 laparoscopic cholecystectomy patients randomized to two groups [238]. Yoga as well was proven able to accelerate healing. Rao et al. [239] compared the efficacy of yoga versus supportive counseling in healing of wounds that remained from breast cancer surgery in 98 patients; they observed faster healings and shorter durations of postoperative hospitalization in the yoga group [239]. Oswal et al. [227] compared bone fracture healing in 30 patients randomized into two groups of yoga (two sessions of 30 min a day for

2 weeks) and control and concluded that although both groups had improvements over the course of the study, the yoga group had better health measurements (the number of united cortices, increased fracture density, and reductions in pain and tenderness) in the end, compared to the control [227].

It is possible to intensify the wound healing impact of relaxation by adopting an integrated protocol: Han [240] divided 47 peptic ulcer patients into two groups; the control group received 15-min tapes of PMR to listen to over four months. The experimental group received seven 1-h sessions of an integrated stress management program over one month. The program consisted of physiological management (autogenic training using a biofeedback instrument and PMR) and cognitive-emotional management and training (counseling). They witnessed faster wound healing in the experimental group [240]. The only study evaluating the moderating effect of timing of relaxation on wound healing was undertaken by Robinson et al. [241] who declared that a brief session of relaxation administered either before or after skin damage might facilitate wound healing in 121 subjects randomized into 2 experimental and 1 control groups [241].

3 Music

The definition of music is equivocal and greatly depending on the culture [242]; it still might be defined in classical terms as an intentional auditory stimulus with organized components such as melody, harmony, rhythm, tempo, pitch, form, etc. [243–246] against noise which is characterized as random and unorganized sounds without control for musical elements and possibly perceived subjectively as unpleasant [247–249]. Listening to one's favorite music can be beneficial due to its pleasant and soothing quality as well as its potential to block out stressful or unfamiliar noises around the person [250]. In medical settings, it can be delivered in two ways: music medicine versus music therapy. Music medicine (or receptive music therapy [251]) is when the patient is offered prerecorded (favorite) music for relaxation or distraction, without any systematic and active monitoring of suitability or elements of the music provided [244, 252, 253]. Conversely, music therapy is an individualized treatment delivered by trained professionals which is modified and optimized based on assessment outcomes and patient preferences and is delivered through various settings including music improvisation, music-based relaxation, music-based life review, chanting, songs (singing, song writing, and lyric analysis), and instrumental participation [244, 252–254].

Music has been used as a cost-effective easily available and appealing therapy to help patients cope with many stressful or painful treatments such as surgery, chemotherapy, dental procedures, injections, or unpleasant operations like colonoscopy, bronchoscopy, or colposcopy [187, 249, 255–267]. Music can reduce anxiety [264, 266, 268–275], blood pressure [264, 274], pulse rate [274], nausea [276], and postoperative confusion [277]. It can also improve the mood [278] and satisfaction during postoperative recovery [277, 279, 280]. Reduced anxiety might possibly be the most robust finding in patients [274, 281, 282] or laboratory subjects [283, 284]. Such physiologic effects of music might stem from its influence on many cognitive, sensorimotor, and emotional processes [285–287] including those modulating stress response [46]. For instance, music can simultaneously activate a multitude of cortical and subcortical limbic/paralimbic systems, multisensory integration, or the mirror neuron system; boost brain neuroplasticity through enhanced dopaminergic activity and neural synchrony; arouse attention, memory, and association; evoke emotions; stimulate imagery; enhance social cognition; or promote relaxation and distraction [244, 252, 285–293]. Classical music might facilitate variability of heart rate (a probable sign of parasympathetic activity), while rock music or noise might decrease this variability [249, 294–296]. It might diminish perceived psychological stress and reinforce coping ability against stressful conditions [297, 298]. Music can as well regulate anxiety (which is an adaptive response to stress experience) through its impact on emotions and cognitive processes modulating

the HPA axis or sympathetic/parasympathetic systems [46, 249, 287, 290–293]. Therefore, music might have physiologic effects. It can increase mononuclear cells and decrease IL-6 levels [299]. It might inhibit or slow down cortisol elevation after stressful medical [300, 301] or laboratory [302] interventions or before/during medical interventions [282, 303, 304]. Nonetheless, some authors could not find significant decreases in cortisol [46, 283, 299, 305] or anxiety [46, 306–308] possibly due in part to methodological factors such as sample sizes or confounding factors. Thoma et al. [46] compared the effects of sound of rippling water versus relaxing music (and silent rest) on salivary cortisol and alpha-amylase (as an indicator of sympathetic activity) as well as heart rate after a stressful task. They asserted that the sound of water had the best effect on cortisol followed by control (no stimulus) and relaxing music as the least effective treatment. On the other hand, relaxing music receded the heightened salivary alpha-amylase level back to normal concentrations faster than did the other treatments. The heart rate and anxiety were not affected by choice of treatment [46].

Music might decrease pain of numerous clinical procedures or chronic pains (such as burn debridement, dental procedures, surgeries, insertion of intravenous lines, colonoscopy or flexible sigmoidoscopy, injection, labor, chronic osteoarthritis, or in cancer patients) [187, 267, 276, 277, 279, 304, 309–329] or subside the need for analgesic/sedative medication [316–318, 330]. Patients needing surgery with spinal anesthesia who listen to music might require less sedative doses to achieve relaxation [265]. Even a study found music more effective than midazolam in reducing anxiety [331], whereas another one did not find its pain- and anxiety-lowering effects to be greater than that of a quiet rest period [332]. It might also be more effective than preoperative counseling, teaching, or relaxation training [249, 333, 334]. The pain-reducing effect of music might exist if music is played either during or after the operation [318]. The mechanism underlying pain-alleviating effects of music might be linked to the gate control theory of pain, in which non-noxious stimuli might suppress pain [280, 335–337]. Listening to one's favorite music might induce relaxation and distraction, while music entrainment together with a professional music therapist might induce a sense of control over pain [338]. In addition, music might upregulate μ opiate receptors and slightly elevate the levels of morphine 6 glucuronide (which is more active than morphine) [299]. Effects of music might vary depending on several factors such as the listener's demographics, mood, age, auditory health, experience, culture, and musical taste, as well as factors related to the music such as musical elements (e.g., rhythm, tempo, melody, harmony, pitch), means of delivery (speakers, earphones, live, or recorded), passive versus active participation, and being alone or in a group [249, 339].

Effects of music have been summarized in multiple reviews and meta-analyses which have demonstrated promising outcomes [244, 252, 258, 280, 340, 341]. Dileo [244] conducted a meta-analysis on 183 articles across 11 medical specialties on the efficacy of music therapy versus music medicine. Both methods were successful in betterment of numerous health indices such as mood, well-being, pain, and nausea/vomiting; and music therapy was more efficacious compared to music medicine [244]. Bradt et al. [252, 258, 340] performed meta-analyses on music interventions for preoperative anxiety [258], in coronary heart disease patients [340], and in cancer patients [252]; they indicated that music is a viable alternative to antianxiety and sedative drugs [258], might have a moderate effect on anxiety and a small but significant influence on psychological distress and pain (in coronary patients) [340], and can alleviate pain, anxiety, and depression rather strongly while decreasing fatigue moderately (in cancer patients) [252]. Also it was effective in reducing heart rate [252, 258] or systolic [252, 340] and diastolic [252, 258] blood pressures and in improving sleep following coronary procedures [340] and quality of life in cancer patients (through music therapy) [252]. Music selection by patients might intensify the favorable impact of music on anxiety [340] and blood pressure [252]. Another meta-analysis [280] regarding the effects of music

intervention on burn patients during treatment procedures found that music can lessen pain, anxiety, and heart rate [280]. Music might as well be used as a cost-effective adjunct to the standard psychiatric treatment [341]. Yet another meta-analysis on 73 trials concluded that music might decrease postoperative pain or analgesic usage and improve satisfaction; it might be effective regardless of the choice of music and timing of delivery, even under general anesthesia [316]. Based on its favorable effects on stress and pain, it seems that music might smooth the progress of healing and recovery. However, its effect on wound healing has not been examined. A case close to wound healing could be engraftment or hospital stay. Engraftment of bone marrow transplants might occur faster in patients having 45-min music-assisted relaxation and relaxation imagery sessions delivered twice a week by a music therapist [276]. On the other hand, a meta-analysis did not find overall significant effects of music on the length of postoperative hospital stay [316]. Owing to its influence on pain and stress, music should be tested and (if found positive) used for wound healing or recovery from injury.

4 Expressive Writing

One of the promising means to reduce psychological stress is expressive writing also known as written emotional disclosure or emotional expression. This is a brief, easily administered session of writing about traumatic and upsetting experiences, thoughts, and emotions allowing one to delve into deepest feelings associated with them and disclose emotions not previously shared [342–347].

Studies testing the effects of written disclosure on health are numerous, but the evidence on the efficacy of therapeutic writing is controversial and still open to more investigation [348]. Many studies have displayed the effectiveness of expressive writing in betterment of psychological and physiological outcomes, such as reducing intrusive thoughts, decreasing stress/anxiety/suffering, improving overall mood, boosting certain markers of immune system, diminishing postoperative complications and clinic visits, attenuating somatic symptoms, and easing wound healing [154, 342–347, 349–355] in various populations such as coronary [356] or cancer patients [154, 353, 357–360], sufferers of chronic pelvic pain [361]/rheumatoid arthritis [362]/or fibromyalgia [363], those undergoing a surgery [345, 364], and athletes under stress [365] or rehabilitating from injuries [345, 366, 367]. In people with fear of rejection who might be prone to lack of perceived social support, expressive writing might reduce negative mood [349]. In post-traumatic stress disorder (PTSD) patients, expressive writing did not lower core symptoms related to PTSD but augmented their capacity to regulate the symptoms or their mood, and lowered their cortisol levels [368]. A meta-analysis on 13 randomized clinical trials on effects of expressive writing (of which 10 were on students) [369] found a moderate overall effect size. The effect size tended to be greater if the percentage of men in the sample was larger, if the interval between the writing sessions was longer, or if the writing was focused on current worries rather than past sources of stress [369].

Expressive writing might work best for people with less severe psychopathologies [370, 371]. In clinical situations, expressive writing has been shown useful for some people (as mentioned above), but not in certain populations such as survivors of trauma and abuse [372–374], cognitively impaired older adults [371], or bereaved individuals [349, 375, 376]; the results have been debated in some groups such as individuals suffering from asthma and rheumatoid arthritis [377, 378], cancer [358, 379], or HIV [380, 381]. In some caregivers, writing emotionally about stressful care giving experiences might not be of much help and even might worsen the conditions compared to the effects of writing about time management or historical events [371, 382]. In a study on caregivers of hospitalized children [382], physical and psychological indices were poorer in the expressive writing group (compared to controls who wrote about summer vacation) immediately after writing and 4 months later. Moreover, in older individuals, a preferable way of regaining positive affect might be selectively

attending to positive information [383] rather than focusing on upsetting experiences [371]. A meta-analysis of 21 studies (on youth aged 10–18 years) [384] presented a positive significant but small overall effect (g = 0.127) with maximum effect sizes not greater than 0.25. The effect size would be larger for adolescents with higher baseline levels of emotional problems and higher dosages of the intervention, i.e., greater numbers or spacings of sessions [384]. A follow-up study on caregivers suggested that writing could be mostly beneficial for those who wrote in optimistic, positive, and future-focused language [385]. Furthermore, some authors did not observe superior quality-of-life outcomes in cancer patients who practiced writing [357, 379, 386]. Expressive writing might work better in some contexts than others. In the case of coping with pelvic pain, cases with higher baseline ambivalence about emotional expression and negative affect were more benefited from expressive writing [361]. It is shown that expressive writing is not an optimal strategy for everyone, and various factors including emotional expressivity (such as alexithymia, dispositional expressiveness, or mixed emotions) might moderate the effect of writing [342, 387–391]. Even many patients might dislike expressive writing [154]. In some others facing bereavement, expressive writing might not necessarily induce changes in meaning-related goals compared to individuals writing about nonemotional topics [392].

The mechanisms underlying the effects of expressive writing are not well understood [342, 350]. It might facilitate cognitive processing and recoding of unorganized, implicit memories and thoughts associated with negative and vague emotions and somatic experiences into clear and organized explicit thoughts and memories dissociated from emotions—which are now labeled and acknowledged; it also might allow reframing the thoughts into more productive, healthy ones possibly charged with positive emotions [114, 154, 344, 345, 347, 370, 393–402]. In this case, the patient can see or create the meaning and derive benefit from what was once perceived solely as painful (i.e., reframing) [154, 395–397]. Additionally, it can facilitate the release of suppressed thoughts, emotions, and behaviors (i.e., the disinhibition theory) and expose the individuals repeatedly to previously avoided emotions and thoughts, leading to the extinction of such disturbances (i.e., the exposure theory) [342, 370, 371]. Frattaroli [342] evaluated moderators of expressive writing effects in a meta-analysis and concluded that the exposure theory might have the higher impact, while the inhibition theory and stress reduction might not have a high impact. The effect of changes in the thought pattern is demonstrated by some research reporting improved physical health in people who used more causal and insightful words in their essays [344, 403, 404], although not all studies found such associations [343]. Compared to neutral writing, emotional disclosure has been linked to immunity enhancements such as higher antibody levels after hepatitis B vaccinations [405], greater proliferation of lymphocytes following mitogen stimulation [346], and increased CD4+ lymphocyte counts together with lower HIV-viral loads in HIV-infected patients [343, 380]. A study found that regardless of the content of writing (disclosing emotions on paper or writing nonemotionally), if subjects' mood improved by writing, their NK cell parameters would improve as well [392]. Such findings can link the psychological effects of writing to the objective indices of physical recovery, such as wound healing. Intriguingly, a number of studies have found improvements in physical healing *without* psychological benefits [342, 345, 347, 357, 362, 406]. A meta-analysis [352] summarizing nine articles indicated that overall, expressive writing might favor physical health (d = 0.21, p = 0.01) significantly more than psychological health ([which itself might have no significant overall effect] d = 0.07, p = 0.17). This might be attributable to methodological limitations such as possibly low sensitivities of psychological assessments [343, 345, 347] or the fact that the baseline psychological conditions were not poor enough in some populations to allow observing significant improvements after the intervention [343, 345]. This effect on physical but not psychological outcomes might also be a true effect, possibly exerted through other pathways in addition to

stress reduction. For instance, expressive writing [359, 407–409] and writing about time management [371] have been revealed to diminish sleep disturbances possibly via decreasing intrusive thoughts, anxiety, autonomic arousal [409, 410], and stress hormones (which might inhibit sleep) [343, 411], noting that sleep disturbances can themselves increase stress hormones [412]. Cytokine profiles and immune system—which are crucial for wound healing—are negatively affected by even modest sleep disturbances [413–415]. Moreover, growth hormone which is necessary for wound healing will diminish by deep sleep deprivation [343, 416].

The influence of expressive writing on wound healing has been evaluated only in four papers. Weinman et al. [406] instructed participants to write emotionally versus nonemotionally (time management) 2 weeks prior to biopsy and found faster wound healing in the experimental group without any significant difference between the groups in terms of psychological indices (depression or stress) [406]. Koschwanez et al. [343] asked two groups of elderly adults to write emotionally about upsetting items versus daily activities (time management) 2 weeks before taking punch biopsies for 3 days (20 min each). Eleven days after wounding, the participants who had written emotionally had healed wounds as twice as did the control group. Better sleep before wounding as well predicted the recovery rate. However, no significant differences were found between the groups in terms of lipopolysaccharide (LPS)-stimulated pro-inflammatory cytokine levels, depressive symptoms, perceived stress, health-related behaviors, and number of doctor visits [343]. Despite this, biological markers measured 2 weeks after writing were associated positively with sleep and lower stress (LPS-induced IL-6 production) and fewer depressive symptoms (lower LPS-induced IL-1 and IL-6) [343] which were in line with the previous literature [417, 418]. As the sole research on the effect of timing of the treatment, Robinson et al. [114] evaluated expressive writing compared to writing about neutral matters, both before and after punch biopsy wounding. They did not discern differences in the outcome of neutral writing before or after the biopsy. However, expressive writing before wounding exhibited the best effect; it would be still effective if done completely until the 6th postbiopsy day [114]. Additionally, it was shown that after writing, the affect first worsens and later rebounds; hence, if expressive writing is performed before wounding, the positive affect will coexist the healing process, while if writing is done after wounding, the lowered mood will overlap healing and possibly disrupt it [33, 113, 114]. This was in agreement with other results on short-term intensifications of negative mood, distress, and physical symptoms following expressive writing [362, 419] which later lead to betterment of mood and physical health probably due to catharsis of bottled up problems [362, 420]. This might be a reason for the delayed effect of therapeutic writing on health [421] compared to the immediate impact of relaxation [422]. Koschwanez et al. [347] asked patients undergoing elective laparoscopic bariatric surgery to either write objectively about how they spent their time or write emotionally about traumatic life events for 3 days (20 min each) 2 weeks before surgery. Interestingly, they noticed escalations in the hydroxyproline deposition (as an indicator of healing) and TNF-α levels in the "daily activity writing" group compared to the "expressive writing" group, 14 days postsurgery. In both groups, levels of perceived stress dropped rather similarly after the surgery [347]. Therefore, perhaps stress reduction as usually suggested was not the mechanism underlying the improved wound healing appeared in the control group. Further, since the stressor (surgery) was much more frightening than stressors in other studies, expressive writing could not help control ruminating about it. Accordingly, they suggested that expressive writing might be more useful once the stress of surgery has passed [347].

Some authors have suggested that the effect of expressive writing might be limited to few weeks and will dissipate over time [342, 378, 380]. Mogk et al. [355] conducted a meta-analysis on 30 studies examining merely long-term effects of expressive writing (between 4 weeks and 8 months of follow-up periods) and found no significant overall effect. Meads and Nouwen

[423] as well performed another meta-analysis on 61 trials, with a focus on longer-term influences, and detected no clear overall improvement. Perhaps, this happens in experimental setups which adopt a small number of short sessions, as it seems that the duration or number of sessions might contribute to the therapeutic effect [369]. There have been attempts to magnify the efficacy of writing. In a form of writing called guided disclosure protocol (GDP) [424], the patient is instructed to write an organized self-reflective narrative under controlled timing and conditions [342, 345, 425]. For instance, the patient is asked to write first about the onset of trauma in a chronological order and then explicitly label the emotions and explain the impact of the trauma and finally to write about future coping and psychological growth [345]. This protocol attempts to integrate the cognitive benefits of expressive writing with the principles of self-regulation, reappraisal, and seeking for benefits through considering various perspectives of an event and finding more adaptive views which can also be used in the future [345, 392, 401–403, 426]. It has been effective in reducing the number of clinic visits [373] and suffering of parents to children with cancer [345, 427]. It seems however that more or less, all written disclosures are already "guided" to some degree and possibly benefited from the same advantages of GDP. Perhaps even limiting the person to a certain framework of emotional disclosing might disallow the occurrence of free associations or the occurrence of a deeper rapport with oneself. A study showed that instructing participants to adopt a narrative writing formation does not boost the effects of writing [428]. There have been other suggestions to make writing more effective. When writing to cope with physical injuries, it is recommended to disclose one's feelings in a private space, possibly at regular intervals, and for short sessions no longer than 30 min [350, 429]. As mentioned earlier, unlike relaxation practice that might have immediate physiological effects [422] and can be successful if done either before or after the injury [241], the effects of expressive writing might take longer to occur [421], and therefore, timing of the intervention might as well matter [114]: while some authors suggest that it should be started after the trauma and not during it [343, 347, 398], some believe that it should have been started before the trauma in order to have optimum effects during and after the trauma [114, 343]. Only one study [114] has compared the impacts of writing before versus after wounding, and it found a greater effect for writing taken place prior to biopsy [114].

Hypothetically, expressive writing might have other merits as well. By writing, many negative and intrusive thoughts are projected onto the paper, feeling as if they are not in the mind anymore and releasing the person from the burden of (probably obsessive) thoughts. It feels like conversing with oneself over the matter, as a set of free association sentences which can facilitate the clarification of vague thoughts and emergence of certain implicit cognitions into the light of consciousness; this resembles a self-hypnotic auto-psychoanalytic experience. The completion of writing and analyzing the emotions allows the feeling of closure, which is usually desirable and soothing. Writing yields a concise and vivid list of few abstracted items at hand now representing the whole complexity of the (once convoluted and elusive) problem, which are easier to remember or use, and are more productive than an ever-changing set of dynamic thoughts. In addition, writing the words is quite slower than talking or thinking. Slowing down the train of thoughts might let the brain better articulate otherwise disorganized, racing, parallel, and rapidly flashing thoughts into a series of sequential and clear-cut sentences. This might be of extra value to individuals having visual or symbolic thoughts. Perhaps even the sensorimotor integration of complex fine motor and visuotactile brain areas involved in writing the letters by a pen helps in more effective recoding of information through global engagement of numerous cortical and subcortical networks; typing the same emotions and thoughts into the computer (which involves fewer fine motor control areas and is much faster) might not feel similarly relieving. Additionally, the placebo effect can account for some favorable effects of writing. These speculations need to be verified scientifically.

5 Hypnosis

The nature and mechanisms of hypnosis are not clearly understood. Hypnosis has been explained as different but partially overlapping theories such as "a way of thinking differently," "the imaginative role enactment," "a fractionation in cognitive systems," or "an altered state of consciousness associated with shifts in neural functions and perception" [430–444]. Spiegel et al. [440, 441] define it as an altered, focused, and receptive state of consciousness characterized by absorption (heightened concentration toward a particular concept or item), dissociation (from one's conscious self), and suggestibility accompanied with a sense of involuntariness and lowered critical judgment. Elman [445] describes hypnosis as a state of consciousness where the critical mind is bypassed and selective thinking established. According to Nash [446], any cognitive phenomenon containing the elements of concentration and relaxation is hypnotic [446]. Similarly, James [447] defines it as a natural mode of focused consciousness accompanied by a relaxed body [448]. Consequently, other concepts such as meditation, relaxation, or guided imagery might fall into the category of hypnotic experiences as well [449, 450]. Despite sharing resemblances with relaxation, meditation, or concentration, hypnosis seems to be a particular state of consciousness which might differ qualitatively from sleep, normal consciousness (awake state), meditation, relaxation, or conscious concentration in terms of the alterations in connectivity and activation of brain networks—at least in highly hypnotizable individuals [442, 450–458]. In addition, hypnosis differs from most other cognitive interventions; e.g., posthypnotic effects are not present in meditation or other interventions [447, 448], and dissociation is not a part of relaxation.

In its classical form, hypnosis begins with an introduction to hypnosis and usually follows by a hypnotic induction, deepening, therapeutic suggestions, posthypnotic instructions, returning to the normal state of consciousness, and discussing the hypnotic experience [435, 440, 459]. It can be delivered directly by the clinician or through a recorded voice (of the subject or somebody else's) [435, 460, 461]; the live and interactive form is more influential because the treatment can be individualized for the current needs of the patient and based on his feedback [188, 435, 460–462]. The effect of hypnosis or the hypnotizability of the patient might be amplified in some other conditions as well, which might be associated with the extent of biologic impact of hypnosis [463, 464]. The effects of suggestions might be intensified by a good rapport and conducting successive sessions, which might induce neurological reorganization and replacement of pain responses with non-painful ones developed to the originally noxious stimuli [52, 453, 461, 465, 466]. A trauma, being in a critical condition, or the expectation of a serious damage (e.g., before surgery) might put the patient into a spontaneous trance, making him more susceptible to suggestions [441, 467]. Another form of delivering suggestions can be during general anesthesia [188, 460, 468], because the perception of sounds and words [466, 469, 470] as well as their further cognitive processing (such as memory formation) might remain functional under general anesthesia [471–475] while the critical judgment is suspended.

Many believe that "all hypnosis is self-hypnosis," i.e., the trance state and its depth depend not on external stimuli but exclusively on internal cognitive processes, mental abilities, and the will of the subject [440, 441, 443, 476]. Some authors argue that certain hypnotic components (such as dissociation) might not be achieved without external stimuli [477]. This author has witnessed few extremely hypnotizable individuals able to dissociate from their conscious self to a great extent without any external intervention. Still, even in such individuals, a part of their conscious, critical, and executive mind needs to stay active in order to decide on, validate, and control the depth and direction of the hypnotic experience, hence the lack of total involuntariness and dissociation (compared to hetero-hypnosis). Also if the bypass of the critical mind is considered to be a necessary component of hypnosis [445], it seems counterintuitive for one to fully bypass his critical mind *intentionally*, i.e., using his conscious and critical mind itself.

Somatic effects of hypnosis are exhibited in numerous meta-analyses on various outcomes [188, 460, 461, 475, 478]. Hypnosis seems to be the most effective cognitive method to improve well-being and recovery [475, 478]. It has been successfully utilized in myriad applications including but not limited to presurgical preparation, reducing anxiety and pain, managing intraoperative blood loss, facilitating postoperative recovery and healing, etc. [21, 479–484]. The mechanisms by which hypnosis may affect the physiology remain unclear [149, 485, 486]. Its influences might depend on the induced relaxation response (regardless of the presence or nature of therapeutic suggestions), the placebo effect, diminishing the pain, and direct effects of hypnotic instructions; deep hypnosis might allow the suggestions direct access to certain unconscious processes such as brain activity, pain perception, endocrine and immune systems, inflammatory responses, autonomic functioning such as electro-dermal activity, blood pressure, heart rate, circulatory changes, oxygen saturation, muscular relaxation, gastric secretions, and basal metabolism, many of which might elude voluntary alteration [21, 119, 149, 184, 447, 448, 486–497]. Two meta-analyses have shown hypnotic suggestions to be stronger than normal-state ones in relieving preoperative distress [460] and postoperative anxiety [188]. Given the deleterious effects of stress on recovery [37, 41, 130], the calming effect of hypnosis is itself therapeutic [498]. A meta-analysis demonstrated that hypnosis has the strongest evidence among other psychological interventions to improve the immune function [184]. Hypnotic interventions may subside immunological dysregulations associated with stress, increase CD3+, CD4+ T, and B cells, and maintain baseline levels of IL-113 during stressful situations [464, 498–500] even compared to relaxation (in terms of B and T cell counts, in highly hypnotizable individuals) [464]. Similar to meditation, hypnosis can activate the left prefrontal cortex (which is associated with self-control and positive emotions) and decrease the activity of the amygdala (associated with fear and negative emotions) and therefore limit the stress response [447, 448]. Rossi [501, 502] proposed that hypnosis might affect the HPA axis, genetic alterations, neurogenesis, and state-dependent memory. Hypnosis effects might be linked to experience-dependent gene expressions characteristic of stem cell growth, reduced oxidative stress, and decreases in chronic inflammation [503]. Changes in gene expression have been observed in leukocytes as well [504].

Another mechanism by which hypnosis can influence biology is pain modulation. Hypnotic suggestions might dilute the sensory and affective components of pain by dissociation of noxious perceptions [465, 505]. It has been successfully used to manage painful symptoms of burn wounds [202, 506]. Since pain can impede the healing response (e.g., via the release of stress hormones, cardiovascular stress, suppression of the immune response, etc. [116–118, 507, 508]), hypnotic pain control might facilitate recovery and healing [119, 479, 505, 506, 509–516].

Hypnotism may also contribute to recovery and healing by mitigating the bleeding and increasing blood flow after the clot formation. Hypnosis cannot influence the bleeding time [517], but might exert a degree of control over circulatory processes (and body temperature) through suggestions indicating very warm or cold temperatures [450, 518–524]. It might reduce bleeding via suggestions of coldness or low blood pressure, and even indirectly by alleviating stress and anxiety [517, 525–528] which might influence platelet aggregation [527, 529]. Suggestions of numbness given before the noxious stimuli have been associated with less severe inflammation and tissue damage compared to controls receiving suggestions of sensitivity [530]. Such interventions might assist in postoperative bleeding control [528, 531] and perhaps in decreasing the inflammation of the area [532]. Abdeshahi et al. [531] compared the duration of post-extraction bleeding under hypnosis versus control and reported that while bleeding continued for at least 48 hours in most of control dental sockets, merely a small portion of experimental sockets continued to bleed over 5 hours. They also reported lower pains in the experimental group [531]. Even some authors proposed that since hypnosis might help in changing blood flow and can even cure

warts, it might also be utilized against cancer [522]; this however, sounds like an excessively optimistic and oversimplistic idea. Beneficial effects of hypnosis on vasodilatation and reduction of vascular resistance (facilitating blood flow) have been documented as well [450, 518–520]. This effect can be used to improve wound healing, as was shown in diabetic patients who have poor peripheral blood circulation [533, 534]. Interestingly, Swope [450] who compared the volitional temperature rise of 20 participants before and after listening to self-hypnotic instructions versus concentration reminders asserted that both groups attained the ability of increasing their fingers' temperature [450].

A hypothesis proposes that glial cells might transmit direct current signals similar to flow of current through a wire and unlike the neuronal signals which are actually a progress of the action potential wave through the axon. According to this hypothesis, such currents might facilitate tissue regeneration and healing [535–539], and thus they might be responsible for some biological influences of hypnosis, anesthesia, acupuncture, or electroacupuncture [535, 536, 539].

There are a number of interesting claims about the potential of hypnosis in controlling autonomic mechanisms. It is suggested capable of aiding in the attenuation of burn wounds' depth, size, inflammation, and complications possibly by inhibiting inflammatory reactions [530, 532, 540–549] although this should be investigated more strictly [532]. Additionally, uses of hypnosis in removal of warts [550] or certain other dermatological diseases such as eczema [551], ichthyosis [552], herpes simplex [553], and psoriasis [463, 554] have been documented [550–556]. There have also been attempts to induce medical conditions by hypnosis, confirming its involuntary influences [21]: hypnosis is suggested to be able to induce autonomic reactions such as migraine headaches, Raynaud's disease, duodenal ulcers, asthma, or eczema and allergic reactions [21, 499, 557]. It is claimed that hypnotic instructions can produce blisters [544, 558, 559] although such claims were mostly based on poor evidence and not reproducible [532, 560, 561].

Many studies have adopted hypnotic or simple instructions to enhance postoperative recovery and health. Hypnosis might control pain, anxiety, and emotional distress, and might reduce nausea, fatigue, pain medication usage, and treatment time [435, 461, 481]. Exposure to therapeutic suggestions under general anesthesia might shorten the hospitalization period [562, 563], although some may disagree [564]. Blankfield [565] summarized 18 clinical trials including five articles on the effects of hypnosis, 11 on the impact of relaxing and positive suggestions (given before or during general anesthesia), and two about relaxation effects on postsurgical recovery. Of the five experiments on hypnosis, all assessed pain alterations, and all [566–569] but one [570] succeeded to moderate pain or analgesic consumption. Also three of them examined some indices of recovery (rehabilitation time, complications, etc.), and all of them were successful [566–568]. Of the studies on suggestion effects, six had evaluated pain reduction, merely two of which detected positive outcomes [571, 572]. Indicators of recovery were tested in nine studies on suggestion effects, of which six reported favorable results [562, 571–575]. Rosendahl et al. [475] undertook a meta-analysis on 32 randomized controlled trials regarding the effects of affirmative or positive therapeutic suggestions given under general anesthesia to patients on their postoperative pain and recovery indices. There were 37 comparisons between 991 patients in control groups and 1111 patients in intervention groups. They calculated insignificant overall effects for pain intensity and mental distress ($g = 0.03$ and 0.04, respectively). Still, small but significant overall effects were observed on analgesic usage ($g = 0.16$) and recovery ($g = 0.14$). They linked the trivial effects to the level of awareness under anesthesia as well as the lack of rapport and therapeutic relationships, which are essential to hypnosis [461, 466, 475] and even might improve healing as independent treatments [576].

Site-specific targeted hypnotic suggestions given for facilitating somatic healing may yield positive results [21, 186, 491, 532]. Moore and Kaplan [543] presented five cases with seemingly symmetric bilateral burns who received hypnotic suggestions of warmness merely for one side. Two cases showed elevated temperatures of the

experimental side, and in four patients, wound recovery was achieved in the experimental side 3 days before the control [543]. Hammond et al. [577] conducted a pilot study on six subjects with experimentally inflicted first-degree burns on each thigh. The authors gave hypnotic suggestions of coolness and analgesia for only one thigh of each subject. Although the temperatures remained similar, the redness was significantly attenuated in the thigh targeted by the suggestion. Mauer et al. [119] divided 60 orthopedic hand surgery patients to the groups "standard care versus standard care plus a brief hypnosis session." They noted declines in pain levels, state anxiety, and postoperative complications as well as a faster recovery progress in the hypnosis group [119]. Ginandes and Rosenthal [578] investigated the effect of multisession hypnotic suggestions (six appointments and taped suggestions to take home) designed to improve fracture healing on signs and symptoms of 11 ankle fracture patients randomized to two groups. Orthopedic and radiographic assessments revealed marginally faster healing as well as improved ability to descend stairs, recovered ankle mobility, and diminished pain and analgesic use in the hypnosis group [578]. Ginandes et al. [21] compared the effects of 8 sessions of adjunctive hypnosis versus usual care as well as eight sessions of another treatment (supportive attention) on early postsurgical wound healing of 18 women to undergo reduction mammoplasty (randomized to three groups of six each). The results derived from objective examinations by clinicians blinded to the interventions suggested the superiority of hypnosis over other groups in terms of improved healing occurred within the 7-week period of study. However, comparisons of subjective scores for postoperative pain, functional recovery, and incision healing did not reach statistical significance [21]. Berger et al. [506] investigated the impact of a pain management protocol in 23 burn wound patients (vs. 23 matched historical controls) on pain intensity, anxiety, and clinical outcomes. Hypnosis reduced pain, anxiety, the number of operations under general anesthesia, and total graft requirements [506].

Hypnosis should be practiced ethically with the knowledge and consent of the patient and by healthcare providers trained (and preferably licensed) to practice it solely within their area of professional expertise [434, 435] and when there is no contraindication [579]. It also should not substitute conventional and approved medications [549]. Certain techniques that are performed without the patient's knowledge or consent (e.g., covert hypnosis) might be regarded as attempts to manipulate or deceive the patient and hence unethical in many situations unless justified medically. Many assume that it is the patient who selects the suggestions to follow and that the so-called protective mechanisms of the unconscious mind always monitor and defend the person; according to them, hypnosis is absolutely safe, as the client can simply refuse to comply with any suggestion he does not find suitable for any reason, such as being dangerous or against his moral code [445]. This implies that any decision made by the client under hypnosis is his own deep desire and thus any hypnotic suggestion eliciting such a response is ethically justified! Aside from sounding fallacious, this hypothesis is irrefutable per se and not scientific. Even if assumed true, this idea still looks overly simplistic and ignoring certain key points. First, many harms or values are not black-or-white concepts to be always identified that easily. Furthermore, definitions of many beliefs or damages can be philosophically complicated and elusive even to a fully critical mind; for example, an act of violence might be acceptable if done for self-defense. Additionally, many of a person's preferences might be at odds with one another (e.g., personal success vs. love and sacrifice for the family). But more importantly, many boundaries and beliefs are not carved in stone; they can be readjusted based on several dynamics such as the person's emotional state or the new information/feedback received. Being a cognitive intervention, hypnosis and rapport can even be utilized to relax or shift the client's very red lines. Taking these into account, it seems that resisting temptations, persuasions, reasoning, justifications, or sophistry can be difficult even outside hypnosis, let alone in deep trance. Therefore, hypnotists should not rely lavishly on a groundless theory of a vigilant and omniscient protective system of the unconscious mind, and instead should practice hypnosis with utmost caution and

responsibility. Elman [445] claimed that no one, out of thousands of his subjects, had ever been damaged by hypnosis, as they refused to act out unwelcome suggestions. Nevertheless, the literature indicates that like any other therapeutic instrument, inappropriate use of hypnosis may accompany a (low but existent) risk of adverse effects, particularly in highly hypnotizable people [441, 579–582]. Moreover, many unsuitable instructions can still inflict psychological harm, even when the subject manages to deny them.

6 The Placebo Effect and Magical Healing

The placebo effect can be defined as favorable effects attributed not to the specific actions of the treatment but to the responses of the patient to the context in which the treatment is delivered [583, 584]. It is a function of the patient's positive expectation toward the treatment as well as behavioral conditioning [585–588], which can elicit subjective effects (e.g., analgesia) [589, 590] or objective results (e.g., type 1 hypersensitivity reactions or blood pressure) [591, 592]. Nocebo is the anticipation of a negative outcome, which might magnify it (e.g., nocebo hyperalgesia) perhaps through the caused anxiety [593–595]. Since learning processes might mediate the placebo effects on physiological responses such as immune system or hormone secretion [596, 597], wound healing might be as well affected by such effects. There is only one study directly assessing the placebo effect on wound healing [598]. Vits et al. [598] applied the same nonactive gel to experimental laser-ablated wounds on both thighs of 23 subjects. They had divided the subjects into two experimental and control groups. The control subjects were told that a nonactive gel would be applied to both thighs. The experimental subjects were informed that one of the two gels will alleviate pain and enhance healing. Vits et al. [598] did not observe any differences in pain or healing rates of both thighs. They linked this finding to potential lack of any placebo effect on mechanisms of wound healing as well as methodological difficulties, such as the lack of adequate baseline pain levels [598]. Maybe with a larger sample of patients having severer wounds, some accelerated wound healing might be discerned, as studies on hypnosis have found so, and there are major similarities between hypnosis and placebo: both instill expectations, rely on trust and faith (i.e., a suspended critical factor), and might share similar neural pathways in pain expectation [550, 599–601]. Additionally, similar to many other psychological interventions, hypnosis as well can be benefited from the placebo effect [601], where the therapeutic relationship, the patient's trust, and his positive expectations might facilitate improvements by mechanisms such as betterment of mood and mitigation of stress or pain [21, 447, 448, 602–605].

A number of interesting theories have been proposed for healing. Some conceptualize healing not as spontaneous and automatic but as a process that needs intentionality to occur [606, 607]. Some believe in the existence of certain (scientifically undetected) "subtle life energies" such as Qi (Chi) or Prana, which should be balanced between the body and the environment to maintain health and can be directed toward the injury site via certain "biofield" practices such as yoga, reiki, or therapeutic touch (TT) in order to aid physical healing [227, 608–611]. TT is said to be attributed to "consciousness-matter" engagement, during which the (altered) consciousness of the healer is assumed capable of affecting the health of the patient directly [609–611]. Although practitioners of such methods claim they can sense and balance this energy, their claims concerning detecting this energy field were not verified as reliable statistically [612, 613]. Still, it is suggested to be useful for various conditions such as reducing pain, fatigue, and anxiety, strengthening the immune system, or accelerating wound healing [609, 614–623]. Wirth et al. [624–628] examined the efficacy of such treatments on the reepithelialization of full thickness wounds in a series of studies; they found no significant results in three studies including a replication of one of the two reports with significant results [624–629]. A recent review [623] investigated and reanalyzed these (which were

five reports of four experiments) and asserted that despite the presence of significant benefit for therapeutic touch in two studies, the other two indicated no statistically significant benefit, and that all of them were at high risks of bias and also not generalizable to the real TT practice (owing to the creative designs adopted by Wirth et al. [624–628] for blinding and reducing biases) [623]. The review found the evidence insufficient [623]. Another review identified little evidence in favor of biofield therapies [630]. On the other hand, two meta-analyses recognized an overall therapeutic effect for TT, despite the high heterogeneity and methodological issues [631, 632]. Another systemic review on 66 clinical studies on biofield therapies (including reiki, therapeutic touch, and healing touch) [622] found a moderate-to-strong overall evidence for reducing pain and a moderate evidence for relieving anxiety (in pain or hospitalized populations) and negative behavioral symptoms (in dementia) [622].

Such interventions resemble hypnotic ceremonies, during which both the therapist and patient are self-hypnotized, deeply believing in the treatment efficacy and the therapist's capability (hence the bypass of their critical factor), and expecting positive outcomes to appear. In this term, the therapeutic effects might be attributed, at least in part, to the placebo effect. However, there are even more interesting anecdotal evidence concerning practices of Sufism, in which some sharp objects such as spikes and daggers are inserted in the body. According to Hall [633], the inflicted wound accompanies minimum or no bleeding, no pain, or no infection, and heals extraordinarily fast [633]. Sufi practitioners attribute these effects to the connection with "higher spiritual energies" that make such miraculous healings possible. Hall [633] argues that this experience differs from hypnotic analgesia, as he did not feel any altered states of consciousness or numbness before the experience. They also monitored the brain activity of a Sufi practitioner using an EEG device and, despite artifacts, identified a slight hypo-aroused altered state of consciousness implied by amplified theta bands and a reduced average alpha power [634]. Hussein et al. [635] succeeded to reproduce this unusual wounding and healing in 28 Sufi practitioners. Similar practices have been reported in other cultures such as Native American healers or Brazilian trance surgeons [633]. Still, more rigorous and comprehensive designs are needed to investigate such thought-provoking phenomena in comparison with the placebo/hypnotic analgesia and bleeding control. As well, it should be researched that what happens at microscopic levels, when such "conscious healing energies" (if any) hypothetically override the natural functions (and the physical laws governing them), forcing the tissue to heal miraculously faster. Is it even possible? Or is it a product of *magical thinking*? What happens to the intricate cellular and molecular machinery involved in healing, during and after such a mystical experience? How can such phenomena activate and precisely regulate physiological and biochemical mechanisms of healing?

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Part III

Wound Healing

Acute Wound Healing: Normal Mechanisms

Melvin A. Shiffman

1 Introduction

A wound is defined as "an injury to the body (as from violence, accident, or surgery) that typically involves laceration or breaking of a membrane (as the skin) and usually damage to underlying tissues" [1]. Wounds can be acute or chronic. The author will deal with acute clean wounds. In the acute wound with normal healing, there are various stages of the healing process including hemostasis, inflammation, proliferation, remodeling, and healing.

2 Classification of Wounds

Classification of wounds was developed initially by the American College of Surgeons and adapted in 1985 by the Centers for Disease Control and Prevention (Table 1) [2].

3 Phases of Wound Healing

3.1 Hemostasis

After injury there is bleeding in minimal or large amounts. Arterial vessels up to 0.5 mm in diameter constrict mediated by increased cytoplasmic levels. This occurs in transverse wounds to the vessel but longitudinal tears may increase the gap [3, 4]. This works for a few minutes until hypoxia and acidosis in the wound cause relaxation of the artery walls and bleeding begins again. Exposure of subendothelial tissues to blood results in activation of factor XII (Hageman factor) that initiates the proteolytic cleavage cascade that results in activation of factor X which converts prothrombin to thrombin that, in turn, converts fibrinogen to fibrin and the formation of fibrin plug [5]. Endothelial damage results in exposure of tissue factor to blood that results in activation of factor VII that results in the clotting cascade that activates thrombin [5]. Angiogenesis is triggered by the hemostatic plug as platelets release growth factors.

Blood and platelets come in contact with exposed collagen and other extracellular matrix components leading to release of clotting factors from the platelets resulting in formation of blood clot composed of fibrin, thrombospondin, fibronectin, and vitronectin [3–7]. The blood clot provides a matrix for cell migration. The cytoplasm of platelets contains α-granules that provide growth factors and cytokines such as platelet-derived growth factor (PDGF), transforming growth factor-ß (TGF-ß), epidermal growth factor (EGF), and insulin-like growth factors (IGF) [4] that activate and attract neutrophils, macrophages, endothelial cells, and fibroblasts [4, 8].

Vasoactive amines like serotonin are released by platelets and result in vasodilatation as well as increased vascular permeability leading to edema [4, 8].

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Table 1 Classification of wounds [2]

Class I: Clean
1. Uninfected operative wound where no inflammation is encountered and respiratory, GI, genital, and urinary tracts are not entered
2. Wounds are primarily closed, and a drain (if needed) is connected to a closed system
3. Risk of infection: 2% or lower
Class II: Clean/contaminated
1. Operative wound that enters the respiratory, GI, genital, or urinary tract under controlled conditions without unusual contamination when no infection or major break in technique has occurred
2. Risk of infection: 5% to 15%
Class III: Contaminated
1. Open, fresh, accidental wound from surgery with a major break in sterile technique or gross spillage from GI tract, incision in which acute, nonpurulent inflammation is encountered (including necrotic tissue without evidence of purulent drainage, such as dry gangrene)
2. Risk of infection: greater than 15%
Class IV: Dirty/infected
1. Old traumatic wounds with retained devitalized tissue, procedures with existing clinical infection (purulence already present in wound) or perforated viscera
2. Risk of infection: greater than 30%

3.2 Inflammation

During the late phase of coagulation and afterward, the inflammation stage begins with activation of complement cascade as well as infiltration of neutrophils. These are attracted to the wound within 24–36 h by chemoattractive substances such as TGB-ß, C3α and C5α, and formylmethionyl peptides (produced by bacteria and platelet products) [7]. Phagocytosis begins with the neutrophils which destroy and remove bacteria, foreign bodies, and damaged tissue. Neutrophils become sticky due to alterations in the regulation of surface adhesion molecules and adhere to the endothelial cells in the post-capillary venules around the wound [4, 9]. Blood flow pushes the neutrophils to roll along the surface of the endothelium. The adhesion and rolling mechanisms are weak attachments [6, 9]. Stronger attachments occur from chemokines secreted by endothelial cells that are mediated by integrins [6, 9]. Neutrophils stop rolling and migrate out of the venules by diapedesis by squeezing between the endothelial cells [6, 9, 10] into the wound environment and phagocytose foreign material and bacteria by releasing proteolytic enzymes and oxygen-derived free radicals [6, 10, 11].

From 48 to 72 h after injury, monocytes are released from the marrow and transform into macrophages that are attracted to the wound and phagocytose cell remnants and apoptotic bodies [11–15]. The attraction of the phagocytes is from clotting factors, complement components, PDGF, TGF-ß, leukotriene B_4, elastin, collagen breakdown products, and platelet factor IV [16]. The inflammatory response provides an abundant reservoir of growth factors including TGF-ß, TGF-α, fibroblast growth factor (FGF), heparin-binding epidermal growth factor, collagenase, activating keratinocytes, fibroblasts, and endothelial cells [6, 7, 10, 11, 13, 17, 18]. Lymphocytes are attracted at 72 hours after injury by interleukin-1 (IL-1), complement components, and immunoglobulin G (IgG) [12, 14, 19].

3.3 Proliferation

On the third day, proliferation begins and ends in about 2 weeks. Fibroblast migration and deposition of new synthesized extracellular matrix appears as granulation tissue [16]. Following injury, fibroblasts and myofibroblasts are stimulated to proliferate for the first 3 days [13], and then they migrate into the wound attracted by TGF-ß and PDGF that are released by inflammatory cells and platelets [20]. Fibroblasts appear in the wound on the third day after injury. They modulate phenotypically, proliferate, and produce hyaluronan, fibronectin, proteoglycans, and type 1 and type 3 procollagen [13, 18]. The collagens act as a foundation for the intracellular matrix. By the end of the third week, extracellular matrix accumulates, which supports cell migration [12, 18]. Fibroblasts change to their myofibroblast phenotype that actively extend pseudopodia attaching to fibronectin and collagen in the extracellular matrix [17, 19]. Contraction takes place as the pseudopodia contract. Redundant fibroblasts are then eliminated by apoptosis [21, 22].

Angiogenesis takes place during all phases of the reparative process even during the hemostatic

phase [9]. Pierce et al. [23] found that recombinant platelet-derived growth factor (PDGF) and transforming growth factor-beta 1 (TGF-beta 1) influence the rate of extracellular matrix formed in acute inflammatory cell influx. PDGF-BB (BB homodimer)- and TGF-beta 1 significantly augmented extracellular matrix formation and healing in 10-day wounds. PDGF-BB markedly increased macrophage influx and GAG deposition, whereas TGF-beta 1 selectively induced significantly more mature collagen bundles at the leading edge of new granulation tissue. Fibroblasts' transforming growth factor-beta (TGF-ß) demonstrated active collagen fibrillogenesis and accretion of subfibrils at the ultrastructural level. TGF-beta 1 preferentially triggers synthesis and more rapid maturation of collagen within early wounds. Takeshita et al. [24] showed that the angiogenic activity of VEGF is sufficiently potent to achieve therapeutic benefit.

Inhibitory and stimulatory agents act on proliferating endothelial cells directly as well as indirectly, by activating mitosis, promoting locomotion, and stimulating the host cells to release endothelial growth factors [25, 26]. Hypoxia causes molecules to be secreted by surrounding tissue that promote proliferation and growth of endothelial cells. Production of proteases by endothelial cells causes degradation of the basal lamina in the parent vessel in order to crawl through the extracellular matrix; chemotaxis occurs with proliferation, remodeling, and differentiation. Growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are trapped in the ECM by various proteoglycans [27]. The proteolytic degradation of these proteoglycans liberates the growth factors allowing them to reach their receptors and influence cellular behavior. Growth factors that indirectly affect angiogenesis are also targets of proteolytic activation. Capillary sprouts from the surrounding edges invade the wound clot, and a microvascular network of many new capillaries is formed.

Chemotactic agents act on cell surface receptors to direct the cell migration involved in angiogenesis during wound healing [28, 29]. This results in neovascularization and vessel repair of the wound. Chemotactic agents also are modulators of cell growth and differentiation. This includes endothelial growth factor (EGF), TGF-α, VEGF, and angiopoietin-1 [16].

Cellular motility requires protrusion at the cell front, adhesion to attach the actin cytoskeleton to the substrate, and traction that propels the trailing cytoplasm forward [30, 31].

In addition to providing a mechanism that allows cells to contact the extracellular matrix, integrins also promote intracellular signals that stimulate and regulate cell movement [29]. Periodic lamellipodial contractions correlate with rearward actin waves [31]. Multiple signaling pathways and regulatory proteins control actin dynamics. The direction of migration requires initial polarization of the cell. Epithelial cells migrate from the wound edges within a few hours of wounding [14, 15, 22].

3.4 Remodeling

Cellular matrix in the proliferative and remodeling is initiated contemporarily with granulation tissue development, and this phase may last up to 1–2 years or sometimes longer [13, 18].

Collagen bundles increase in diameter and hyaluronic acid and fibronectin are degraded [14, 22].

Matrix metalloproteinase enzymes are produced by neutrophils, macrophages, and fibroblasts in the wound and are responsible for degradation of the collagen. Fibroblasts' interactions with the extracellular matrix cause connective tissue to shrink and bring the wound edges closer together. The process is regulated by a number of factors including PDGF, TGF-ß, and FGF [23, 32].

4 Wound Healing Suppression

Those things that can influence wound healing include the patient's medical conditions (i.e., diabetes, etc.), thoroughness of wound debridement, microorganisms, dead tissues, and inflammation. Correct clinical management may positively influence the course of wound healing [16].

Growth factors are essential for wound healing (Table 2).

Table 2 Growth factors in wound healing

Growth factor	Source	Function
Epidermal growth factor (EGF)	Macrophages, platelets, endothelial cells (keratinocytes)	Cell growth, proliferation, differentiation, migration
Fibroblast growth factors 1 and 2 (FGF-1, FGF-2)	Macrophages, mast cells, fibroblasts, T lymphocytes, endothelial cells	Angiogenesis, cell proliferation, chemotaxis, wound contraction
Vascular endothelial growth factor (VEGF)	Mesenchymal cells, endothelial cells	Endothelial cell proliferation, vascular permeability, smooth muscle cell proliferation
Transforming growth factor-α (TGF-α)	Macrophages, T lymphocytes, keratinocytes	Expression of antimicrobial peptides and chemotactic cytokines, epithelial cell proliferation
Transforming growth factor-ß (TGF-ß)	Fibroblasts, T lymphocytes, platelets, macrophages, keratinocytes, endothelial cells, smooth muscle cells	Angiogenesis, fibroplasia, chemotaxis, keratinocyte proliferation, tissue inhibitor of metalloproteinase (TIMP) synthesis, fibronectin and proteoglycan synthesis
Platelet-derived growth factor (PDGF)	Platelets, macrophages, endothelial cells, smooth muscle cells	Angiogenesis, cell proliferation, chemotaxis, matrix metalloproteinase, fibronectin and hyaluronan production, wound remodeling, integrin expression regulation
Insulin-like growth factor-1 (IGF-1)	Fibroblasts, hepatocytes	Fibroblast and endothelial cell proliferation and chemotaxis
Tumor necrosis factor-α (TNF-α)	Macrophages, lymphocytes	Inflammatory process, fibroblast activity
Keratinocyte growth factor (KGF)	Keratinocytes	Keratinocyte migration, proliferation, and differentiation

Modified from Nabavian R, Garner WL. Normal wound healing. LAC/USC Burn Center. 2002 file.lacounty.gov/dhs/cms1_205424.pd. Accessed 9/21/16 and Wikipedia. Wound Healing. https://en.wikipedia.org/wiki/Wound_healing. Accessed 9/21/16

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Stem Cell Therapies for Wound Healing

Ayman Grada and Vincent Falanga

1 Introduction

A chronic wound is defined as a barrier defect that has not proceeded through an orderly and timely repair to reestablish structural and functional integrity [1]. Vascular insufficiency, diabetes mellitus, and prolonged external mechanical pressure are the leading causes of chronic cutaneous wounds. Furthermore, systemic factors and other comorbidities such as compromised immunological status and nutritional deficiencies, and aging are also contributing to poor wound healing.

The number of patients suffering from chronic cutaneous wounds is reaching epidemic proportions and will become even more burdensome in both human health and economic terms. The prevalence rate for chronic wounds in the USA is roughly 2% of the general population, and the incidence is expected to increase due to the rise in healthcare costs and growing aging population, especially in individuals with comorbidities such as diabetes, vascular disorders, and morbid obesity [2]. Chronic wound care consumes roughly 2–3% of health budgets in the Western countries. In the USA, care for chronic cutaneous wounds costs about $10 billion annually [3], twice the annual budget of the World Health Organization [2]. Moreover, non-healing wounds have a significant negative impact on the quality of life of patients [3]. Chronic unemployment and psychosocial issues are quite common among patients with chronic wounds [3, 4].

Most chronic wounds occur on the lower extremities. Venous leg ulcers (VLU) are the most common type, with an increasing incidence among the elderly of up to 3–4% [5]. Chronic venous insufficiency leads to structural and functional alterations of the skin in the lower leg, manifesting as lipodermatosclerotic changes, and ultimately skin ulceration. The cellular and molecular mechanisms, however, remain unknown, although recent findings provide several hypotheses including chronic inflammation, disruption of keratinocyte migration, and misregulated signaling and expression of specific microRNAs [6]. Atherosclerotic disease is the second most common underlying cause of non-healing skin wounds of the lower leg. Arterial ulcerations are a consequence of reduced arterial blood supply resulting in tissue hypoxia and structural damage and necrosis. Diabetes mellitus is the most common metabolic disease associated with impaired wound healing. Currently, the prevalence of diabetes is 9.3% in the USA [2]. The prevalence of type 2 diabetes is 6.4% worldwide and is anticipated to increase to close to 8% in the year 2030 [7]. Among diabetic patients, 1–4.1% will develop a foot ulcer annually [7, 8], and as high as 25%

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will develop a foot ulcer during their lifetime [7]. The 5-year mortality rate for patients suffering from DFU or ischemic ulcers is much higher than that of prostate or breast cancer [9].

2 Therapeutic Modalities for Non-healing Wounds

Traditional therapeutic modalities may include treating the underlying disease and providing conventional wound management (Table 1), and, when conservative approaches fail, bioengineered skin constructs may be utilized in selected cases. Reconstructive surgery using tissue flaps and grafts often becomes the last resort. In spite of recent advances in recombinant growth factors and bioengineered skin, up to 50% of chronic wounds that have been present for more than a year remain resistant to treatment [31]. Stem cell-based therapy offers new possibilities to address several deficiencies in wound treatment.

Table 1 Conventional therapeutic modalities for chronic wounds

Treatment	Mechanism of action	Levels of evidence	Source
Debridement (mechanical and chemical) Biosurgical debridement	Removed foreign debris and devitalized or contaminated tissues from a wound bed	Level III[a] Level I[a]	[10] [11]
Compression	Decreased venous backflow and capillary leakage	Level II[b]	[12]
Pressure-relieving devices and surfaces	Prevention and offloading	Level III[c]	[13–15]
Negative pressure	Remove exudate, reduce edema, increase local perfusion, decrease bacterial count, and enhance granulation tissue formation	Level III[a]	[16]
Electrical stimulation	May promote migration of various cell types to the wound, reducing the size of venous leg ulcers	Level III[a]	[17–19]
Hyperbaric oxygen therapy	Improve neovascularization, reduce production of pro-inflammatory cytokines, and increase synthesis of growth factors and collagen	Level lll[a]	[12, 20–22]
Lasers, phototherapy, shockwaves, and ultrasound	Decrease inflammatory cells, increase fibroblast proliferation, stimulate angiogenesis, promote formation of granulation tissue, and increase collagen synthesis	Level III[a]	[23, 24]
Antimicrobials	Antimicrobial		
• Silver		Level III[a]	[25, 26]
• Cadexomer iodine		Level II[a]	[25]
• Others (povidone-iodine, peroxide-based preparations, ethacridine lactate, chloramphenicol, framycetin, mupirocin, ethacridine, or chlorhexidine)		Level III[a]	[25, 26]
Dressings (alginate, foam dressing, hydrocolloids, hydrogels)	Provide appropriate moist wound environment, which contributes to faster wound reepithelization	Level III[a]	[27–30]

Levels of evidence according to Oxford Centre for Evidence-Based Medicine 2011 (available from www1.wfh.org/publications/files/pdf-1502.pdf)

Level I, systematic review of randomized trials; Level II, randomized trial; Level III, nonrandomized controlled cohort/follow-up study. Level of evidence was graded down on the basis of study quality and imprecision, because of inconsistency between studies, small sample size, high risk of bias, and unclear randomized method used, or because the absolute effect size is very small

[a]Level of evidence in promoting wound healing

[b]Level of evidence in decreasing recurrence

[c]Level of evidence in preventing ulcer development/reduce pressure

3 Stem Cells in Wound Healing

Stem cells have two key properties: self-renewal and potency (potential for differentiation into different cell lineages). Based on potency, stem cells are classified into totipotent, pluripotent, multipotent, oligopotent, and unipotent (Table 2). Furthermore, based on the source of origin, stem cells are categorized into embryonic and adult stem cells. Candidate cell populations for therapeutic applications include adult mesenchymal stem cells (MSCs), skin stem cells (SSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs).

There is a long-held hypothesis that stem cells have a distinctive capacity to restore tissue structure and function because they can sense their environment and differentiate in a manner that repairs any defect. It is indeed possible that certain subpopulations of multipotent stem cells may "teach" the wound microenvironment how to rectify the cellular and molecular phenotypic flaws that lead to impaired or failed healing.

However, most published data are derived primarily from small, uncontrolled trials plus a few well-controlled, randomized trials have not reliably generated adequate evidence for the effectiveness and safety of stem cell treatments [32]. More scientific evidence is required to ensure that innovation in this field delivers on its promise for wound healing.

Table 2 Classification of stem cells based on potency

Potency	Feature	Example
Totipotent	Capable of giving rise to all the cell types of the body. Capable of division and differentiation to produce a complete organism	Cells from early (1–3) days embryo
Pluripotent	Not capable of producing a complete organism, but able to divide and differentiate into all of the various cell types. Not capable of making extraembryonic tissues such as the amnion, chorion, and other components of the placenta	Some cells of blastocyst (5–14 days)
Multipotent	Capable of dividing and differentiating into more than one cell type from different lineage	Fetal tissue cord blood and adult stem cells
Oligopotent	Ability to differentiate into few cells	Adult lymphoid or myeloid cell
Unipotent	Ability to produce cells of their own type, self-renewal	Adult muscle stem cells

4 Mesenchymal Stem Cells

MSCs are adult multipotent, fibroblast-like cells, which were first isolated from bone marrow but are now known to be present in other tissues, such as adipose tissue. The International Society for Cellular Therapy (ISCT) proposed the following minimal criteria to define human MSCs: (1) they must be plastic adherent when maintained in standard culture conditions; (2) they must be lineage negative and express CD73,CD90, and CD105 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules; and (3) they must have the ability to differentiate into at least osteoblasts, adipocytes, and chondroblasts in vitro. Using a homogenous and well-characterized MSC population is crucial.

5 Sources for MSCs

MSCs can be isolated from bone marrow, adipose tissue, umbilical cord blood, nerve tissue, and skin. However, bone marrow and adipose tissue are presently the most reliable sources for MSCs.

6 How Do MSCs Promote Wound Healing?

MSCs have shown the ability to orchestrate cellular and biochemical changes in all phases of normal wound healing. Previous studies described different mechanisms: (1) immune

Table 3 Summary of growth factors and cytokines critical for MSC-mediated wound healing

Growth	Functions	Source
PGE2	Reduce inflammation	[36–38]
IDO	Reduce inflammation	[36, 39–41]
KGF	Wound healing	[42–44]
FGF	Tissue repair, intrinsic stem cell survival, and regeneration	[45, 46]
IGF-1	Wound healing, neurogenesis	[45, 47]
TGF-β	Wound healing	[48, 49]
VEGF	Angiogenesis, vascularpermeability	[49–51]
EGF	Angiogenesis	[45, 52–54]
PDGF	Angiogenesis, endothelial cell proliferation	[44, 55]
HGF	Angiogenesis, anti-fibrotic, inhibition of myofibroblast differentiation	[56, 57]
SDF-1	Angiogenesis	[58–60]
Ang-1	Angiogenesis	[44, 51, 61]

Ang-1 angiopoietin-1, *EGF* epidermal growth factor, *FGF* fibroblast growth factor, *HGF* hepatocyte growth factor, *IDO* indoleamine 2,3-dioxygenase, *IGF* insulin growth factor-1, *MSC* mesenchymal stem cell, *PDGF* platelet-derived growth factor, *PGE2* prostaglandin E2, *KGF* keratinocyte growth factor, *SDF-1* stem cell-derived factor-1, *TGF-β* transforming growth factor β, *VEGF* vascular endothelial growth factor

modulation, (2) promotion of angiogenesis, (3) differentiation to skin cells, (4) paracrine signaling, and (5) antimicrobial effect. Under the stimulation of different inflammatory cytokines at the wound microenvironment, the newly transplanted MSCs release a plethora of growth factors (Table 3). These growth factors coordinate activities of fibroblasts, endothelial cells, as well as stem cells to promote tissue repair by enhancing angiogenesis, inhibiting leukocyte transmigration, and stimulating intrinsic progenitor cell/stem cell differentiation.

7 Immune Modulation

Inflammation is a necessary phase in normal wound healing. Persistent inflammation in the wound microenvironment, however, is a hallmark of healing impairment in chronic wounds [33]. Present research demonstrated that in most systems, allogeneic MSCs are "immunoprivileged," which means they do not induce a significant host immune response. Additionally, MSCs release immunosuppressive factors that inhibit proliferation of immune cells such as T cells, B cells, mast cells, and natural killer cells, thus reducing inflammation in the wound bed. MSCs have shown to decrease fibrosis in a mouse model. This anti-inflammatory effect might help by optimizing tissue remodeling and reducing scarring following the repair process. Low immunogenicity and immunosuppressive features, combined with their availability, ease of propagation, and storage, suggest that allogeneic MSC grafting from healthy donors may be used for chronic wound therapy and could be especially useful for situations when the host's endogenous MSC population is defective, such as in diabetes, aging, and autoimmune diseases. The immunosuppressive effects of MSCs mainly rely on their ability to secrete various soluble factors, such as indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), and prostaglandin E2 (PGE2). Whether tissue-resident MSCs play a physiological part indirectly modulating immune responses in vivo is still unknown. Utilizing economy of scale for the production of ready-to-use allogeneic stem cells would also make treatment less expensive than the use of autologous treatment. However, one animal study suggested that IL-6 downregulation during MSC differentiation may result in loss of immunoprivilege and lead to a host response to the allogeneic MSCs [34]. MSC-derived IL-6 contributes to the immunosuppressive activity. The use of autologous MSCs, however, has not shown to induce an immune response at any level of differentiation.

8 Potency

MSCs can differentiate into keratinocytes, fibroblasts, endothelial cells, osteoblasts, chondrocytes, and adipocytes when cultured under specific conditions. It appears that MSCs represent a safe treatment, although large studies are

still needed. While concerns that MSCs might transform into tumorigenic cells still exist, there is general agreement that BM-MSCs can be safely cultured in vitro with no risk of malignant transformation. To date, there have been no reports in humans of formation of tumors by in vitro cultured cells, thus making MSCs a reasonable therapeutic option.

9 Paracrine Effects

The current hypothesis is that paracrine factors secreted by MSCs provide protective microenvironmental cues and promote the activation of local tissue-resident progenitor populations [35]. MSCs secrete several cytokines and growth factors that have paracrine activities. These factors enhance angiogenesis and stimulate mitosis and differentiation of tissue-intrinsic reparative or stem cells (Table 3). Hypoxia in the wound microenvironment has a stimulatory effect on MSCs [62]. MSC-conditioned medium has been shown to accelerate wound healing through a chemoattractant effect on epidermal keratinocytes and dermal fibroblasts [63]. The conditioned medium also induces dermal fibroblasts to increase production of collagen type-l [64]. MSCs secrete mitogens that stimulate proliferation of keratinocytes, dermal fibroblasts, and endothelial cells [42, 64, 65].

10 Antimicrobial Effect

MSCs contribute to the innate immune response against Gram-negative bacteria through the secretion of antimicrobial peptide LL-37 [66]. This antimicrobial effect, when combined with slow-release antiseptics, might further decrease bacterial colonization in chronic wounds.

11 Stem Cell Delivery

MSCs can be delivered to target tissue by direct topical/spray, scaffold loaded constructs, or subcutaneous injection. Ideally, MSCs should be able to retain their potency after being delivered. Engraftment appears to be affected by the delivery protocol of MSCs into the wound. It seems that timing of delivery, MSC number, and the delivery site all affect the efficiency of MSC engraftment [67]. The number of delivered stem cells may be critical. In our MSC studies in humans, we found that a cell concentration of at least $1 \times 10^6/cm^2$ of wound surface was required to achieve a beneficial outcome [68]. Presently, we have reason to believe that much higher MSC doses (higher than $5–6 \times 10^6/cm^2$) are probably required (Falanga, unpublished, 2016).

12 Topical Application

Acceleration of wound closure in both human and mouse models can be achieved by topical delivery of MSCs with a modified fibrin spray system. In 2007, we demonstrated accelerated healing of acute surgical wounds in human subjects treated with autologous BM-MSCs delivered in a fibrin spray [68]. Wounds were biopsied and histology suggested that MSCs migrated through the upper layers of the wound bed and differentiated into a fibroblast phenotype [68]. Chronic venous and diabetic ulcers were also treated, and a substantial decrease in size was observed at 4 months following three topical applications of MSCs. This work, for the first time, supported the concept of MSC effectiveness. For the first time in humans, Falanga et al. used a fibrin spray system, which works by mixing diluted fibrinogen and thrombin together with MSCs in a single syringe system. This system allows for immediate polymerization into fibrin and gluing of the stem cells to the surface of the wound. Initially, we focused on chronic lower extremity ulcers. Figure 1 demonstrates that MSCs delivered in a fibrin construct system promote healing of acute surgical wounds. Subsequently, we developed data suggesting that this approach could help other types of wounds. Figure 2 shows that MSCs delivered using a fibrin spray promote healing of sclero-

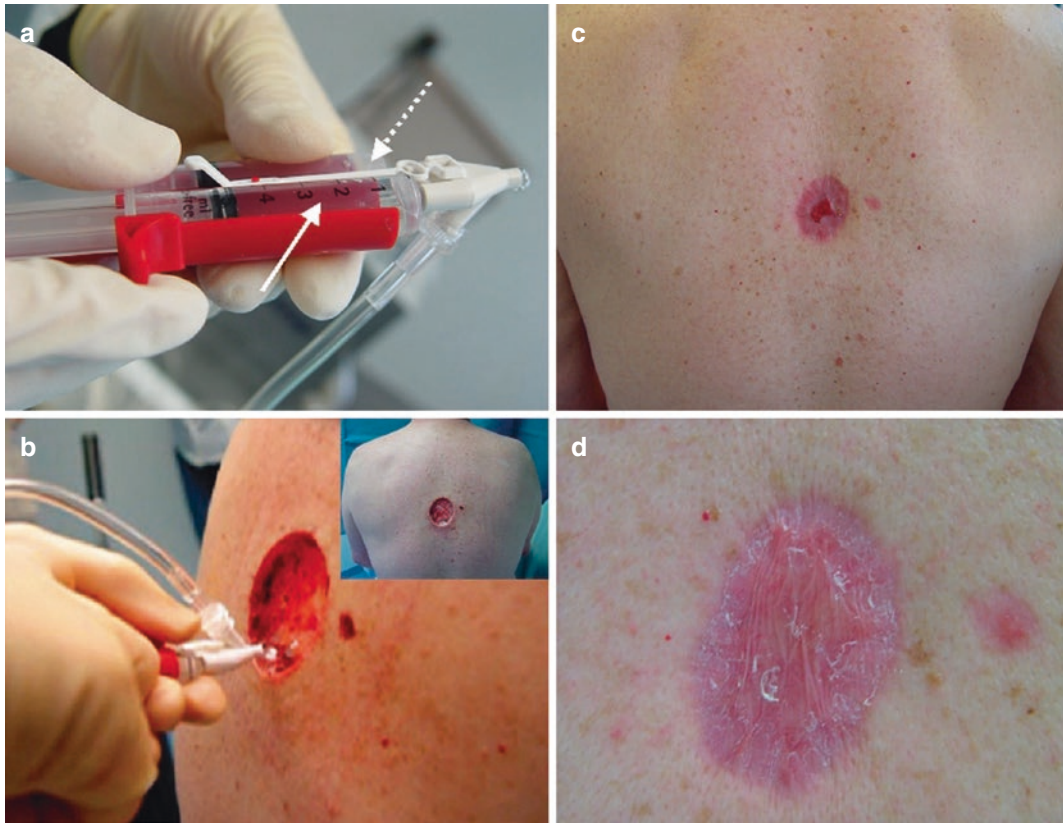

Fig. 1 Application of bone marrow-derived cultured MSC to acute surgical wounds. A) The cells were applied directly to the wound using a fibrin polymer spray delivered from a double-barreled syringe. The arrows point to the individual barrels filled with either thrombin or the cell-containing fibrinogen solution. The tubing, attached to the common spray jet area below the syringe, was connected to CO2. B) Application of the cultured cells to the wound, in this panel at baseline and immediately after surgery, was done by pressing on the common plunger of the double-barreled syringe shown in (A) and approximately 2 cm away from the wound bed. The inset shows the large wound on the back of the subject, who was sitting up. No runoff of the sprayed material is observed. C) Appearance of the wound at week 6, showing complete filling of the wound bed and almost complete epithelial resurfacing. D) Complete healing of the wound occurred by week 7, and the wound remained healing by week 12, as shown. The pink area to the right of the healed wound indicates a healed biopsy site (Copyright Vincent Falanga, MD)

derma (systemic sclerosis) digital ulcers within four weeks. In fibrin construct system, the cultured stem cells were mixed with fibrinogen and placed in a double-barreled syringe already containing thrombin. As the preparations simultaneously exit the two chambers, they mix and form a gel-like substance (fibrin) that can be administered topically to cover the wound bed. This spongy layer serves as a microenvironment to enhance the attachment and proliferation of the epithelial cells found at the edge of the wound.

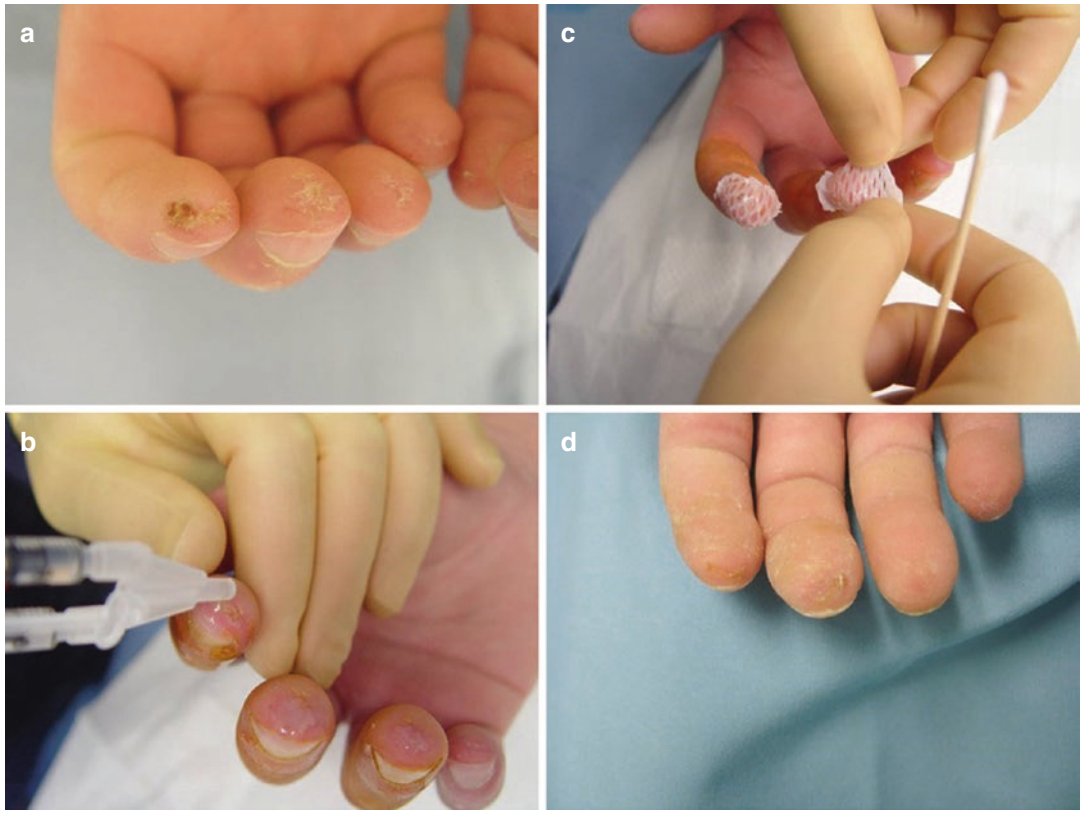

Fig. 2 Systemic sclerosis (scleroderma) digital ulcers treated by MSCs delivered in fibrin. Scleroderma digital ulcers treated with MSCs. Ulcer on the index and a pitting scar on the middle fingertips, respectively. MSCs were delivered in a fibrin construct system, appearing as a gel on the fingertips: A) digital ulcers and pitting scars at baseline; B) MSCs in fibrin being applied to the digital ulcers; C) the digital ulcer was dressed with bioengineered skin (Apligraf); and D) healed digital ulcers by 4 weeks (Copyright Vincent Falanga, MD)

13 Injection

One randomized trial reported a significant decrease in diabetic foot ulcer size in patients treated with BM-MSCs delivered via intramuscular/subcutaneous injections [69].

14 Bioscaffold

Synthetic extracellular matrix (ECM) composed of collagen, hyaluronic acid, or other naturally derived or synthetic materials can be used as a bioscaffold to enhance engraftment and paracrine activity and provide controlled spatial cues for seeded stem cells in hopes of establishing a functional cell niche [70].

15 Systemic Delivery

Although relatively convenient, systemic delivery is—at least in humans—not an efficient method for delivery; this is because of limited

cell engraftment and lower viability, especially when the target is a cutaneous wound in a particular anatomic location. Stem cells delivered intravenously can be sequestered in various internal organs such as the lungs, spleen, liver, bone marrow, thymus, and kidney, hence limiting the number of cells that can reach a cutaneous wound. However, there could be some indirect benefits from systemic delivery of stem cells. Interactions with nontarget tissues such as the spleen and liver may promote stronger immunomodulatory effect. It remains unclear whether systemically administered stem cells can influence cutaneous wound healing in humans.

16 Adipose-Derived Stem Cells

Adipose tissue has proven to be a reliable source for MSCs [71]. Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is easily isolated. Adipose-derived stem cells (ASCs) are an abundant supply of multipotent adult stem cells, which are easily harvested from adipose tissue through minimally invasive procedures, such as limited liposuction or excised fat samples, and with minimal donor morbidity [72]. Whereas only 0.001–0.002% of cells found in the bone marrow are MSCs, up to 1% of adipose cells are estimated to be stem cells [73]. High cell yield from lipoaspirate (as many as 1×10^7 cells from 300 mL of lipoaspirate and with at least 95% purity), as compared with bone marrow aspiration, makes ASCs a particularly attractive cell source for the acute wound setting. Moreover, ASCs have been tested in multiple preclinical trials on wound healing and have been found to enhance cutaneous wound healing significantly and increase blood vessel formation. Despite their increasing popularity, more clinical evidence is needed to substantiate a role for ASCs in humans. Previous studies have demonstrated that human ASCs, after prolonged culture in vitro, are capable of forming tumors in immunodeficient mice [74]. If confirmed, this potential tumor formation would prove to be a substantial setback.

17 Skin-Derived Stem Cells

MSCs have also been identified in all compartments of the skin. In the epidermis, there are three distinct stem cell populations responsible for the homeostasis of the superficial layers of the skin: interfollicular, sebaceous gland, and bulge area stem cells [75]. In the dermis, two MSC populations have been identified so far: stem cells from the dermal papilla of hair follicles and a population of perivascular stem cells [75]. While cutaneous MSCs are less appealing as a source for cell-based therapeutics for wound healing applications, their contribution to local skin repair is still significant [76].

18 Strategies to Enhance Therapeutic Effectiveness of MSCs

We have proposed a "didactic paradigm" whereby certain subpopulations of multipotent stem cells may "instruct" the wound microenvironment how to correct the cellular and molecular phenotypic flaws that lead to impaired healing. In this paradigm, the delivery of MSCs would be accompanied by the administration of a more mature tissue component, such as autologous grafting or bioengineered skin. In essence, a temporary organ system is applied. We have shown this "didactic paradigm" can heal ischemic digital ulcers in patients with systemic sclerosis (scleroderma; Falanga, unpublished). Moreover, we have shown that a living bioengineered skin construct would produce up to a 200-fold increase in wound repair-related mediators when preincubated in ideal tissue culture conditions for 24 h prior to application [77]. Possibly, therefore, an in vitro priming step may be required just before the administration of cell therapy [77]. This "priming" step may enhance the expression of essential genes and mediators such as epidermal growth and migration mediators.

19 Limitations to MSC-Based Therapy

Treatment of diabetic foot ulcers with autologous MSCs is of great clinical significance. However, the compromised potency of MSCs from diabetic patients restricts its value [78–80]. The diabetic wound microenvironments may also have a negative impact on the function of MSCs. Compared with MSCs isolated from nondiabetic mice, MSCs from diabetic mice were significantly less effective in accelerating epithelialization, granulation tissue formation, and angiogenesis in diabetic wounds [81]. However, ASCs and umbilical cord-derived cells have shown promise in clinical trials of diabetic ulcers [82].

20 Embryonic Stem Cells

ESCs are pluripotent cells derived from the inner wall of the blastocyst. However, the embryo must be destroyed in order to obtain the ESCs, which brings about serious ethical considerations and substantial legal restrictions surrounding the use of ESCs in any capacity, particularly in humans. Furthermore, there is still the potential for immunogenicity and tumorigenicity. Regulatory issues and problems derived from ESCs' vast differentiation capacity must be addressed before clinical applications become feasible.

21 Induced Pluripotent Stem Cells

iPSCs are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and transcription factors necessary for maintaining the defining properties of embryonic stem cells [83]. In a breakthrough discovery in 2007, Takahashi and Yamanaka [84] generated iPSC by reprogramming human adult fibroblasts into an immature, pluripotent state. The use of iPSC technologies allows for the generation of autologous pluripotent stem cell populations derived from differentiated adult tissues, thereby avoiding the ethical issues associated with human ESCs. A recent landmark study described the successful treatment of age-related macular degeneration (AMD) in a woman by means of transplantation of a sheet of retinal pigment epithelial (RPE) cells derived from autologous iPSCs [85]. The iPSCs were generated from skin fibroblasts from the patient.

Although iPSCs have been useful tools for drug development and modeling of diseases, there are legitimate safety concerns about utilizing them in wound repair. Moreover, we may not need all that pluripotentiality to treat chronic wounds safely, especially with the teratoma (even teratocarcinoma) concern and regulatory hurdles associated with iPSCs. The possibility of immunological rejection remains to be seen. Viral vectors are currently more commonly used for genetic reprogramming. Incorporation of the viral genome into that of the host poses a serious cancer risk [84]. In animal experiments, the virus used to introduce the stem cell factors sometimes causes malignant tumors [86]. A recent study reported that human iPSC clones derived from elderly adults show accumulation of somatic mitochondrial DNA (mtDNA) mutations and that structural damages to the mtDNA genes in iPSCs will likely diminish their metabolic function in energy demanding differentiated cells and, therefore, limit their therapeutic potential [87]. These findings suggest that using autologous iPSCs derived from elderly patients may not be a good therapeutic choice for treating chronic wounds in older adults.

22 Very Small Embryonic-Like Stem Cells (VSELCs)

An interesting development has been the discovery of what was ultimately called "very small embryonic-like stem cells" or VSELs by

Ratajczak et al. [88]. These very small cells can be derived from the bone marrow and can also circulate in the peripheral blood. The number of VSELs in an organism is probably very small, and extensive purification is required. Apparently, because of their small size (about the size of platelets), these cells may have been overlooked in the past in the course of purification from bone marrow or blood. The VSELs are of interest because they apparently are "conditionally pluripotent" and may offer an advantage over multipotent cells (like MSCs) and greater safety than embryonic stem cells. For example, VSELs are not known to cause teratomas when injected in mice and when using common assays. We have found that, in our mouse model of full-thickness tail injury, as little as 250 VSELs delivered in a fibrin construct could achieve equal acceleration of healing compared to 500,000 MSCs (Falanga, 2013, unpublished). There has been some controversy about the very existence of VSELs, mainly from one laboratory [89]. However, the collective strength of available published evidence points to their being a promising type of stem cells. Thus far, VSELs have not been successfully cultured in the laboratory, but recently this obstacle may have been overcome by the use of gonadotropins (Dr. Ratajczak, personal communication, 2016).

23 Secretome-Based Therapy

A secretome refers to the totality of secreted organic molecules and inorganic elements by biological cells. Stem cell-conditioned medium has been shown to promote wound healing in several preclinical studies [63, 90, 91]. The stem cell secretome contains a wide variety of growth factors and cytokines. Many of the therapeutic properties of MSCs can be mainly attributed to their secretome, which has been shown to regulate several processes in vitro and in vivo, such as cell proliferation, differentiation, survival, angiogenesis, immunomodulation, anti-apoptosis, and stimulation of tissue adjacent cells [92]. A few recent studies suggest that using stem cell secretome may open future therapeutic options for cell-free-based therapies [93, 94]. However, there are still some inherent limitations related to preparation of the secretome such as issues with pharmacokinetics, heterogeneity, and protein stability in vivo. Moreover, although MSC secretome is partly characterized [95], it seems unlikely that specific growth factors and cytokines alone give MSCs their remarkable repair abilities. More evidence needs to be generated from adequate well-controlled clinical trials in order to evaluate the efficacy of secretome-based therapy in wound healing.

24 Safety Regulations

The process of developing cell-based therapies is complicated and must be done correctly to ensure a safe application of stem cells. Recent developments and ongoing research in stem cell-based therapy has led to great interest in MSCs as a potentially useful therapy [96]. A Good Manufacturing Practice (GMP) facility is required for manipulating and manufacturing human cells in vitro [97, 98].

The following are other considerations to take into account with stem cells:

1. Cost-effectiveness of acquisition, testing, storage, and subsequent use in humans.
2. Stem cell delivery must be easily accomplished and without the use of sophisticated laboratory techniques that may require components and reagents that are not FDA approved.
3. The treatment must be such that it can be used in the clinic and not just within the confines of a laboratory-associated clinical research unit.
4. The presence of competent laboratory personnel.
5. The availability of off-the-shelf stem cell product for immediate use in cases involving burns and/or trauma.

Thus, it is urgent to resolve these limitations in order to support cell-based therapeutic commercialization.

The International Society for Stem Cell Research (ISSCR) has recently released guide-

lines for clinical translation of stem cells [99]. The guidelines highlight the distinction between the innovative therapies that are based on meticulous preclinical evidence, proven in rigorous clinical trials, and approved for marketing after regulatory review and the unproven interventions that are offered by practitioners who are ill-informed about the biologic complexities of stem cells [100].

25 Future Directions

Mesenchymal stem/stromal cells (MSCs) are of great interest in the field of regenerative medicine. However, we need a better mechanistic understanding of MSCs, including investigations to define the interactions between MSCs and the other cell types present in the wound microenvironment. Determining whether MSC's production of cytokines and growth factors is regulated during wound healing will lead to studies of whether the MSC supernatant can be used to enhance healing.

Progress continues in our ability to isolate stem cells, to characterize them, and to think of ways to deliver and use them in translational and interventional research. For now, multipotent stem cells are more useful for the acceleration of healing, particularly owing to their favorable risk/benefit ratio. Nevertheless, we do not reject the notion that pluripotent stem cells may ultimately find a role in wound healing. When properly handled, such cells could bring the field of tissue repair closer to regeneration or true wound healing.

Conclusions

The use of stem cells in wound healing was a natural step, given the potentially immense benefit that a variety of stem cells, whether they are multipotent or pluripotent, could bring to the problem of impaired healing and scarring. As one approaches this field, it must be kept in mind that the use of these cells needs to be placed in the context of their safety and regulatory environment for eventual approval. Hence, in our opinion, multipotent stem cells, like MSCs, have much to offer. We see considerable obstacles in the use of pluripotent stem cells, such as ESCs and iPSCs, due to safety/regulatory issues and, in the case of ESCs, persistent ethical considerations in some countries like the USA. It is true, however, that many decisions have to do with risk/benefit ratios and that pluripotent stem cells might be a game changer in particularly serious conditions. If the controversy about VSELs is satisfactorily resolved, which we believe it will, we will have a new and ideal weapon that enjoys the power of being at least conditionally pluripotential and apparently without the development of teratomas or teratocarcinoma. Not to be forgotten is that some stem cells, like MSCs, may truly alter the approach to scarring. Therefore, much more work needs to be done to address the true potential of stem cells in wound healing and, hopefully, regeneration.

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Stem Cells and Ear Regeneration

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and Ali-Mohammad Karimi

1 Introduction

Reconstruction of ears after trauma or due to congenital malformation (microtia-anotia) is a heavy and time-consuming procedure, and it needs several sessions of surgery. In spite of obvious progress in reconstruction techniques during the last six decades, still some problems have not been solved in this issue [1].

2 Etiology of Missed Ears

Besides the microtia or anotia, there are other categories of patients who have lost their external ear due to trauma, accident, frostbite, cancer removal, animal or human bites, or burns [1–5]. Congenital malformation of ear or microtia is a deformity that there is underdeveloped ear. A completely missed pinna is called anotia. Because microtia and anotia have the same origin, it usually referred to as microtia-anotia. Microtia can be unilateral or bilateral. Incidence of microtia is 1 out of about 8000–10,000 births. In unilateral microtia, the right ear is most commonly affected.

2.1 Classification

There are four grades of microtia [1]:

1. Grade I: A less than complete development of the external ear with minimal structures and a small external ear canal
2. Grade II: A partially developed ear (usually the top portion is underdeveloped) with a closed (stenotic) external ear canal with conductive hearing loss
3. Grade III: An absence of the external ear with a small peanut-like vestige cartilage and an absence of the external ear canal and eardrum. The most common form of microtia
4. Grade IV: An absence of the total ear or anotia

2.2 Pathophysiology

The pathophysiology of microtia is still unknown but most probably is multifactorial, and it happens during week 8–12 of gestation period, when the external ear is forming [1].

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3 Traditional Methods for Ear Reconstruction

For reconstruction of the ear, several methods have been advised.

1. Reconstruction with rib costal cartilage (Table 1)
2. Reconstruction with Medpor (porous polyethylene) and placing a skin flap (or multiple flap) over it
3. Using an external ear prosthesis

One of the most popular one is reconstruction with costal cartilage; some authors recommend this technique at the age of 7 (before entering the primary school) and some others recommend at the ages of 8–10 as the ear in this age is completely developed and reached to its maximum size (Fig. 1). The major advantage of this surgery is that the patient's own tissue is used for the reconstruction. But the technique involves from two to four stages depending on the surgeon's preferred method. The main disadvantages of this method are that it is a heavy and time-consuming procedure and it needs several sessions of surgery (at least three) sessions of operations, removal of costal cartilage (donor-site morbidity) (Table 2), lack of enough cartilage elasticity in adults (calcification of cartilage in adults), and shortage of and small size costal cartilage in some patients, remaining of scar in the chest, hypertrophic scar in the chest, some respiratory problems after the surgery specially during exercise, lack of enough cartilage for both sides (bilateral missed ears), lack of reserve cartilage for a third surgery

Table 1 Summary of total autologous auricular reconstructive techniques

Surgeon	Technique	Pros	Cons
Tanzer	Four stages:		
	1. Rotation of the lobule into a transverse position		
	2. Fabrication and placement of a costal cartilage framework	– First stepwise total auricular reconstruction	– Multiple operations
	3. Elevation of the ear from the side of the head	– Good results	– Transposing lobule first poses risk of vascular compromise of skin flap
	4. Construction of a tragus and conchal cavity		
Brent	Four stages:		
	1. Rib cartilage framework fabrication and placement		– Multiple operations
	2. Lobule transposition	– Good contour	– Lack definition of conchal bowl
	3. Elevation of framework and creation of a retroauricular sulcus	– Postoperative drain limits complications of bolster dressings	– Composite skin/cartilage tragal grafts can contract
	4. Conchal excavation and tragus construction		
Nagata	Two stages:		
	1. Fabrication of costal cartilage framework including the tragus, conchal excavation and rotation of the lobule	– Less operations	– More cartilage needed
			– Detailed framework so long learning curve
		– High-definition framework to create a good tragus	– Minimum age 10 years
	2. Elevation of framework, placement of cartilage graft in auriculocephalic sulcus, covered with temporoparietal fascial flap and skin graft		– Partial necrosis of posterior flap
			– Wire sutures increase extrusion

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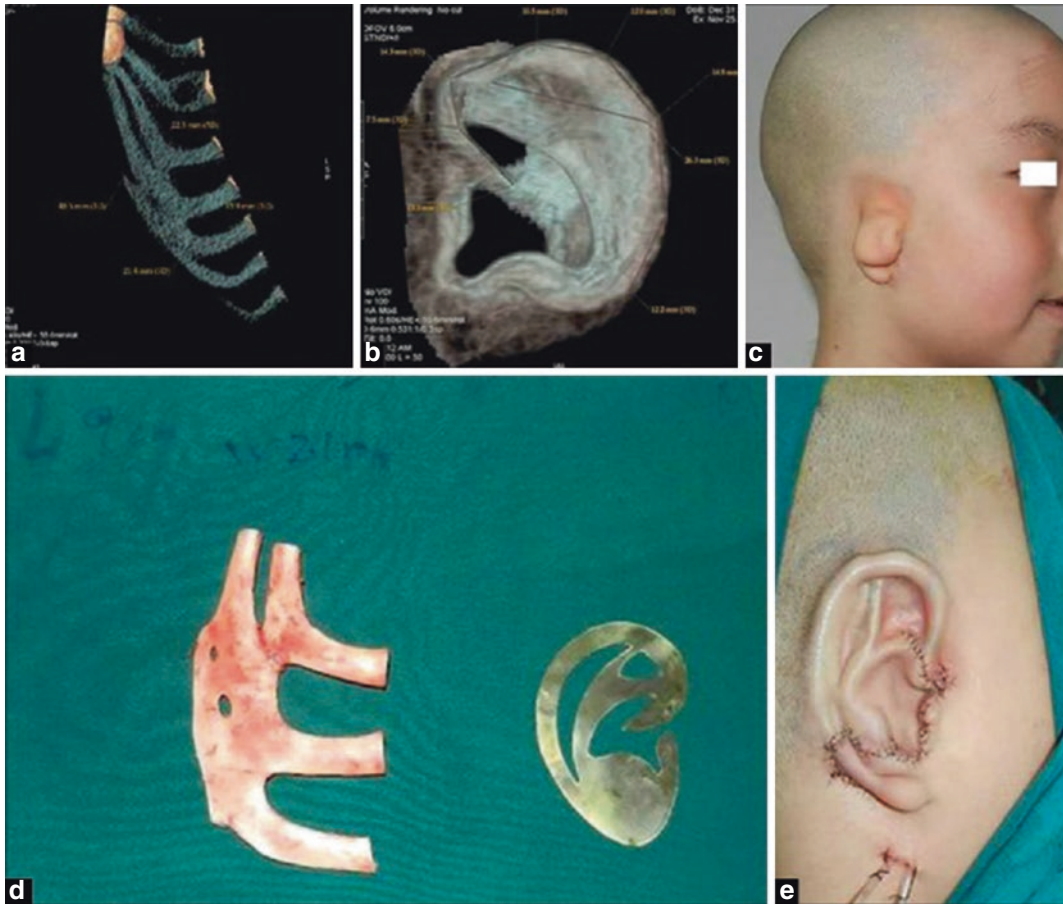

Fig. 1 Reconstruction of microtia ear using rib cartilage. Permission to reprint (Kang SS, Guo Y, Zhang DY, Jiang DY. Rib cartilage assessment relative to the healthy ear in young children with microtia guiding operative timing. Chin Med J (Engl). 2015;128(16): 2,208–14)

Table 2 Donor-site morbidity associated with total autologous auricular reconstruction

	Donor-site morbidity	Incidence	Total number of patients per study
Early	Pneumothorax	3 (1%)	270
		19 (22%)	88
	Atelectasis	4 (22%)	18
		7 (8%)	88
	Pleural effusion	–	–
Delayed	Persistent pain	6 (14%)	42
	Thoracic scoliosis	4 (25%)	16
	Seroma	9 (8%)	108, rhinoplasty group
	Clicking	3 (7%)	42
	Abnormal scarring	0 (0%)	42
		3 (2.7%)	110
		12 (14%)	88
		14 (5.3%)	264
		21 (6.5%)	322
	Contour deformity	3 (7%)	42
		16 (50%)	32
		22 (25%)	88

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(re-sculpturing of a new costal cartilage for secondary reconstruction), the shape and configuration of the reconstructed ear sometimes are not completely perfect and the rate of resemblance to normal ear is low [6–11]. Some of the normal configurations of normal ears cannot be reproduced, and some of them have no long-term durability (Table 3).

Table 3 Long-term limitations of autologous auricular reconstruction

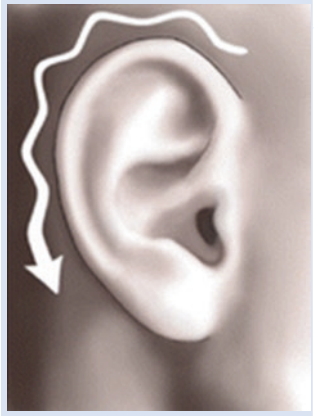
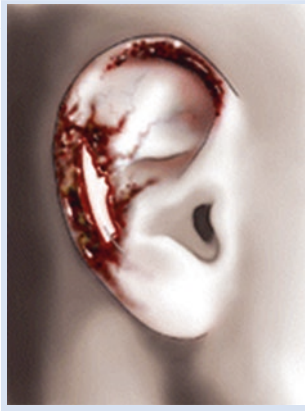
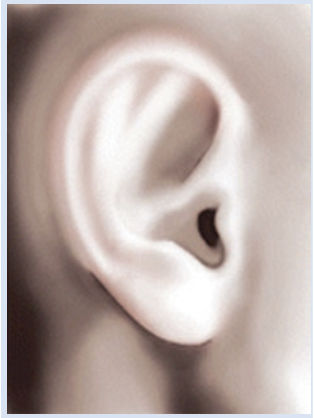
Long-term limitations		Reasons
Stiffness		1. Different biomechanical properties of fibrocartilage donor 2. Heterotopic calcification
Extrusion		1. Skin flap necrosis 2. Wire sutures to assemble cartilage framework 3. Wound infection or pressure dressings
Projection loss		1. Effacement of postauricular sulcus due to contraction of skin grafts

Table 3 (continued)

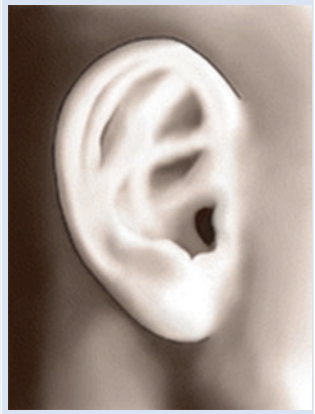
Long-term limitations		Reasons
Distortion		1. Constriction of skin and soft tissue overlying the construct due to scarring or ischemia 2. Cartilage degradation and resorption leading to loss of definition

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Fig. 2 Medpor ear framework

Fig. 3 Medpor ear framework

Reconstruction with Medpor has some advantages, like one-stage procedure, no donor-site morbidity, and good shape and size (Figs. 2 and 3). But the disadvantages are using a synthetic material, infection, extrusion of framework, inflammatory reactions, inability to grow with patient, and so on [12].

For external prosthesis only one stage of fixator insertion is enough, but some inflammatory response to the screws or fixation site, easily dropping of loose prosthesis, incompatibility of other children in school (they may make a joke by removing the ear and playing with it in the class), changing the color during the time, changing of quality during the time, need for buying a new one after some years, inability to grow with patient's age (Fig. 4).

Therefore these problems promote some surgeons to find another option.

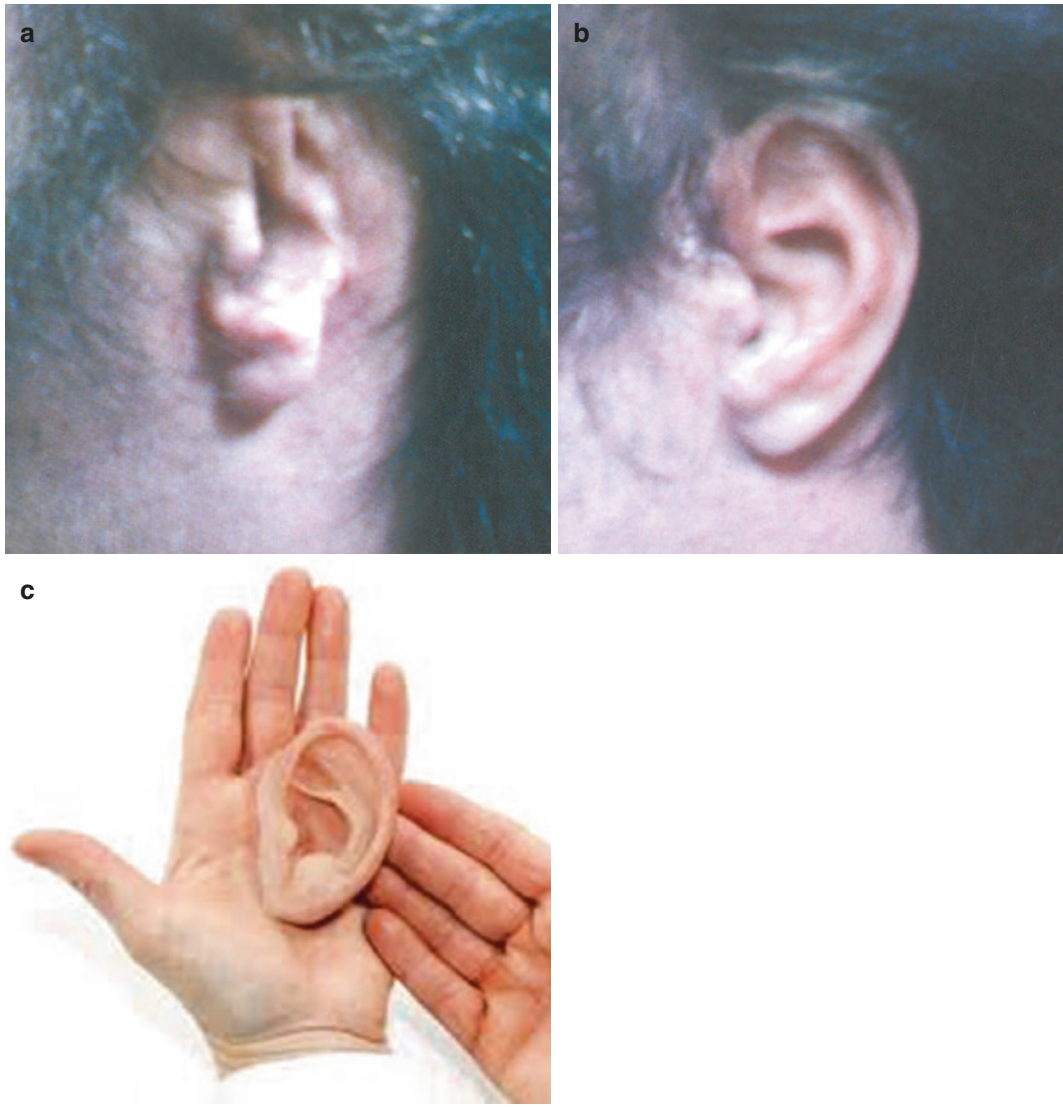

Fig. 4 (**a**–**c**) Artificial ear prosthesis

4 New Concepts for Ear Reconstruction

For reconstruction of the ear, two different tissues have to be restored, skin envelope and cartilaginous ear framework and skin and ear framework.

1. Skin reconstruction:
   The skin can be repaired with:
   (a) Using the postauricular skin + skin graft
   (b) Reproducing with a flap from superficial temporal fascia which covers the framework as a sandwich and putting total skin graft over it
   (c) Free fascial flaps (such as radial forearm flap) and total skin graft of it
   (d) Placing tissue expander in postauricular skin and expansion of skin [12]
   (e) Using multiple flaps

4.1 Reconstruction of Ear Framework

For reconstruction of framework using new methods, surgeons from 15 years ago started to use cell expansion techniques and cell cultures. One of the first steps in this regard was using the chondrocytes of the patient from normal side. The scientists extracted the chondrocytes and cultured it in vitro, and after expansion they used it over a scaffold and placed it in vitro or in vivo and tried to build a cartilaginous framework. The more scaffold resembles to the patient's ear, the more the reconstructed ear resembles to the normal ear. Although it is one stage and has no morbidity in the chest, it needs another operation for harvesting the chondrocytes.

4.1.1 Animal Chondrocytes

In one of the reports from Japan in 2004, the authors used bovine articular chondrocytes and expended it and seeded over PLLAEC (poly(L-lactic acid-epsilon-caprolactone)) copolymer scaffold (which resembles to human ear). Then they inserted it in the back of mouse for 40 weeks. The resulted ear was very similar to human ears [13].

In 2007 from the USA, the authors used chondrocytes of the joint, septum, or ear of the rabbit and reconstructed a new cartilage over a scaffold. They found high proteoglycan, collagen type II, and glycosaminoglycan in the cartilage and used them for reconstruction of trachea cartilage. They concluded that only ear cartilage chondrocytes maintained their biomechanical characteristics [14].

In a report from Germany in 2003, the authors used septal chondrocytes and expended them and seeded over hyaluronic acid scaffold (Hyaff 11), and in 4 weeks they reconstructed a new cartilage [15].

In 2003 there is a report that authors used bovine chondrocytes with scaffold and acrylic internal support. And in 12 weeks in rats, they reconstructed new human shape ear and human nasal-tip cartilages which had good rigidity (Fig. 5) [16].

In 2002 there is a report that stated that rabbit chondrocytes (autograft or allograft) and a rabbit decellularized cartilage can be cultured together (they used decellularized cartilage as a framework or scaffold). They found that new ear had viable chondrocytes [17].

For evaluation of scaffolds, a study in 2014 was done. The authors used rabbit articular chondrocytes with swine articular scaffolds and used the new cartilage in a defect in rabbit trachea. They concluded that allogenic chondrocytes with xenogenic scaffolds would result in a cartilage that have good shape and function [18].

These reports have shown us that a new cartilage can be regenerated from xeno-scaffolds. But still there are other reports that the new cartilages are not durable [19] or stable [20], although still other reports emphasized over rigidity [16], biocompatibility [21], and proper biomechanical characteristics of new cartilages [14].

Then researches focused on the origin of chondrocytes. It was found that ear chondrocytes will produce elastic cartilage and articular chondrocytes will produce articular cartilages [22]. Besides it is found that costal chondrocytes will calcify during a long-term period and the best chondrocytes for tissue engineering are those extracted from the ear, septum, and joints [23].

4.1.2 Human Chondrocytes

Gradually after successful results with animal chondrocytes, the studies went for the culturing of human chondrocytes. There are reports that used human chondrocytes and cultured them in the lower abdomen. The result is a block of cartilage that can be used for sculpturing and reconstructing ear framework [24, 25]. The authors stated that the cartilage has a neoperichondrium, and it can also be used as chondro-fat composite graft too. They reported the cartilages were stable for 1–5 years [4]. These works are suitable as there is no donor site in the chest and rib cartilages. In some other studies, normal human chondrocytes were cultured in special media and used for reproduction of ear cartilage in lab (Fig. 6) [5].

There is another report from the USA that children ear chondrocytes can be cultured for

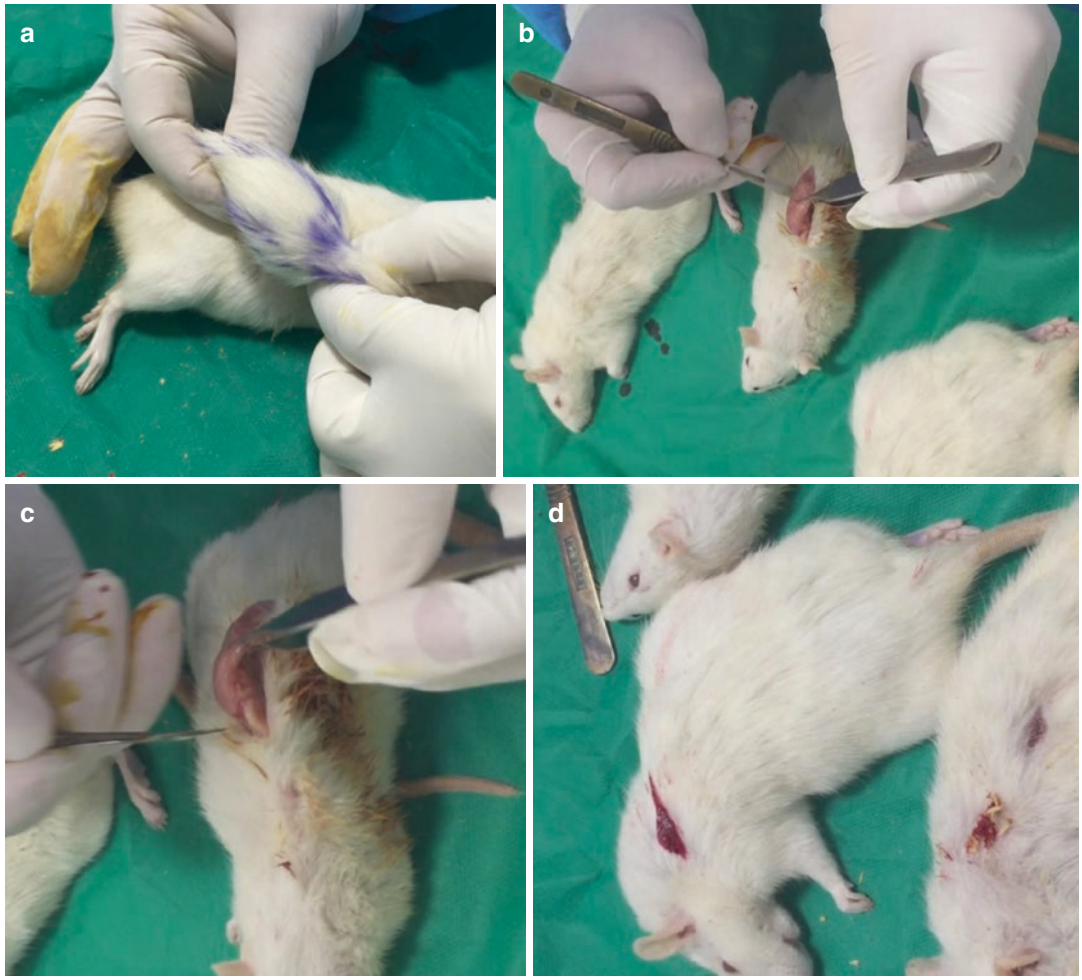

Fig. 5 (**a**–**d**) Reconstruction of human ear cartilages in the back of the rats, in vivo

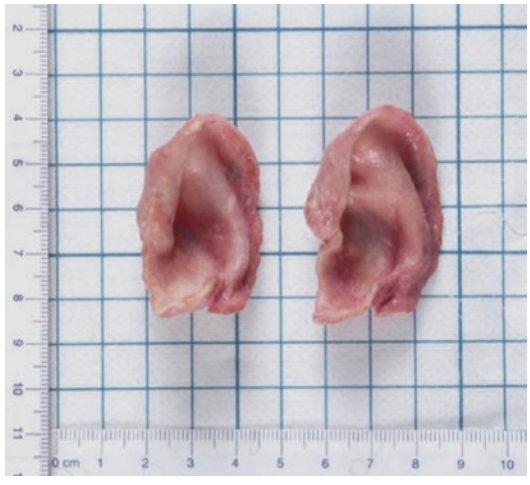

Fig. 6 Reconstructed ears with chondrocytes

framework reconstruction. The cells have a better speed for doubling, and it has longer biocompatibility [26]. There is also another report from the USA that human chondrocytes were seeded over PGA/PLA (polyglycolic acid-polylactic acid) scaffolds and the regenerated cartilages had no differences with normal ones regarding the mechanical and histological properties, unless the cells were smaller and there was a fibrous capsule around the cartilage [27]. In a report from Switzerland in 2014, the authors mentioned that they used human septal chondrocytes, and after 4 weeks, the results were proper hyaline and fibrous cartilages which remained stable and functional for 1 year [28].

4.1.3 Chondrocytes from Microtia Ear

The problem of taking chondrocytes from a normal ear has led the scientists to another option. They used chondrocytes from microtia ear, as they are expandable and with no donor morbidity and no morbidity for normal ears. These new cartilages had proper physical characteristics and abundant amount of proteoglycans and collagen type II [29]. It is reported that for human chondrocytes, FGF-2 promotes more proliferation and OP-1 has induced and maintained the phenotypic characteristics of ear chondrocytes [30]. In this option, surgery is one stage with no donor morbidity in normal ear and finding a way of use for microtia cartilage.

4.1.4 Stem Cell Regeneration

After microtial chondrocytes, another step was to culture and expand the stem cells and differentiate them into the chondroblasts. Obviously the researches begun with animal stem cells. Stem cells have the ability to increase into number of millions, and they can also be kept in liquid nitrogen for further culture and use. And after multiplication, they can differentiate into target cell, i.e., cartilage cells. These processes can be performed in the lab and have no stage in the patient's body. Many stem cells and many ear cartilages can be regenerated from one stem cell, so if there is any need for re-sculpturing and redoing of ear reconstruction, there would always be another cartilage for this purpose.

Many sources for stem cells have been used in this regard: bone marrow stem cells (BMSC) [22, 31–55], adipose-derived stem cells (ADSC) [52, 56–59], mesenchymal stem cells (MSC) [32, 60–63, 64], dental pulp stem cells [5, 56], perichondrial stem cells [65, 66], chondrocyte-derived progenitor cells (CDPC) [67], and fetal cartilage-derived progenitor cells (FCPC) [68].

The stem cells from these origins are harvested and cultured and expanded; then they will set in a chondrogenic culture media in order to produce the chondroblasts [38]. Then the new chondroblasts are placed in vitro, and with the help of external molds or internal scaffolds, they will gain the shape of a human ear.

The chondrogenic culture media (like DMEM/F12 media) should have special characteristics, and it should have the ability to induce stem cells into chondroblast [38]. These inducing media are very specific for the type of cells. For example, in a report in 2013, ADSC and ear chondrocytes of rat were cultured separately in the same media. ADSC produced proper cartilage, while ear chondrocyte did not produced proper cartilage [59]. Some specific cell mediators and growth factors should be added to this media for promoting the stem cells. Some of them are for increasing the number of stem cells such as FGF-2 [30] and some of them are necessary for changing into chondroblasts such as TGF-B3 [33], OP-1 [30], PTH [36], b FGF [69], and PRP [44] and some are needed to maintain the phenotype of cartilage like chondromodulin-1 [45].

Problems with a pre-induced media have led the scientists to a new innovation. Some of the authors used mature chondrocytes as a co-culture for stem cells and chondrogenic microenvironment that was produced had an induction effect on the stem cells and after induction, the chondroblasts and chondrocytes were produced and could be seeded to the scaffolds. It has been shown that co-culturing of ADSC with osteocytes can produce osteoblasts and co-culturing with chondrocytes can produce chondroblasts [56]. Some of the authors used xenograft chondrocytes [32, 61, 62] and some others used allograft human chondrocytes [37] and some others used autograft chondrocytes [34, 41, 42, 44, 46, 49, 54, 56, 57].

About the time of induction in the induction media or co-culture, several studies have been done. Some scientists advise from 7 to 10 days and some others up to 3 weeks and others even 8–12 weeks. It is obvious that there is no consensus about this issue [37, 38].

What should be the ratio or the percentage of stem cells vs. chondrocytes in co-culture media? There are many reports from 1:2 to 1:1 to 7:3 to 75:25 to 80:20. Most of the authors advised 75:25 ratio [32, 40–42, 54, 57].

4.1.5 Neonatal Chondrocytes Vs. Adult Chondrocytes

There is very good study from the UK that the authors compared the quality of cartilage after co-culturing of BMSC with human neonatal

chondrocytes versus adult chondrocytes. They found that there were no difference and even the quality of cartilage in adult cartilages in some issues was better than neonatal cartilages. So it seems that power of reproduction in chondrocytes themselves is not an important issue and only chondrogenic effect is important [37].

4.1.6 Choosing the Stem Cells

There is a very good study in 2010, and the authors reported that among BMSC, ADSC, and chondrocytes, the best cartilages were obtained from BMSCs. Therefore it seems that the choice for regeneration of ear cartilage is BMSCs [52].

4.1.7 Scaffolds

There are some few reports that did not use the scaffolds and still reported the good results. Although the longevity of these cartilages and quality of them are in question, they stated the proper results, while some of them reported that the cartilages were not stable [20, 29, 36]. Some of the authors who did not use scaffolds used fibrin sealant in order to put the regenerated cells together. This may have some minor effects of scaffolds, but the regenerated cartilages would be with the shape of a block [29, 36, 39, 47].

For using a stable structure that put every cell together, some authors used external molds, and more authors advised internal scaffolds.

There are reports about externals molds that will shape the cartilage into a human ear cartilage [70, 71]. These authors reported good and proper results after 8–12 weeks of in vivo implantation. But the difficulty of using an external mold for a long time in an animal model has led the scientists to use an internal scaffold that would shape the regenerative cartilage. There are several kinds of scaffolds: absorbable, nonabsorbable, double scaffolds, and so on. These scaffolds can help scientists to produce a 3D cartilage which resembles the ear framework. Several characteristics have been written for a good and proper scaffold: it should provide good surface for adhesion of the stem cells, it should provide good structure for cell support, it should have mechanical properties similar to the ideal cartilage, scaffolds and its degradation should not be toxic to the host, the scaffold has to have the ability for 3D reproduction, it should have high (90%) porosity for cell-to-polymer interaction, it should have enough space for matrix production, it should help to prevent loss of phenotype, it is better to be absorbable after regeneration of cartilage, degraded scaffolds should not be toxic to stem cells, and it should be easily replaced by the new cartilage tissue. The scaffolds should have the ability for cell induction in early steps and ability to support cell differentiation in the next stages (Table 4).

Absorbable scaffolds are PLA (polylactic acid), PLLA (poly-L-lactic acid) [35], PLA/PGA [22, 27, 40, 62], PGA (polyglycolic acid) [34], collagen scaffolds [65], collagen type I hydrogel [34], PLCG (polylactic-co-glycolide) [37], PLLAEC poly(L-lactic acid-epsilon-caprolactone) copolymer [13], collagen type I glycosaminoglycan [38], Hyaff 11 (a hyaluronic acid derivative) [15], swine scaffold PCS [18], hexanolactone/carbonate, decellularized ear cartilage (only collagen and elastin) [48], cadaver ear framework [55], acellular dermal matrix (ADM) [49], PLA/PLEC poly(L-lactic acid) and poly(L-lactide-epsilon-caprolactone) [23], silk fibroin [43], GT/PCL (gelatin/polycaprolactone) [42], PCL (poly-e-caprolactone) [61], and polycaprolactone-based polyurethane [72].

Nonabsorbable scaffolds include chitosan nonwoven [31], polyethylene [73], silk polymer [60], acrylic internal support [16], and porous coral scaffold [46].

Double scaffolds are also numerous such as chitosan nonwoven/PLGA(poly(DL-lactide-co-glycolide)) [31], PLAEC/PLA (poly(L-lactic acid-epsilon-caprolactone)/polylactic acid) [23], alginate and silk polymer [60], porous collagen/titanium wire [69], and silk fibroin/chitosan [43]. In some mixed scaffolds, both scaffolds are absorbable; in others one is absorbable and nourishing for the cells, and the other is nonabsorbable and provides heavy and stable structure for a long time, and in other less frequent types, both are nonabsorbable. Liu et al. [5] also used double mesh framework, one as structure and one as nourishing mesh, and have done it

Table 4 Different type of scaffolds

Absorbable	Poly-L-lactic acid Polyglycolic acid Polyglactin Polyurethane	Dexon Vicryl
Nonabsorbable	Polyvinyl alcohol Polytetrafluoroethylene Polyethylene Nylon Polyester Carbon fiber meshwork	Porous sponge Teflon Dacron
Biomaterial non-synthetic	Collagen type I sponge Decalcified bone Fibrin polymer Hyaluronic acid Meniscus ADM Decellularized cartilage Cadaver cartilage	Our previous report (Karimi H, Emami SA, Olad-Gobad MK)

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in vitro. After 12 weeks they had good results for elasticity and shape.

In a study by Nayyer-Leila et al. [74], they used human stem cells and collagen-polyester mesh for reconstruction of the ear. But they had done it in only one patient, and this type of study needs further cases and researches.

Selection of scaffolds depends on experience of surgeon or scientists, type of stem cells that they want to use, and type of regenerative cartilages that are needed to be regenerated. For example, for reconstruction of the ear, most frequent scaffolds that are used include PLA/PGA and PLA.

Scaffolds are widely used to reconstruct cartilage. Yet, the fabrication of a scaffold with a highly organized microenvironment that closely resembles native cartilage remains a major challenge. Some authors suggested that scaffolds derived from acellular extracellular matrices are able to provide such a microenvironment.

There is a report specifically on decellularization of full-thickness ear cartilage. In this study, decellularized ear cartilage scaffolds were prepared. The authors removed cells and cell remnants from elastic cartilage. And the obtained scaffolds retained their native collagen and elastin contents as well as their architecture and shape. High magnification scanning electron microscopy showed no obvious difference in matrix density after decellularization. However, glycosaminoglycan content was significantly reduced. Then the authors used BMSC over this scaffold, and the new cartilages had good and proper characteristics [48]. In another report from our center, we used ear framework of cadaver with BMSC. The resulted ears have very good elastic and biomechanical properties (Figs. 7, 8, 9, 10, and 11), and the shape, weight, and size of the ears were remained and were stable. This was the first time in the published literature that the weight of ears was measured, and with BMSC, a new cartilage was formed with proper shape and weight and size. In that study we concluded that using ear cadaver framework seeded with bone marrow stem cells for reconstruction of the ear is a feasible, fast, one-stage technique and the elasticity, shape, size, and weight of the framework would be preserved. Using the patient stem cells also would provide the most HLA compatibility for the reconstructed ear [55]. There is another report in 2015 that used human cadaver ear framework for reconstruction of ear too [48].

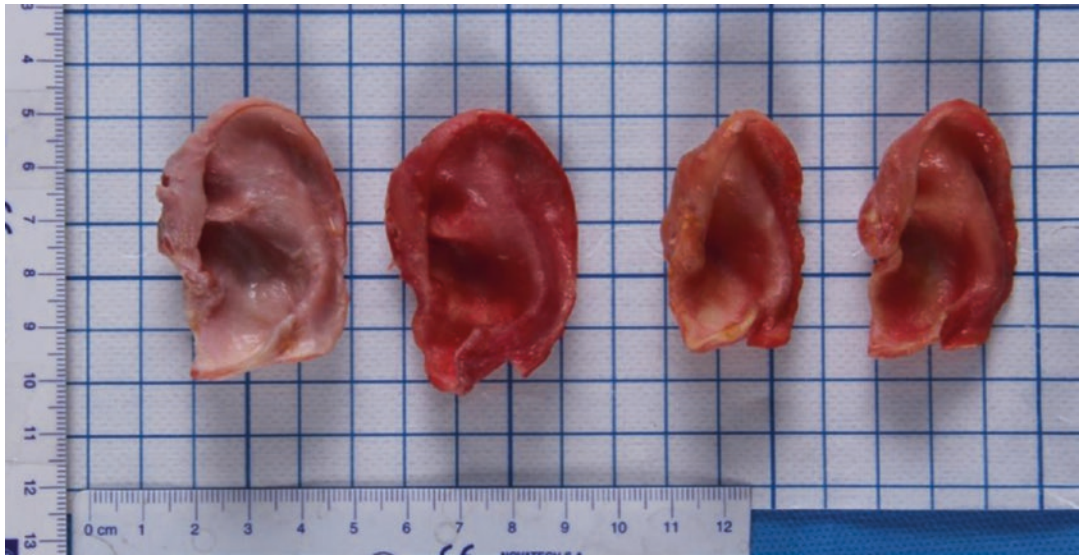

Fig. 7 Reconstruction of human ears with BMSC and cadaver framework

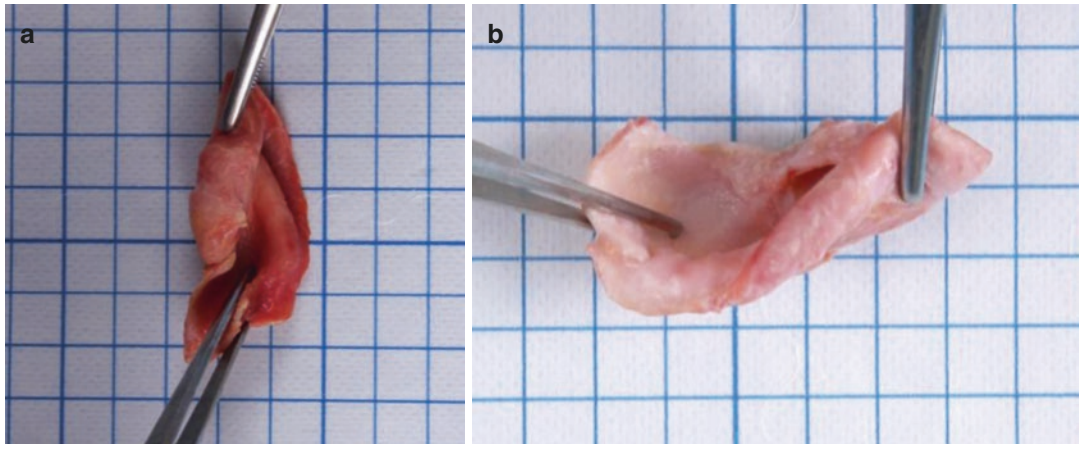

Fig. 8 (**a**, **b**) Testing the elasticity of reconstructed ear that was regenerated from the patients' own BMSC and cadaver framework

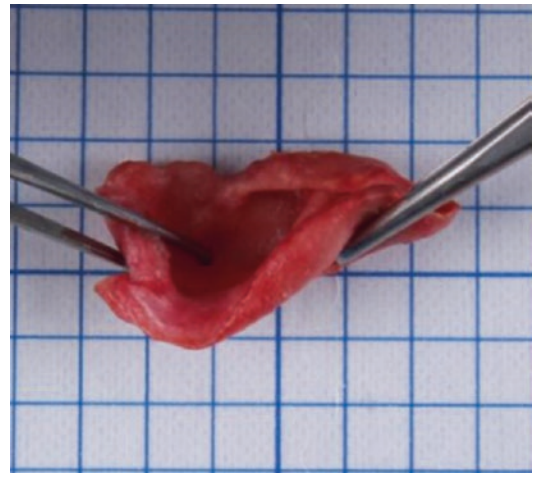

Fig. 9 Testing the flexibility of reconstructed ear that was regenerated from the patients' own BMSC and cadaver framework

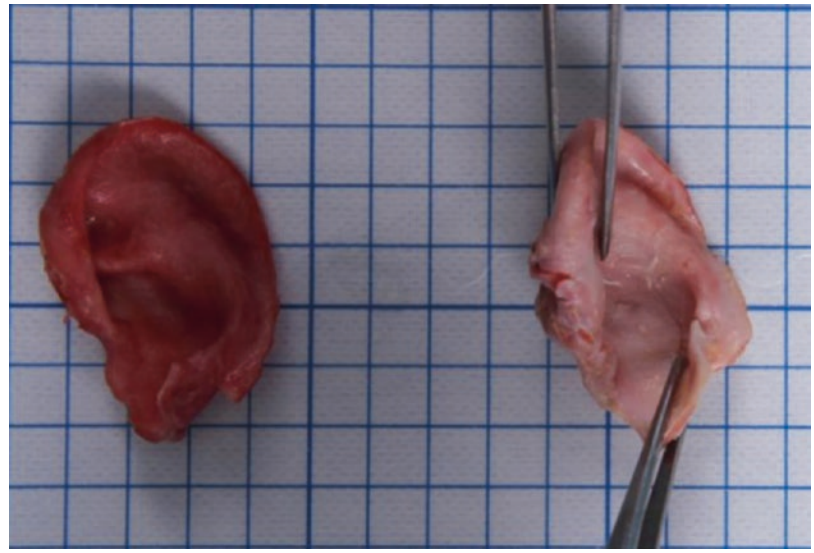

Fig. 10 Testing the flexibility of reconstructed ear that was regenerated from the patients' own BMSC and cadaver framework

Fig. 11 Measuring the weight of frameworks in two groups after 12 weeks in the back of the rat, in vivo. Our results showed that BMSC and cadaver framework will maintain the weight and shape and elasticity of the ear framework, and after 12 weeks, the reconstructed ear can be used for reconstruction of missed ear in the patients

4.1.8 Growth Factors

For culturing and expanding of the stem cells, it is needed to have a chondrogenic media and provide some growth factors and cytokines [40, 56] to facilitate transformation of stem cells into chondroblasts. The stem cells can differentiate into chondrogenic cells by the help of chondrogenic media with growth factors or co-culturing with chondrocytes or chondrogenic matrix.

Some of these growth factors are:

1. FGF-2: for promoting cell proliferation.
2. OP-1: for promoting cell phenotypes into chondroblasts.
3. TGF-ß3: for transforming into chondrocytes.
4. ß FGF: for cell proliferation.
5. Chrondromodulin-1: for stabilization of phenotype and prevention of calcification of cartilage.
6. TGF-ß: for cell differentiation and matrix formation and transformation to chondroblast. It works synergistically with IGF-1.
7. Dexamethasone: for transformation to chondroblast.
8. Ascorbic acid: as adjuvant.
9. TGF-ß3: for transforming into chondrocytes.
10. IGF-1: for transforming into chondrocytes and matrix formation.
11. TGF-ß1: for chondrogenicity.
12. BMP: bone morphologic protein.
13. Basic FGF (FGF-2): for cell proliferation.
14. PDGF (platelet-derived growth factor): for cell proliferation.

4.1.9 Maturation Process

It is proven that tissue-specific stem cells can produce a new auricular cartilage, but this new cartilage structure is immature phenotypically; advantage of this issue is that it is highly active metabolically and it can grow and develop into mature adult ear cartilage, but disadvantage is that it may be liable to resorption. In the first and highly innovative study of auricular tissue engineering by Vacanti et al. [75], a new ear-shaped cartilage was made from bovine chondrocytes and biocompatible scaffolds that were xenografted into a nude mouse. The shape of the cartilage was supported by an externally fixed mold, but after removal of the mold, the cartilage deformed and started shrinkage. So the new cartilage was not stable in long term (Fig. 12).

Newer studies have shown that the process of resorption may be due to an intrinsic property of new cartilage or due to extrinsic factors, e.g., cell mediators or inflammatory mediators or cell-to-cell interactions.

Advantage of an internal permanent support, like coiled wire or titanium wire or nylon scaffolds, is that it is shown in animal studies that it helps to prevent or to reduce shrinkage of the neo-cartilage [76], but disadvantage is that implanted synthetic materials may be extruded [77–82].

The extracellular matrix of cartilage has collagen and elastin framework and can last forever, as it has extensive chemical cross-linking that stabilizes the structural of the cartilage. The maturation process of cross-linking starts after the birth and in 15 years will be completed. It is called functional adaptation (Figs. 13 and 14) [83–85].

Gradual increasing of the number of cross-linking would increase the biomechanical strength of cartilage [86]; in this way a durable cartilage will be produced [87, 88]. Until now there was no solution for promoting maturation in a new cartilage. The lack of maturation is one of the major causes of the failure for production of a durable tissue-engineered ear cartilage. There is a marvelous study that fibroblast growth factor (FGF)-2 and transforming growth factor beta-1 (TGF-β1) may induce maturation in the cartilage in immature articular cartilage [89, 90].

No one knows as to what extent does auricular cartilage undergo tissue maturation. But the goal of newer studies is to accelerate the maturation process and have a functional and durable cartilage (Fig. 15).

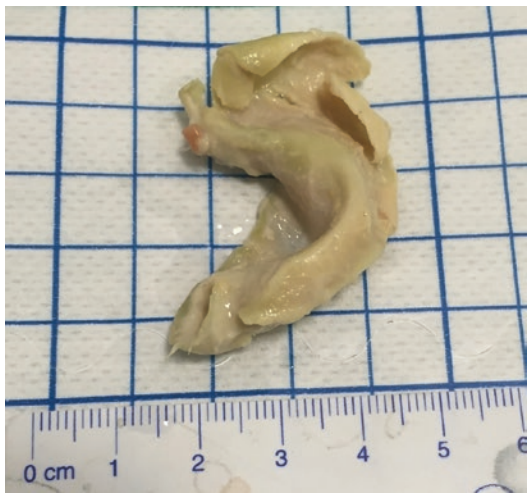

Fig. 12 Deformity and shrinkage of the regenerated ear framework

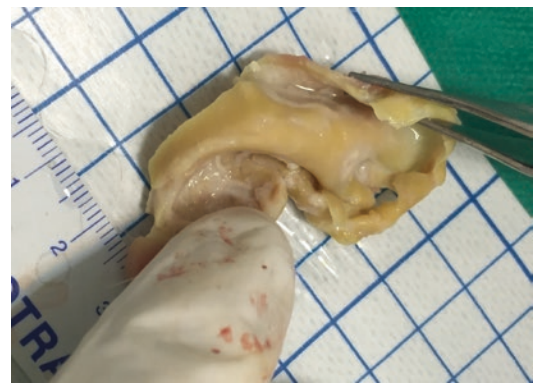

Fig. 13 The shape and flexibility of the framework have been lost

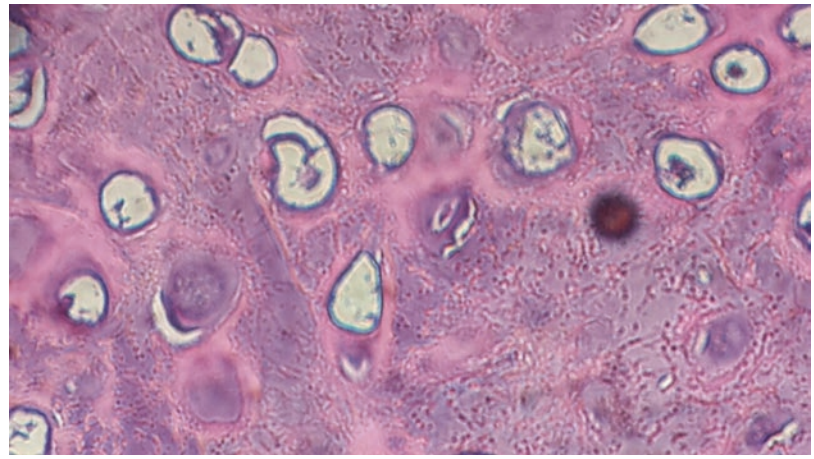

Fig. 14 Microscopic examination of cartilage after shrinkage

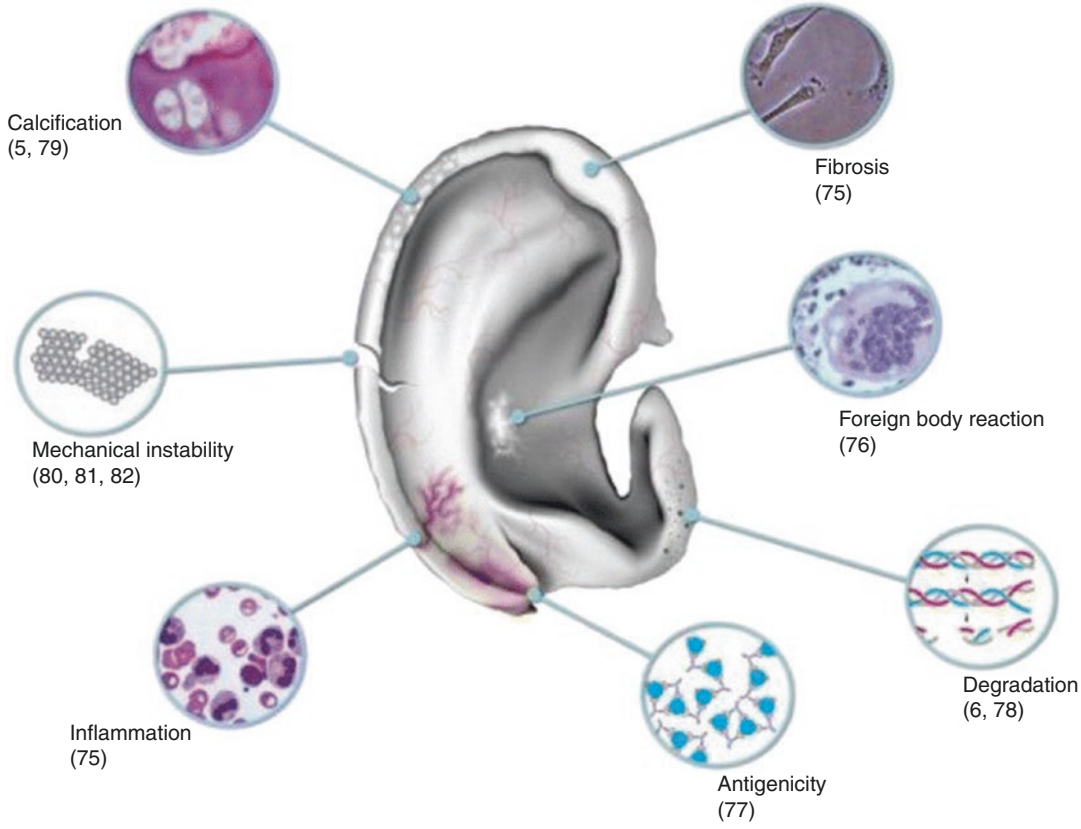

Fig. 15 Limitations of current tissue-engineered auricular cartilage constructs. Reprinted with permission (Jessop ZM, Javed M, Otto IA, Combellack EJ, Morgan S, Breugem CC, Archer CW, Khan IM, Lineaweaver WC, Kon M, Malda J, Whitaker IS. Combining regenerative medicine strategies to provide durable reconstructive options: auricular cartilage tissue engineering. Stem Cell Res Ther. 2016;7:19)

Table 5 Potential future benefits and challenges of combining regenerative medicine with additive manufacturing

	Feature	Benefits	Challenges
Bioprinting	Control over macrostructure and microstructure of tissue produced	Replicate anatomical formReduce surgical technique learning curve	Biomechanical properties of bioinksEffect of printing on cellsPrinting resolution
	Patient-specific macrostructure from image acquisition (CT/MRI)	Reduce variability in surgical outcomes	Macrostructure may alter during bioreactor maturation
	Manufacture ex vivo	Avoid donor-site morbidityReduce operating time	Potential for contaminationRegulatory constraints
Regenerative medicine	Tissue-specific stem cells to improve quality and functionality of engineered tissue	True "like for like" replacementRestoring native anisotropy allows improved matching of mechanical properties	Genetic stability and differentiation capacity of cells after prolonged expansion in culture
	Tissue maturation utilizing growth factors	Reduce degradation and constriction	Optimal growth factor combinations and temporal effects

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CT computed tomography, *MRI* magnetic resonance imaging

5 Achieved Results

Up to now several progressions have been made by scientists to produce ear cartilages by tissue engineering. These cartilages can be formed in vivo or in vitro. If you want to use in vivo option, there would be no need for a two-stage operation for the patients. And if you want to use in vitro option, there would be one-stage operation, but the mechanical properties of the new cartilages are weaker than the in vivo one.

The 3D manufacturing of the scaffolds is a new concept that has many successful results, and the regenerated ear would have a natural 3D configuration very similar to a normal ear.

6 Horizons of Research

The new research can be focused over reconstructing cartilage in fewer days, promoting faster maturation for new cartilages, having strong mechanical properties, and regenerating ear framework with bilayer skin over both sides of framework (Table 5).

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The Role of miR-205 During Skin Wound Reepithelialization

Tao Wang, Na Zhao, Shuang Long, Lan Ge, and Yongping Su

1 Introduction

Skin wound healing is one highly orchestrated process that involves coordinated interactions among tissue repair cells, growth factors, and extracellular matrix [1, 2]. The process to repairing damaged skin can be divided into three sequential and overlapping phases: an initial inflammatory phase, followed by a proliferative phase, and a concluding with a remodeling phase. As the primary objective is to quickly reestablish barrier function of skin after wounding, reepithelialization is one essential component of wound healing [3]. Actually, epithelialization is a defined parameter of a successful wound closure, and a wound cannot be considered healed in the absence of reepithelialization. Recent advances in wound healing studies have clarified the mechanisms of reepithelialization in multiple levels including cross talks between keratinocytes and other cell types, microenvironment in the wounds, extracellular matrix, integrins, growth factors, and so on [3, 4]. And there is no doubt that keratinocytes play central roles in reepithelialization. Reepithelialization involves migration, proliferation, and differentiation of keratinocytes to cover the denuded dermal surface. Since the defects in migration, but not in proliferation or differentiation, are associated with the clinical phenotype of chronic non-healing wounds [5, 6], the mechanisms that regulate keratinocyte migration are central issues of reepithelialization. However, the molecular mechanisms of keratinocyte migration during reepithelialization are still not fully elucidated.

microRNAs (miRNAs) are endogenous small noncoding RNAs (19–22 nt in length), which play pivotal roles in diverse physiologic and pathologic processes, including development, proliferation, differentiation, apoptosis, and carcinogenesis [7, 8]. Recently, the roles of miRNAs during skin wound healing are gradually revealed, and many miRNAs are involved in reepithelialization by regulating keratinocyte migration [9–16]. miR-205 is one abundant keratinocyte-specific miRNA in the epidermis [17, 18]. It has an essential role in promoting neonatal expansion of skin stem cells during early development by modulating the PI(3)k pathway [19]. In vitro studies using both primary human epidermal keratinocytes (HEKs) and corneal epithelial keratinocytes (HCEKs) indicate that miR-205 can promote keratinocyte migration via targeting the lipid phosphatase SHIP2 and KIR4.1, respectively [20, 21]. While, as to the role of miR-205 in cell migration, different studies have reported con-

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flicting results in different models, some indicate miR-205 can promote migration [22, 23]; however, others show opposite results [24–28]. Nevertheless, these reports suggest that keratinocyte-specific miR-205 may participate in reepithelialization by modulating keratinocyte migration during cutaneous skin wound healing.

Previously, we identified miR-205 changed for more than twofold at the stage of granulation formation during skin wound healing by miRNA microarray [11]. To further ascertain miR-205 function, its possible role in reepithelialization during skin wound healing was investigated. Unexpectedly, we found that miR-205 expression is significantly downregulated in the leading edge of the migrating epithelial tongue by in situ hybridization. In HaCaT keratinocytes, miR-205 could be downregulated by TGF-β1 stimulation. And similar to the effect of TGF-β1, miR-205 knockdown could promote keratinocyte migration in wound scratch model in vitro. Furthermore, tropical inhibition of miR-205 by administrating Pluronic gel containing antagomir-205 could accelerate reepithelialization in mouse skin wound model in vivo. Moreover, we identified integrin alpha 5 (ITGA5) as one key functional miR-205 target in the reepithelialization process, and epidermal downregulation of miR-205 may desilence ITGA5 to promote keratinocyte migration. And knockdown of ITGA5 would abolish the pro-migratory effects of miR-205 inhibition in vitro. Furthermore, we found dysregulation of miR-205 and its target ITGA5 in clinical chronic wound samples with persistence of high-level miR-205 and absence of ITGA5. Our findings indicate that downregulation of miR-205 in the leading migrating keratinocytes is critical for reepithelialization and miR-205 may be a potential therapeutic target for chronic wounds.

2 Technique

2.1 Wound Model

Male, 6–8-week-old C57BL mice were obtained from the Center for Experimental Animals, Third Military Medical University (TMMU, Chongqing, China). All the animal experiments were performed according to local ethical guidelines and were approved by the local Administration District Official Committee of Third Military Medical University (permit number: 81,372,061). Mice were anesthetized with 1% pentobarbital (ip, 30 mg/kg), and then hairs of the dorsal skin were shaved. Four equidistant full-thickness excisional wounds with diameter of 4.0 mm were made with a biopsy punch (Acuderm, USA) in the shaved dorsal skin at four separate sites. To ensure the homogeneity of the wounds, the punch was dipped into the inkpad before stamped onto the skin, and then the wounds were made with scissors according the stamp. At indicating days after wounding mice were killed, the wound and the surrounding unwounded skin were surgically removed with a 6-mm biopsy punch. Specimens were then fixed in 4% paraformaldehyde for further histological and in situ hybridization assays.

2.2 miRNA In Situ Hybridization Analysis

miRNA in situ hybridizations of paraffin-embedded skin sections were performed according to previous report with a slight modification [16]. Signals were detected using the TSA Plus Fluorescein System (Perkin Elmer) for fluorescent signal. The miRCURY LNA miR-205 detection probe (Exiqon) was 5'-DIG and 3'-DIG double labeled and hybridized at 61 °C. For co-staining with loricrin, the developed in situ slides were treated incubated with primary antibody against loricrin (1/100 dilution; GeneTex, Inc., USA). Subsequent antibody co-staining was performed as described previously.

2.3 Inhibition of miR-205 In Vivo

To prepare the 5 μM antagomir-205 in 30% Pluronic F-127 gel, 250 μL antagomir-205 oligonucleotides (RiboBio Co., Guangzhou, China) with concentration of 20 μM were mixed with 750 μL PBS. Then, 300 mg Pluronic F-127 were added into the 1 mL antagomir-205 solution and

incubated into 4 °C freezer overnight to get fully dissolved gel. To inhibit miR-205 level in the topical wound sites, a single 60 μl application of antagomir-205 Pluronic F-127 gel chilled was added and smeared into each pair of wounds immediately following wound induction. After treatment, the mice were fed separately. At 1–3 days after wounding mice were killed, the wounds and their surrounding area, including the scab and epithelial margins, were harvested with a 6-mm biopsy punch either for isolation of RNA (the upper two wounds) or histological analysis (the below two wounds) (Fig. 1). To determine the epidermal tongue length, the tool of segmented line of ImageJ software was used to measure the length of migrating tongue from the site of the epidermis with the broken dermis to the distal end. A minimum of five mice were used for each time point examined.

2.4 Cell Culture and Transfection

Human immortalized keratinocytes HaCaT cells and HEK293 cells were grown in DMEM medium (Gibco) supplemented with 10% fetal bovine serum. Cultures were maintained at 37° in humidified atmosphere of 5% CO_2. For TGF-β1 stimulation, HaCaT cells were cultured in the absence or presence of 2 ng/mL TGF-β1 (PeproTech) for 24 h. Antagomir-205 for inhibiting miR-205, agomir-205 for activating miR-205, and corresponding negative control oligonucleotides were purchased from RiboBio Co. (Guangzhou, China). The concentrations of miRNA-related oligonucleotides used in subsequent experiments were 20 nmol/L. siRNAs for ITGA5 have previously been described. In addition, a nonspecific scrambled siRNA duplex was used as control. All the siRNAs used in experiments were custom synthesized by GenePharma (Shanghai, China). The target sequences were listed as the following: si-ITGA5–1, CTCCACAGATAACTTCACCCGAA, and si-ITGA5–2, CCTCACTTACGGCTATGTC. The concentrations of siRNAs used in subsequent experiments were 50 nmol/L. SiRNA and miRNA transfections were performed using Lipofectamine 2000 (Life Tech, USA) according to the manufacturer's protocol.

2.5 Scratch-Wound Assay

Cells were seeded and grown to sub-confluency in standard culture medium and conditions. Then

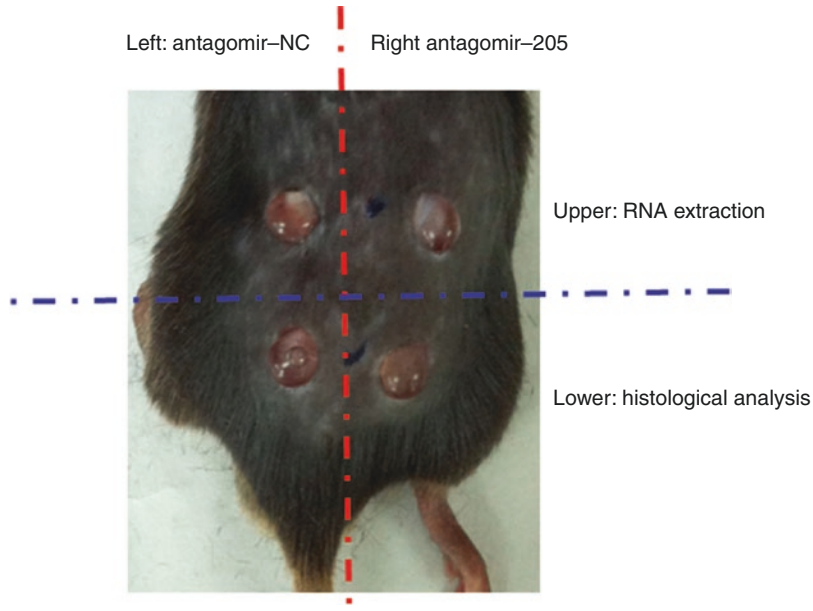


Fig. 1 The schematic diagram of inhibition of miR-205 in vivo

cells were transfected with miRNA or siRNA oligonucleotides. At 24 h post-transfection, cells were incubated for 2 h with mitomycin C (10 μg/mL) to prevent migration by cellular proliferation. For scratch-wound assay, a p200 pipe tip was used to scrape the cell monolayer in a straight line. Cells were washed twice with PBS to remove the debris. Wound assays were observed after 24 h. The percentage decrease in the wound gaps was calculated using the TScratch software and normalized to the time 0-h wounds. To make up the difference of initial wound gaps, the wound closure of the control group was set up as 100%, and the wound closures of other groups were shown as relative percentages to the control group.

2.6 RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted by the RNAiso Plus Reagent (TaKaRa, Japan) according to the manufacturer's protocol. For regular quantitative real-time PCR to examine ITGA5 and β-actin, the method was performed as described previously [29]. Primer pairs used are listed as the following: ITGA5 F, CTACAATGATGTGGCCATCG; ITGA5 R, GGATATCCATTGCCATCCAG; β-actin F, TCCCTGGAGAAGAGCTACGA; and β-actin R, AGCACTGTGTTGGCGTACAG. For detecting miR-205, Bulge-Loop miRNA qRT-PCR Primer Set (RiboBio Co.) was used according to manufacturer's instructions. The PCR results were normalized with U6 snRNAs as an internal control and then expressed as relative expression compared with related control samples.

2.7 Western Blot

Protein extracts and western blots were performed as described before [30]. The antibodies used were anti-ITGA5 (1/200 dilution; Sigma, St. Louis, MO, USA) and anti-β-actin (1/1000 dilution; Beyotime Biotechnology, Shanghai, China).

2.8 Luciferase Reporter Assay

The whole 3′UTR of human ITGA5 gene was cloned downstream of the luciferase reporter gene of pMIR-REPORT vector (Ambion). Mutation of the predicted target site of miR-205 was generated using the KOD-Plus-Mutagenesis Kit (Toyobo Co., Ltd.) according to the manufacturer's instructions. The PCR primers for mutation are listed as the following: hITGA5 mut F, ACTTCCTCCCTTGTTTACACATACCCTC, and hITGA5 mut R, CAGTGCATGGGGGGGAGGGATCCCC. The wild-type and mutation vectors were verified by sequencing (Invitrogen, Shanghai). For reporter assays, the luciferase vectors (150 ng/well) and pMIR-β-gal vector (150 ng/well) were co-transfected with agomir-205 or agomir-NC (20 nm) into HEK293 cells in 24-well plates using Lipofectamine 2000. Cells were harvested 24 h after transfection. Luciferase activities were measured using the Promega assay system normalized to β-galactosidase activities. The transfections were performed in triplicate, and all experiments were repeated three times.

2.9 Immunohistochemistry

Immunostaining of ITGA5 was performed on mice wound sample of 3 days after wounding and clinical sample of chronic wounds on paraffin-embedded sections as described previously [30]. The rabbit anti-ITGA5 (1/300 dilution; Sigma) was used and detected in 3,3′-diaminobenzidine tetrahydrochloride (Zhongshan Golden Bridge Bio., Beijing, China). Images of areas of interest were collected by Olympus IX73-A21PH microscope (Olympus, Japan).

2.10 Human Skin Specimens

Skin biopsies from consented venous ulcer patients were collected during surgical debridement procedures. Samples were collected from patients (n = 10) between the ages of 45 and 63 years old. This study was approved by the

local, domain-specific ethical review board in accordance with the Declaration of Helsinki. All participants gave written, informed consent.

2.11 Statistical Analysis

All data are presented as mean ± SD. Statistical analysis was performed by two-tailed Student's t-test to determine the significance between two groups, except for paired t-test. *P*-values <0.05 were considered to be statistically significant.

3 Results and Discussion

3.1 Downregulation of miR-205 in Migrating Epithelial Tongue After Wounding

Our previous study indicated that miR-205 increased for more than twofold at the stage of granulation formation during skin wound healing by miRNA microarray [11]. The in situ hybridization results showed strong miR-205 signals in the hyperplastic epidermis surrounding the wound but reduced signals in the leading migration keratinocytes (Fig. 2), which is one very interesting expression pattern. The results of miRNA microarray reflected the total expression change but less information of localization. The results of miR-205 in situ hybridization indicate strong induced expression of miR-205 in the hyperplastic surrounding epidermis, which may contribute the more than twofold change of miR-205 in the miRNA microarray, although there is obviously reduced expression of miR-205 in the migrating epithelial tongue. Similar situation is miR-203, which also upregulated more than twofold in our microarray but detected downregulated in the migrating epithelial tongue [16]. It has been reported that miR-205 plays essential role in promoting neonatal skin stem cell expansion during early development [19]. We also found miR-205 can promote HaCaT keratinocyte proliferation (unpublished data). Both epidermal proliferation and migration contribute to wound healing, but they appear to be somewhat mutually exclusive in space [4]. Thus, the downregulation of miR-205 in the keratinocytes of wound edge may be one common feature of wound healing and benefits keratinocyte migration.

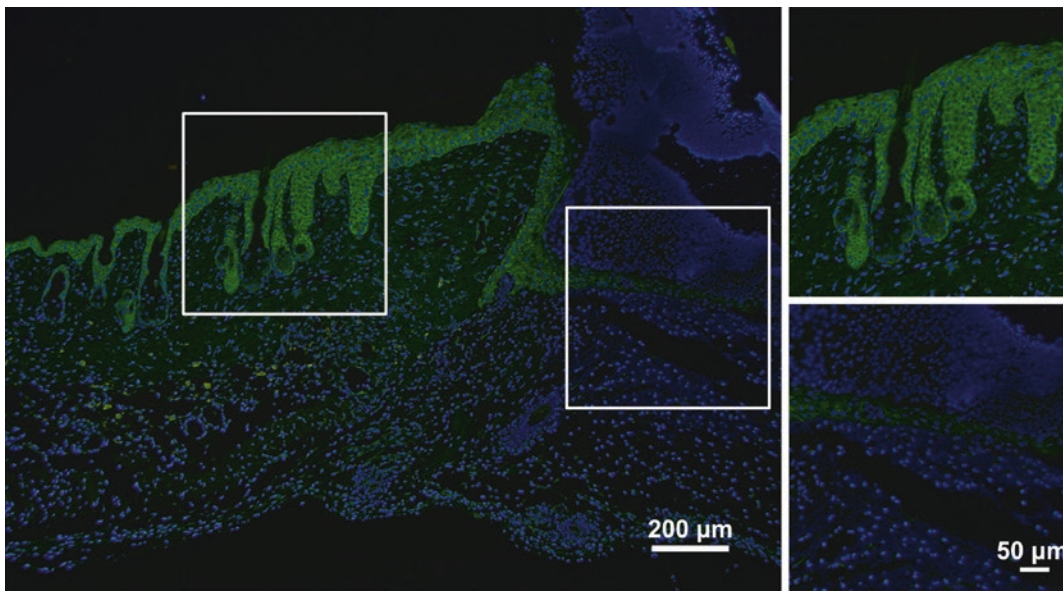


Fig. 2 Expression pattern of miR-205 in mouse skin wound healing. In the 3-day wound, miR-205 was downregulated in the leading edge of the migrating epithelial tongue

3.2 Downregulation of miR-205 Promotes Keratinocyte Migration

It has been reported that miR-205 is downregulated by TGF-β during epithelial to mesenchymal transition (EMT) [31]. Cutaneous wound reepithelialization is regarded as a partial and reversible EMT, in which TGF-β/Smad pathway plays an important role [4]. In accord with the downregulated miR-205 in the leading migration tongue, miR-205 was significantly reduced after TGF-β exposure in HaCaT cells [32]. This suggests that TGF-β is key factor involving downregulation of miR-205 in the migrating keratinocytes during reepithelialization, which is similar to the regulation of TGF-β on miR-198 during skin wound healing [13].

Given that miR-205 is markedly downregulated both in the leading migrating keratinocytes during reepithelialization in vivo and in the TGF-β1-treated HaCaT cells in vitro, we further investigated the role of miR-205 in keratinocyte migration. As expected, miR-205 knockdown can promote keratinocyte migration in both HaCaT keratinocyte wound scratch model in vitro (Fig. 3) and mouse skin wound model in vivo (Fig. 4). These results were in agreement

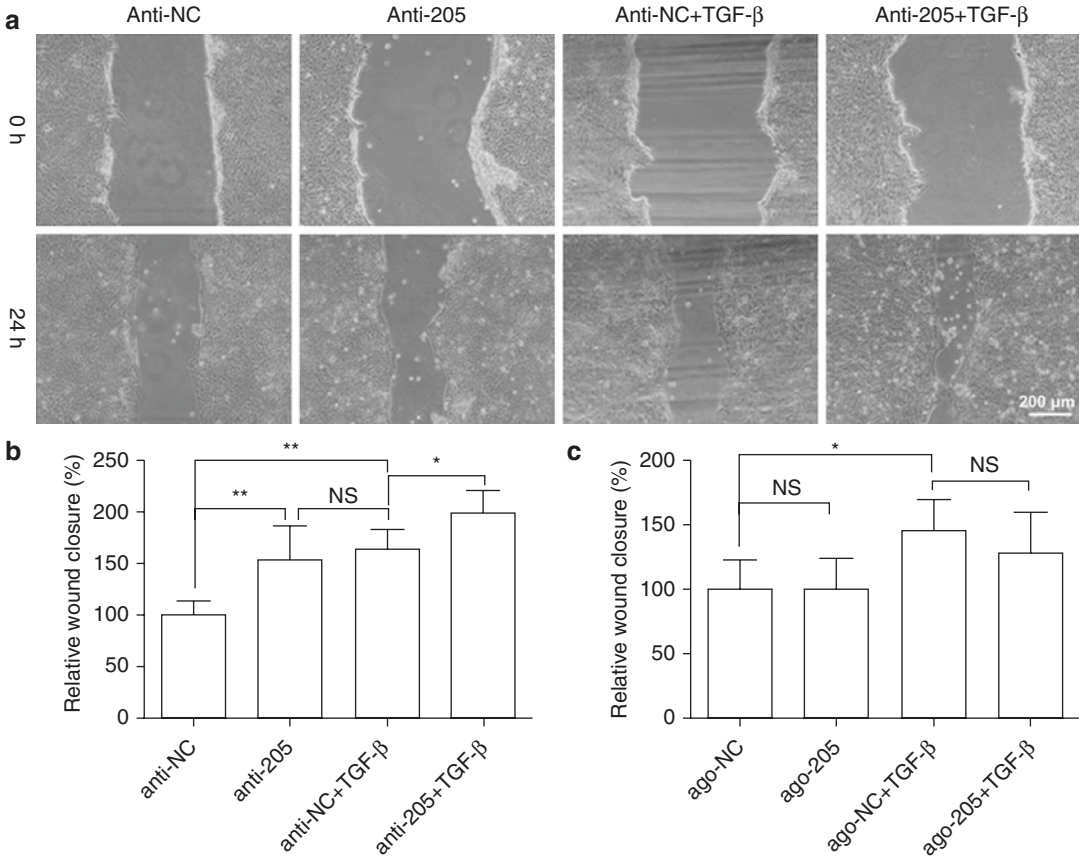


Fig. 3 Downregulation of miR-205 promotes keratinocyte migration in vitro. (**a**) Scratch assays to assess the migration rate of HaCaT cells transfected with antagomir-205. (**b**) To calculate the healing rate, wounds treated with antagomir-205 (in triplicate) were photographed, and the percentage of wound closure from a representative experiment ($n = 6–8$) was measured in indicated time points using TScratch software. And the wound healing area of cells treated with control oligonucleotides was taken as 100%. Then the relative wound closure was calculated. (**c**) Scratch assay to assess the migration rate of HaCaT cells transfected with 20 nM agomir-205

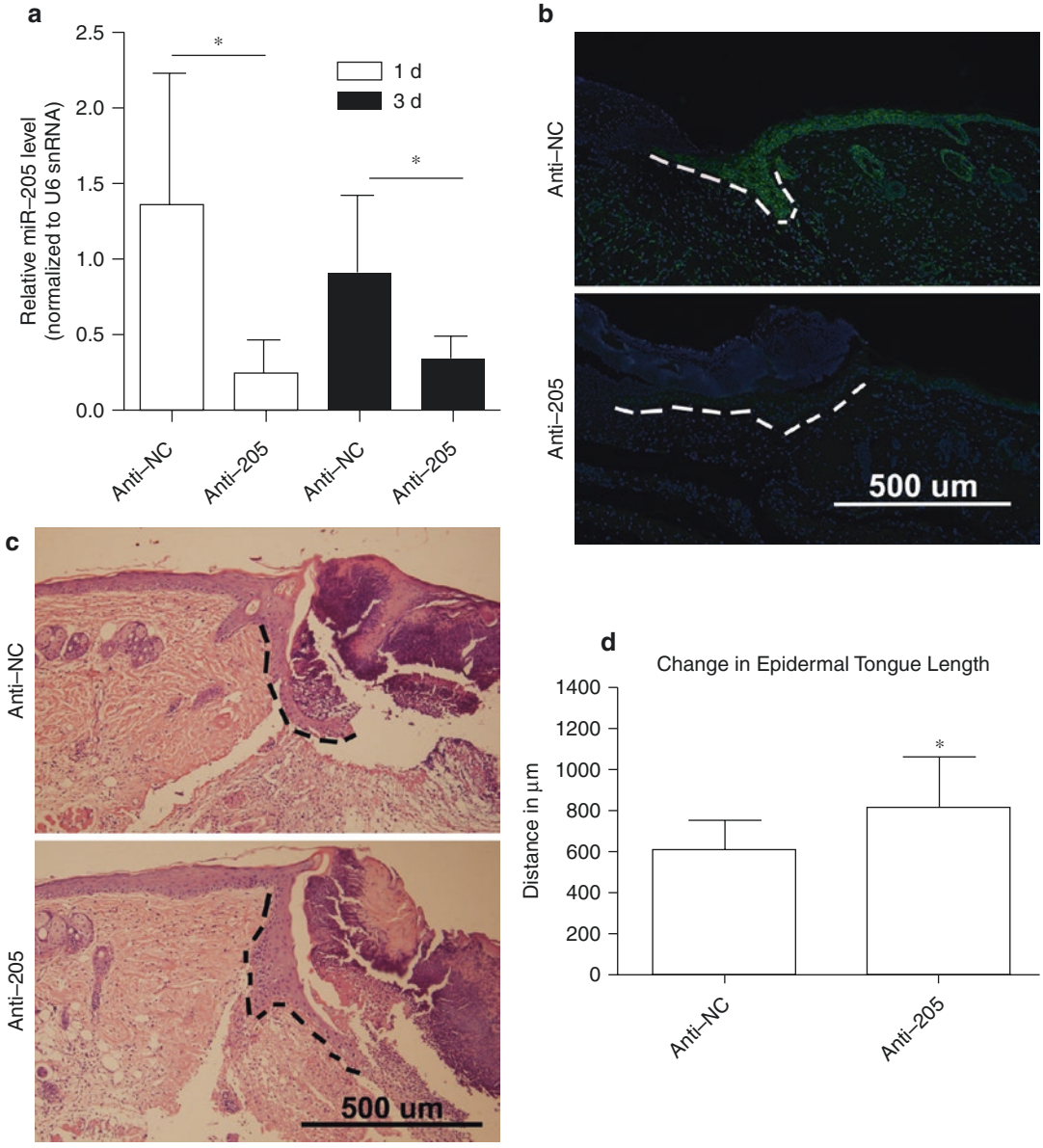


Fig. 4 Inhibition of miR-205 promotes reepithelialization in vivo. (**a**) Real-time PCR results showed that miR-205 at wound edge treated with antagomir-205 were effectively reduced compared with that at control wound edge at both 1–3 days after injury. (**b**) In situ hybridization result indicated that antagomir-205 treatment reduced signals significantly at wound edge 3 days after injury. (**c**) H&E sections of the day 3 wounds with migration tongues. The lengths of the migrating tongues were shown with corresponding black dotted lines. (**d**) Quantification of the migrating tongue lengths. A minimum of eight tongues from each group were measured

with the previous studies in cancers that loss of miR-205 contributes to migration and invasion [24, 28]. Recently, results of others indicated that miR-205 can promote migration of both primary human epidermal keratinocytes (HEKs) and corneal epithelial keratinocytes (HCEKs) [20, 21]. We used to think the contradiction came from different medium used for cell culture. We presumed that the HaCaT DMEM medium has relatively higher-level calcium ion to induce the intercellular

junctions between cells, which is much closer to the actual situation of the skin. However, the HaCaT cultured without calcium also showed coincident results (unpublished data). So, the reason of the different effects of miR-205 on migration of HaCaT and HEKs needs to be further investigated.

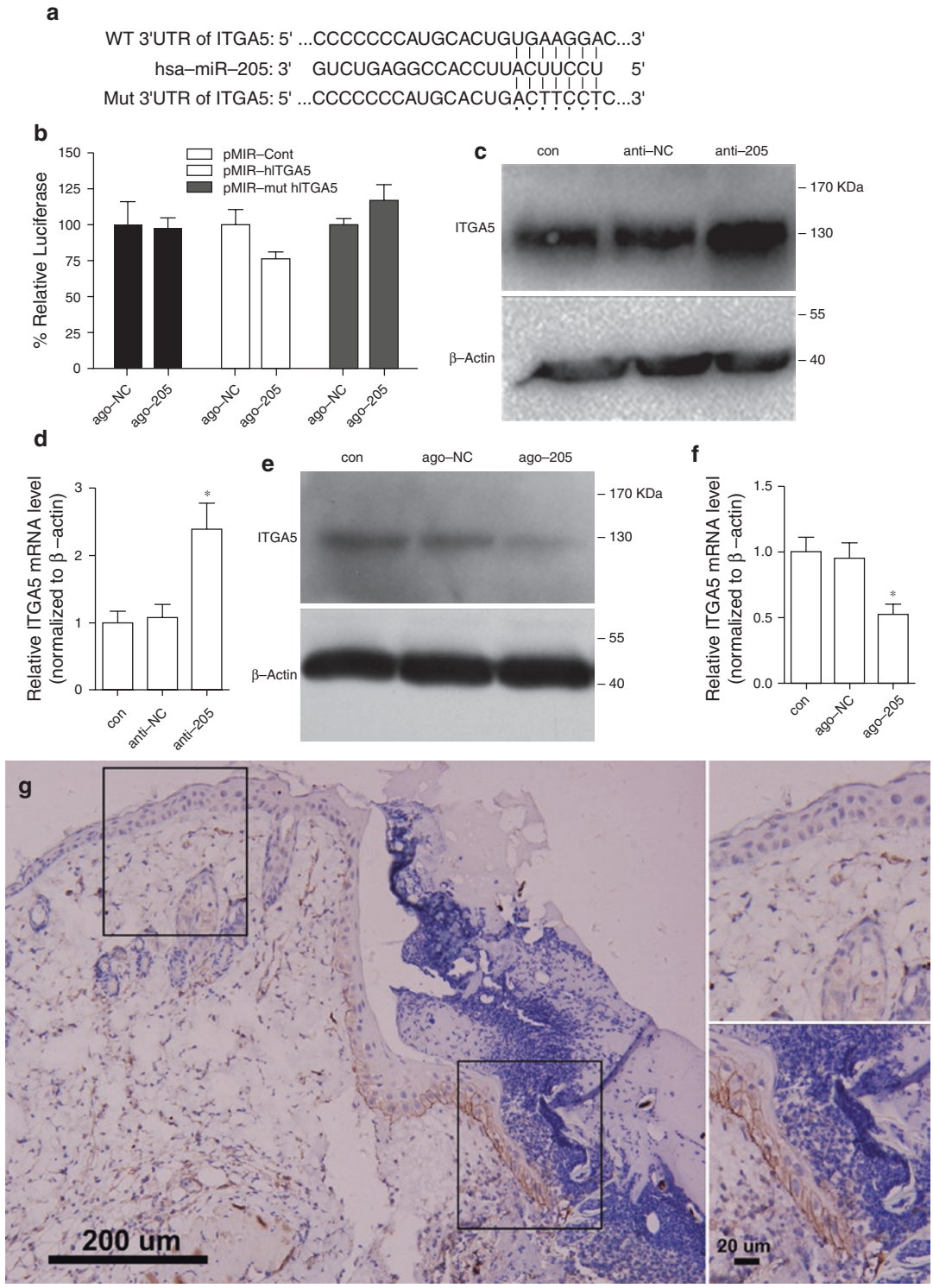
3.3 ITGA5 Is One Functional Target of miR-205 in Keratinocytes

To interpret the role of miR-205 in regulating reepithelialization, we identified ITGA5 as one important functional target gene (Fig. 5). Integrins are heterodimeric membrane glycoproteins involved in cell-ECM and cell-cell interactions. Among them, integrin $\alpha_5\beta_1$ is the classical fibronectin (FN) receptor, and the interaction between ITGA5 subunit and FN is very important for keratinocyte migration during skin wound healing process [33, 34]. Indeed, epidermal keratinocytes under homeostatic condition do not express ITGA5 and FN, but their expressions are highly induced following skin injury [35–37]. The interaction between $\alpha_5\beta_1$ receptors expressed on the surface of keratinocyte and FN localized in the basement membrane is crucial for keratinocyte migration. The deficiency of $\alpha_5\beta_1$ receptors would interfere with epidermal keratinocytes to close the wound. It has been showed that although the level of FN is heavily upregulated in chronic wounds, the ITGA5 subunit is not induced in the epidermis of chronic wounds [38]. Thus, the failure to express appropriate levels of ITGA5 on epidermal keratinocytes in chronic wounds may contribute to the healing defect in these wounds. Recently, miR-205 has been shown to target ITGA5 gene to inhibit lung cancer cell invasion and metastasis [28]. We showed that knockdown of miR-205 could induce ITGA5 mRNA and protein expression in keratinocytes in vitro. And also, we observed that miR-205 is downregulated in the migrating epithelial tongue along with induction of ITGA5 in these migrating keratinocytes. While in our study, knockdown of ITGA5 in HaCaT cells has no effect on migration, which is in line with the wound scratch results of agomir-205-treated cells. We presume that the relatively low basal level of ITGA5 in HaCaT cells may account for these results, which is just like the homeostatic keratinocytes without ITGA5 expression. While when these siRNAs co-transfected with antagomir-205, the pro-migratory effects of miR-205 knockdown were abolished, which suggest ITGA5 is one important target in modulating the effects of miR-205 on keratinocyte migration [32]. Of note, some growth factors having important roles in wound healing like VEGFA, TGFA, and epiregulin also predicted targets of miR-205, which suggests downregulating miR-205 in the migrating epidermal tongue may be beneficial to inducing these growth factors during wound and then promotes healing in paracrine and/or autocrine models.

3.4 Dysregulation of miR-205 and ITGA5 in Venous Ulcers

In contrast to the normal acute wound healing characterized by activation of the TGF-β pathway, studies have demonstrated attenuation of TGF-β signaling in the epidermis of non-healing chronic wounds [13, 39–41]. Also, it has been reported that ITGA5 is significantly induced in the active migration keratinocytes during acute skin wound healing, but not induced in the epidermis of chronic wounds [38]. As we have

Fig. 5 ITGA5 is one direct target of miR-205 in keratinocytes. (**a**) The miR-205 binding site in the 3′UTR of ITGA5 mRNA predicted by TargetScan Human 6.2 and the mutation used in the luciferase reporter construct. Dots indicate the nucleotides that were mutated in binding site. (**b**) Luciferase assay showed that miR-205 inhibited wild-type but not mutated ITGA5–3′UTR reporter activity. (**c**, **d**) HaCaT cells were transfected with antagomir-205 or control at 20 nM for 24 h. ITGA5 expression was analyzed by western blot (**c**) and real-time PCR (**d**). (**e**, **f**) HaCaT cells were transfected with agomir-205 or control at 20 nM for 24 h. ITGA5 expression was analyzed by western blot (**e**) and real-time PCR (**f**). (**g**) IHC analysis of ITGA5 expression at day 3 after wound


a
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hsa–miR–205: 3' GUCUGAGGCCACCUUACUUCCU 5'
Mut 3'UTR of ITGA5: 5' ...CCCCCCCAUGCACUGACTTCCTC...3'
b
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pMIR–mut hITGA5
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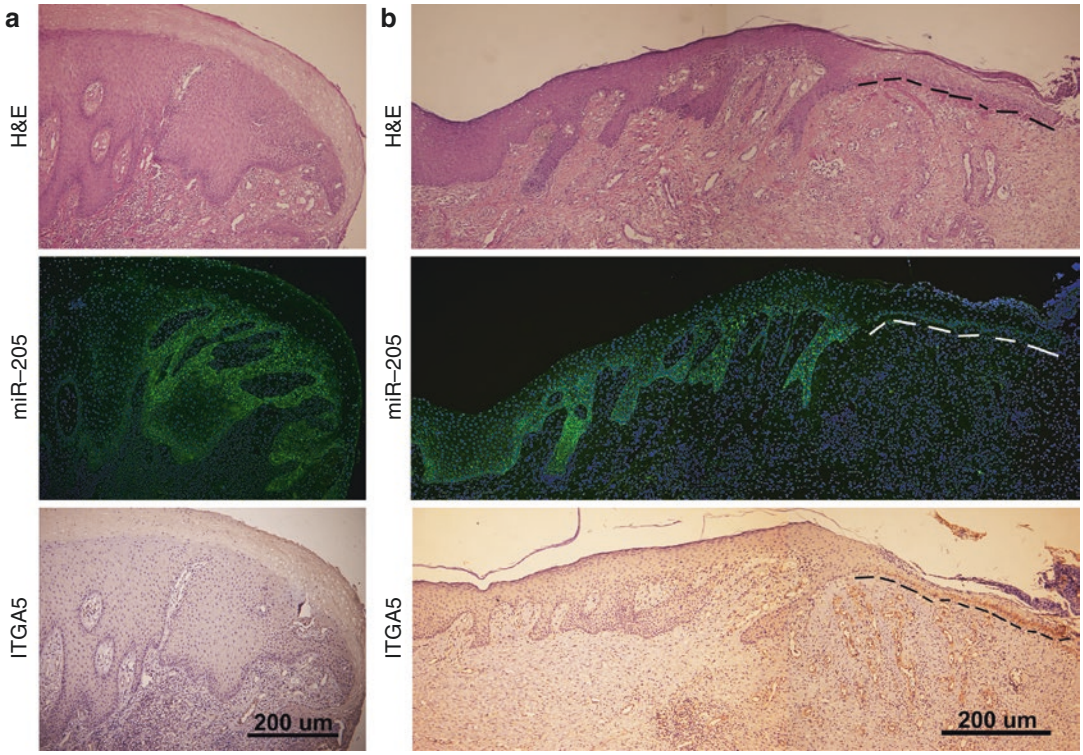

Fig. 6 Expression of miR-205 and ITGA5 in human chronic venous ulcer. Histological analysis of both (**a**) typical and (**b**) atypical human chronic venous ulcer samples. From the top: H&E, in situ hybridization of miR-205, IHC analysis of ITGA5

shown that TGF-β stimulation could downregulate miR-205 level in HaCaT keratinocytes [32], we speculated that dysregulation of miR-205 and ITGA5 may contribute to the non-healing chronic wounds. It is as expected that there are persistence of high-level miR-205 and absence of ITGA5 in the typical clinical chronic wounds (Fig. 6), which is similar to the expression pattern of miR-198 and its targets [13]. Meanwhile, we introduced some atypical chronic venous ulcers featured with relative thinner epidermis, distinct migrating epithelial tongue, and more ITGA5-positive vessels in the granulation (Fig. 6), which further confirmed the reciprocal expression pattern of miR-205 and ITGA5 in the migrating epithelial tongue during wound healing. As to clinical prognosis of these two types of chronic venous wounds, it will take a time for us to collect enough clinical samples to analyze and draw a conclusion.

Conclusions

The present study has demonstrated the involvement of miR-205 in regulating reepithelialization during wound healing. Our findings indicate that downregulation of miR-205 in the leading migrating keratinocytes is critical for reepithelialization by desilencing ITGA5. What's more, there is dysregulation of miR-205 and its target ITGA5 in the epidermis of clinical venous ulcer samples with persistence of high-level miR-205 and absence of ITGA5. Further investigation into the expression pattern of miR-205 in other types of chronic wounds like diabetic foot ulcer and pressure ulcer is necessary. And, given that miR-205 may be a potential therapeutic target for chronic wounds, it is also necessary to test the effects of miR-205 inhibition on wound healing in animal chronic wound models.

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Porcine Acellular Lung Matrix in Wound Healing and Hernia Repair

Vishwanath Chegireddy, Koby D. Caplan, and Joseph S. Fernandez-Moure

1 Introduction

Hernias are among the most common conditions of surgical care with more than 300,000 surgeries occurring annually in the USA alone [1–3]. The volume and complexity of hernia surgery continues to remain a challenge because of increasing life expectancy, a proportionally older surgical population, and a growing population of morbidly obese and diabetic patients with other comorbidities, which influence native strength, and perfusion of tissues [4, 5]. Hernias necessitating emergent surgery are on the rise [6]. Five to 27% of people will develop a hernia over the course of their lifetime [7, 8]. Although hernia repairs are performed very frequently, they remain vulnerable to numerous complications such as infection and recurrence.

This socioeconomic burden would greatly benefit from a reduction in these complications; by reducing the rate of recurrence alone, the healthcare system would save $32 million for every 1% reduction in repeat operations [1]. Many of these issues are due to the lack of ideal materials used as mesh in the site of herniation. Reinforcing a repair mesh is the standard of treating incisional hernias given the high likelihood of recurrence with suture repair alone; however the material may migrate from the hernia site, become infected, or erode into adjacent structures [9–13]. Chronic pain due to complications related to mesh is an issue and is generally relieved by surgery to remove it [14–16]. To try and overcome these shortcomings and combat the problems plaguing the industry of synthetic mesh, manufacturers and researches have created acellular biological prosthetics to be used in hernia operations [4, 17, 18]. Although expensive, bioprosthetic meshes—constructed from human, bovine, or porcine tissue—have yielded promising results so far, particularly in contaminated fields and have therefore received much clinical and commercial attention [4, 19, 20].

2 The Ideal Mesh

The introduction of synthetic polypropylene mesh in hernia repair revolutionized abdominal wall repair, winning Natta and Ziegler the Nobel Prize in 1954 [7, 21]. Yet synthetic mesh is far from a perfect solution. To introduce mesh is to

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introduce foreign bodies that can impact the human body. They may lead to inflammation, infection, fibrosis, calcification, seromas, or adhesions to vital organs such as the bowel [22–25]. Meshes cause abnormal physiologic wound healing and scar formation, altering the ratio of type I to type III collagen, which decrease mechanical stability regardless of the mesh used [26–28]. In order to minimize these complications from an immune response, recognizing synthetic mesh as a foreign body, a new question emerged; would it be possible to transplant already existing architecture into which vessels can regrow and fibroblasts can remodel? To address the problems of synthetic mesh, researchers sought to find acellular matrices for reconstruction.

An ideal mesh is characterized by its strength, flexibility, and host tissue compatibility; no synthetic or biologic mesh has yet to fulfill all these criteria, and currently there is no true gold standard [21, 29, 30]. The metrics of a biological prosthetic mesh's success is revascularization and cell repopulation in the tissue [31–33]. An ideal mesh must cause a significant enough of an inflammatory response to signal fibroblasts to deposit collagen, but tame enough to limit excessive scarring, graft encapsulation, and degradation [4]. Angiogenesis must occur to allow for tissue remodeling; otherwise the graft will be replaced by scar tissue [4]. Vascularization and cell repopulation are signs that demonstrate a mesh will incorporate well, hereby decreasing the odds of recurrence and infection [32]. Through the body's natural healing processes, biological meshes are exposed to proteinases and collagenases that degrade them over time, thus weakening the repair. Cellular infiltration and angiogenesis of decellularized tissue constructs is not only dictated by the tissue source but inherently the microarchitectural cues innate to that tissue. Thus, much work in the realm of tissue engineering and regenerative medicine has been performed to understand the structure and biochemistry of acellular scaffolds.

3 Tissue Engineering and Hernia Repair

The fields of regenerative medicine and tissue engineering hold the promise of revolutionizing the practice of medicine and surgery. Many patients in need of tissue and replacement organs may stand to benefit from the advancement being made in the field of tissue engineering. By combining the principles of engineering and life sciences, tissue engineers seek to develop biological substitutes capable of not only providing a scaffold for innate cellular infiltration but in some ways facilitating drug delivery and cellular delivery in hopes of promoting enhanced healing by the patient's own body. Specifically, in the realm of hernia repair, many investigators have sought new tissues or tissue constructs to be adapted to the repair of hernias in hopes of overcoming the adverse effects of synthetic material foreign body reactions that lead to the complications seen following hernia repair. Of those, the use of decellularization technology has emerged to generate acellular tissue constructs capable of maintaining adequate mechanical strength while simultaneously providing a biological scaffold that would allow the body to remodel and replace with its own living tissue.

4 Bioprosthetic Mesh

In response to growing evidence of the adverse outcome associated with synthetic mesh repair, physicians and researchers alike sought to investigate alternative approaches to hernia repair with a focus on the prosthetic used. Bioprosthetic meshes, generated from source organs such as the dermis or small intestine, have emerged as commercially available products for use in hernia repair. There are several commercially available bioprosthetic meshes with new products emerging annually. Currently, they are indicated for ventral hernias in contaminated settings but can be used in a variety of scenarios and have been advocated for as an alternative to the more

Table 1 Commercially available bioprosthetics

Material	Product	Manufacturer	Properties
Human dermis	AlloDerm	LifeCell	Non-cross-linked, aseptic
Human dermis	FlexHD	Ethicon	Non-cross-linked, aseptic
Human dermis	AlloMax	Bard Davol	Proprietary process
Porcine dermis	Permacol	Covidien	Chemically cross-linked Terminally sterilized
Porcine dermis	Strattice	LifeCell	Non-cross-linked Terminally sterilized
Porcine dermis	XenMatrix	Bard Davol	Non-cross-linked, electron beam sterilized
Porcine intestine	Surgisis	Cook	Modified submucosal matrix, non-cross-linked
Bovine pericardium	Veritas	Synovis	Multidirection dense connective tissue
Bovine pericardium	Tutopatch	Tutogen	Non-cross-linked bovine pericardium
Bovine dermis	SurgiMend	TEI Biosciences	Fetal dermis, non-cross-linked
Porcine liver	Miromesh	Miromatrix	Non-cross-linked
Porcine urinary Bladder matrix	Gentrix Surgical Matrix	ACell	Porcine derived extracellular matrix

Adapted from (http://plasticsurgerykey.com/biologic-mesh-choices-for-surgical-repair)

commonly used synthetic meshes [4, 34]. Unfortunately, the lack of high-quality scientifically rigorous studies inhibits our ability to determine a gold standard for bioprosthetic meshes [29]. Further, with the lack of American Society for Testing and Materials (ASTM) International standard for biological tissues, considerable heterogeneity is seen from product to product of the same tissue and even within the same product line [35]. Commercially available biological products include homografts and xenografts from bovine and porcine sources (Table 1). In addition to different species, brands may be classified by organ of origin.

5 Dermis

There have been major advances in the field of bioprosthetics for ventral hernia repair using allogenic and xenogeneic materials. AlloDerm™ (LifeCell Corp, Branchburg, NJ) has been one of the most utilized and most studied homographs available today. Made from human cadaveric acellular dermal matrix (hADM), AlloDerm has been used as a tissue-grafting substitute for decades, although it has not been used for abdominal wall reconstruction and has decreased recently [36]. It has yielded promising results, helping to spur the movement for biomesh [36]. Some authors, however, have described laxity of the material and the propensity to stretch over time, leading to the development of pseudo-recurrences [29, 36]. The processing of bioprosthetics involves decellularization via a variety of methods to influence the native biochemical and biomolecular structure of the collagen scaffold [37]. The processing of hADM is one example of how processing of a tissue may change its architecture [38]. Compared to native hADM, the processed tissue underwent noticeable changes to the ultrastructure (Fig. 1) [38]. The loss of the collagen integrity after processing may lead to an increased inflammatory reaction and increased fibrous capsule [38]. While the mechanical properties may not be ideally suitable for incisional hernia repair where strong mechanical forces are applied to the prosthetic, hADM has been used extensively in breast reconstruction and tissue expansion prior to definitive reconstruction because of its robust ability to neovascularize [36, 39–41].

Porcine dermal tissue has also been a common biological mesh used in abdominal

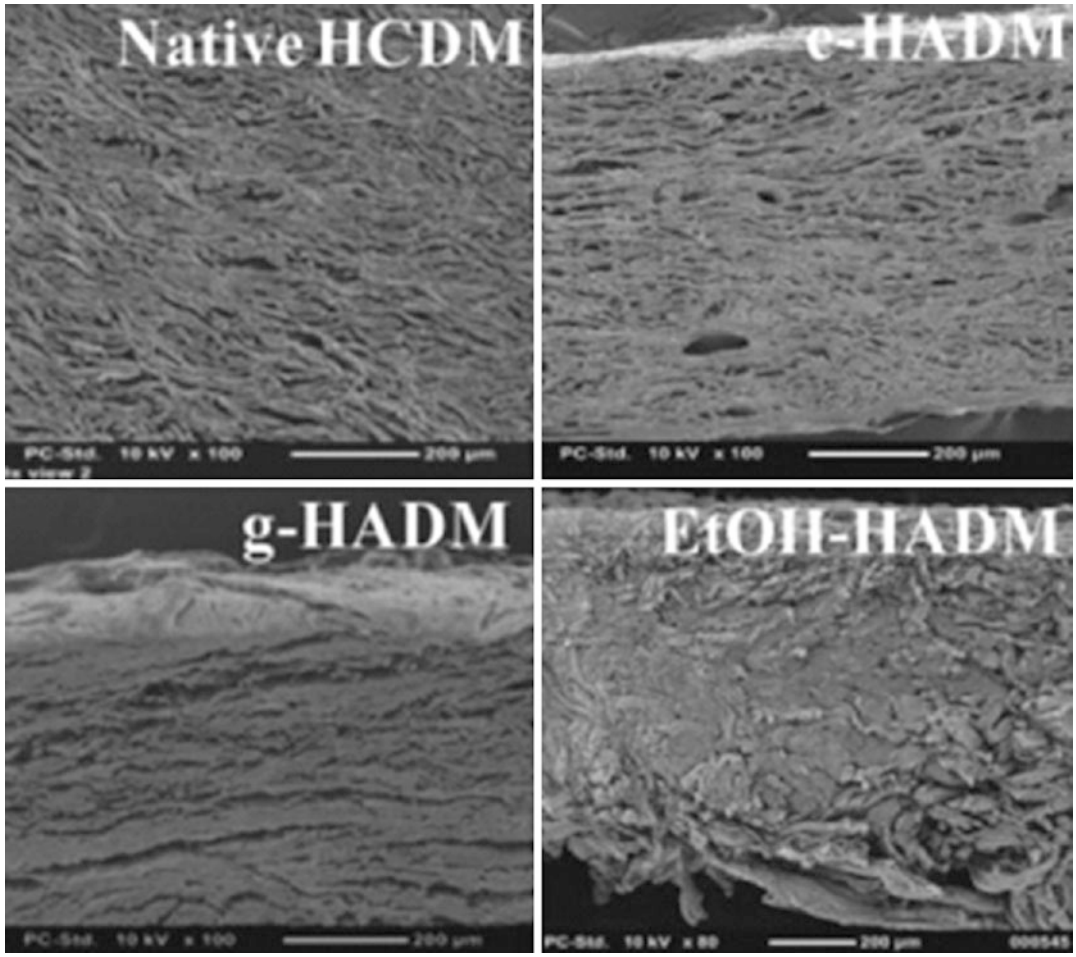

Fig. 1 Scanning electron micrograph of out-of-package morphology of hADMs (100×). Native hADM, electron beam-irradiated hADM (e-HADM), γ-irradiated hADM (g-HADM), ethanol-stored hADM (EtOH-hADM) [38]

wall repair, Strattice™ (LifeCell Corporation, Bridgewater, NJ, USA) [42]. This non-cross-linked porcine ADM has been shown to have minimal adhesion formation and bowel erosions, complications associated with synthetic mesh repair, but is predisposed to recurrence, particularly when used as a bridge [4]. Butler et al. [43] reported non-cross-linked PADM to have a quicker infiltration with host cells and vessels in comparison to cross-linked PADM, which were encapsulated. Also, non-cross-linked PADMs had weaker intraperitoneal adhesions at repair sites with increasing mechanical strength at an earlier time at the bioprosthesis-musculofascial junction. More recently the use of non-cross-linked porcine ADM has increased as studies continue to demonstrate its ability to resist infection and withstand mechanical forces of the abdominal wall [44, 45]. The rates of recurrence are similar to those with synthetic mesh repair when used in conjunction with component separation. The use of acellular dermal matrices in hernia repair and abdominal wall reconstruction was associated with 11.5–14.6% hernia recurrence rates at 3-–5-years follow-up [46].

Permacol™ (supplemental cross-linked; Covidien, New Haven, CT, USA) is a bioprosthetic made from porcine dermal collagen with post-processing cross-linking of proteins. The

cross-linking is performed in order to add strength to the material and reduce its inflammatory response [47]. Traditionally, porcine acellular dermal matrices (PADMs) were noted to induce greater immune response than human acellular dermal matrix (hADM) and thus have been processed to chemically cross-link the collagen fibers [43]. Collagen cross-linking has been noted to have a key role on tissue response to biologic meshes, which alters the extracellular matrix structure and possibly inhibits cellular infiltration, revascularization, and matrix remodeling potential [48]. The structure, similar in structure to human dermis, can support fibroblast infiltration and neovascularization [49]. However, its strengthened cross-linked architecture may actually hinder the material's success in remodeling and neovascularization [29, 50]. Integration of Permacol™ into host tissue and angiogenesis, though delayed, help to facilitate antibiotic diffusion and help to resist infections [51]. Some studies noted the rate of complications in cross-linked porcine dermal collagen mesh was double than that of their non-cross-linked porcine dermal counterparts [43].

6 Small Intestinal Submucosa

Small intestinal submucosa (SIS) is another tissue for decellularized matrices used in abdominal wall reconstruction. Originally investigated as a bioprosthetic for use in vascular repair, SIS emerged as a potential prosthetic for use in hernia repair [52, 53]. Today, the most commonly placed SIS mesh in abdominal wall repair is constructed from porcine SIS, Surgisis™ (Cook Surgical, Bloomington, IN). It notably has a lower recurrence rate than AlloDerm and Permacol in a comparative study (8.0% rather than 20.8% and 10.9%, respectively) [54]. Some authors note that Surgisis starts strong at first, but loses strength with remodeling [26, 55]. The small intestine is a more vascular organ than the dermis, and neovascularization could play a role. Studies investigating its use in laparoscopic hernia repair were promising, and more recently its use in the contaminated field has been shown to be safe [56, 57].

7 Bovine Pericardium

Bovine pericardium consists of collagenous connective tissue with three-dimensional intertwined fibers. Initial studies showed that the bovine pericardium might not have stood up to the test in hernia repair with either early resorption or poor incorporation in animal models [58]. Tutomesh® (Tutogen Medical GmbH Germany) has been praised for retaining multidirectional strength and keeping the elasticity of the original tissue, yielding good results [29, 59]. The product has less elastin relative to dermal products, resulting in a higher ratio of mature collagen to elastin and reducing pseudo-recurrence [60]. When compared to fascia lata, it was found to be superior in burst strength and adhesion formation [61]. More recently, attention has been paid to its potential use as a prosthetic in contaminated hernia repair. It has been demonstrated to be safe and effective in repair in the contaminated field and particularly in the setting of bowel resection [62].

8 Liver

Biologic mesh has been an available alternative to permanent synthetic mesh for over 20 years. Various biologic meshes, specifically porcine tissue prostheses, have been evaluated. More recently, porcine liver has been decellularized for use in both transplantation science and in decellularized implant tissue engineering (Fig. 2) [63–65]. Petro et al. [66] have demonstrated application of porcine liver prosthesis for hernia repair. They utilized a novel prosthetic, Miromesh, a biologic mesh derived from porcine liver. They were able to demonstrate the efficacy of Miromesh in comparison to Strattice in regard to cellular infiltration, acute inflammation, chronic inflammation, granulation tissue, foreign body reaction, and fibrous capsule formation

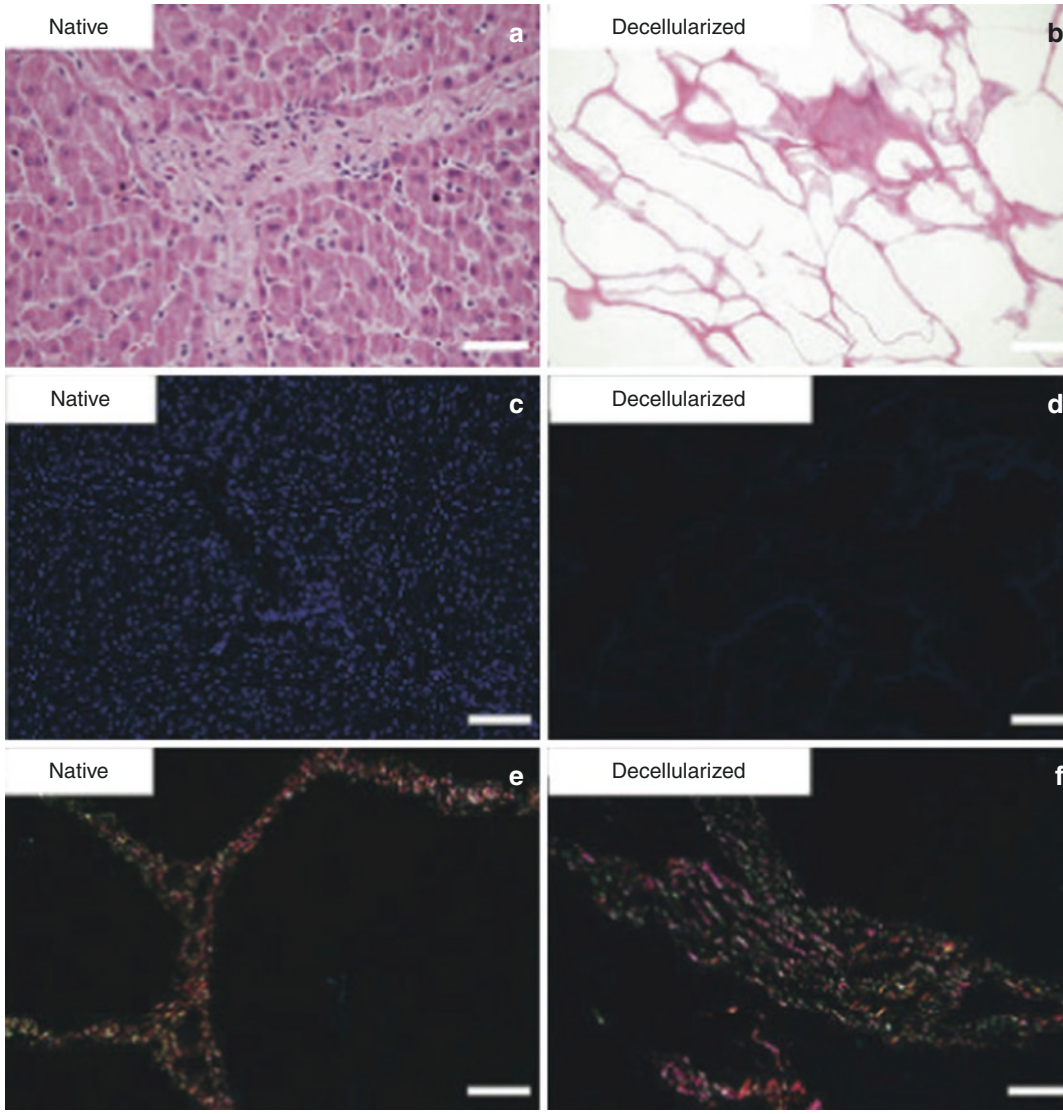

Fig. 2 Native and decellularized liver. Representative H&E staining of the ultrastructure of native liver (**a**) and decellularized liver matrix (**b**). Scale bars = 100 μm. DAPI staining demonstrated a lack of nuclear components suggestive of complete decellularization (**c**, **d**). Sirius red staining of different types of collagen and proteoglycan shows retention of important ECM substrates (**e**, **f**). Scale bars = 200 μm [63]

[66]. The advantage in using Miromesh compared to Strattice and other dermal prostheses is its proprietary perfusion decellularization with intact portal triads that provide optimal collagen scaffold for cellular infiltration. The study conducted revealed that Miromesh had greater cellular infiltration with comparable clearance of bacteria. However, there are some weaknesses associated with the use of Miromesh, which include heterogeneity, large porosity, and lower density matrix, which could negatively impact the longtime durability and mechanical ability of the inserted mesh. Further studies are needed to investigate the use of porcine liver prostheses.

9 Use in the Contaminated Field

Since the introduction of biologic mesh material, it has been viewed as a promising alternative to synthetic mesh by providing cellular infiltration, neovascularization, and potentially regeneration into native tissue [67, 68]. These specific properties of biologic material may lead to superior outcomes over synthetic material in the setting of contamination [67]. In addition, biologics have been used with some success to repair complex abdominal wall defects in clean-contaminated and infected fields when synthetic mesh is contraindicated. Management of contaminated ventral hernia repairs has been evaluated over the years; however, there is still no consensus about the most optimal and durable repair. Some authors have argued for a multistage reconstructive approach, which includes delayed definitive reconstruction 6–12 months later with component separation when inflammation and dense adhesions have resolved [69]. Rosen et al. [67], in a retrospective study, were able to demonstrate that biologic mesh reinforcement can be safely performed in the repair of ventral hernias in contaminated fields in a single-stage approach. Despite a high rate of wound morbidity in the study, this did not lead to complete mesh excision nor did it include mesh infections. The study evaluated patients undergoing single-staged ventral hernia repairs in a contaminated field using biologic mesh over a 5-year period. The outcomes included postoperative wound complications in 47.7% and hernia recurrence in 31.3% of the patient population. The high rate of wound morbidity can be accounted for based on the ASA score, recent history of smoking, diabetes mellitus, number of previous abdominal surgeries, number of previous hernia repairs, hernia defect size, bridged defects, and long operative times [67]. Short-term efficacy can be noted with the probability of recurrence at 1 year at 8%. When taken to 24 months, patients who underwent a hernia repair in the contaminated setting had successful repair as a single-stage procedure using porcine ADM [70]. Few randomized controlled comparative studies have compared complication rates on all biomeshes on the market, and currently there is no gold standard [71]. More research is needed to find the most cost-effective mesh with the fewest instances of recurrence. As the source tissue for decellularization continues to expand, we must continue to broaden our view of what is considered a possible tissue. Many organs because of their inherent mechanical strength may not initially be thought of as candidate for hernia repair. Many of the tissues used are connective in nature, but because of this property may not be optimized for the biologic response required for long-term repair. An alternative approach, focusing on the potential for biologic optimization and incorporation, may provide answers as to the expansion of potential sources such as the lung.

10 Porcine Acellular Lung Matrix

The generation of decellularized lung tissue began with an effort to address the growing need for tissue-engineered approaches for whole lung regeneration [72]. Of the organs procured for transplantation, the lung is the most sensitive to ischemia and the most damaged as a result of the retrieval and preservation process [73]. While transplantation wait list times continue to go down, the need for organs has not and the need is as great as ever. One approach to this grave problem is the regeneration of whole lung tissue. While other organs have undergone decellularization processes in order to make them suitable for implantation, the lung, with a complex and multifunctional system, had not been investigated for this purpose in humans. In his seminal work, Ott et al. [74] described and perfected the process of lung decellularization and recellularization with autologous cells and implantation (Fig. 3). This work paved the way for more advanced approaches and scaling up of the model for human use.

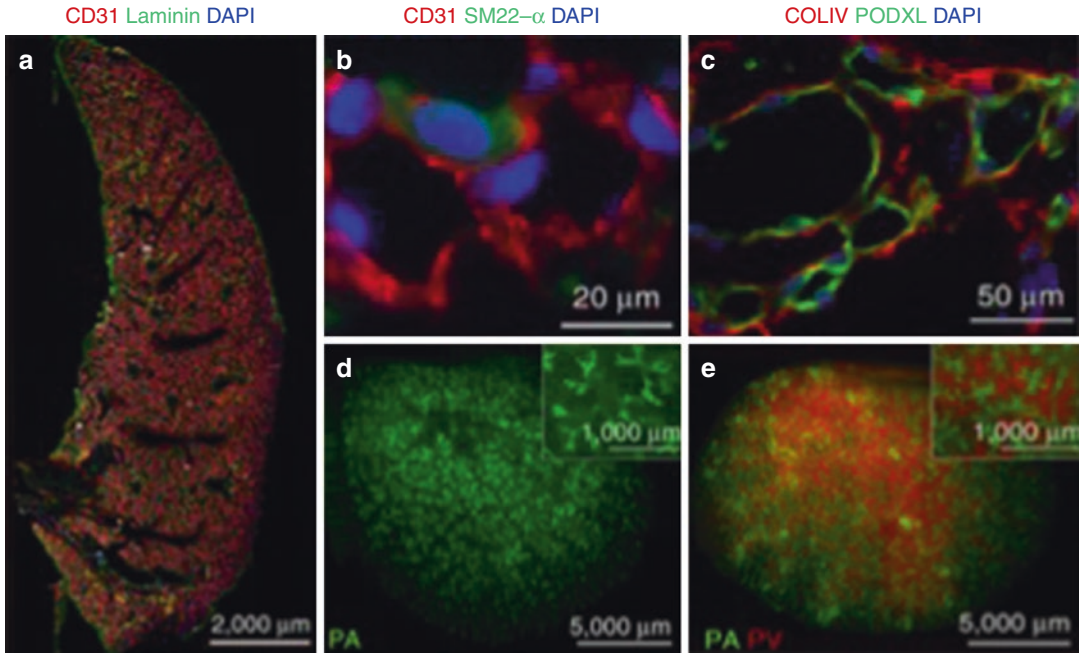

Fig. 3 Decellularized lung is capable of cell adherence and proliferation. (**a**) A representative stitched image showing endothelial coverage of a HUVEC-hMSC regenerated lung lobe after two-phase culture (CD31, red; laminin, green; DAPI, blue). (**b**) Interconnected vascular network structures formed by endothelial cells (CD31, red) with individual hMSCs (SM22-α, green) adhering to the network. (**c**) Establishment of apical-basal polarity shown by localization of PODXL (green) on the luminal surface and COLIV (red) on the basement surface. (**d**) A representative whole-mount image of decellularized rat lungs perfused with green-fluorescent microspheres (0.2 µm) through the PA. (**e**) A representative whole-mount image of decellularized rat lungs perfused with green-fluorescent microspheres (0.2 µm) through the PA and red-fluorescent microspheres (0.2 µm) through the PV

Nichols et al. have described porcine acellular lung matrix (PALM) as a natural scaffold capable of cell attachment while maintaining cell viability [72, 75]. Porcine lungs were taken and processed through both mechanical infusion of decreasing gradient of SDS (Fig. 4) [75]. In their work, they were able to demonstrate that PALMs can sustain forces of mechanical ventilation for prolonged periods of time without changes to macro- or microstructure of the tissue due to its predominance of collagen I and elastin [75]. In addition, PALMs were noted to have minimal inflammatory response with minimal apoptosis of mesenchymal stem cells or human alveolar epithelial cells. The extracellular matrix (ECM) proteins play an important role in influencing lung strength, flexibility, and elasticity [72]. It is vital for production of decellularized lung to retain key ECM components while removing cell debris and nucleic acid through exposure to detergents and physical methods. More recently, Dr. Ott's team also demonstrated the capacity of PALM to sustain human cells, survive implantation in a pig model of lung transplantation, and withstand the forces of mechanical ventilation allowing for gas exchange [76]. Various detergents are used for decellularization of the lung including sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). SDS-based perfusion decellularization has been shown to produce acellular lung scaffold with loss of DNA while preserving ECM composition and architecture [77]. The preservation of this architecture and ECM composition is vital to the success of any tissue-based scaffold, and in this case the PALM. Thus, with a the lack of optimal bioprosthetics material for ventral hernia repair, there is an ongoing

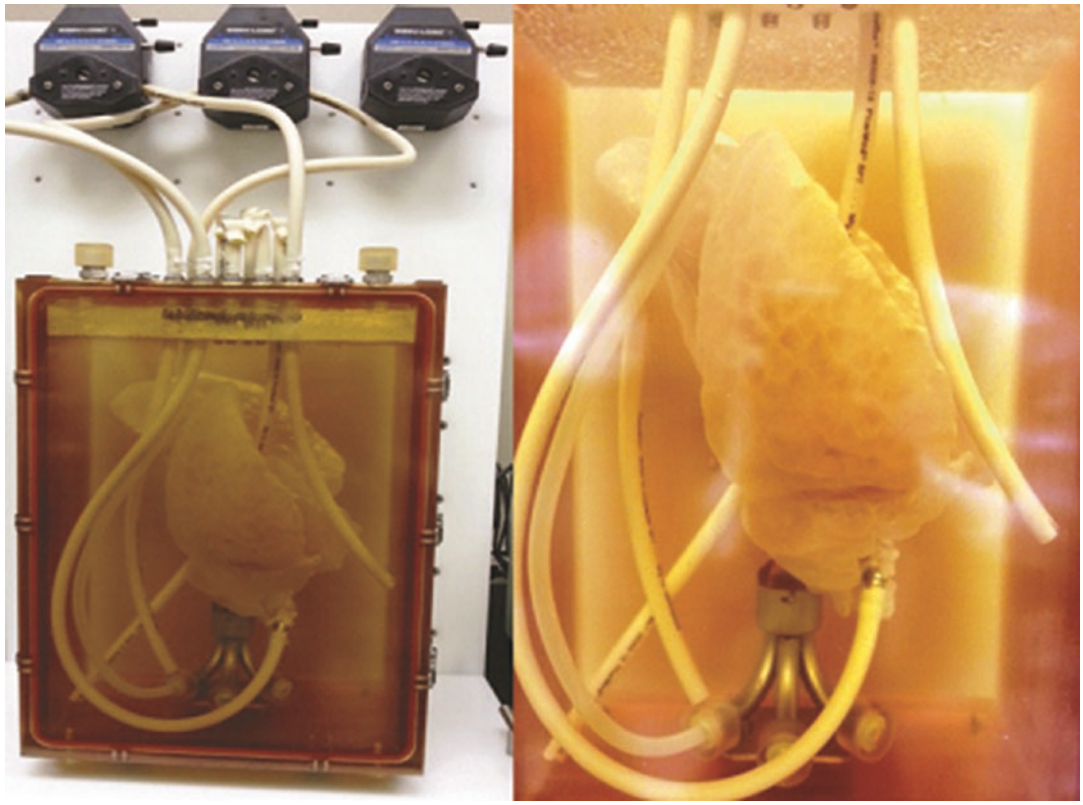

Fig. 4 Decellularization bioreactor. Porcine lung in initial phases of decellularization within the decellularization chamber which is comprised of a perfusion system infusing the processing detergent through the pulmonary artery into the lung

search for ideal mesh materials that will provide long-term efficacy with improved biological activity and incorporation into native tissue.

11 PALM in Hernia Repair

Critical to the success of any tissue repair is the robust infiltration of reparative cells and blood vessels. With their rich vascular architecture, the lung theoretically makes an ideal matrix suitable for cell and blood vessel infiltration. Biomedical engineers have long investigated the ideal microarchitecture for angiogenesis. To achieve this, the material must possess a controlled interconnectivity of pores that supports the invasion and proliferation of progenitor cells that will ultimately recapitulate the natural environment [78]. Not only must the porosity be ideal for the migration of cells but also for the diffusion of nutrients and product of cellular activity and to maintain cell-cell and cell-scaffold interaction [78, 79]. Researchers have developed patterned biomaterials mimicking the natural environment with intricate architectures and variable porosity in an attempt to promote angiogenesis [80, 81]. Likewise, investigators have sought out natural materials with high vascularity in order to promote cellularization and neovascularization, two metrics of incorporation [18, 19, 36, 59, 82]. As mentioned above, the dermis, pericardium, submucosa, and liver have all been investigated as tissue sources for enhanced incorporation. With this in mind, we sought to identify another source of highly vascularized tissue to test in the setting of bridging ventral hernia repair.

To this end, Fernandez-Moure et al. evaluated the efficacy of PALMs in chronic hernia repair in

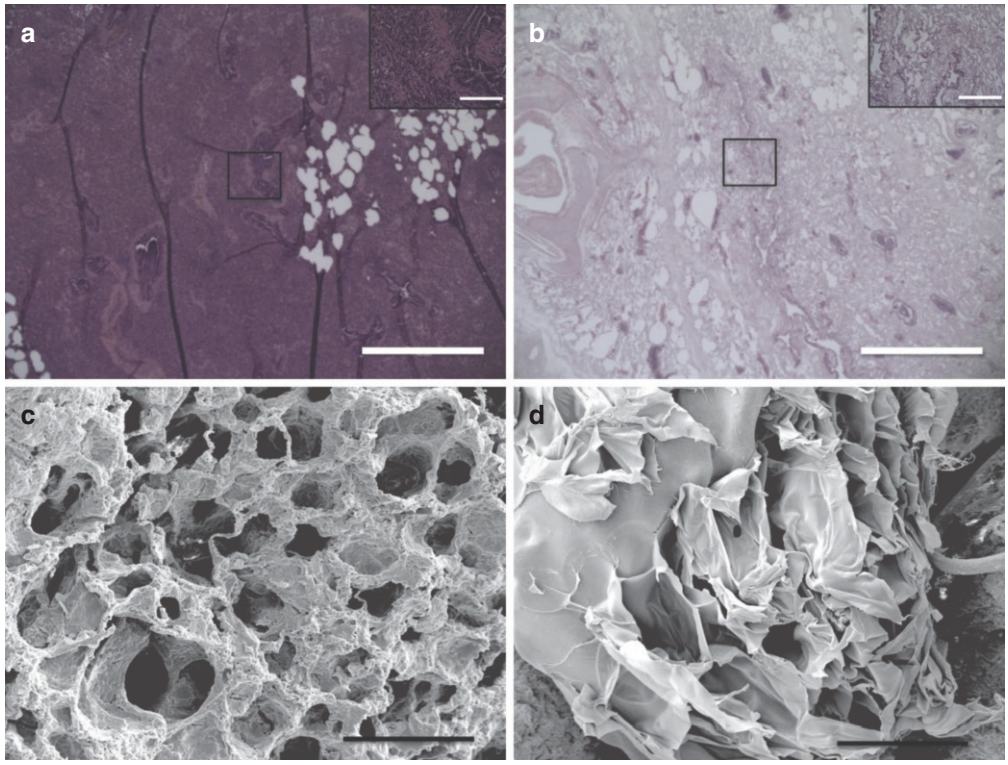

Fig. 5 Characterization of decellularization. Hematoxylin and eosin staining at (**a**) day 0 and (**b**) day 7 confirms complete decellularization following SDS baths (scale bar, 150 μm; inset 40× magnification scale bar 20 μm). (**c**) Well-formed and cellularized tubular vascular structures are in lung tissue prior to processing (scale bar, 100 μm). (**d**) Decellularized vascular structures retain tubular morphology and architecture (scale bar, 100 μm)

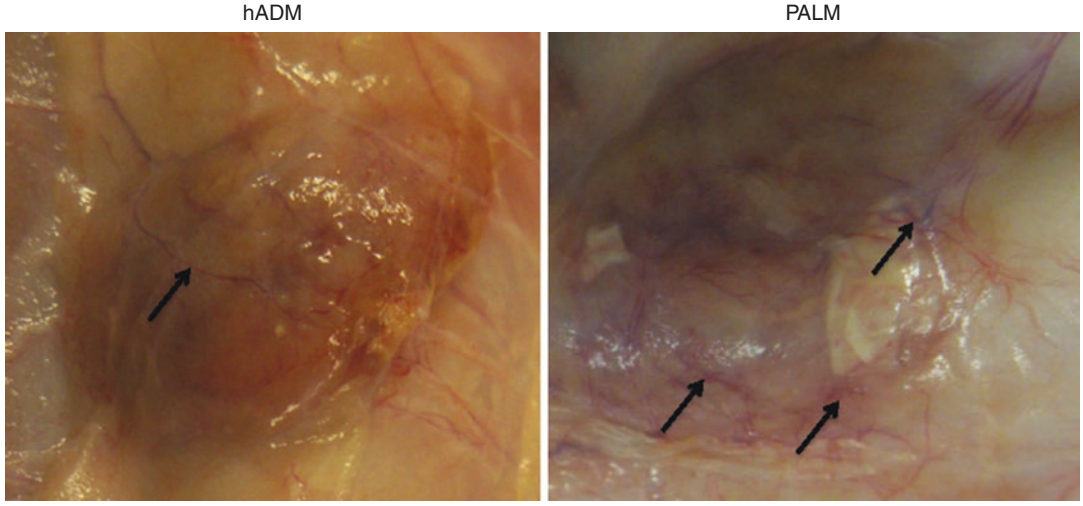

Fig. 6 Subcutaneous implantation. Human acellular dermal matrix and (hADM) and porcine acellular lung matrix (PALM) following implantation. Macroscopic vessel infiltration (arrows) is seen following 6 weeks of subcutaneous implantation

rat models in comparison to the human acellular dermal matrix (hADM) [83]. In the study, the porcine lungs were processed via a perfusion decellularization process using SDS solution, and decontamination was performed by perfusing streptomycin, penicillin, and amphotericin B prior to dissection. Effectiveness of the decellularization of the porcine lungs was evaluated using hematoxylin and eosin (H&E) staining, which showed loss of cells throughout the tissue. Also, scanning electron microscopy (SEM) showed the highly organized structure of the lung that contributes to its high potential for neovascularization (Fig. 5). Subcutaneous implantation of PALM and hADM showed PALM was capable of robust macroscopic neovascularization compared to hADM (Fig. 6). The animal model was one of a chronic hernia fixed with a bridging repair. The authors feel this approach while not necessarily suitable for human translation is optimal for evaluating mesh under mechanical forces. The rats were divided in two groups, and each animal had hernia repair with bridging repair with implantation of either PALM or hADM. After 6 weeks, the PALMs group demonstrated significantly greater cell infiltration and cell density compared to the hADM group. Also, the PALMs group showed a greater number of vessels per 1 mm^2 and up to six times the number of vessels per field of view compared to hADM (Fig. 7). Given

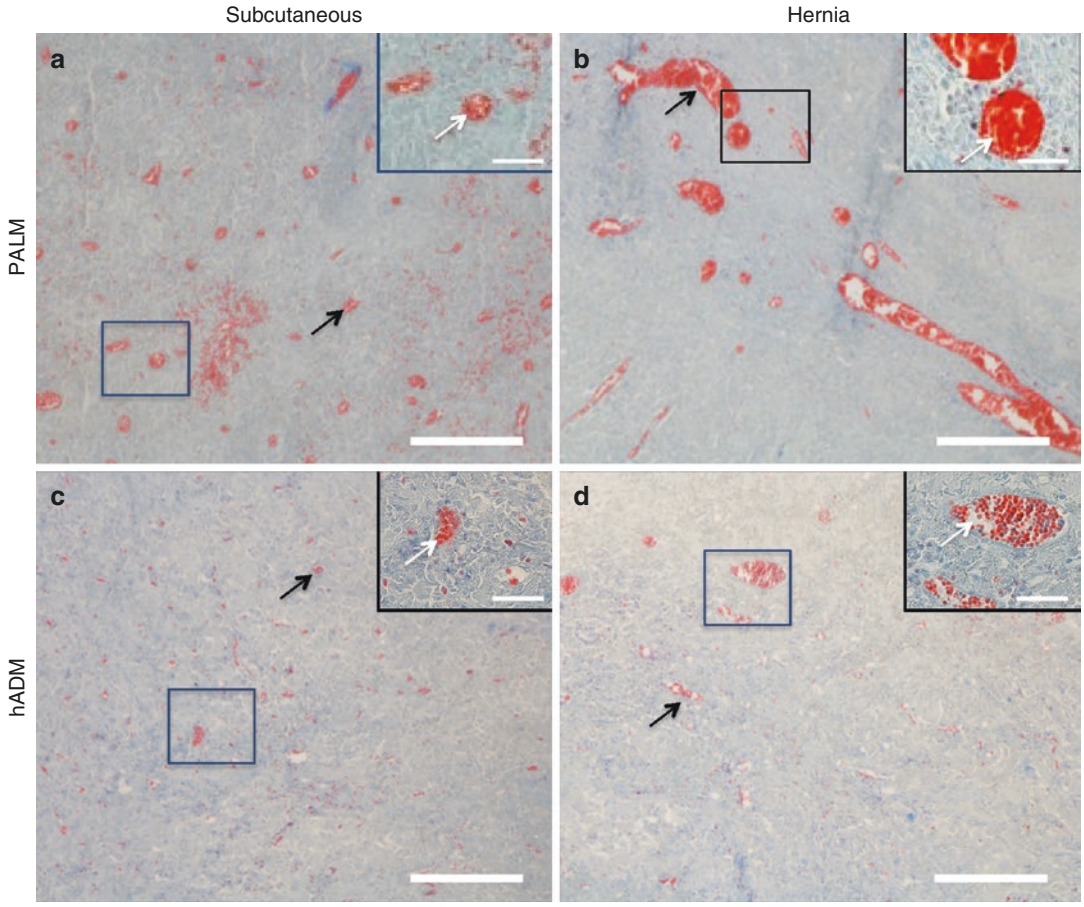

Fig. 7 PALMs demonstrate greater vessel formation. Representative images of Masson's trichrome-stained implants following (**a**, **c**) subcutaneous implantation and (**b**, **d**) implanted for hernia repair. PALMs had greater vessel infiltration (white and black arrows) compared with hADM (scale bar, 50 μm) (insert 40× magnification; scale bar, 20 μm)

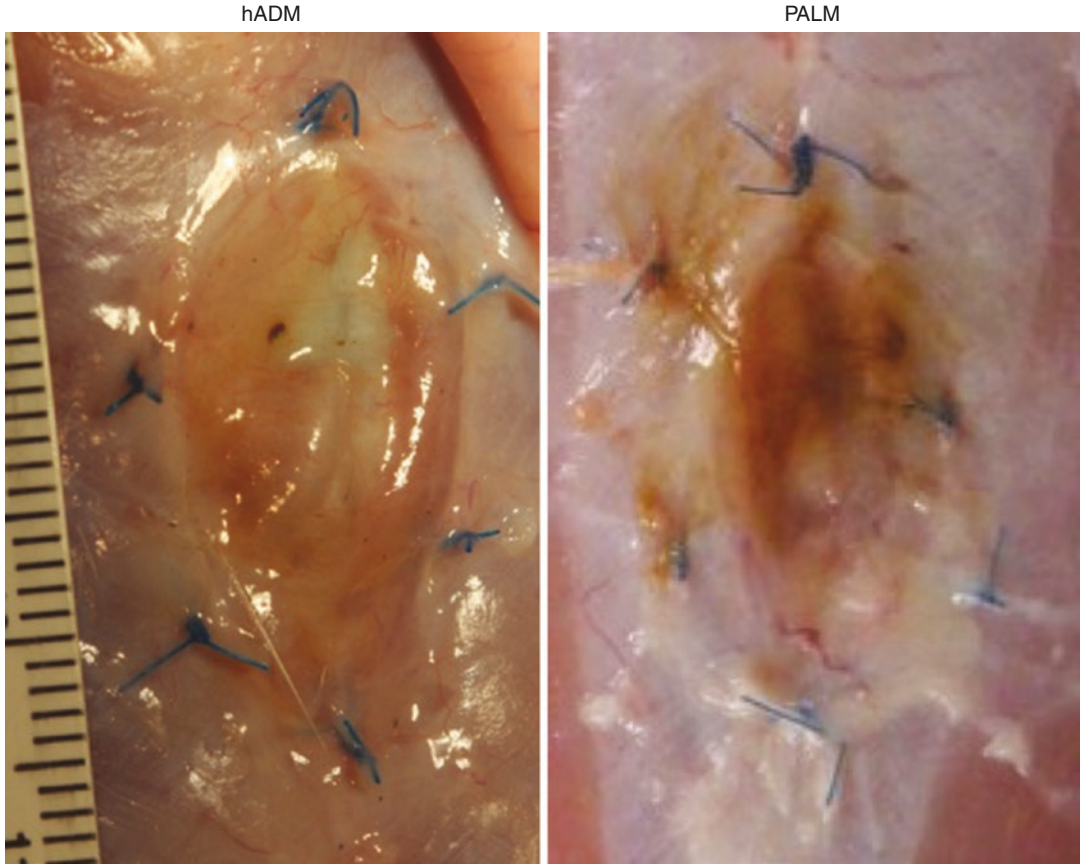

Fig. 8 PALM and hADM following 6-week implantation. Both Palm and hADM were intact following 6 weeks. Minimal bulging was seen in PALM with none of the animals demonstrating any mesh failure at that time

the innate architecture of the lung, the authors felt this along with the preserved ECM components contributed to the significant incorporation and the lack of re-herniation or scaffold breakdown. The lung, unlike other bioprosthetics, does not maintain its mechanical properties when decellularized. After processing the lung tissue becomes very soft and fragile. Another critical finding they noted was that PALM implants did not undergo significant bulge or mechanical failure, which is clinically relevant since bulge is a predictor of long-term mechanical failure (Fig. 8) [84]. Based on the characteristics for metrics of incorporation with cell infiltration and vessel formation, the study demonstrated that PALMs have superior surgical outcomes compared to hADM.

Conclusions

Abdominal wall hernias continue to be a large socioeconomic burden in the USA. Currently, the most commonly used prosthetics are purely synthetic in nature and carry significant risks with them. To address this issues investigated have looked to nature for biologically derived tissues as prosthetics. Various decellularization technologies have emerged, as have various tissue sources for prosthetic repair. The dermis remains the most commonly used and most studied although it may not be ideal for repair as evidenced by unacceptable long-term failure rates in bridging repair. Thus, research have sought new tissue sources based on the microarchitecture of the substrate organ in order to maximally promote

the metrics of incorporation, namely, cell infiltration and vascularization. PALM has been investigated as a novel prosthetic for repair and has demonstrated enhanced incorporation and short-term mechanical stability in a chronic ventral incisional hernia model with bridging repair. We hope this focus of pre-existing microarchitecture and gentle decellularization technologies will foster a new avenue of thought when evaluating current prosthetics and those to come.

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Wound Healing Problems in the Mouth

Constantinus Politis and Gaétan Van De Vyvere

1 The Specific Characteristics of Wound Healing in the Oral Cavity

1.1 Healing of Jawbone

Jawbone fractures can heal by primary or secondary intention. Bone healing by primary intention, or direct bone healing, occurs without callus formation, necessitates the bone fragments to be in direct contact with each other with a fracture gap of up to 1 mm, whereas bone healing by secondary intention, or indirect bone healing, is characterized by callus formation. Secondary bone healing is by far the most important form of bone healing in maxillofacial surgery [1, 2].

1.2 Alveolar Bone Healing

The healing of an empty socket after a tooth extraction follows the same pattern as secondary bone healing [3, 4]. Minutes after the tooth is extracted, the alveoli are closed via blood clotting. A low-level ooze for 12–24 h after tooth extraction is normal as an organized clot forms in the tooth socket. In healthy patients, reepithelialization starts 24 h post-extraction. In just after 1 week, the blood clot is replaced with granulation tissue. After 8 weeks, the extraction cavity is filled with bone [3, 4]. The bone remodeling process continues for 6 months after the extraction and is accompanied by a loss of alveolar width and length due to resorption and remodeling [3]. The amount of bone loss varies among individuals and depends on the location, the presence of adjacent teeth, the treatment protocol, smoking behavior, and the use of membranes and bone substitutes [5].

1.3 Healing of the Periodontal Ligament

The healthy periodontal ligament (PDL) connects the cementum (a thin layer of mineralized tissue covering the roots of the teeth) to the alveolar bone and retains human teeth fixed in their alveolar socket throughout life both in rest and during function. The periodontal ligament covers the roots up to the cemento-enamel junction and does not only have an important role in supporting teeth but also contributes to tooth nutrition, homeostasis, and repair of damaged tissues [6]. A vital PDL residing on the root surface exhibits bone-inducing capacities which allow complete regeneration of a labial bone plate after surgical removal [7]. Isolated removal of the part of the periodontal ligament

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(PDL) facing the alveolar bone leads to complete healing of the PDL, whereas isolated removal of the cemental part of the periodontal ligament only leads to complete healing if the size of the defect is no larger than 4 mm^2. Yet the different periodontal ligament stem cell populations do not represent a gradient from cementum to alveolar bone, but are rather paravascular located [8].

1.4 Healing of Cementum

Loss of cementum occurs in trauma (root fractures) and in surgical procedures (segmental osteotomies, apicoectomy, placement of osteosynthesis screws). Cementoblasts in the vicinity of the defect will cover the exposed dentine to form new cementum.

1.5 Healing of Pulp

In immature teeth with open apex, revascularization and reinnervation of the dental pulp will occur. As teeth mature and the apical foramen closes, the chances of pulp revascularization diminish accordingly and become very unlikely below an apical diameter less than 0.3 mm, whereas revascularization is very likely above 1 mm. Pulp ischemia is followed by the deposition of reactive dentine in the pulp chamber leading to pulp canal obliteration on dental radiographs [9]. A further consequence can be ingrowth of PDL and bone through the apical foramen into the pulp. The remaining pulp in the canal becomes necrotic. This pulp necrosis can remain sterile or can be infected. Persistent inflammation of pulp tissues leads to pulp necrosis. Pulp ischemia and pulp necrosis in maxillofacial surgery mainly occur after dental trauma, after surgically assisted rapid palatal expansion (Fig. 1), and after osteotomies too close to the apical area of the tooth.

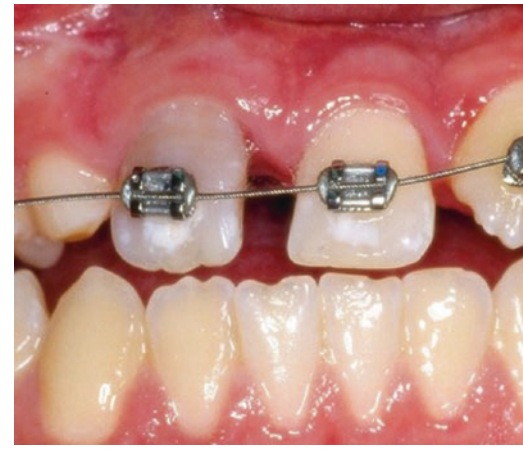

Fig. 1 Discoloration of the upper right incisor and necrosis of the interdental papilla after a surgically assisted rapid palatal expansion in adult causing pulp ischemia and subsequent pulp necrosis

1.6 Healing at Dental Implant Interfaces

A specific form of wound healing occurs around dental implants. In biphasic dental implant procedures, the implant is placed directly under or at the same level as the bone surface. The cover screw is overlaid with soft tissues that heal without substantial granulation tissue formation. Healing in the bone occurs between the edge surface of the implant and the prepared osteotomy edge [4, 10]. Blood clots form mainly at the inner side of the implant grooves and are then infiltrated by granulocytes and macrophages. Fibroblastic progenitor cells migrate into the provisional matrix, enabling formation of granulation tissue, which is then vascularized by endothelial cell migration. Finally, the cells in the granulation tissue differentiate into osteoblasts, creating bone. This bone formation starts 4 days after placement of dental implants, achieving maximum bone-implant contact after 3 months [4]. Depending on the mechanical

stress caused by occlusal forces, bone remodeling around the dental implant persists for at least 1 year.

1.7 Healing of the Palate

Wound healing in the oral cavity is typically characterized by healing of the palate and gingival tissue in the presence of healthy underlying bones and without scar tissue formation [11]. This is due to early onset of the inflammatory phase, decreased levels of immunity mediators, fewer blood vessels, more cells originating from the bone marrow, rapid reepithelialization, and rapid fibroblast proliferation [11]. In fetal wound healing, the inflammatory phase is absent [12].

Wound healing in the palate is more difficult in the absence of healthy underlying bone. In such cases, wound healing might be accompanied by perforation to the nose and antrum or by serious scarring. This can lead to narrowing of the transversal width of the maxilla if the patient is in their growing phase, as is observed in cleft patients who have surgery of the palate [1, 4].

2 Oral Tissues, Saliva, and Gingival Crevicular Fluid

Barriers against the continuous exposure of microorganisms are found all over the oral mucosa, in the saliva, and in the gingival crevicular fluid surrounding the teeth. These barriers consist of antimicrobial proteins, antimicrobial peptides, chemokines, cytokines, and neuropeptides [13]. Apart from its immunoprotective role, the oral epithelium further acts as an important mechanical barrier to penetration.

The two tissues that in the skin are known as epidermis and dermis are known in oral mucosa as oral epithelium and lamina propria. The transition from oral mucosa to the underlying tissues depends on its location: it is firmly attached when the underlying tissue is periosteum and bone (mucoperiosteum), and it is loose submucosa in areas where flexibility is necessitated. Wounds over mucoperiosteum usually do not spread open and need no suturing if the mucoperiosteum is not detached.

3 Clinical Manifestations of Disturbed Wound Healing in the Mouth

Clinical manifestations of a disturbed wound healing can include excessive bleeding or absence of blood clot formation as seen in alveolitis sicca. Other manifestations can include the granuloma formation, sinus polyps (Fig. 2), fistulas, wound dehiscence, ulcers, perforations, wound necrosis, flap necrosis, pus formation, chronic infections with or without granulation tissue formation, keloid formation (Fig. 3), fibrosis, and trismus [2, 11, 14–16]. Hom et al. [17] state that the following clinical signs indicate poor wound healing: persistent inflammation for longer than 7 days, malodorous wound, increased exudate, delayed epithelialization, maceration of the surrounding skin, wound dehiscence, and necrotic tissue. A traumatic eosinophil granuloma of the tongue can also be considered a manifestation of poor healing progress.

Cases of nerve damage in the trigeminus area exhibit a special form of tissue damage due to disturbed wound healing. Damage to the branches of the trigeminal nerve due to nerve crushing or pressure can cause dysfunction in the form of neuropathic pain with or without hypoesthesia of the innervation area [18]. Although the mechanisms have not yet been elucidated, it is hypothesized that this effect occurs when the nerve and its surrounding environment (vasa nervorum,

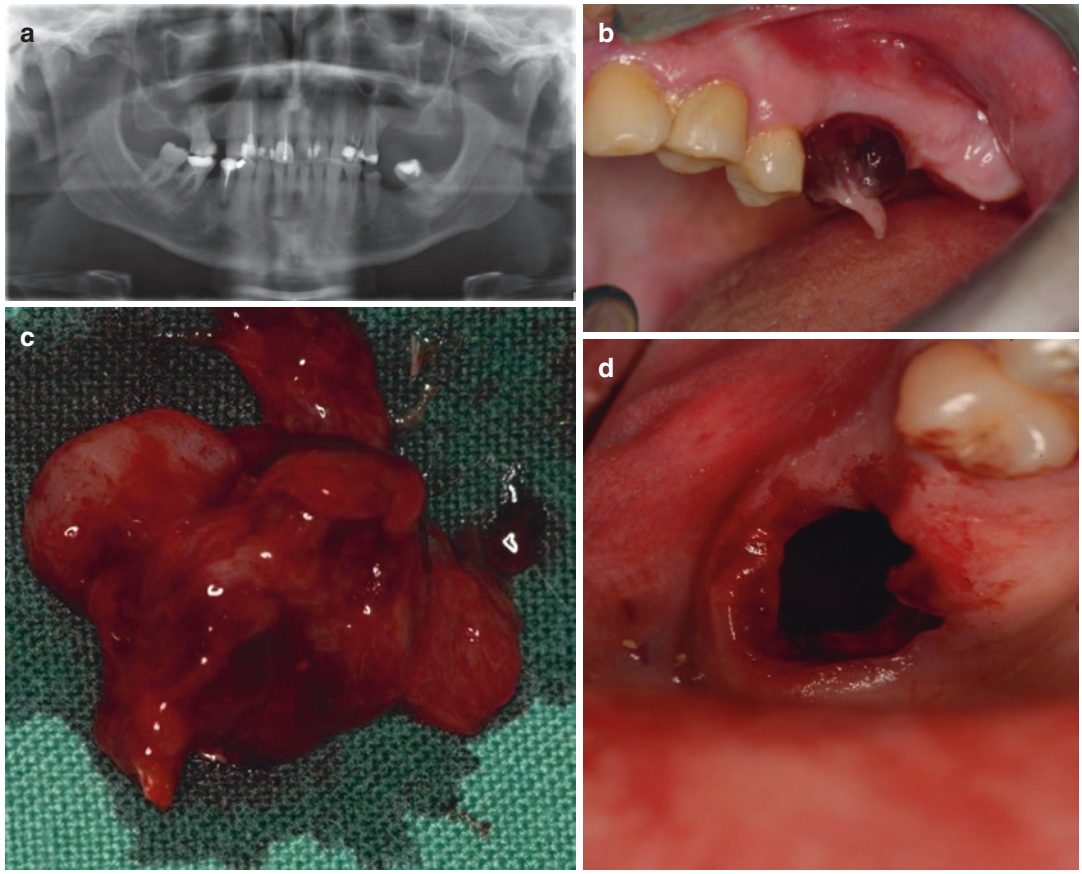

Fig. 2 Sinus polyp emerging from the maxillary sinus as sign of an oroantral communication after removal of the first upper molar in the left maxilla. The width of the oroantral fistula becomes apparent after curettage and removal of the granulation tissues

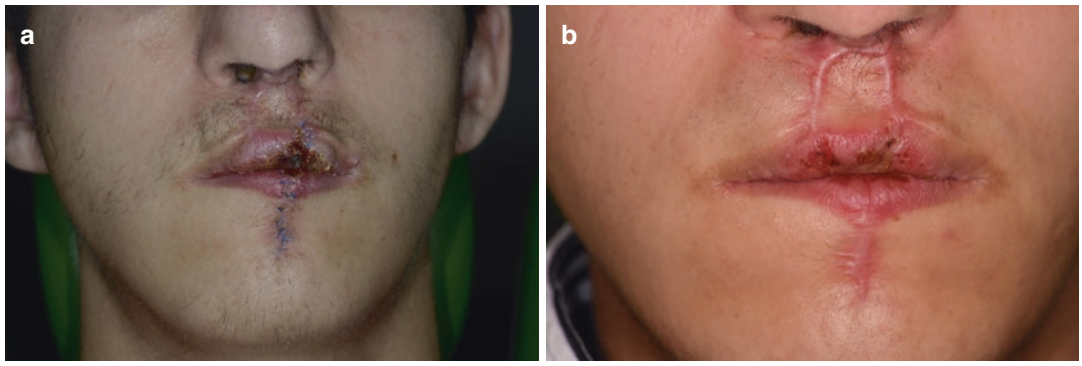

Fig. 3 Hypertrophic scarring after an Abbé flap procedure in a cleft patient. (**a**) One week postoperative. (**b**) One year postoperative

bone, and adipose tissue) do not sufficiently recover during wound healing.

A typical form of disturbed wound healing involves the root resorption of an element following tooth trauma, tooth transplantation, or reimplantation of an element. Severe damage to the periodontal ligament and cementum which transcends healing capacity is giving rise to tooth ankylosis and replacement resorption (Fig. 4) [19, 20].

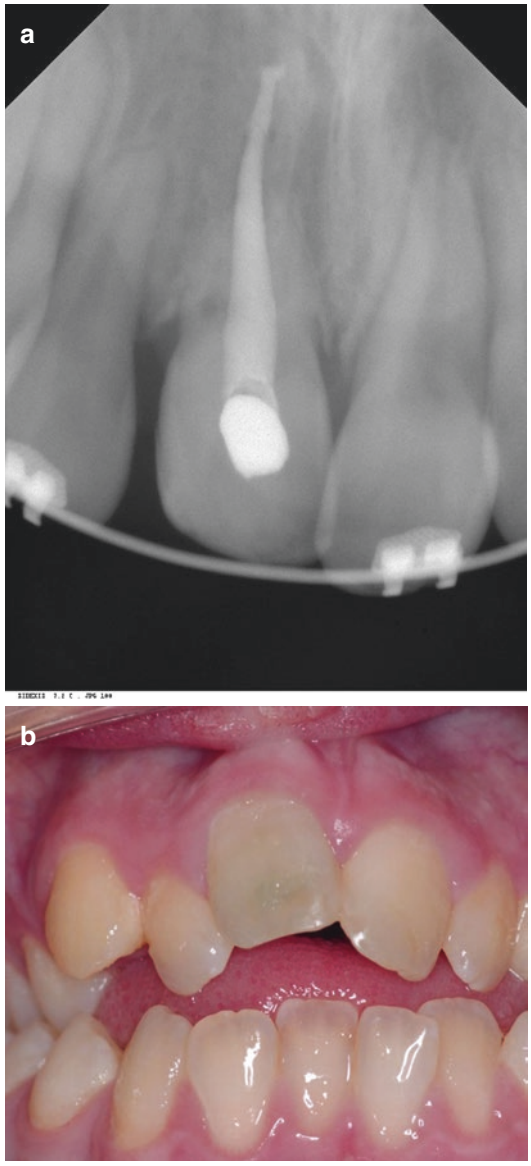

Fig. 4 (**a**) Avulsion and reimplantation of the right upper central incisor resulted in pulp necrosis and tooth ankylosis with subsequent root resorption. (**b**) Impaired growth of the alveolar process and development of an anterior open bite

3.1 Disturbed Oral Wound Healing by Local Factors

Oral wound healing most often is disturbed by local conditions of the environment or the underlying tissues:

1. Poor oral hygiene
2. Injudicious flap design in surgery
3. Wound size
4. Wound localization
5. Postoperative bleeding
6. Thermal damage
7. Perforation to the sinus maxillary
8. Sharp bone edges
9. Local anesthesia
10. Infection
11. Hypoperfusion
12. Ischemia
13. Foreign body
14. Smoking
15. Venous insufficiency
16. Mechanical trauma
17. Local toxins
18. Head or neck irradiation
19. Cancer of the mouth
20. Presence of necrotic tissue
21. Local stem cell injections
22. Underlying pathological fractures
23. Edema
24. Pathological mobility
25. Tooth in the line of a jaw fracture
26. Traumatic occlusion

3.1.1 Poor Oral Hygiene

Poor oral hygiene with calculus formation, gingivitis, and periodontitis is a frequent cause of infections and of poor intraoral wound healing (Fig. 5). As long as oral hygiene is not properly addressed, surgical interventions intraorally sometimes become surgical adventures. It is ill-advised to place dental implants in a mouth where gingivitis or periodontitis is still present.

3.1.2 Injudicious Flap Design

Most errors in intraoral flap design relate to misjudgment of the pattern of vascularization of the raised flap or to failure to adhere to basic principles, including ensurance that the flap base is suffi-

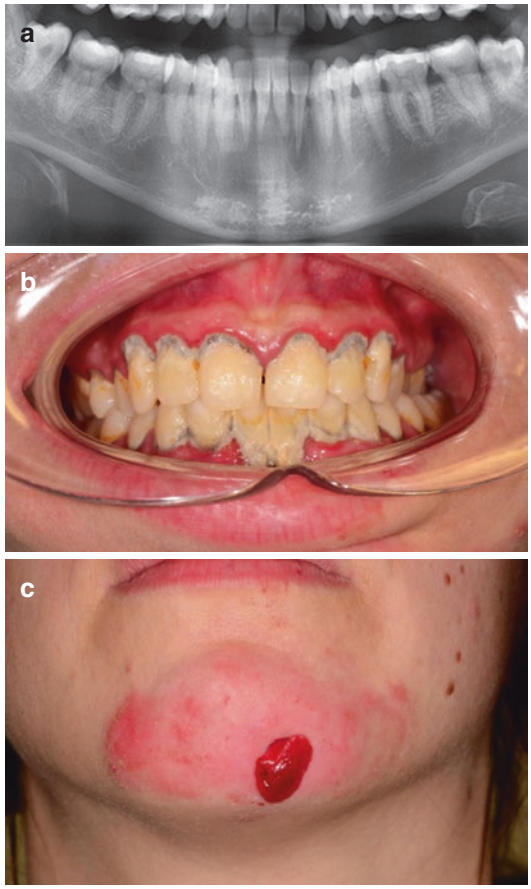

Fig. 5 (**a**) Poor oral hygiene with calculus formation and periodontal defects causing (**b**) infection with osteolytic zones on the panoramic radiograph. (**c**) Cellulitis and external pyogenic granulation formation at the chin

ciently wide, keeping the wound edges on healthy bone, limiting the use of monopolar electrocoagulation, and creating no excessive tension on a wound edge. Moreover, incisions should never be made on an open junction to the maxillary sinus, but rather on healthy bone edges. Primary closure over a site of active infection or closure over necrotic bone, over exposed dental implants (Fig. 6), keeping a flap stretched over a sharp bony wall, and free grafting on cortical bone are all causes of failure.

In oral cancer surgery, a mandibular split should be avoided to gain access to the oral cavity, whenever the area of the mandibular split received or will receive a radiation dose of >60 Gy. The area of the mandibular split becomes a predilection place for subsequent osteoradionecrosis (Fig. 7).

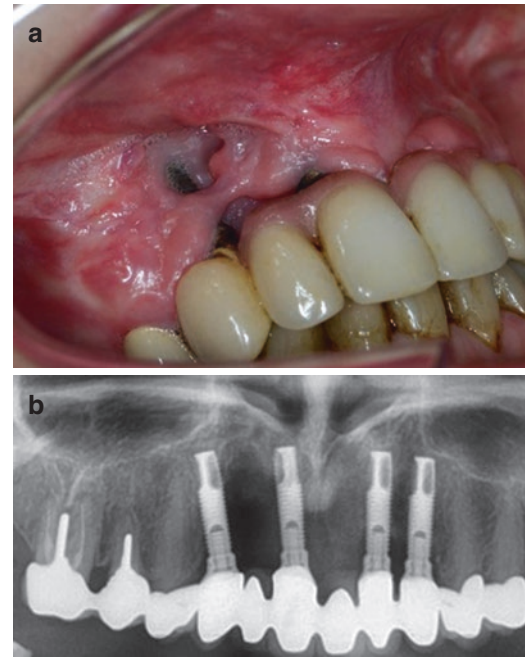

Fig. 6 (**a**) Surgery over exposed implants enhances the risk of breakdown of a thin mucosal flap. (**b**) Panoramic radiograph

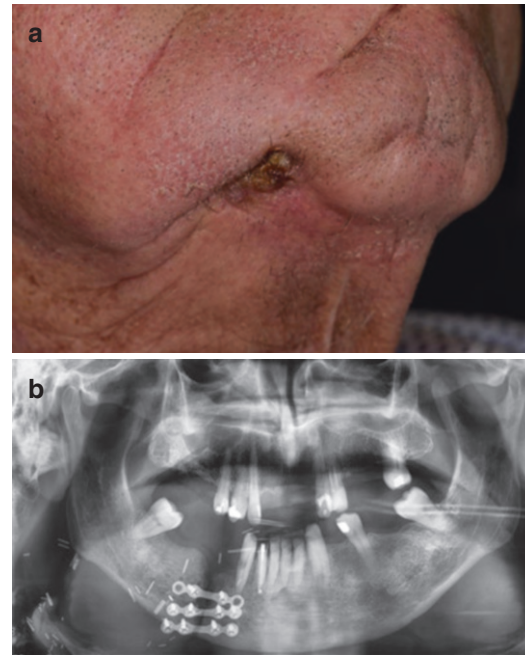

Fig. 7 (**a**) External fistula due to osteoradionecrosis and failure to heal after a tumor resection of a malignant spinocellular carcinoma of the floor of the mouth using a mandibular split osteotomy to gain surgical access. The area of the mandibular split is a predilection place for osteoradionecrosis. (**b**) Panoramic radiograph

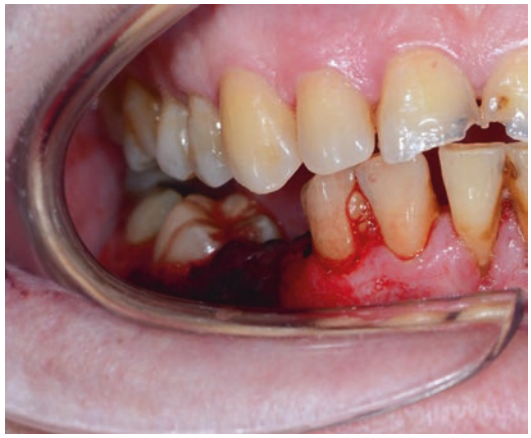

Fig. 8 Continued bleeding over a false clot formation impedes normal wound healing

3.1.3 Postoperative Bleeding

Postoperative bleeding disturbs granulation tissue formation, slowing the healing process [4] (Fig. 8). Hereditary and acquired bleeding tendencies only give rise to disturbed wound healing in the presence of pathological bleeding [21] . In daily clinic bleeding after tooth extraction is most frequently seen due to local infection, poor oral aftercare (early forceful rinsing and smoking), and in patients under dual antiplatelet therapy or with an INR exceeding 3.5 or during bridging [22].

Absence from bleeding or early loss of the blood clot before maturation in the extraction socket after tooth extraction leaves the bony alveolar walls exposed to the oral fluids, inducing a "dry socket" or alveolar osteitis. Patients with symptoms caused by alveolar osteitis usually present about 5 days after the tooth extraction. Due to the low incidence of alveolar osteitis in <1% of extracted teeth, both third molars and other teeth, routine prophylactic use of antibiotics is not recommended because of the high number needed to treat and the moderate tolerability of the antibiotics [22].

3.1.4 Hypoxia and Oxygen Tension

Wounds must have a minimum oxygen tension of 30 mmHg for normal cell division and a minimum of 15 mmHg for fibroblast proliferation [23]. Bacterial destruction by phagocytosis relies on a high partial oxygen pressure in the tissues. Sufficient oxygenation is also required for cell proliferation, angiogenesis, collagen synthesis, and reepithelialization. The role of oxygen has mainly been investigated through in vitro or animal experiments, and there is not yet sufficient in vivo data from humans to truly understand the role of oxygen in wound healing [24]. However, it is clear that hypoxia is associated with disturbed wound healing and with bacterial colonization in chronic wounds [23].

3.1.5 Ischemia

Following dental trauma (avulsion, extrusive luxation, or lateral luxation) or tooth transplantation, a special form of local ischemia can arise in which neovascularization occurs through an open apex and is followed by osteodentin deposition with a small central pulpal channel that contains a blood supply—termed root canal obliteration. This predominantly occurs in teeth with an open apex, within the first year after trauma. The role of odontoblasts in this process remains unclear [20]. Ischemia also is known to occur in drug addicts to cocaine. Cocaine is a strong vasoconstrictor and can endanger axial pattern flaps in surgery (Fig. 9). Ischemia and occlusion of vessels in free flap surgery is a serious postoperative complication in patients treated for head and neck cancer (Fig. 10).

3.1.6 Antrum Perforation

Antrum perforation can lead to bad wound healing following extraction of a molar or premolar in the upper jaw.

3.1.7 Local Infection

Infection keeps a wound in an inflammatory state. Such infections are not necessarily prominently visible. A chronic maxillary sinusitis can lead to recurrence of a buccosinusal connection following closure with a Rehrmann flap [15].

3.1.8 Thermal Damage

Excessive monopolar electrocoagulation of the bone, or drilling without cooling, can lead to bone necrosis and the formation of bone sequesters [14, 15].

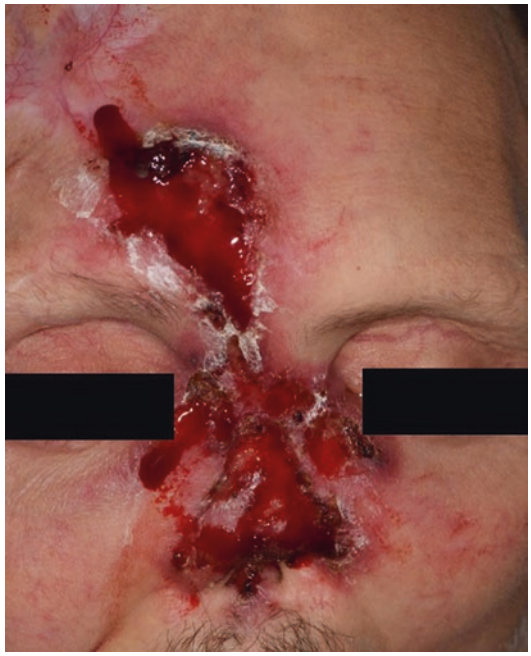

Fig. 9 Necrosis of a forehead flap to reconstruct a nasal defect in a patient with severe addiction to cocaine

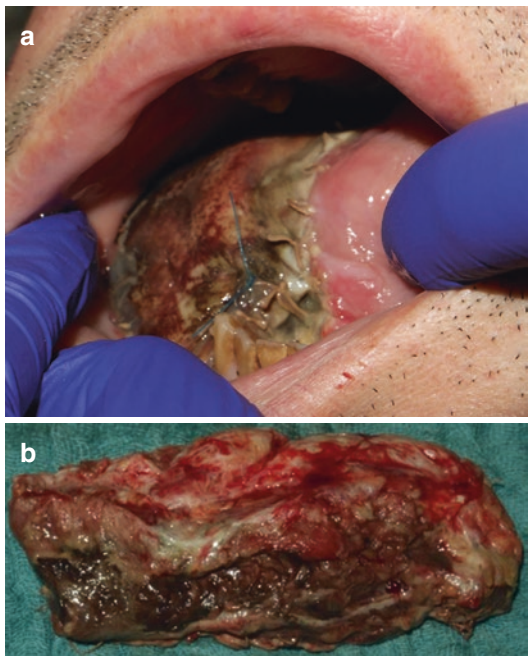

Fig. 10 (**a**) Necrosis of a free vascularized osteomyocutaneous fibular flap to reconstruct a defect after partial mandibulectomy for spinocellular carcinoma. (**b**) Excisional specimen

3.1.9 Edema

Wound edema restricts the oxygen and nutrient supply to the wound by enlarging the diffusion distance.

3.1.10 Foreign Body (Corpus alienum)

Common corpora aliena in the mouth can vary from a piece of gutta-percha or root canal cement to inserted hydroxyapatite granules or osteosynthesis screws. Residual tooth elements, a radix relicta, residual pieces of crown, and necrotic bone sequesters (Fig. 11) can also act as corpora aliena and lead to chronic wound infections and wound breakdown (Fig. 12). Remaining wicks and compresses (Fig. 13) can also lead to poor wound healing and latent infections.

Advanced peri-implantitis can surround implants which then act as foreign bodies causing infections and fistulas. As long as these implants are not removed, tissue healing will not occur (Fig. 14).

Following removal of the wisdom teeth in the mandible, wound healing can be delayed by ischemic necrosis of the buccal edge of the alveolus [25]. On some occasions, dental implants can act as a corpus alienum and cause severe wound healing problems, such that removal is the only option. Although a corpus alienum is often visible on radiography, this is not always the case—amalgam tattoos, crown cement around an abutment, and small residues of broken reamers and files can be easily overlooked and cause chronic problems.

3.1.11 Pathological Mobility

Following a Le Fort I fracture or a Le Fort I osteotomy, a pseudarthrosis can develop due to either bruxism or insufficient rigid fixation. In the mandible pseudarthrosis develops after insufficient or inadequate fixation following a mandibular fracture or osteotomy of the mandible [2]. When opting for a load-bearing fixation (the plate resists the functional masticatory forces and bears the functional load to the bone) or a load-sharing fixation (sharing the load

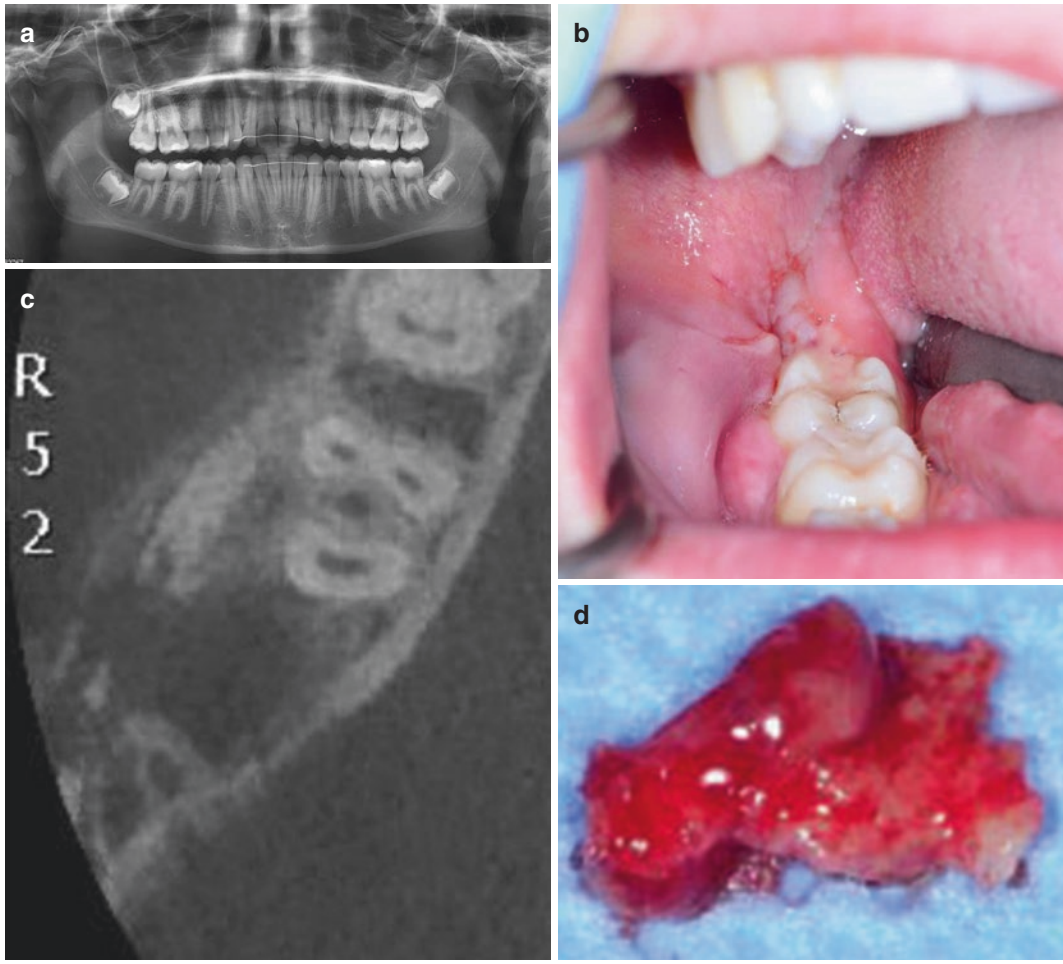

Fig. 11 (**a**) Chronic infection and beginning osteomyelitis after wisdom tooth removal on panoramic radiograph. (**b**) Exploration of the wound revealed a large necrotic bone sequester with chronic swelling. Removal of all necrotic tissue and antibiotics resulted in healing; (a) preoperative condition. (**c**) Cone beam computed tomography (CBCT) with periostitis and necrotic bone. (**d**) Bone sequester

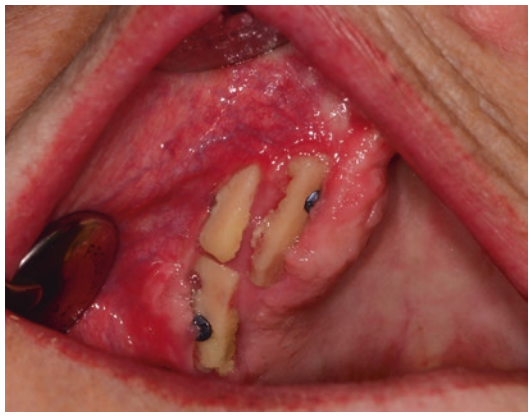

Fig. 12 These cranial bone grafts failed to integrate and became necrotic. Both the necrotic basis and the sharp edges of the bone cause breakdown of the overlying mucosa leading to exposure of the bone graft in the mouth

between osteosynthesis plate and bone), the choice should be for load-bearing plates in case of fractures of the extreme atrophic mandible, comminuted fractures, and fractures with avulsed or missing segments.

Pathological fractures amount to less than 2% of all mandibular fractures and occur in an area where the bone is weakened by an underlying pathological process, most often ORN (osteoradionecrosis) (Fig. 15), MRONJ (medication-related osteonecrosis of the jaws), an odontogenic or a malignant tumor, and extreme mandibular atrophy (Fig. 16), following osteomyelitis of the lower jaw or following third molar removal.

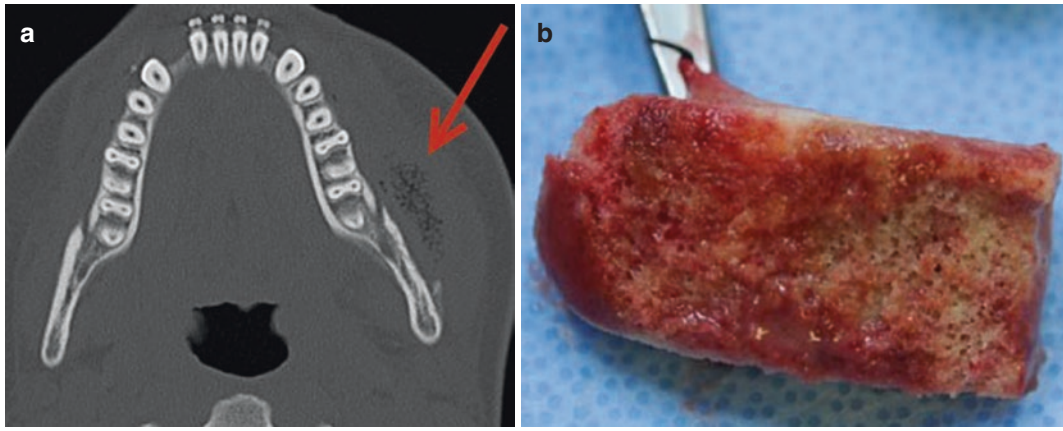

Fig. 13 A forgotten compress, left behind in the surgical wound, impaired soft tissue, and bony healing. (**a**) CBCT (red arrow shows compress and impaired soft tissue). (**b**) Specimen

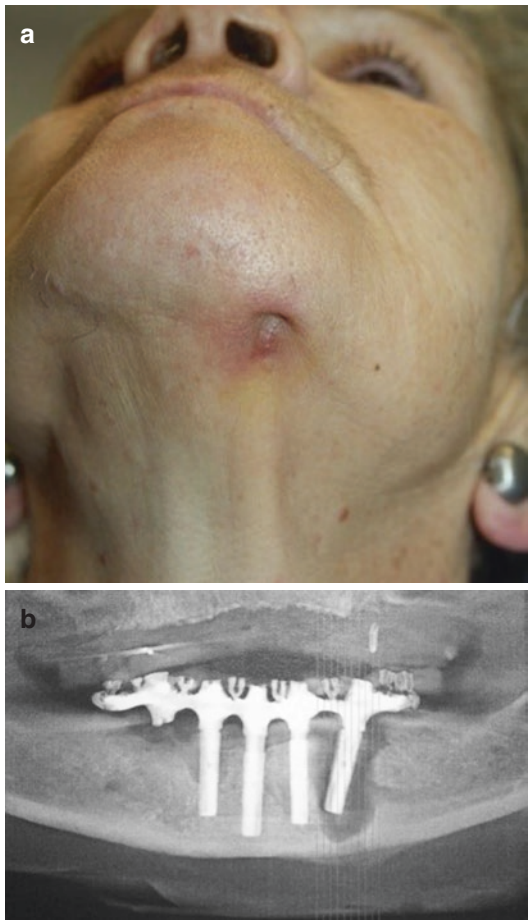

Fig. 14 (**a**) Untreated peri-implantitis surrounding the entire implant is causing an external fistula to drain the infection. Once the osteointegration of the implant is lost, it becomes a corpus alienum. (**b**) Panoramic radiograph

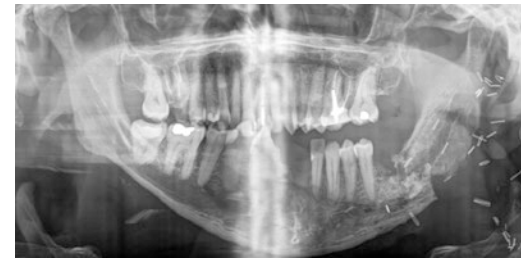

Fig. 15 Pathological mandibular fracture at the left side due to osteoradionecrosis of spontaneous origin after radiation therapy with the area receiving >70 Gy

3.1.12 Tooth Element in the Line of a Jaw Fracture

A non-union can be caused by a tooth element in the line of a mandibular fracture, especially when the tooth exhibits a root fracture.

3.1.13 Traumatic Occlusion

When a toothless maxilla is opposed with natural teeth in the lower jaw, the occlusal forces can threaten a reconstruction when the elements bite on the thin mucosa. Exposure of a bone fragment shortly after reconstruction threats the integration. A patient will often present not one but multiple simultaneous contributory factors. Clinical situations that carry higher risk include Kelly syndrome and an egression of the lateral parts of the maxilla with an edentate antagonistic jawbone. Also, after transplantation of teeth, traumatic occlusion can dislocate the transplanted tooth causing failure.

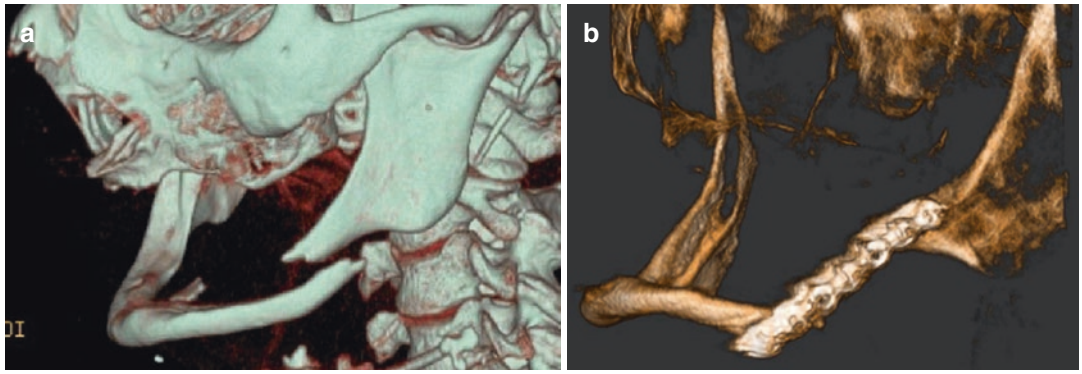

Fig. 16 78-year-old patient, with multiple sclerosis and smoker, developed extreme atrophy of the mandible, Cawood class VIII, with (**a**) spontaneous fracture at the mandibular angle. (**b**) Referred because of pseudarthrosis after fracture reduction with a load-sharing plate

3.1.14 Sutures

Intraoral wounds are usually sutured with resorbable suture. With regard to resorbable sutures, the tissue reactivity ranges from polydioxanone (PDS) and polyglyconate (Maxon) being the least reactive, to polyglycolic acid (PGA) and polyglactin acid (Vicryl) showing mid-level reactivity, to chromic catgut as the most reactive with tissues [26].

3.1.15 Local Anesthesia

In cell cultures and laboratory animals, local anesthetics exhibit an inhibitory effect on wound healing, which is mainly reflected in the inflammatory and proliferation phase of wound healing [27]. The use of epinephrine underlines this effect [28]. Significant clinical effects have not been proven in humans. However, it is recommended to not use large volumes of local anesthetics (tumescent infiltration) in close proximity to surgical incisions in the gingiva.

3.2 Disturbed Oral Wound Healing by General Factors

In spite of careful tissue handling and sound surgical approaches, general factors can significantly affect wound healing and surgical outcome. These factors concern:

1. Old age
2. Obesity
3. Hereditary defects of wound healing
4. Nutritional deficiency
5. Vitamin A
6. Bone resorption inhibitors
7. Corticosteroids
8. Anemia
9. Diabetes mellitus
10. Ethanol abuse
11. Smoking
12. Hyperbaric oxygen
13. HIV
14. Chemotherapeutics
15. Immunosuppressives
16. Radiation therapy for cancer
17. Jaundice – bilirubin
18. Uremia
19. Hypothyroidism

3.2.1 Age

Although age has been proposed to be a cause of bad wound healing, this is not supported by evidence [14].

3.2.2 Obesity

Obesity is considered to be a general factor contributing to disturbed wound healing [15]. However, the literature does not include data supporting this association with regard to oral wounds.

3.2.3 Hereditary Factors

In the literature, Ehlers-Danlos syndrome and Marfan syndrome are stated to be associated with

bad wound healing [29]. However, at our tertiary center, we have not noted any problems with wound healing in the mouth among these patients over the last 30 years. Wound healing in the mouth is problematic among patients with osteogenesis imperfecta or epidermolysis bullosa.

3.2.4 Nutrition

Several epidemiological studies report that conditions related to chronic inflammation can be improved by a diet rich in bioactive fat mediators—as present in fish oil, e.g., polyunsaturated fatty acids, eicosapentaenoic acid, and docosahexaenoic acid—together with low-dose aspirin [30]. A diet rich in omega-3 fatty acids can be recommended for treatment of chronic inflammatory processes [16, 31].

Undernutrition is a problem in wound healing, as is established in daily practice among some patients with mouth cancer combined with ethanol abuse, and in depressed elderly patients who are socially isolated. Protein and vitamin deficiencies particularly influence wound healing [32].

3.2.5 Vitamin A

Macrophage numbers increase with vitamin A intake. A lack of macrophages leads to reduced collagen synthesis and inhibits wound healing.

3.2.6 Bone Resorption Inhibitors

Osteonecrosis of the jaw (ONJ) is a relatively uncommon but potentially serious adverse event of treatment with bone resorption inhibitors (BRIs) such as bisphosphonates and denosumab. BRIs decrease the risk of skeletal-related events, such as fractures, in patients with cancer and bone metastases. ONJ is defined by the American Association of Oral and Maxillofacial Surgeons (AAOMS) as exposed bone with or without extraoral fistulae in the maxillofacial region for more than 8 weeks in patients currently or previously treated with BRIs or antiangiogenic agents. Furthermore, patients may not have a history of radiation therapy to the jaws or obvious metastatic disease to the jaws [33]. BRIs are also used in the osteoporosis patient population to prevent fractures but at a much lower dose compared to cancer patients, with an estimated incidence of ONJ at 0.001–0.01%, which is marginally higher than the incidence in the general population (<0.001%). The incidence of ONJ is the greatest in the oncology patient population (1–15%), where high doses of these medications are used at frequent intervals.

Besides the use of BRIs, risk factors for ONJ include dental extraction, which is by far the most important precipitating factor in the ONJ population. Other risk factors are radiation therapy, chemotherapy, antiangiogenic drugs, periodontal disease, glucocorticoid therapy, diabetes, denture use, tobacco use, and increasing age [34, 35]. Symptoms of ONJ include pain, tooth mobility, mucosal swelling, erythema, ulceration, paresthesia, or even anesthesia of the associated branch of the trigeminal nerve. The necrotic mandible or maxilla (Fig. 17) can become secondarily infected, and fistulae can develop to the nasal cavity, to the antral cavity, and to the skin. Wound healing of the oral mucosa remains impaired as long as underlying necrotic bone remains present. Preventive measures include preoperative and perioperative antibiotics, atraumatic extraction technique, avoidance of deperiostation, avoidance of vasoconstrictors in local anesthetics in these patients, and avoidance of depositing the local anesthetic subperiosteally. The advantage of a drug holiday is controversial.

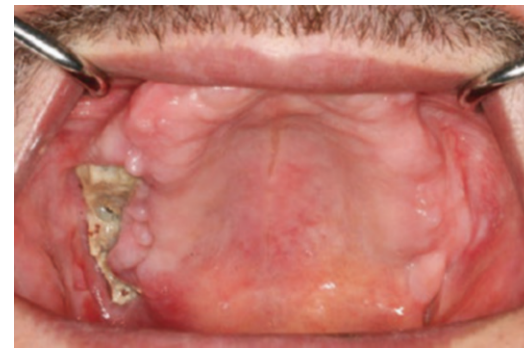

Fig. 17 A patient with lung cancer and osteolytic bony metastases received monthly IV Zometa therapy, developing bisphosphonate-induced osteonecrosis in the right maxilla. Over the area of bony necrosis, a breakdown of the overlying mucosa will expose the necrotic bone

3.2.7 Corticosteroids

Corticosteroids have an inhibitory effect on macrophages, leading to a decline in collagen synthesis. Acute administration of high doses of corticosteroids should not impair wound healing, as opposed to chronic corticosteroid administration [36].

3.2.8 Anemia

In maxillofacial reconstructions using free flaps, low hemoglobin levels should be avoided, especially when the flap exhibits characteristics of ischemia [37].

3.2.9 Diabetes Mellitus

Diabetes mellitus can severely disturb wound healing, likely due to toxic sorbitol accumulation in tissues, pericapillary albumin deposition that hampers nutrient and oxygen diffusion, and disturbed collagen synthesis and collagen maturation (Fig. 18) [1]. Patients with diabetes also exhibit macrophage dysfunction that causes the inflammatory phase to last longer [16].

3.2.10 Ethanol Abuse

Alcohol metabolism leads to formation of acetaldehyde, reactive oxygen radicals, and other molecules that damage healthy tissue. Almost all phases of wound healing are adversely affected by ethanol consumption [38]. Exposure to ethanol prior to injury in mice caused a significant decrease in wound breaking strength by directly impairing fibroblast function, leading to decreased collagen production [39]. Since alcohol intoxication occurs frequently in trauma patients, this mechanism could provide an explanation for the increased healing complications seen in these patients.

3.2.11 Smoking

Smoking has severe negative effects on all phases of wound healing [40] (Fig. 19). Quitting smoking 4 weeks before surgery has positive effects on the inflammatory phase, but the proliferation phase remains disturbed. Administration of vitamins C and E can decrease the damage in smokers, particularly related to collagen synthesis [40].

3.2.12 Hyperbaric Oxygen

Hyperbaric oxygen therapy is not used for normal wound healing, but is indicated in complex compromised acute injuries with tissue crushing, as well as in necrotic infections, osteomyelitis, chronic ulcers, and late complications of radiotherapy [41]. Its role in prevention of ORN in irradiated patents is disputed.

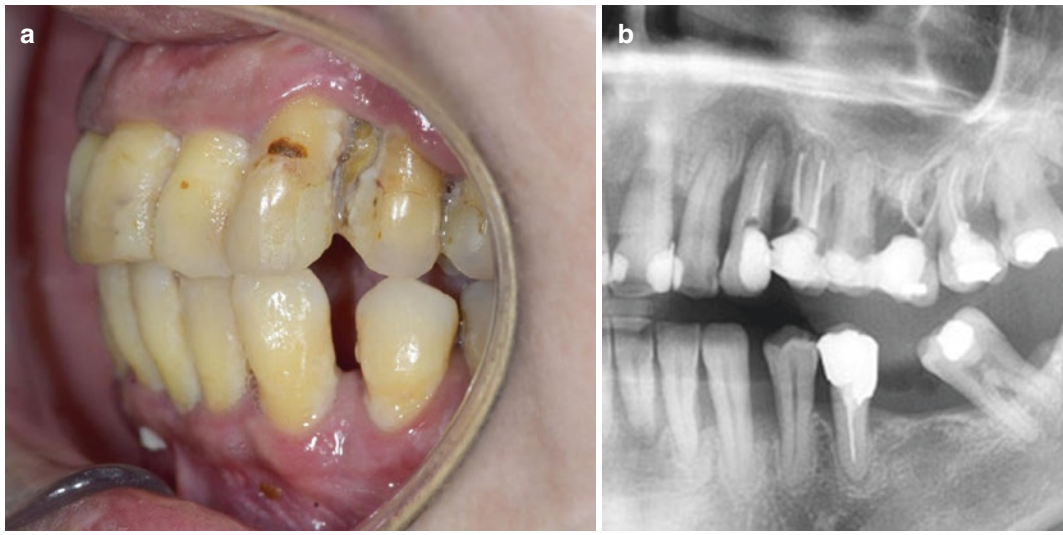

Fig. 18 (**a**) Patient with diabetes mellitus demonstrating periodontal breakdown, periodontitis, and soft tissue necrosis. (**b**) Panoramic radiograph

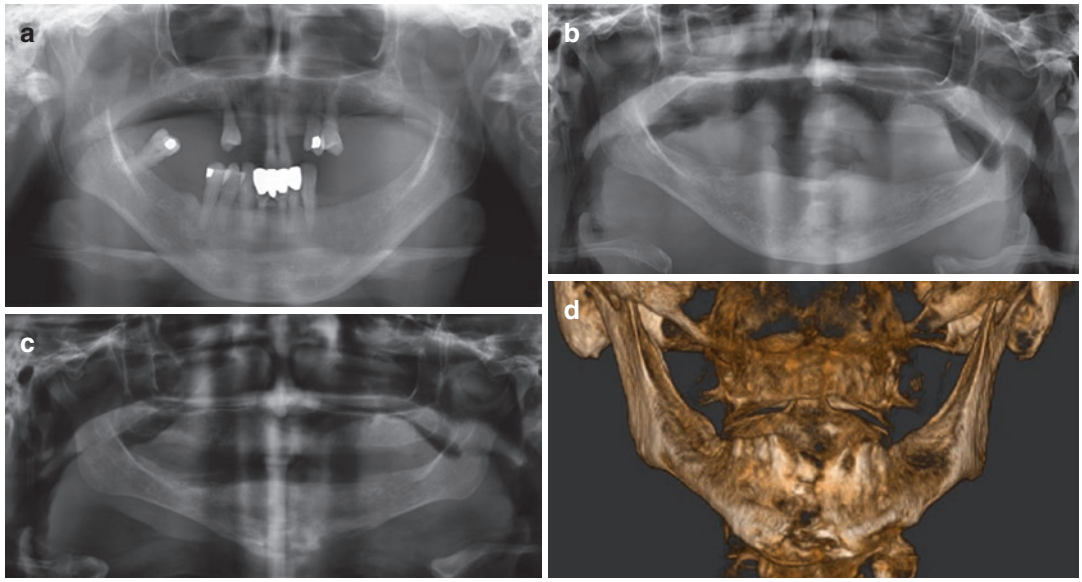

Fig. 19 (**a**) Pre-extraction status of patient—panoramic radiograph. (**b**) Six years later and 1 month after total extraction of the lower teeth—panoramic radiograph. Seven months later spontaneous fracture due to osteomyelitis in a malnourished patient with impaired wound healing and smoking >40 cigarettes per day. (**c**) Panoramic radiograph. (**d**) CBCT

3.2.13 HIV

HIV-positive patients do not seem to have more complications following extraction therapy [42], but do show more complication after mandibular fracture treatment [43]. The risk for complications significantly increases when the number of CD4 T lymphocytes is less than 400 per microliter [42].

3.2.14 Chemotherapy: Immunosuppression

Chemotherapy for mouth cancer can result in oral mucositis during the active treatment phase, and in vitro studies indicate disturbed wound healing during chemotherapy. However, disturbed wound healing is not a clinically significant finding after extractions in patients with a history of chemotherapy [44]. Blood platelets and white blood cells are highly important in wound healing; therefore, wound healing complications are observed during active immunosuppression or chemotherapy [45]. However, this disadvantageous effect on wound healing is not permanent.

3.2.15 Radiation Therapy

At our institution, radiation therapy with IMRT for oral cancer is complicated with osteoradionecrosis (ORN) in about 8% of the patients, usually in non-dentated areas or more rarely due to a progressive periodontitis. ORN occurs mostly within the 2 first years after radiation therapy, preferably in the mandible and only in areas receiving >60 Gy. We did not observe ORN in areas irradiated <60 Gy.

Jacobsen et al. [46] recorded implant survival rates of 86% in non-irradiated grafted fibular bone and 38% in irradiated grafted fibular bone. Fibrosis, atrophy, contraction of oral mucosa, fistula formation, wound dehiscence, skin flap reconstructive failure, non-healing wound, and skin necrosis are documented side effects of radiation therapy [47]. Intraorally the formation of hypertrophic scars is extremely rare, but development of fibrosis is not, especially in irradiated tissues and whenever a tissue plane containing fat is excised. Immobilization of muscles and oral mucosa by scarring causes manifest limitation of the maximal interincisal opening of the mouth.

3.2.16 Jaundice: Bilirubin

The literature is conflicting. Both impairment and enhancement of wound healing have been vindicated [48, 49].

3.2.17 Uremia

Studies which imply uremia as a cause of impaired wound healing give conflicting results. Only in rat models of uremia, wound healing is significantly impaired [50].

3.2.18 Hypothyroidism

Postoperative thyroid dysfunction has been vindicated as a cause of impaired wound healing and fistula formation in cancer patients after surgical procedures including laryngectomy and ipsilateral thyroid lobectomy, with or without radical neck dissection, resulting in hypothyroidism. Thyroid function should be monitored postoperatively in head and neck cancer patients and treated accordingly with thyroid hormone therapy [51, 52].

Conclusions

Oral wound healing in the mouth usually goes undisturbed. In case of a disturbed healing process, it is recommended to eliminate underlying local or general factors. The paradox of progress is that new medication which allows humans to survive longer or better, at the same time can disrupt oral wound healing. Forewarned is forearmed, but without easy solutions to these challenges. Well-educated and well-trained maxillofacial surgeons and dentists are able to differentiate between risk factors which are merely a nuisance and those which pose a serious threat to oral wound healing and dental tissue repair.

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Skin Substitutes in Wound Healing and the Stimulatory Effects of Adipose-Derived Stem Cells for the Proliferation of Keratinocytes on Chitosan

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1 Introduction

The skin is the protective barrier of muscles, bones and internal organs from external insults; therefore, any damage or injury to the skin should be efficiently mended. Wound-healing biology involves the signaling pathways that trigger relatively inactive cell lineages to the wound margin for the proliferation, invasion, and reconstruction of new matrices in the wound gap [1]. The interference caused by loss of tissue, inadequate or restriction in blood flow, and high comorbidity in different stages can lead to chronic wounds that become very complex to treat [2]. Studies have established that the incidence of severe burns in the United States is estimated to be 70,000 per year [3]; the occurrence of venous leg ulcer is between 600,000 and 1,500,000 [4]; and the prevalence of chronic foot wounds in people suffering from diabetes is 15–20% [5]. Thus, the dressing cost alone for the abovementioned cases has been calculated as $5 billion per year [6, 7].

During the re-epithelialization phase in wound healing, the proliferation of keratinocytes is increased in order for the cells to migrate and cover the entire surface of the wound. In severe or chronic injuries, the keratinocytes on the wound edges are not able to migrate and therefore the wound cannot be closed. This is because the non-healing keratinocytes on the wound edges are unresponsive to the activation signals that promote cell migration [8–10]. Over the past few years, stem cell therapy has emerged as a novel clinical approach for several diseases including wound repair and regeneration. The abundant availability of fat tissue, ease of isolation, excellent proliferative capacity, and the ability to secrete various growth factors make adipose-derived stem cells (ASCs) an ideal cell type for treating non-healing wounds [11]. It has been shown that ASCs have the capacity to transdifferentiate into keratinocyte-like cells (KLC) [12]. Since, ASCs help secrete various growth factors, it might help activate the signals in non-healing keratinocytes at the wound edges to proliferate and cover the wound surface.

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Autologous skin grafts such as split- or full-thickness skin graft were used widely for wound coverage, but the factors like donor site morbidities, limited availability, and scarring are the main drawbacks of these autograft applications. Over the past two decades, the emergence of bioengineered skin substitutes has paved way for the advancement in wound management technologies. The main focus of these skin substitutes was to replace allograft, autograft, and xenograft for acute and chronic burn injuries [6]. The timely restoration of lost functions in burn victims with various levels of severity is the important issue to be addressed. There are two main catogories of skin substitutes, namely, synthetic and biological skin substitutes. The morphology of biological skin substitutes closely resembles the intact human skin, whereas the synthetic skin substitutes do not mimic the skin, but they can be modified and produced as per specific demands [13].

The skin substitutes can be custom designed to treat different types of wounds such as extensive burns, diabetic ulcers, and trauma-induced wounds [3, 14–16]. These skin substitutes are of three types, namely, full-skin substitute, epidermal substitute, and dermal substitute. The usage of living keratinocytes on the epidermal compartment and living fibroblasts in the dermal compartment on top of acellular dressings has been largely accepted. These living skin substitutes secrete growth factors, chemokines, and cytokines that accelerate wound healing and also provide an excellent coverage for wound [17–19]. Even though tremendous advancements have been made in the composition of skin substitutes, extensive full-thickness injuries are associated with mortality due to minimal availability of donor sites. Although multiple treatments such as skin grafting and skin reconstruction with biomaterials exist, further development and refinement of skin substitutes or biomaterials are essential for perfect skin reconstruction.

The focus has then been moved to chitosan, a biopolymer that are biodegradable, biocompatible, non-allergenic, and nontoxic in nature. It also exerts anti-inflammatory and antimicrobial properties when used as wound dressings [20]. Studies have shown that the chitosan scaffold acts as an excellent template for ASCs [21]. Adipose tissues are available in abundance and are the source of adult stem cells for translational clinical approaches and also in skin tissue engineering [22, 23]. It has also been reported that ASCs and their conditioned medium have stimulatory effects on keratinocyte proliferation [24]. Therefore, the study of interaction between ASCs and keratinocytes on the chitosan scaffold would contribute to the advancement of skin tissue engineering.

2 Wound-Healing Process

Wound healing is a complex mechanism that begins with an inflammatory phase followed by re-epithelization and ending with a remodelling phase. Tissue injury disrupts vascular vessels and initiates extravasation. The inflammatory response begins with the release of growth factors, cytokines, extracellular matrix (ECM) components, and the migration of inflammatory cells such as neutrophils, macrophages, and lymphocytes into the wound area. Neutrophils are the first inflammatory cells that appear in the wound area. Neutrophils start to attract chemoattractive agents in the wound and activate their phagocytosis functions to destroy bacteria and necrotic tissue. Neutrophils release inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) to further stimulate inflammatory response. Neutrophils activity will gradually deplete and they undergo apoptosis after the process of phagocytosis. Then the apoptotic neutrophils will be engulfed by macrophages [25–27]. Finally, lymphocytes migrate into wounds following the neutrophils and macrophages to promote fibroblast proliferation and collagen biosynthesis [28].

The inflammatory phase generally follows the tissue re-epithelization phase. The re-epithelization phase is characterized by the involvement and proliferation of keratinocytes to resurface the wound area with a layer of new epithelium. As this epidermal layer continues

migrating, the keratinocytes at the wound margin begin to proliferate and migrate to contact the wound margin [1, 29–31]. This process is activated by the epithelial and non-epithelial cell signaling pathways which release different cytokines, matrix metalloproteinase (MMP), and growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), insulin-like growth factor − 1 (IGF-1), nerve growth factor (NGF), and TGF-α [27, 28].

The final phase of the wound-healing process is the remodelling phase. During this phase, the collagen fibers reorganize and mature to gain tensile strength [31]. As the wound heals, the fibroblast and macrophages undergo apoptosis, the components of the ECM get changed, the angiogenesis and the wound blood flow diminish, and the wound metabolic activity slows down and finally stops producing a fully mature scar. In summary, the wound-healing mechanism is a complex chain of events involving different cell-to-cell interaction and interactions among tissues that are impaired due to a number of medical conditions. Skin injuries could be healed more quickly, but re-epithelization is not always perfect and leaves a connective tissue scar. Therefore, the study of the proliferation of keratinocytes, which plays a major role in re-epithelization, is important for tissue reconstruction.

Biological wound-healing therapies facilitate innate repair mechanisms and involve the components of active biomolecules from plants that have antioxidant, anti-inflammatory, and antimicrobial properties. Biological wound dressings prevent heat loss, evaporative water loss, electrolytes and protein loss, and also contamination. Biological skin equivalents and their role in wound healing should be extensively studied in the field of tissue engineering [7].

3 Survival of Skin Graft and Healing

The successful skin graft depends on the vascular ingrowth and uptake of nutrients from the graft bed that involves three phases. The first phase is called as plasmatic imbibition: fibrin layer is formed during the initial contact of the graft to the recipient site and the nutrients are diffused by the capillary action from the graft bed. The second phase is called as the inosculation process: the recipient and donor end capillaries align to form a vascular network. The third phase is revascularization: ingrowth of new vessels and capillaries by neovascularization followed by the ingrowth of nerve fibers from the surrounding tissue and graft bed [32, 33].

The degree of contraction after harvesting skin from the donor site is directly related to the amount of dermis in the graft that is followed by revascularization in the graft bed. The contraction of skin graft occurs in two stages, namely, primary and secondary contraction. When the grafts are freshly harvested from the same area of the body, it contains elastin in the dermis resulting in instant recoil during primary contraction. Since full-thickness skin graft contains more amount of dermis, it exhibits higher chances of primary contraction. The split-thickness grafts display less contraction because of the lower elastin content in dermis and epidermal resulting in unsuccessful contraction [32].

The secondary contraction occurs in the healed graft due to the activity of myofibroblast. It has been observed that full-thickness skin grafts contract less than the split-thickness skin grafts when placed on recipient bed [34]. Therefore, full-thickness grafts are mostly preferred in areas that have significant impact by scarring, such as the hands, head, neck, breast, and genitals. Later, collagen gel models were widely used for studying contraction. However, these collagen gels failed to replicate cell-matrix interactions in the human skin. Therefore, in vitro models that have an exact replica of the human skin are needed [35]. In any case of injuries, the epidermal component keratinocytes should undergo stratification and differentiation on the basement membrane called ECM for wound closure [36, 37]. Therefore an ideal skin substitute that mimics the ECM is in high demand for excellent proliferation of keratinocytes.

4 Skin Substitutes for Wound Healing

The initial treatments for severe injuries and burn cases include autografts, allografts, and xenografts. However, these treatments often have limited donor sites. Based on the depth of the injuries, wounds can be classified into epidermal, superficial partial thickness, deep partial thickness, and full thickness [38]. The therapeutic approaches for the treatment of deep dermal and full-thickness injuries remain unsatisfactory; therefore, more effective treatment strategies are needed [39].

In the 1980s, when the patients with extensive burn injuries and with inadequate sources of autologous grafting required an early coverage of burn injuries, the importance of tissue-engineered skin substitutes and cell-based therapy for wound healing was highly focused [3, 6]. With advancement in tissue engineering, more recent studies showed that bioengineered skin substitutes have a wide range of applications in wound healing [6]. The main goal behind the use of skin substitutes is to accelerate wound healing using the normal repair mechanism and provide a surface for cells to proliferate and prevent bacterial infection [40]. Based on the requirements of skin injury, different types of skin substitutes are used for specific purposes [16, 35, 41–47].

Bioengineered skin substitutes can offer four functions such as protection, creating a defense barrier to microorganisms; procrastination, achieving permanent wound closure particularly in case of extensive burn injuries; promotion, delivering matrix components, growth factors, and cytokines; and provision, incorporating dermal collagen or cultured cells at the site of wound [48]. Although the meshed skin graft covers a greater area, most of the skin grafts and skin substitutes that have been used have various disadvantages, such as slow epithelialization, delayed wound healing, graft contraction, scarring of tissues, slow vascularization, and inadequate acceleration of wound healing [49].

Additionally, skin substitutes should be cost-effective, readily available, and resistant to infection and have a longer shelf life. Unfortunately skin substitutes with all of these properties are unavailable in the market. Because of the great importance and high demand of skin substitute products, research should be carried out to develop an ideal skin substitute [7, 46]. Table 1 shows some of the widely used skin substitutes with their own advantages and disadvantages [13, 61].

5 Chitosan in Wound Healing

Chitosan is an abundantly available biopolymer composed of (1–4)-2-acetamido-2-deoxy-b-D-glucan (N-acetyl D-glucosamine) and (1–4)-2-amino-2-deoxyb-D-glucan (D-glucosamine) units, which are partially derived from the deacetylation of chitin polymers [62]. Chitin is most commonly found in invertebrates such as crustaceans or insect cuticles, mushrooms, green algae, yeasts, and the shells of shrimp and crab [63–66]. However, the poor solubility of chitin limits its practical usage. The presence of amino groups differentiates chitosan from chitin and gives unique properties to the chitosan polymer, which has more clinical and nonclinical applications (Fig. 1) [67, 68].

Chitosan biopolymers have been widely used in the fields of biotechnology, cosmetics, biomedicine, food, and agriculture [65]. In tissue engineering, chitosan that accelerates wound healing has been used for wound dressings and creating artificial skin [66]. Chitosan is found to be biodegradable and biocompatible and is an excellent hemostatic and analgesic agent with antioxidant properties [65]. Chitosan enhances the functions of inflammatory cells and growth factors, thereby promoting granulation and remodelling of damaged tissues in large, open wounds of animals [69]. It has been shown that chitosan hydrogel interacts with fibroblast growth factor (FGF-2) on an open-wound surface in a

Table 1 Various types of skin substitutes

Types	Product and description	Advantages	Disadvantages
Acellular skin substitutes	Biobrane®: discovered in 1970, used as a temporary skin substitute; has a nylon mesh and a silicon membrane	Nylon mesh acts as dermis, silicon membrane acts as epidermis; temporary coverage for superficial or partial-thickness wounds, burns, donor sites, and congenital diseases; readily available, low pain, accelerates wound healing, temporarily covers the wounds until the graft material is available	• Risk of infection • Occurrence of toxic shock syndrome due to the accumulation of exudate underneath it [48, 50–53]
	Integra®: bilayered skin substitute made of silicone membrane; bovine collagen and shark chondroitin-6-sulfate glycosaminoglycan	Silicone membrane acts as an epidermal layer; impermeable to water, thereby protects against infection; revascularization of wounds occur within 2–3 weeks; immediate availability, neo-dermis formation and aesthetic results	• Requires two-step operation • Expensive • The accumulation of exudate underneath it can lead to infection [50, 51, 54, 55]
	Alloderm®: formed from acellular matrix derived from cadaveric dermis; epidermis and any cellular material are removed using salt	Provides a medium for fibroblast and endothelial cells to regenerate from the neodermis	It has no epidermis [50, 51, 55]
Cellular allogenic skin substitutes	TransCyte®: tissue-engineered skin substitute made from nylon mesh and a silastic semi-permissible and biocompatible layer; allogenic fibroblasts from neonatal foreskin are embedded in the mesh	Fibroblasts are allowed to grow for 3–6 weeks to produce a cellular matrix of collagen and growth factors; enhance wound healing	Requires surgery in case the wound is unhealed [48, 52, 55]
	Dermagraft®: similar to TransCyte but it lacks silicone layer; contains viable fibroblasts	The neonatal foreskin fibroblasts are mixed with biodegradable mesh from polyglycolic acid in a bag with circulating nutrients; cryopreserved fibroblasts can be used and when implanted to the wound, they start to proliferate and produce growth factors and extracellular collagen matrix components; the polyglycolic acid mesh is absorbed within 3–4 weeks and used effectively in vestibuloplasty after mucogingival junction and supra-periosteal dissection	• Used for temporary coverage • 6-month shelf life: may cause clinically infected ulcers • Ulcers with sinus tracts • Hypersensitivity to bovine products [50, 51, 56, 57]
	Apligraf®(Graftskin®): also known as composite skin graft, skin equivalent, or organo-typical skin substitute; prepared by mixing fibroblasts from neonatal foreskin with bovine collagen type I and exposed to heat to produce a loose matrix; dense fibrous network is formed on 2 weeks' time; the keratinocytes from the same or different donor are then seeded on the surface of the dermal fibrous matrix, and after 4 days, they proliferate and differentiate; within 7–10 days, stratum corneum is formed which is then ready for clinical use	Has both living dermis and epidermis; first true composite skin graft for the treatment of non-infected or full-thickness venous ulcers; it is clinically effective due to its occlusive properties and biological mediators; also helps managing wounds in epidermolysis bullosa, donor sites, surgical excision of skin cancer and burns	• Multiple applications are required for larger wounds • Expensive • Viral transmission [50, 51, 54, 57]

(continued)

Table 1 (continued)

Types	Product and description	Advantages	Disadvantages
Cellular autologous skin substitutes	Cultured epidermal autograft (CEA): the keratinocytes isolated from skin biopsy are cultured on irradiated 3 T3 mouse fibroblasts; they are processed to sheets and placed on petroleum gauze	Culture medium contains essential growth factors; commercially available; minimal or no chance of rejection because autologous cells are used; permanent wound coverage for extensive burns	• Long culture time required for keratinocytes (3 weeks) • Not effective in full-thickness wounds or burns. Therefore, they need a supporting dressing • Expensive and poor long-term results • May cause blisters, contractures, and infection [50, 51, 58, 59]
	Cultured skin substitutes (CSS): they have both epidermal and dermal components	Since it is an autologous graft, there is minimal risk of transmission of infection; acts as a permanent coverage; can be handled easily; do not cause blisters	Expensive [50, 51, 55]
Permanent skin substitute	Autologous fibroblasts and keratinocytes isolated from skin biopsy are cultured on a laser-micro-perforated biodegradable matrix of benzyl esterified hyaluronic acid	Micro-perforations allow for drainage of wound exudate; less chance of rejection; can be produced in shorter period of time: less chance of infection; ease of handling	• Limited availability • Shorter shelf life (2 days): expensive [60]

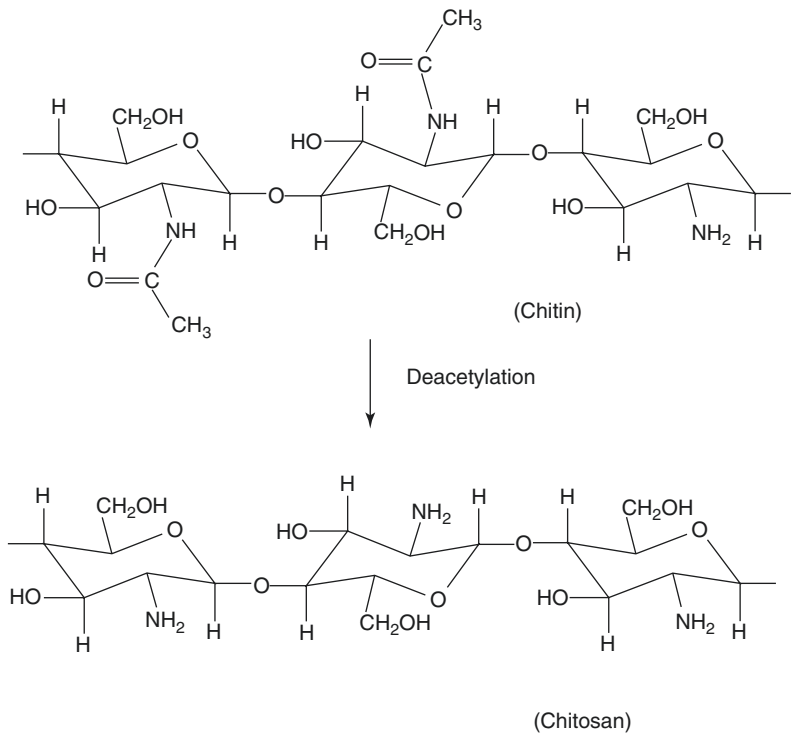

Fig. 1 Chitosan derived from deacetylation of chitin [67]

mouse model. This interaction resulted in contraction of the wound, formation of granulation tissue, and closure and healing of the wound [70]. Recent studies have shown that a bilayer chitosan membrane, which consists of an upper chitosan film layer attached to an inner layer of porous membrane, serves as an efficient skin-regenerating template for treating third-degree burns and cutaneous wounds. This chitosan bilayer has the potential to enhance the proliferation of fibroblasts, thereby forming a monolayer to cover the wound surface [71]. The three-dimensional structural organization of chitosan is essential to serve as a vehicle for delivering and retaining the cells at a specific site and to initiate appropriate cell-to-cell interactions [72]. Chitosan supports the adhesion and activation of platelets, which are enhanced by plasma and ECM proteins [73].

Several other reports have also described the beneficial effects of chitosan for wound healing. The study by Okamoto et al. [74] observed that chitosan powder promoted re-epithelization and inflammatory cell infiltration at 28 days when used to treat full-thickness wound created on dorsal midline of a dog. In a different study, Mi et al. [75] prepared an asymmetric chitosan membrane that contained several advantages including the ability to evaporate water loss, absorb oxygen, promote fluid drainage, inhibit the invasion of microorganisms, and had inherent antimicrobial property of chitosan. Open skin wounds created on rats and covered with this asymmetric chitosan membrane were found to heal faster with increased rate of epithelialization and the collagen deposition in the dermis also well organized. Additionally, the effect of a chitosan acetate bandage on *Staphylococcus aureus*-infected wounds in mice was also studied. The results found that application of the chitosan acetate bandage promoted wound closure, reduced inflammatory cells, and stimulated effective wound healing [76].

It has also been shown that in comparison to heparin powder, chitosan greatly prevented the early extension of skin burns [77]. Moreover,

histological analysis revealed that lack of granulomatous inflammatory reaction in burn wounds treated with chitosan hydrogel [78]. A similar study also found that the chitosan hydrogel promoted good tissue regeneration and induced inflammatory cell migration, angiogenesis, and collagen synthesis when applied on third-degree burn skins [79]. Biagini et al. [80] found that treatment of the surgical site using the modified chitosan, N-carboxybutyl chitosan, promoted better histo-architectural order, improved vascularization, reduced the inflammatory cells, and promoted proliferation of the Malpighian layer at the epidermal layer. The N-carboxybutyl chitosan also promoted an organized cutaneous tissue and reduced an abnormal healing. Evaluation of wound healing at the split skin graft donor site dressed with chitosan dressing showed rapid re-epithelization and nerves regeneration with reduced scars compared with the conventional dressing [81]. A similar study by Azad et al. [82] found that mesh chitosan membrane applied on the fresh wound of skin graft donor site promoted cell adherence, hemostasis, healing, and re-epithelialization. The application also reduced itching and pain sensitivity. The new type of biological chitosan dressing which consists of lyophilized human placenta was designed. The evaluation of the wounds dressed with this invented dressing showed improved rate of demarcation, growth of granulation tissue, and epithelialization [83].

Based on the literature, tissue-engineering scaffolds should (1) be biodegradable to favor the cured tissue in replacing the biomaterial, (2) not trigger acute or chronic inflammatory responses, (3) have surface properties that enhance the attachment, proliferation, and differentiation of cells, (4) mimic the skin *in vitro*, (5) have suitable mechanical properties, and (6) be suitable for manufacturing into different shapes [68]. Chitosan when compared with the abovementioned skin substitutes has all of these remarkable properties which make chitosan scaffold highly potential for the management of wound healing. Also, its availability in different forms would serve as efficient scaffolds in the treatment of acute and chronic wound injuries [7].

6 Keratinocytes, Skin Substitutes, and Chitosan

Keratinocytes are the predominant cell components of the epidermis. These cells play a significant role in the wound-healing process because they are involved in the complex mechanisms of initiation, proliferation, and re-epithelialization of wound healing. Normal and healthy keratinocytes differ from the keratinocytes at the non-healing chronic wound edges. In cases of injuries, the migration of basal keratinocytes from the wound margin and cutting epidermal appendages to the denuded wound surface are essential to carry forward or move over the newly reconstructed dermal scaffolding. The stratified keratinocytes proliferate and differentiate to produce neoepidermis, which covers the entire wound surface and restores the functions of the skin [84]. For the successful closure of wounds, the proliferation of keratinocytes is essential to communicate with other cell types that are involved in wound healing [85].

In case of extensive, full-thickness burn injuries, permanent wound closure remains as a challenge. This is because recovery from chronic burn injuries requires intricate critical care that involves cardiovascular and respiratory support, fluid resuscitation, management of microbial contamination and infection, nutritional support, psychosocial adaptation, and physical therapy. However, complete recovery depends on closure of wounds with the help of autologous epidermis and connective tissue that aid in wound healing with minimal scar [86, 87]. Keratinocytes initiate strong adhesions of intercellular components. When the epidermal sheets contain confluent keratinocytes, rapid contraction takes place up to 70% of their original area. Keratinocytes are also very effective in contracting collagen gels when they are seeded on top. As this mimics the in vivo environment, the keratinocytes start migrating across the surface of the wound during re-epithelialization [88, 89].

Cultured keratinocytes have also been sprayed as cell suspensions on partial-thickness burns or used in dermal substitute, but the healing time is lengthy because of the slow transformation of

cultured keratinocytes to form stratified epidermal layer [90–93]. In dermal skin substitutes, both fibroblasts and keratinocytes are cocultured and used. Keratinocytes and fibroblasts strongly interact with each other maintaining the integrity of the skin [17, 18]. Fibroblasts have a great influence on the proliferation, differentiation, and migration of keratinocytes occurring in the process of epidermal homeostasis and re-epithelialization. Fibroblasts and keratinocyte interactions form a basement membrane for the attachment of epidermis to the dermis. So when keratinocytes are cocultured with fibroblasts, they secrete growth factors, cytokines, and chemokines that are involved in wound healing [19, 31, 94, 95].

StrataGraft human skin substitute (Table 2) contains the culture of keratinocytes which forms a stratified epidermal layer that closely resembles the human skin [2]. Keratinocytes are the predominant cell type in the skin and are characterized as "non-professional" antigen presenting cells. This is because the human keratinocytes express major histocompatibility complex (MHC) class I antigens on the cell surface but the expression of MHC class II antigens is absent [101–103]. The expression of MHC class I antigens HLA-A (human leukocyte antigen complex), HLA-B, and HLA-C and MHC class II antigen, HLA-DR, is related to tissue rejection in humans, and when these molecules are closely matched between the donor and recipient, the clinical output can be highly improved. The structure, expression of differentiation markers, development of basement membrane, barrier function, and the expression of MHC of StrataGraft tissue closely resemble human skin [104].

Table 2 Types of skin substitutes used in combination with ASCs

Description	Production method	Advantages
Skin substitutes without external matrix [96, 97]	They imply on endogenous production of ECM components by different skin cells under controlled specific culture conditions. The fibroblasts or ASCS start to produce ECM proteins after the stimulation by ascorbic acid. This results in the formation of cellular sheet which could be manipulated and folded to obtain the desired thickness of a graft	• More advance than using dermal fibroblasts • Desired epidermal thickness and stratification could be reached • Three-layered skin substitutes were developed
Collagen type I hydrogel-based skin substitute [22, 98]	The collagen type I from animals is the most commonly used polymer for the production of skin substitutes. Bovine type I collagen with the whole human stromal vascular fraction (SVF) has been used to develop a vascularized dermal component of the skin. The skin constructs were cultured with stromal or dermal cells and also help to form capillary network by endothelial cells	• Low immunogenicity and excellent properties to support cell growth • Possible prevascularization of collagen type I hydrogel-based skin substitutes in vitro • The preformed human capillaries allowed fast blood flow throughout the graft
Vascularized skin substitutes [98, 99]	Cells seeded on collagen type I matrix were differentiated into fibroblast-like cells in one layer. The same cells when seeded on fibrin-based layer developed a network of blood capillaries. The ASCs were differentiated into adipocytes in the third collagen type I-based layer of construct forming the hypodermis	ASCs in different lineages were used in one skin substitute with various biomaterials
Fibrin hydrogels [99]	The skin substitutes were developed using three skin layers which includes epidermis, dermis, and hypodermis. ASCs were seeded on the hypodermis layer where they successfully differentiated to adipocytes	The layers interact effectively influencing the behavior of epidermis in vitro
Tropoelastin-based scaffold [100]	Tropoelastin highly expressed in *E. coli* is used in the development of this scaffold by electrospinning procedure. This bimimetic network was seeded with ASCs in vitro and transplanted on SCID (severe combined immunodeficiency) mice	• Rapid wound closure • Increased epidermal thickness

Bilayered, allogenic skin substitutes were used as a temporary wound coverage before autografting. Also cultured epithelial autografts were applied as partial stratified keratinocyte sheets but were reported having various disadvantages such as causing blister, ulcers, and fragile texture due to the absence of basement membrane [105–107]. There are currently not many keratinocyte-based skin substitutes in the market, and the advancements have to be made with existing substitutes with keratinocytes [86].

Because chitosan has been employed as the best biomaterial for wound dressing, studies have been conducted to determine the interactions between chitosan and keratinocytes. The degree of acetylation (DA) is a term used to define chitin and chitosan. DA is an essential structural parameter that influences biological and wound-healing properties [108–110]. Cultures of keratinocytes were analyzed on five different chitosan films with DAs ranging from 2.5% to 47%, and the cell adhesion and proliferation of the keratinocytes on these chitosan films were investigated. The DA does not influence the cytocompatibility of chitosan films with keratinocytes in vitro; however, fully deacetylated chitosan films allowed for better adhesion to keratinocytes, resulting in their better proliferation. This finding indicates that chitosan films with low DA could act as efficient biomaterials because they would adhere to fibroblasts and induce the proliferation of keratinocytes and re-epithelialization [111].

The effects of chitin and chitosan on the proliferation of keratinocytes in vitro were studied. Primary human keratinocytes and an immortalized human keratinocyte cell line (HaCaT) were cultured with and without an irradiated fibroblast feeder layer. Chitosan and the primary keratinocytes with the irradiated feeder layer supported the growth and proliferation of keratinocytes in vitro [112]. This study proves that highly deacetylated chitosan has more potential for wound healing. However, the mechanism of interaction between chitosan and keratinocytes is not clear [7, 113]. Hence it is important to study the proliferation of keratinocytes on chitosan scaffold.

7 Adipose-Derived Stem Cells (ASCs), Skin Substitutes, and Chitosan

The epidermal skin substitutes containing keratinocytes were found to be not very effective and also the esthetic results were not satisfying due to scars, graft contracture, and infections [43, 114, 115]. Even then, these skin substitutes are improvised by adding cellular dermal component that enhances the function and esthetic appearance. Fibroblasts are the main cellular dermal component which helps produce ECM proteins such as laminin, elastin, collagen, and fibronectin, thereby creating a stability to dermis and regulating the functions of epidermis, melanocytes, and keratinocytes. So, the dermo-epidermal tissue-engineered skin substitutes (DESS) were extensively studied and used as an alternative in treating deep burns [100, 116–118].

Previous research findings reveal that bone marrow-derived stem cells (BMSCs) were found to play an important role in tissue repair. BMSCs secrete growth factors that enhance the stimulation, proliferation, and regeneration of damaged cells [119]. The transplantation of BMSCs requires harvesting of large numbers of bone marrow cells under general anesthesia, which leads to several complications that limit its use [120, 121]. ASCs are pluripotent stem cells that are derived from adipose tissue and have characteristics that are similar to mesenchymal stem cells derived from bone marrow.

ASCs were found to have potent applications in the repair and regeneration of damaged tissues by helping in wound healing and in the treatment for scarring and photoaging. Therefore, ASCs can be used therapeutically to treat chronic wounds and other conditions. However, for massive tissue damage, the available ASCs are insufficient to efficiently repair the damaged tissue, but this could be overcome by using adipose tissue obtained from liposuction procedures [122, 123]. The microenvironment for skin regeneration mainly depends on interactions between stem cell progenitors and their niche [124]; therefore, any tissue-engineered reconstruct should provide a suitable

microenvironment for the cells to proliferate and differentiate. Therefore, the selection of suitable biomaterials for the culture and differentiation of ASCs is very much required [96]. It has to be compatible, support growth, and proliferation of skin cells and most importantly, they should mimic the nature of the human skin. Various biomaterials and scaffolds have been studied for creating artificial skin substitutes. Below are a few examples of the skin substitutes that are used in combination with ASCs.

These ASCs, when combined with chitosan, have been found to increase the repairing and healing potential of damaged tissues. ASCs were seeded on silk fibroin chitosan (SFCS), and the impact of ASC-SFCS on wound healing was evaluated. Because of its biocompatible nature, silk fibroin was hybridized to chitosan, and the resulting hybrid matrix mimicked the constituents of the ECM and served as a substrate for cell adhesion and migration and the incorporation of tissues [125, 126]. In a murine cutaneous injury model, ASC engrafts proliferated and differentiated into vascular, fibroblastic, and epithelial cell phenotypes in their newly established microenvironment. The ASCs-SFCS also showed vascular enhancement, and SFCS acted as a delivery vehicle that provided a supportive niche for the migration, proliferation, and differentiation of the cells. This study elucidated that ASC-SFCS supports the engraftment of stem cells and their differentiation into epithelial and fibrovascular components; therefore, it could be applied to clinical applications [127].

It has also been found that ASCs induce osteogenic and chondrogenic differentiation in chitosan-agglomerated scaffolds, which are used as a substitute for the ECM [128]. In a similar study, human hair follicle stem cells (HFSCs) combined with fibroblasts were seeded in chitosan and found to be successful in accelerating wound healing in full-thickness wounds of irradiated rats [129]. Porous chitosan scaffolds were proven to be compatible for the attachment and proliferation of ASCs [21]. Studies also revealed that ASCs enhance the proliferation of HaCat cells (immortalized human keratinocytes) and accelerate in vitro wound healing [130]. ASCs and their conditioned medium secrete type I collagen, IL-10, and other growth factors such as KGF-1 and platelet-derived growth factor that play a vital role in wound re-epithelialization by having stimulatory effects on keratinocyte proliferation [130–132]. Therefore, it is critical to study the proliferation of human keratinocytes that is enhanced by ASCs on a chitosan scaffold in vitro.

Conclusions

The cell-based wound therapies were found to be successful in accelerating wound healing but require advanced preservation methods to enhance shelf life. The mechanism of therapeutic applications of ideal skin substitutes should be extensively studied. Studies have shown that keratinocytes, fibroblasts, and ASCs play a vital role in wound healing, and when they are used in combination with skin substitutes, they accelerate wound healing. The unique properties of chitosan may increase the proliferation of keratinocytes when seeded with ASCs. It is therefore important to study the proliferation of human keratinocytes induced by ASCs using the in vitro model of the chitosan scaffold. This scaffold may maximize the healing potential, which aims for perfect skin regeneration. Research should be carried out to establish the stimulatory effects of ASCs on a chitosan scaffold and the involved cellular and molecular mechanisms of these cells. Furthermore, importance should be placed on the efficient development of chitosan skin substitutes with long-term safety and longer shelf life. Thus, this review has highlighted the various skin substitutes that are available and the importance of chitosan for the proliferation of ASCs and keratinocytes in tissue reconstruction, which paves the way to novel therapeutic strategies.

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Why Isn't This Wound Healing?

Rose L. Hamm

1 Introduction

An initial evaluation of a patient with a wound is designed to answer two questions. First, what is the wound etiology? And second, why is the wound not healing?

Even when the etiology is obvious, reasons for slow or lack of healing may be more difficult to identify. The purpose of this chapter is to present both common and uncommon factors that can impair wound healing as well as characteristics that may be present, offering a red flag to the clinician that other intrinsic or extrinsic issues need to be addressed. Factors will be discussed in the following categories: comorbidities, infections, medications, nutritional deficits, and psychosocial behaviors.

The most helpful clues in identifying factors that impair wound healing come from the patient and/or caregivers; however, laboratory values are of value in many cases. Table 1 lists the laboratory tests, normal values, and clinical presentations of wounds that are affected by either an increase or decrease in the test results.

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2 Comorbidities

2.1 Diabetes

Diabetes is universally recognized as a disease that involves delayed wound healing by impaired leukocyte function with resulting chronic, intense inflammation and higher risk of infection, as well as impaired macrophage and fibroblast function [1, 2]. These impairments are in addition to the known higher risk for peripheral arterial disease and coronary artery disease with diabetes, which can also interfere with timely wound healing (Figs. 1 and 2). These changes appear to occur not only in tissue involved with the typical diabetic foot ulcer but also with wounds of other etiologies such as surgical incisions, pressure ulcers, and venous wounds that occur on patients with diabetes. Further, the effect of diabetes on wound healing is evident in all phases of acute wound healing (hemostasis, inflammation, proliferation, and remodeling). Although the exact mechanisms for the impaired cellular function are not well understood, studies primarily on animal models are giving more insight to the dysfunction of cellular and noncellular components of wound healing that occurs in patients with diabetes. During the normal hemostasis phase, there is an infiltration of platelets which both form a fibrin plug and release growth factors and cytokines which are responsible for attracting the cells for the inflammatory phase (neutrophils, macrophages, mast cells,

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Table 1 Laboratory values with implications for wound healing

Laboratory test	Normal range	Values affecting wound healing	Clinical presentation
CBC			
WBC	4.5–11 × 10^3/mm^3	Increased	Signs of infection, inflammation, necrosis, trauma, or stress
		Decreased	Failure to initiate immune response against bacteria
RBC	M 4.5–5.5 × 10^6/mm^3 F 4.1–4.9 × 10^6/mm^3	Decreased	Pale or anemic granulation or failure to progress
Hemoglobin	M 13.5–18 g/dL F 12–15 g/dL	Decreased	Pale or anemic granulation or failure to progress
		Increased	Failure to progress (patient may show signs of congestive heart failure or COPD)
Hematocrit	M 37–50% F 36–46%	Decreased	Pale or anemic granulation or failure to progress
		Increased	Signs of thrombi or emboli
Platelet count	150–400 × 10^3/mm^3	Decreased	Bleeds easily, fatigue
		Increased	Signs of infection or inflammation
Automated differential			
Neutrophil Rel	57–67% of leukocyte count	Increased	Bacterial infection Chronic inflammation
Lymphocyte Rel	25–33% of leukocyte count	Increased	Signs of bacterial infection
		Decreased	Opportunistic infections
Monocyte Rel	3–7% of leukocyte count	Increased	Tissue injury Early healing response
Eosinophil Rel	1–4% of leukocyte count	Increased	Allergic reaction Parasitic infection
		Decreased (with corticosteroid use)	Delayed inflammatory response
Basophil Rel	0–0.75% of leukocyte count	Increased	Allergic reaction
Neutrophil Abs	4300–10,000 cells/mm^3	Increased	Signs of infection
Lymphocyte Abs	2500–3300 cells/mm^3 2000–2500/μL	Increased	Signs of bacterial infection
		Decreased	Opportunistic infections
Monocyte Abs			
Eosinophil Abs			
Basophil Abs	0–1000 cells/mm^3	Increased	Allergic reaction
Coagulation studies			
PT (prothrombin time)	12.3–14.2 s	Increased (>2.5 × reference range)	Bleeds easily
PTT (partial thromboplastin time)	25–34 s		
INR	Normal 0.9–1.1 Therapeutic range 2–3 Mechanical heart valves 2.5–3.5	Elevated	Bleeds easily Skin bruising
Routine chemistry			
Sodium	135–145 mEq/L		
Potassium	3.5–5.3 mEq/L		
Chloride	95–105 mEq/L		
CO_2	22–29 mEq/L 35–45 mmHg (arterial)		

Table 1 (continued)

Laboratory test	Normal range	Values affecting wound healing	Clinical presentation
Glucose	70–115 mg/dL	Decreased (<70)	Headache, dizziness, altered mental status, malaise
		Increased (>200)	Arrested healing processed Signs of infection Increased risk of abscess formation
Calcium	8.8–10.5 mg/dL		
Phosphate	2.5–5.0 mg/dL		
BUN	7–18 mg/dL	Increased (renal failure)	Edema Poor healing
		Decreased (liver failure)	Jaundiced skin Yellow fluids
Creatinine	0.1–1.2 mg/dL	Increased (renal failure)	Edema
		Decreased	Decreased lean body mass
Albumin	3.5–5.5 g/dL	Decreased	Lack of granulation tissue Bilateral edema Muscle wasting
		Increased	Signs of dehydration
Prealbumin level	15–36 mg/dL	Decreased	Poor wound healing Lack of granulation formation
Globulin	2.5–3.5 g/dL		
Fibrinogen	200–400 mg/dL		
A/G ratio	1.5:1—2.5:1	Decreased (liver damage)	
		Increased (iron deficiency)	
Bilirubin total	0.1–1.0 mg/dL	Increased	Yellow wound fluids Jaundiced skin
Alkaline phosphate	30–85 U/L		
Others			
HbA1c	4–6%	Increased	Delayed healing
C-reactive protein (CRP)	0–1.0 mg/dL or <10 mg/L	Increased	Inflammation Infection
Retinal binding protein	10 mg/L 0.002 g/kg body wt.	Increased	Delayed healing due to protein deficits
Magnesium	1.5–2.5 mEq/L		
Phosphorus	2.5–4.5 mg/dL		
Iron		Decreased	Lack of granulation
		Increased	Hemochromatosis
Ferritin		Decreased	Anemic granulation Lack of granulation
		Increased	Hemosiderosis
Transferrin	204–360 µg/dL <0.1 g/kg body wt.	Decreased	Delayed wound healing due to protein deficits
		Increased	Iron deficiency
Zinc		Decreased	Delayed wound healing Lack of epithelialization
Microbiology			
Wound culture	Negative	Positive	Poor wound healing Wound degradation
Blood culture	Negative	Positive	Systemic signs of infection

From Hamm R. Factors that impede wound healing. In Hamm R (Ed). Text and Atlas of Wound Diagnosis and Treatment. New York: McGraw Hill; 2015: 297–316

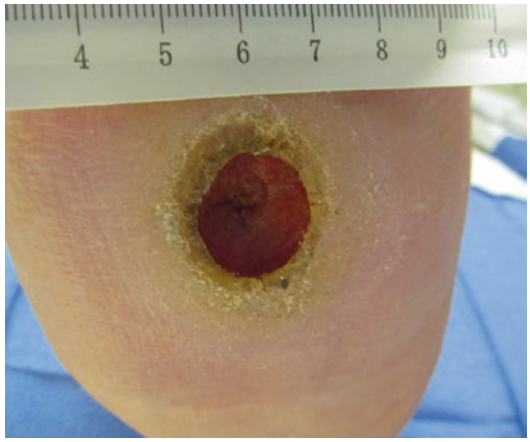


Fig. 1 A typical diabetic foot ulcer occurs on the plantar or weight-bearing surface of the foot and has hyperkeratotic edges. Drainage, granulation tissue, and other wound characteristics are variable

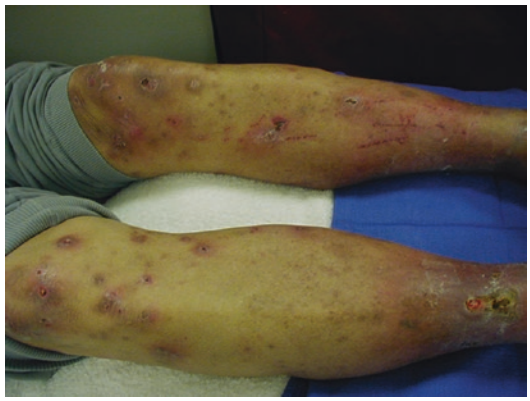


Fig. 2 Patient with diabetic dermopathy, a condition characterized by hyperpigmentation and bilateral asymmetrical atrophic skin lesions. They are caused by microvascular disease associated with diabetes. Other complications of diabetes include nephropathy, retinopathy, and peripheral neuropathy (motor, sensory, and autonomic)

and ultimately fibroblasts). In a hyperglycemic state, there is a delay in the fibrin plug formation which leaves the wound open to contaminants. This, along with the delay in the phagocytic cell migration, leads to an increased risk of infection in patients with diabetes [3].

Keratinocytes are a key cell in instigating the inflammatory process and cell proliferation; however, in the injured tissue of diabetic patients, there is diminished keratinocyte proliferation and migration. Lan et al. [2] used cultured human keratinocytes and a diabetic rat model to show that interleukin-8 production and neutrophil infiltration are both increased in a high-glucose environment due to elevated reactive oxygen species (ROS) levels, resulting in impaired wound healing in diabetic skin. Using the same animal model for another study, Lan et al. examined the potential influence of keratinocyte-derived thrombospondin-1 (TSP1), an antiangiogenic molecule, on diabetic wound healing. They showed that a hyperglycemic environment increased the expression of TSP1 in keratinocytes; furthermore, the early administration of antioxidants normalized the TSP1 expression and improved wound healing in vivo [4].

It is known that increased inflammatory cytokines (e.g., tumor necrosis factor and interleukin-6) lead to dysregulated leukocyte influx to wounded tissue, thus resulting in chronic inflammatory responses and poor wound healing. The expression of pro-inflammatory cytokines occurs partly as a result of the activation of Toll-like receptors (TLRs); overexpression of TLRs leads to an overproduction of pro-inflammatory cytokines which in turn can result in tissue destruction and/or a chronic inflammatory state such as that observed in diabetic ulcers [5, 6]. Wu and his associates also found that REG3A, an antimicrobial protein that regulates uncontrolled inflammatory responses, was decreased in epidermal keratinocytes around acute wounds in patients with diabetes [5].

During the inflammatory phase, neutrophils form extracellular traps (termed NETs), and patients with diabetes have been shown to have higher levels of NETs with impaired wound healing. In an animal study with diabetic mice, it was found to be a major determinant in delaying wound healing [7].

Fibroblasts are active during proliferation in the formation of extracellular matrix which anchors the new capillaries produced by the endothelial cells. Patients with diabetes are known to have abnormal fibroblast function. Khamaisi et al. [8] studied the fibroblasts of subjects with type 1 diabetes (T1D group) as compared to subjects without diabetes and found the following fibroblast abnormalities: lower basal VEGF protein secretion and mRNA levels in the

T1D group, no increased production of VEGF and mRNA in the T1D group with hypoxia and insulin stimulation, decreased fibroblast migration in the T1D group, and increased levels of TGF-ß, fibronectin protein, and TGFB in the T1D group. Furthermore, they observed threefold greater neovascularization in the control group than in the T1D group. Several tests also demonstrated increased PKCδ expression in the T1D group with decreased VEGF secretion and delayed wound healing in vivo.

These studies show that in addition to the macro- and microvascular disease, cardiovascular disease, obesity, neuropathy, and reduced resistance to infection, there are unique and detrimental cellular activities which contribute to the tissue environment that impairs wound healing in patients with diabetes.

2.2 Autoimmune Diseases

Connective tissue disorders, also termed collagen vascular diseases, include several related inflammatory conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), scleromerma (systemic and localized), Sjogren's syndrome, dermatomyositis, polymyositis, and mixed connective disease. Other autoimmune disorders include pyoderma gangrenosum, vasculitis, cutaneous microthrombic ulcers, antiphospholipid syndrome, cryoglobulinemia, cryofibrinogenemia, cholesterol embolism, and calciphylaxis [9]. All of these have been associated with delayed wound healing or high risk of ulcer formation; however, they each have a different autoantibody as well as a different wound presentation [10, 11]. Table 2 presents the

Table 2 Wounds associated with autoimmune diseases

Disorder	Wound presentation	Pathology	Medical management
Rheumatoid arthritis	Angular shape or undulating border	Venous insufficiency due to decreased ankle motion; medications; vasculitis	Controlling the RA; compression therapy for LE edema; pinch grafting; adalimumab with methotrexate; moisture retentive dressings
Systemic lupus erythematosus	Over digital joints or on the malleolar, surpamalleolar or pretibial areas; painful; sharp or punched out borders; adjacent skin is erythematous, purpuric, rolled or violaceous; skin rash after UV exposure	Multifactorial; can be vasculitic or thrombotic, venous insufficiency, lupus profundus, lichen planus overlap or drug-induced lupus syndrome	Control the SLE; meticulous wound care; mesenchymal stem cell therapy (experimental)
Scleroderma	Induration around the wound; hypo or hyperpigmented skin; small wound aperture; telangiectasia; usually on the fingertips or dorsa of the IP or MCP joints; painful; tend not to respond to standard care	Fibrotic skin; vascular compromise with ischemia; abnormalities in coagulation; tissue calcium deposition; Raynaud's phenomenon; cutaneous calcium deposits (CREST)	Moisture retentive dressings; enzymatic debridement; Nifedipine; Iloprost; patient education to avoid cold environs; avoid smoking; rehabilitation therapies to optimize function
Sjogren's syndrome	Raynaud's phenomenon, dry skin, angular stomatitis, livedo reticularis, purpura, erythema nodosum, LE ulcers	Associated with cryoglobulinemia, vasculitis, anticardiolipin antibody, Felty's syndrome	Immunosuppressive agents, supportive wound care
Dermatomyositis	Heliotrope rash, periungual telangiectasias, dystrophic cuticles, violaceous erythema, Gottron's papules, dermal calcinosis after trauma	Vasculitis	Poor prognosis; I cyclophosphamide pulse therapy to induce DM remission; colchicine and calcium channel blockers for calcinosis
Mixed connective tissue disease	Raynaud's phenomenon with hand edema; sclerodactyly; calcinosis; telangiectasia; photosensitivity; malar rash		

Adapted from Dabiri G, Falanga V. Connective tissue ulcers. *J Tissue Viability.* 2013;22(4):92–102

diagnoses, wound presentation, pathology, and medical/wound management for some of the connective tissue disorders. The other autoimmune disorders, which have specific etiologies that sometimes lead to wound formation and atypical wound presentations, will also be discussed in this chapter.

One of the common denominators which affects wound healing in these disorders is the use of steroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and other medications that are necessary to control the underlying systemic disease. In these cases, wound care is challenging and requires discernment from the clinician and physician team as to the most efficacious management of medications, methods of debridement, types of dressings, amount of compression, and application of biophysical agents in order to facilitate healing while optimizing function.

2.2.1 Rheumatoid Arthritis

Although the primary effect of RA is deforming arthropathy, extra-articular nodules and ulcerations can also occur (Fig. 3). Typical locations are the digits as a result of trauma (such as repeated friction or pressure from poorly fitting shoes); venous ulcers on the lower extremities as a result of impaired ankle function, decreased gastrocsoleus contractions (also termed the venous pump), and resulting chronic edema; and pressure ulcers over bony prominences as a result of immobility.

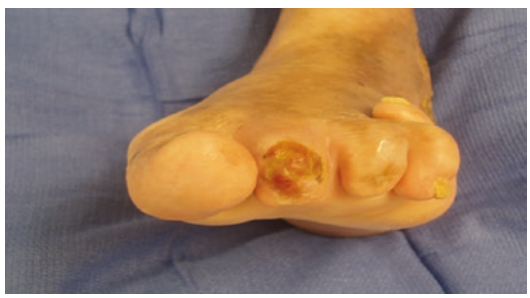


Fig. 3 Pressure ulcer on the distal digit of a patient with severe rheumatoid arthritis as a result of poorly fitting shoes

Several studies have looked at the risk factors that lead to adverse surgical outcomes in patients with RA. Kadota et al. [12] reviewed elective orthopedic procedures and found that patients with foot and ankle surgeries had a higher incidence of surgical site infections and patients with total knee arthroplasty and longer disease duration had a higher risk of delayed wound healing. Menchaca-Tapia [13] found that patients with RA who had hand surgery were more likely to have impaired wound healing after the first surgery with longer disease duration and more aggressive treatment, suggesting that earlier and less aggressive interventions led to improved surgical outcomes.

Because forefoot deformities occur in many patients with RA, surgery of the forefoot is common and has a high rate of delayed wound healing. A longer operative time [14], longer duration of RA, preoperative dorsoplantar deformity, and perioperative tissue damage were found to be risk factors for delayed wound healing [15]. Other reviews have studied the perioperative management of antirheumatic medications, and the only definitive result was that methotrexate appears to be safe in the perioperative period [16]. Based on the review, Club Rhumatismes et Inflammation suggests that drugs be discontinued several weeks prior to surgery and they should not be restarted until after complete wound healing [14]. More discussion on the effects of medications are included later in the chapter.

2.3 Pyoderma Gangrenosum

Pyoderma gangrenosum (PG) is a rare, chronic neutrophilic dermatosis of unknown etiology that causes painful and progressive skin necrosis. PG occurs most often in patients who have systemic autoimmune disorders such as inflammatory bowel disease, hematologic malignancies, rheumatologic disease, vasculitis, lymphoproliferative disorders, and autoimmune hepatitis [17–20]. It is thought to be immune-mediated, and studies have shown that the predominant cells

Table 3 Subtypes of pyoderma gangrenosum

Most common variations
Vegetative
• Also known as superficial granulomatous pyoderma • Localized predominantly to the torso, head, and neck • Presents as superficial wart-like projections with a nonpurulent base • Does not always occur with associated systemic diseases
Bullous
• Acute onset with superficial papules and purple/blue bullae that hemorrhage • Associated with leukemia
Ulcerative
• Most common type • Begins with a small pustule surrounded by inflamed, painful, and rapidly progressive halo • Occurs on the head and neck; associated with systemic vasculitis • May be malignant PG which is aggressive and possibly fatal
Pustular
• Rare, associated with fever and joint pain • Occurs more in patients with inflammatory bowel disease • Presents with pustules that may become ulcerative lesions, primarily on the external surface of the extremities • May also occur with ulcerative lesions
Less common variations
Peristomal
• Occurs after ostomy formation
Pyostomatitis vegetans
• Presents as pustular rash of the oral mucosa • Associated with inflammatory bowel disease
Atypical
• Presents as bullous lesions, usually on the lower extremities • Associated with hematological and/or malignant diseases

Adapted from Soncini JA, Salles AG, Neto JAF, Gemperli R. Successful treatment of pyoderma gangrenosum after augmentation mastopexy using vacuum therapy. *Plast Reconstr Surg Glob Open.* 2016;4(11):e1072, Published online 2016 Nov 9. doi: https://doi.org/10.1097/GOX0000000000001072

present in wound biopsies are neutrophils with chemotaxis dysfunction and hyperresponsiveness [21]. Four types and three other variations have been reported and are listed in Table 3. Postsurgical PG has also been described as rapidly progressing skin ulceration at a surgical site, occurring on the average of 7 days postoperatively (Fig. 4) [22].

Diagnosis of PG begins with a thorough patient history and most often starts as a small lesion such as a scratch or bite that rapidly progresses in size and severity or after a medical procedure. A study by Zuo et al. [22] found that wound complications occurred on average 7 days postoperatively. Again, history of a systemic inflammatory disease is a red flag, especially if

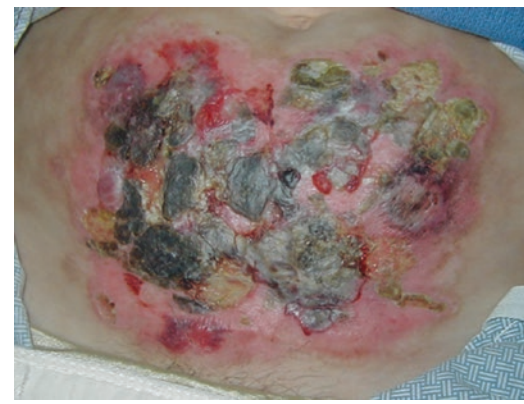


Fig. 4 Postsurgical pyoderma gangrenosum on the abdomen of a patient with diabetes who recently had a hysterectomy

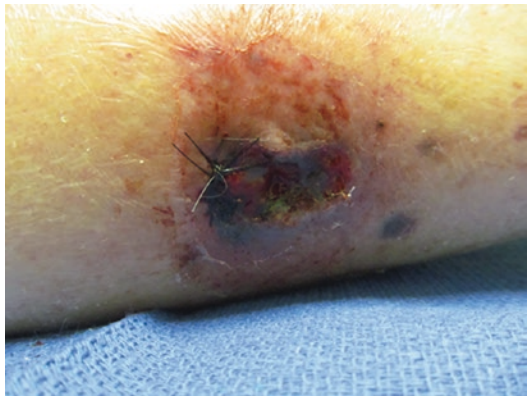


Fig. 5 Pathergy resulted in a larger wound around a biopsy site

other skin disorders are ruled out. The usual presentation is a full-thickness ulcer with undermining and violaceous borders; however, the initial lesion may be a hemorrhagic nodule or papular-pustular lesion with rapid progression to ulceration into the subcutaneous tissue. The only definitive histologic finding is neutrophilic infiltration of the dermis [19, 23]. Therefore, PG is primarily a diagnosis of exclusion, depending primarily on patient history.

Treatment of suspected PG with corticosteroids, if there is a positive response, can help with the diagnosis. Control of the underlying systemic disease, usually with steroids or anti-inflammatory medications is imperative [15, 24], along with meticulous wound management in order to prevent infection. Surgical or sharp debridement is generally contraindicated as it may lead to further pathergy and poor outcome (Fig. 5). A study by Thomas et al. [25] suggested that topical corticosteroids and tacrolimus combined were an effective first-line treatment for mild to moderate cases. Wound management should be performed without trauma or the use of caustic topical agents, again to prevent further tissue destruction [19].

2.4 Vasculitis

Vasculitis is an inflammatory disease of the arterial system that can affect vessels of any size or location, thereby resulting in organ damage (Table 4). When the microvascular system is affected, skin ulceration and delayed wound healing will occur, usually on the lower leg and foot. This condition is also referred to in the literature as cutaneous leukocytoclastic angiitis (CLA) (Figs. 6, 7, 8, and 9). Vasculitis is classified as either primary with no known etiology or secondary in which the inflammation occurs in conjunction with an infection, a toxin, another inflammatory condition (e.g., rheumatoid arthritis, systemic lupus erythematosus), presence of a paraprotein (e.g., cryoglobulinemia or fibrinogenemia) [26], or a malignancy (e.g., lymphoma) [27, 28].

Any vasculitic skin disorder confirmed histologically may be an indication of a systemic vasculitis and needs to be investigated for a definitive diagnosis and optimal treatment [29]. For example, histopathological features of cutaneous or small vessel vasculitis such as papillary dermal edema and perivascular C3 deposition, clinically evident edema, and lesions above the waist may indicate renal or gastrointestinal involvement in Henoch-Schonlein purpura [30].

Vasculitic ulcers may initially appear as self-limited palpable purpuric lesions that progress to deep, subcutaneous ulcerations. Because of the associated anoxia, the wounds are intensely painful, unlike venous leg ulcers that are usually accompanied by a heavy, aching feeling. The chronic inflammatory state of the vasculitic ulcers can cause chronic edema as well, and effective wound management requires treating the edema with appropriate compression, depending on the patient's arterial status and sub-bandage pressure tolerance. However, the most important factor in treating patients with any type of vasculitic wound is the treatment of the underlying systemic disease. Life- or organ-threatening disorders are treated with cytotoxic immunosuppresants and high doses of corticosteroids, usually for 3 to 6 months, and then adjusted to maintain remission. The ultimate goal is to use the least amount of immunosuppresants for as long as needed and to taper and eliminate the corticosteroids and replace with methotrexate or azathioprine [31].

Table 4 Vasculitic syndromes based on affected vessels

Name	Typical vessels involved	Symptoms
Behcet's disease	Small vessels	Recurrent painful lesions in the mouth, on the genitals, on the skin (acne-like), or in the eye (uveitis); limb claudication
Buerger's disease (thromboangiitis)	Vessels to the hands and feet	Typically in smokers; thin shiny skin, thick nails, pain in hands and/or feet; cyanosis that may result in tissue necrosis
Churg-Strauss syndrome (eosinophilic granulomatosis with polyangiitis)	Small and medium vessel	3 stages: 1. Airway inflammation, asthma, allergic rhinitis 2. Hypereosinophilia 3. Vasculitis with tissue necrosis, beginning with purpura
Cryoglobulinemia	Small vessels	Often associated with hepatitis C; appears as painful purpura that progresses to full thickness, often infected wounds; usually on the distal digits. Precipitated by exposure to cold environment that leads to presence of coagulated cryoglobulins that clog the small vessels.
Giant cell arteritis	Temporal and cranial arteries	Headaches, temporal pain, visual disturbances, scalp sensitivity, dry cough with respiratory symptoms, fever, upper extremity weakness and sensory changes, unequal BP measurements or unequal/absent pulses in the limbs. May be associated with polymyalgia rheumatica
Henoch-Schönlein purpura (IgA vasculitis)	Small vessels	Purpura, arthritis, abdominal pain (usually in children)
Hypersensitivity vasculitis (allergic vasculitis, cutaneous vasculitis, or leukocytoclastic vasculitis)	Small vessels to the skin	Purpura, usually on the lower extremities or back (in bedridden patients) due to an allergic reaction to a medication
Immune complex-associated vasculitis	Small vessels to neurons	Peripheral neuropathy
Kawasaki disease	Any of the vessels, any size	Skin erythema, enlarged lymph nodes, red mucous membranes, and in some cases heart problems; occurs in childhood
Microscopic polyangiitis	Small vessels to organs	Ischemia, hemorrhage, loss of organ function
Polyarteritis nodosa	Small and medium arteries	Subcutaneous nodules or projections of lesions; fever, chills, tachycardia, arthralgia, myositis, motor and sensory neuropathies
Primary angiitis of the CNS	Small and medium vessels in the brain and spinal cord	Brain: headache, altered mental status, focal CNS deficits; spinal cord: lower extremity weakness, bladder dysfunction
Takayasu's arteritis	Aorta, aorta branches, pulmonary arteries	Inflammatory phase with flu-like symptoms, pulseless upper extremity, claudication, renal artery disease; fatigue, night sweats, sore joints, and weight loss may occur first.
Wegener's granulomatosis (granulomatosis with polyangiitis)	Small and medium vessels	Organ failure (lung and kidneys), variable including skin, depending on the vessels involved

Adapted from Hamm R. Atypical wounds. In Hamm R (Ed). Text and Atlas of Wound Diagnosis and Treatment. New York: McGraw Hill; 2015: 227–253 and https://www.nhlbi.nih.gov/health/health-topics/topics/vas/types
http://www.merckmanuals.com/professional/musculoskeletal-and-connective-tissue-disorders/vasculitis
Note: The trend is to classify the diseases by descriptive names rather than by names referring to the discovering physician

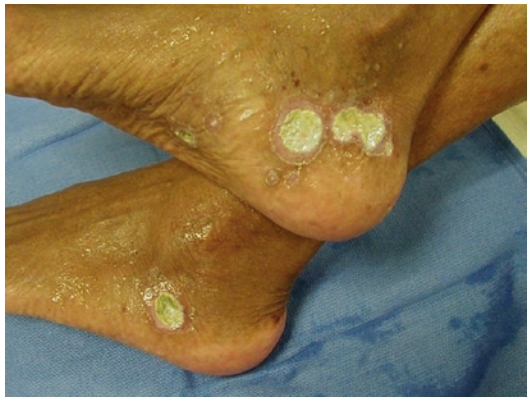


Fig. 6 Vasculitic wounds on the feet of a patient with systemic lupus erythematosus

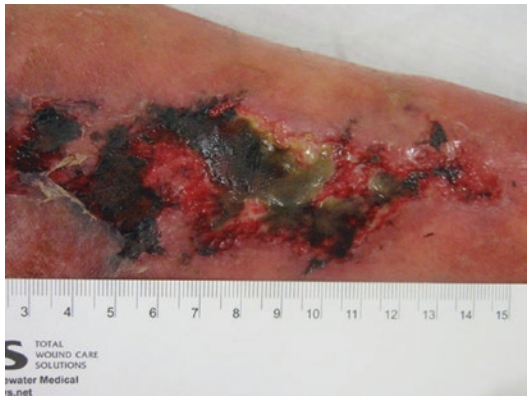


Fig. 7 Vasculitic wound on the posterior leg of a male who had 6 weeks previously experienced CVA symptoms without diagnosis of confirmed cerebral artery disease

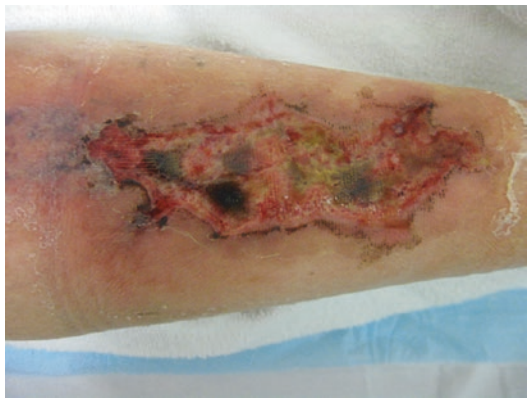


Fig. 8 Two weeks later the patient is showing signs of continued evolution of the vasculitic dermal necrosis at the proximal edge and wound healing at the central and distal wound bed

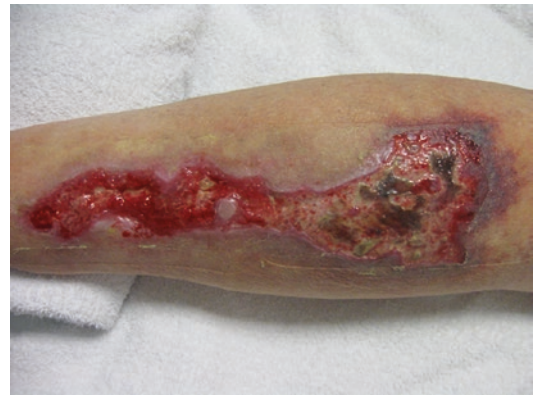


Fig. 9 Four weeks later, the distal wound is granulating, the proximal wound is still in inflammatory phase. Patient was treated with noncontact low-frequency ultrasound, silicone-backed moist wound dressings, and multilayer compression, along with systemic anti-inflammatory medications and progressed to full closure

2.5 Microvascular Occlusion

Several disorders can cause occlusion of the microcirculation (those vessels too small to be named), resulting in dermal changes and/or necrosis. Occlusions that have been discussed in the literature include thrombi, emboli, cholesterol emboli, hydrophilic polymers, and calcium deposits.

Antiphospholipid syndrome (APS) is an acquired autoimmune disorder in which antibodies are directed against one or more phospholipid-binding proteins or their associated plasma proteins, resulting in hyper-coagulation within the small vessels. APS can be with or without associated rheumatic disease (e.g., systemic lupus erythematosus) [32]. Several hypotheses have been proposed for the pathophysiology of APS; however, the end result is that the autoantibodies displace the protective phospholipid proteins that prevent excessive coagulation, thus producing procoagulant endothelial cell surfaces and subsequent arterial or venous thrombosis [31, 33]. Cutaneous manifestations include livedo reticularis, purpura, splinter hemorrhages, skin necrosis, and ulceration. If the thrombosis is venous, the result may be peripheral DVT with lower extremity edema, tachypnea due to pulmonary emboli, and ascites. Arterial occlu-

sions may result in digital ulceration and/or gangrene (Fig. 10) [32]. Because cutaneous manifestations are the first sign of APS in up to 41% of patients, a full medical examination is advised for any patient showing the clinical symptoms in order to identify and treat the underlying pathology [34, 35].

Cholesterol crystal embolization (CCE) is a complication of radiology, vascular surgery, vascular angiography, endovascular procedures, and anticoagulation in patients with atherosclerosis and ulcerated aortic plaques [36, 37]. The initial sign of CCE is often the gradual onset of peripheral cutaneous changes (e.g., livedo reticularis, blue toe syndrome) accompanied by progressive increases in blood urea nitrogen and creatinine levels due to associated renal involvement [38]. Skin biopsy of the middle to deep dermis is diagnostic in 92% of the cases, revealing needle-shaped spaces left by the dissolved crystals within the lumen of the affected arterioles [39]. Because of the renal and cardiac complications, mortality rate is high (81%) and early diagnosis crucial to survival [39].

Microvascular occlusion with cutaneous manifestations has also been reported from hydrophilic polymers after endovascular procedures [40, 41]. Symptoms include sudden onset of lower extremity livedo racemosa, purpuric rashes, or both which occur hours to days after the endovascular procedure involving the aorta. Histopathologic evaluation reveals basophilic lamellated material (hydrophilic polymer gel emboli) within the small vessels [40].

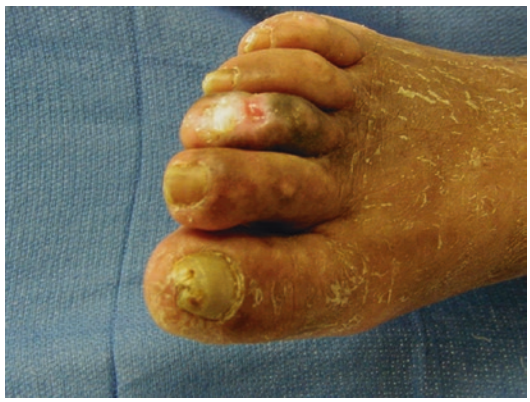


Fig. 10 Foot of a patient with arterial insufficiency. Typical characteristics include shiny or scaly skin, thick yellow nails, presence of fungal infection (termed onychomycosis), and skin ulceration

2.6 Calciphylaxis

Calciphylaxis, also known as calcific uremic arteriolopathy, is a disease of unknown etiology that results in calcification of arterioles in the dermis with subsequent skin and subcutaneous tissue ischemia and necrosis (Figs. 11 and 12). It has typically been associated with end-stage renal disease and long-term dialysis; however, it has also been reported to occur from non-uremic causes, e.g., primary hyperthyroidism, malig-

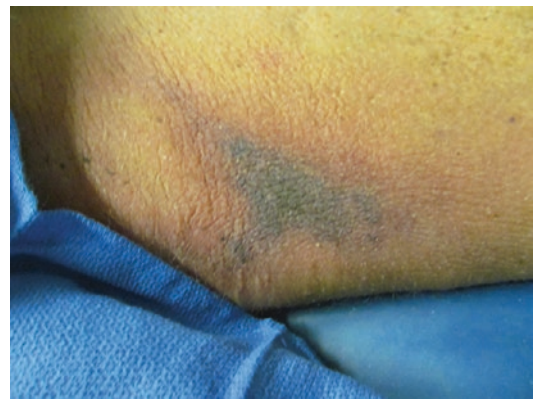


Fig. 11 Initial presentation of a wound due to calciphylaxis

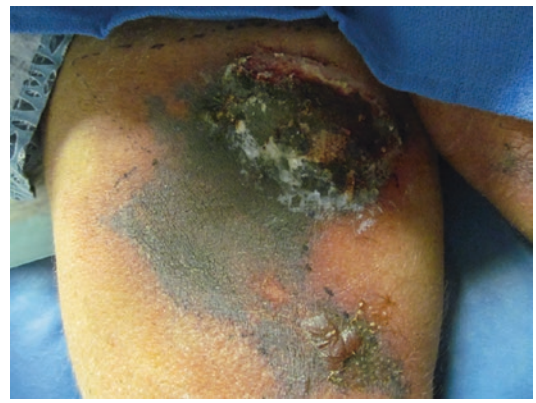


Fig. 12 Advanced calciphylaxis wound on the upper thigh

nancy, alcoholic liver disease, connective tissue disease, previous corticosteroid use, and protein C and S deficiencies [42]. The pathology remains unclear; however, some studies have shown deficiencies in vascular calcification inhibitors, such as fetuin-A and matrix Gla protein, as well as dysregulation of extraskeletal mineralization [42].

The disease initially manifests itself with painful purpuric plaques and nodules that progress to skin necrosis with thick leathery eschar. Most of the lesions occur on the legs (60%), abdomen (23%), buttocks (9%), and feet (7%) [43] and are usually bilateral and symmetric. Calciphylaxis has a reputation of having a high mortality rate; however, with improved medical and wound care, mortality rates are hopefully decreasing. Multiple interventions, including the use of IV sodium thiosulfate to lower calcium-phosphorus by calcium-free phosphate binders, have been reported [43]. A study by Baldwin et al. [44] reported the use of IV sodium thiosulfate plus trigger-agent cessation (calcium-based phosphate binders, alfacalcidol, and warfarin), meticulous wound management, antibiotic therapy, intensified hemodialysis, and hyperbaric oxygen therapy, with six of seven subjects showing complete recovery. Pain management is crucial, although pain medications may interfere with appetite and adequate diet resulting in a need for nutritional supplements in order to get adequate resources for healing. Surgical debridement, negative pressure therapy, and skin grafting with autologous or tissue-engineered skin may provide the most expeditious approach to healing [31].

2.7 Obesity

Obesity is known to increase the risk of other diseases such as type 2 diabetes, cardiovascular disease, hypertension, hyperlipidemia, chronic venous insufficiency, and atherosclerosis, as well as overall physical and mobility limitations. There is now evidence that obesity also contributes to delayed wound healing by creating an aberrant low-level inflammatory state [45]. In addition, studies have shown that obese patients have higher risks for surgical site infections, wound dehiscence, and delayed wound healing [46–48]. This may be in part due to the decreased vascularity of adipose tissue with relative decreased oxygen tension, resulting in decreased collagen synthesis, decreased immunity to infection, overall decreased ability to support the processes of wound healing, and increased tissue necrosis [49]. The decreased vascularity may be inherent (with chronic low-grade inflammation and increased glucocorticoids that suppress angiogenesis) or acquired (as a result of surgical and trauma tissue injury that disrupts the adipose tissue); however, either mechanism can disrupt the normal healing process [49]. The heightened inflammatory state can also result in part from chronic venous insufficiency and thereby affect wound healing, especially in the lower extremities.

The specific mechanisms involved in the chronic low-grade inflammatory process have been studied primarily in obese mice studies and have consistently shown increased pro-inflammatory cytokine production resulting from the activation of invariant natural killer T cells by the excess lipid [49]. Other inflammatory mediators that have been shown to increase with obesity include angiotensinogen, tumor necrosis factor alpha, leptin, interleukin 6, and transforming growth factor beta [45, 50]. In addition, decreased adiponectin concentration (which occurs with obesity even though it is produced by adipocytes) impairs adequate perfusion and wound epithelialization [49].

Other factors that may cause wounds or impede wound healing in the obese population are the prevalent comorbidities, nonadherence to treatment plans, poor nutrition, increased risk of friction/shear with resulting pressure ulcers, and confirmed infection [51]. Most strategies to address poor wound healing in the obese have concentrated on maintaining blood glucose levels, proper nutrition for optimal healing, preventing infections, and meticulous wound care. However, one animal study using obese mice suggests that physical exercise may help improve cutaneous healing in obese individuals [45].

Research on both cellular causes and interventions and holistic treatments such as exercise is still investigational but much needed, given the increased prevalence of obesity in our society.

2.8 Cancer

The three factors involving treatment of cancer patients that relate to poor wound healing are the use of radiation to target and destroy specific malignant cells, the use of chemotherapy to treat or prevent metastasis, and the overall decline in general health that results from both the disease and the treatment [52]. In addition, many of these patients have surgical interventions that present problematic considerations for the timing of radiation and/or chemotherapy, as well as the individual ability to support wound healing.

2.9 Radiation Therapy

Radiation targets and destroys cancer cells by ionizing electrons to higher energy orbits around the nucleus; when the electrons return to their original, more stable orbits, the process causes irreparable damage to the cells, including their DNA [53]. While every effort is made to target only the malignant cells, damage to adjacent tissue cannot always be avoided. The effects of radiation on the tissue can be classified as immediate, acute, or chronic and are summarized in Table 5 [54]. Further, the effects of radiation are dependent upon the following factors: dose (measured in rads or grays), treatment volume, daily fraction size, energy and type of radiation, total treatment time, individual cellular differences, and host overall well-being [55]. Lower amounts of exposure will result in improved tissue survival [53].

Cellular changes that occur with different dosages can be summarized as follows:

1. Low doses (up to 100 mGray) – single-strand DNA breaks in the cell; however, damage can be repaired as a result of the remaining DNA in the surrounding area.
2. Medium doses (0.5–5 Gy) – double-strand DNA breaks causing irreparable damage and cellular death.
3. High doses (> 5 Gy) – cellular death occurs immediately [52].

Table 5 Immediate, acute, and chronic effects of radiation

Immediate (within 1 week)
• Transient, faint erythema
• Dilated capillaries
• Epilation
• Dry skin
Acute (days to weeks)
• Erythema localized to radiation field
• Warm and tender skin
• Edema
• Arteriole obstruction by fibrin thrombi
• Small foci of hemorrhage
• Hyperpigmentation
• Dry desquamation
• Wet desquamation
• Ulceration
Chronic (months to years)
• Thin, dry, semitranslucent skin
• Lack of hair follicles and sebaceous glands
• Fibrosis of the skin
• Dyspigmentation
• Induration with loss of range of motion
• Accumulation of fibrinous exudate under the skin
• Telangiectasia
• Delayed ulceration due to ischemia
• Radionecrosis

An inflammatory reaction occurs with radiation that results in edema which subsequently leads to occlusion of the small blood vessels, decreased influx of platelets, increased matrix metalloproteinases, decreased cytokines and fibroblast infiltration, and decreased angiogenesis, all manifested as poor wound healing. The lack of angiogenesis can result in skin changes (e.g., thin translucent appearance, absent hair follicles, ergo fewer stem cells, lower tensile strength) that result in poor healing of traumatic wounds that may occur years after radiation (Fig. 13) [52, 53].

Timing of the radiation for patients who also require surgical management of tumors is crucial and individualized. Generally, preoperative

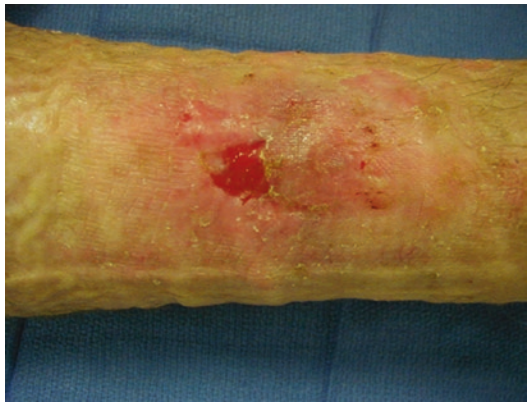


Fig. 13 Traumatic wound radiated tissue approximately 20 years after treatment for Kaposi sarcoma on the lower extremity. The patient also had severe venous insufficiency treated with multilayer compression and new onset of diabetes treated with diet and medication. The wound was ultimately healed with pinch grafts

radiation is given 3 to 6 weeks prior to surgery. Wound healing is most affected if doses larger than 50 Gy are administered, if treatments are given less than 3 weeks before surgery, or when the surgery is performed 6 or more months after radiation therapy, during which the irradiated tissue becomes hypoxic and fibroblasts become dysfunctional [53].

Two therapies that have been reported as potentially beneficial for healing late radiation tissue injury include hyperbaric oxygen therapy [56, 57] and stem cell therapy [58, 59]. The purpose of both therapies is to increase tissue oxygenation and resulting revascularization of the injured tissue and thereby improve wound healing. Treatment of radiated wounds is much like that of burns: manage the drainage with absorbent dressings, remove necrotic tissue in the least painful way for the patient, prevent infection, use nonadherent dressings such as silicone-backed foams, and optimize quality of life during a very difficult time for the patient.

2.10 Chemotherapy

Chemotherapy, intended to kill circulating metastatic cells, also affects the normal cell function, including the cells involved in wound healing. Myelosuppression, or decreased bone marrow production of red blood cells, can result in three conditions that affect wound healing – anemia, neutropenia, and thrombocytopenia. Consequently, patients may have tissue hypoxia, increased risk of infection, and increased risk of bleeding [52]. Chemotherapy has also been reported to inhibit protein synthesis, decrease angiogenesis, decrease extracellular matrix formation, and ultimately delay surgical wound healing or impede healing in other wounds such as pressure ulcers. Common categories of chemotherapeutic agents and their effects on wound healing are presented in Table 6 [53]. The optimal time to deliver chemotherapy to patients requiring surgical removal of a tumor has been the focus of numerous human and animal studies. Historically it was felt that impaired wound healing occurred most commonly when drugs were administered preoperatively or within 3 weeks postoperatively (specifically those such as adriamycin) [60]. In an extensive discussion of wound healing in patients with cancer, Payne et al. conclude that “delaying the initiation of chemotherapeutic regimes until 7 to 10 days postoperatively seems to have minimal effects on wound healing in this patient population” [53].

However, the similarities of wound healing cellular processes and metastases of malignant cells are well-documented. Surgical removal of a tumor initiates the wound healing cascade in order to heal the incision; however, the inflammatory process that ensues involves cellular activity that forms “premetastatic niches” where tumor cells can successfully metastasize to and develop into tumors [61, 62]. Both reviews conclude that postponing chemotherapy until after the surgical wound has healed may facilitate metastatic spread of cancer and that more studies are needed to determine the efficacy of pre- and perioperative chemotherapy.

The monoclonal antibody bevacizumab is frequently used to decrease vascularity to the tumor by targeting vascular endothelial growth factor and thereby decreasing the blood supply to the tumor. The consequence of this treatment, however, may also be impaired wound

Table 6 Chemotherapies and their effects on wound healing

Chemotherapy agents	Effect on wound healing	Recommendations
Alkylating agents Cyclophosphamide Chlorambucil Thiotepa Mechlorethamine Cisplatin	Inhibits fibroblast function Attenuates vasodilation and later neovascularization Delayed wound closure Decreased wound tensile strength	Use as low a dose as possible during the wound healing process
Antimetabolites Methotrexate 5-Fluorouracil 6-Mercaptopurine Azathioprine	Decrease in wound tensile strength especially in days 3–7	Administer leucovorin with methotrexate Optimize nutrition Begin administering drug 1–2 weeks post-operatively
Plant alkaloids Vincristine Vinblastine	Transient early decrease in wound tensile strength	Administer preoperatively
Antitumor antibiotics Cleomycin Doxorubicin Actinomycin D Mitomycin C	Limits skin fibroblast production with delayed closure Decreased wound tensile strength	Delay administration 1–2 weeks post-operatively

Adapted from Payne WG, Naidu DK, Wheeler CK, Barkoe D, Mentis M, Salas RE, Smith DJ, Robson MC. Wound healing in patients with cancer. *ePlasty.* 2008:e9. Available at http://ncbi.nih.gov/pmc/articles/pmc2206003. Accessed 4/14/2017

healing [52, 61]. A meta-analysis by Zhang et al. [63] found that bevacizumab increases the incidence of wound healing complications for patients with colon cancer, but not for breast cancer, metastatic renal cell carcinoma, non-small-cell lung cancer, and gastroesophageal adenocarcinoma. However, a randomized phase 2–3 trial by Cunningham et al. [64] on the use of bevacizumab with perioperative chemotherapy for patients with resectable gastric, esophagogastric junction, or lower esophageal adenocarcinoma reported more wound healing complications in the bevacizumab group as compared with chemotherapy alone (12% versus 7%). Berger and Jager [65] recommend that for patients with gastric and esophageal cancer, "bevacizumab treatment should be stopped at least 5 weeks before surgery to reduce the risk of thromboembolic events, bleeding, and wound healing complications." A study on patients with unresectable stage III lung cancer detected no wound healing problems when treated with bevacizumab in conjunction with chemotherapy every 3 weeks for four cycles, followed by surgery (either pneumonectomy or complete resection) [66].

2.11 Nutrition

The effects of radiation, chemotherapy, surgery, and fighting cancer leave patients in a weakened state of general health, often with nausea, vomiting, loss of appetite, pain, and other symptoms that limit ability to participate in an active life, which in turn may lead to depression. Patients who are malnourished will also have a greater susceptibility to developing infections [54]. In addition, pain medications used to alleviate the symptoms (e.g., corticosteroids, NSAIDs, opioids) may further complicate nutrition and/or wound healing. All of these factors are discussed in greater detail later in this chapter but must be taken into consideration when developing wound care plans, setting goals, and guiding cancer patients with realistic expectations of wound healing.

2.12 Aging

Many of the factors that impede wound healing in the older population (especially those over 60 years of age) involve their comorbidities,

medications, fall risks, poor nutrition, and general decline in health; however, there are changes in the skin that occur with aging that make it more vulnerable to injury and slower to heal. These changes can be intrinsic (due to changes in the cellular and tissue structure) and/or extrinsic (due to exposure to environmental hazards).

As the skin ages, the epidermis becomes thinner, and the rete pegs between the epidermis and dermis flatten, leading to an increased risk for shearing and friction tissue injury (e.g., blisters, skin tears). There are also fewer hair follicles, sebaceous glands, and oil glands in the skin which affects both its vulnerability to tears and cracks due to being drier and its ability to reepithelialize both partial- and full-thickness wounds. During reepithelialization, epithelial cells migrate from the wound edges and from the epithelial lining of hair follicles; thus, with fewer follicles, there is decreased ability to form new epithelium [67].

In addition to the skin changes that occur in the aged, the immune system also changes, losing its ability to protect against infection and thereby supporting normal or timely wound healing [68]. This decline in immune system function leads to increased inflammatory diseases such as rheumatoid arthritis (RA) and the subsequent use of medications that also inhibit healing. It has been shown that T cells from patients with RA tend to differentiate into pro-inflammatory effector cells, thus sustaining chronic, persistent inflammatory lesions in the joints and other organs, including the skin [68]. The altered inflammatory response includes delayed T-cell infiltration into the wound area with alterations in chemokine production and reduced macrophage phagocytic capacity [69]. Other intrinsic changes include enhanced platelet aggregation, increased secretion of inflammatory cytokines, delayed migration of macrophages and lymphocytes, decreased secretion of growth factors, delayed angiogenesis, decreased collagen deposition, and decreased tensile strength [70, 71].

Extrinsic changes in the skin are primarily a result of exposure to ultraviolet light, termed photoaging. In contrast to the thinned epidermis and fine wrinkles caused by intrinsic changes, photoaging is characterized by deep wrinkles, skin laxity, telangiectasias, and lentigines (clearly defined areas of hyperpigmentation caused by melanocytic hyperplasia) [72].

In summary, the primary change in healing of aged skin, in the absence of other diseases, is delayed healing rather than abnormal healing. The other factors discussed in this chapter, however, are more prevalent in the elderly population and must be systematically explored when evaluating the patient with a non-healing wound.

2.13 Arterial Insufficiency

Tissue that is poorly oxygenated as a result of arterial insufficiency (termed ischemia) will show specific clinical signs, beginning with mild claudication and progressing to critical limb ischemia with gangrene (Figs. 14 and 15). The most common causes of peripheral arterial disease (PAD) are arteriosclerosis and atherosclerosis, often linked to the presence of type 2 diabetes. Other risk factors include age, smoking, hypertension, hypercholesterolemia, dyslipidemia, family history, and obesity.

PAD can be categorized into three critical phases which help determine appropriate vascular testing and interventions. The first critical phase occurs when the collateral circulation is

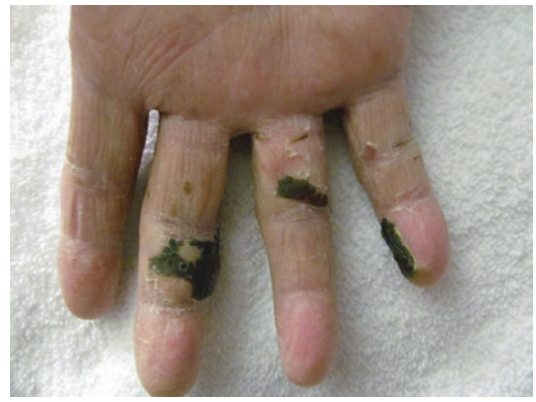


Fig. 14 Nonhealing traumatic wounds on the hands of a patient with diabetes, renal failure, and dialysis access on the same extremity

insufficient for the metabolic needs of the affected extremity; therefore, the blood supply is shunted to the muscle arteries where flow resistance is low rather than to the skin where resistance is high. Frequently the patient is asymptomatic until a traumatic wound occurs on the distal toes and poor healing ensues. The second critical phase occurs when activity or exercise causes relative ischemia and pain, termed intermittent claudication, as a result of increased oxygen demand from the muscles and moderate insufficiency during activity. When PAD becomes severe, the third critical phase, also termed critical limb ischemia, results in resting pain, gangrene, non-healing wounds in the extremity below the occlusion, dependent leg syndrome, and positive test for rubor of dependency [73]. More specifically, moderate PAD is defined as having an ABI between 0.5 and 0.9 or a toe pressure between 50 mmHg and 70 mmHg and severe PAD as having an ankle pressure < 70 mmHg or a toe pressure < 50 mmHg [74].

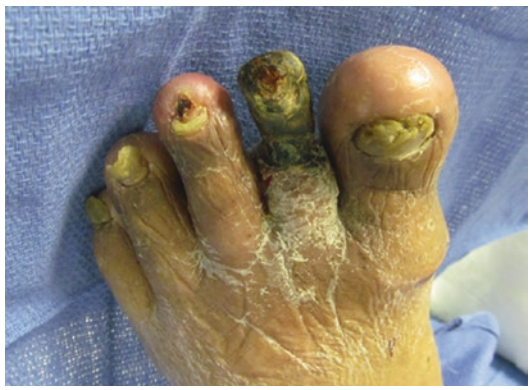


Fig. 15 Necrotic toes on the foot of a patient with critical limb ischemia

Any patient with a non-healing wound on the foot or lower leg is advised to have a vascular screening, beginning with pulse examination of both the dorsalis pedis and posterior tibialis arteries, followed by Doppler and Duplex studies if the pulses are absent or faint, and referral to a vascular surgeon before beginning aggressive wound care. This is recommended as well for patients with diabetes who present for routine podiatric care due to the compromised immunity and increased risk for infection. Guidelines for interpretation of the ankle-brachial index (ABI), a study used to assess the severity of large vessel disease, are presented in Table 7. Microcirculation disease, or decreased blood flow to the small unnamed arterioles and capillaries, is assessed by measuring transcutaneous oxygen tension ($TcPO_2$). Guidelines for interpreting this study are presented in Table 8.

An extensive literature search was conducted by an advisory panel, chaired by Drs. Harriet Hopf and Cristiane Ueno, to determine guidelines for treatment of arterial wounds. The guidelines are formulated in the following seven categories: diagnosis, surgery, infection control, wound bed preparation, dressings, adjuvant therapy (device, systemic, local/topical), and long-term maintenance. A synopsis of the guidelines is presented in Table 9 [75].

Table 7 Interpretation of the ankle brachial index for peripheral arterial disease

1.0–1.2—normal
0.8–1.0—minimal peripheral arterial disease. Compression for edema control is safe to use
0.5–0.8—moderate peripheral arterial disease, often accompanied by intermittent claudication. Referral to a vascular specialist is advised. Compression therapy is contraindicated if <0.6; modified compression is indicated if 0.6–0.8
<0.5—severe ischemia with resting pain. Compression therapy is always contraindicated
<0.2—tissue death will occur
1–1.3—may occur with venous hypertension
>1.3—non-reliable in patients with diabetes due to calcification of the arteries

Ankle-brachial index (ABI) is used to determine the severity of peripheral arterial occlusive disease and to select interventions that will promote healing without causing more tissue necrosis, e.g., by using too much compression for lower extremity edema or by debriding a poorly perfused wound with eschar. The test can be easily performed in any clinical setting with a blood pressure cuff and handheld Doppler.

Table 8 Interpretation of periwound transcutaneous oxygen tension

<20 mmHg—unlikely for healing to occur
20–30 mmHg—healing can be expected, but may be delayed
>30 mmHg proximal to the toes—wound can be debrided
<30 mmHg proximal to the toes—wound should not be debrided until revascularization is accomplished
>40 mmHg—desired for healing of minor amputation site
60–90 mmHg—normal chest measurement

Transcutaneous oxygen tension ($TcPO_2$) is a measure of oxygen tension in the skin and is used to determine severity of microvascular disease. The readings also serve as indicators for wound healing potential and safe debridement of necrotic tissue.

Table 9 Guidelines for the treatment of arterial insufficiency ulcers

Diagnosis
• Guideline: All patients with lower extremity ulcers should be assessed for arterial disease. Principle: Pure arterial ulcers are unusual. Arterial insufficiency frequently contributes to poor healing in ulcers with another primary etiology such as diabetic neuropathy or venous insufficiency.
• Guideline: Patients presenting with risk factors for atherosclerosis who have ulcers are more likely to have arterial ulcers and should be carefully and broadly evaluated. Guideline: In ischemic-appearing ulcers, look for contributing factors other than atherosclerosis that involve the arterial system such as thromboangiitis, vasculitis, Raynaud's, pyoderma gangrenosum, thalassemia, or sickle cell disease. Principle: Patients with ulcers that appear "ischemic" should be evaluated for diseases beyond large vessel occlusive disease if the clinical presentation is not completely consistent the atherosclerotic occlusive disease.
• Guideline: Patients presenting with rest pain or gangrene should be promptly referred to a vascular specialist. Principle: Ulcers in patients with rest pain and gangrene may progress rapidly and delay in referral increases the risk of limb loss.
Surgery
• Guideline: Prior to revascularization, an anatomic roadmap should be obtained, including angiogram, duplex angiography, magnetic resonance angiography, contract tomography angiography. Principle: The goal of revascularization (open or endovascular) is to restore in-line arterial blood flow to the ulcer, which may be manifested by a pulse in the foot and/or improved ABI. • Guideline: Adjuvant therapies may improve healing of the ulcer but do not correct the underlying vascular disease. Principle: Approximately 10–20% of the patients with PAD will need revascularization surgery. • Guideline: The risk of surgery should be weighed against the likelihood of success given the patient's comorbidities. Principle: Care must be individualized with risks and benefits fully discussed with patients.
Infection control
• Guideline: Necrotic tissue needs to be debrided to facilitate a more normal wound healing process except in the case of dry gangrene or eschar in which case arterial inflow needs to be reestablished first. Principle: Necrotic tissue is laden with bacteria; devitalized tissue is a rich environment for bacteria growth that leads to infection. • Guideline: Patients with neuro-ischemic ulcers may benefit from a short course of antibiotics even if signs of infection are not present; however, chronic treatment with systemic antibiotics does not prevent infection and may worsen outcome if infection does develop. Principle: Patients with diabetes have an impaired immune system; thus careful and evidence-based use of antibiotics is required. (Reference Section 3.2 of the Guidelines.) • Guideline: Wounds heal and infection is better prevented and controlled in an adequately oxygenated environment. Principle: Revascularization is the ideal way to increase wound oxygen delivery, but warmth, correction of dehydration, and increased inspired oxygen can increase impaired flow and improve oxygen delivery. For patients with ischemic diabetic ulcers, hyperbaric oxygen therapy has been show to be beneficial. • Guideline: Topical antimicrobial dressings may be beneficial in management of chronically/heavily colonized wounds, decreasing their bacterial load and aiding wound healing. Principle: There is evidence that hypoxia, impaired blood flow, and bacterial load are associated with healing impairment, and that topical antimicrobial dressings and bacterial proliferation is lower under occlusive dressings. However, further research is required to clarify the relationship between healing and wound colonization and infection.

Table 9 (continued)

Wound bed preparation
• Guideline: An arterial ulcer is a component of multiple diseases and a full evaluation of the patient as a whole (including systemic diseases, medications, nutrition, tissue perfusion, and oxygenation) is required. Principle: All factors in an individual's medical history need to be addressed to facilitate optimal wound healing. This includes nutrition, hydration, weight loss, oxygenation, and social history (e.g., smoking). • Guideline: Debridement of necrotic tissue is advised AFTER revascularization. Principle: Debridement in the absence of oxygenation will result in a larger wound; however, once the limb is revascularized, removal of necrotic tissue, excessive bacteria, senescent cells and cellular debris will promote wound healing. • Guideline: There is no consensus on the best agent for debridement. Principle: It is common to combine debridement methods in order to maximize the healing rates. • Guideline: Compression therapy may be beneficial in ulcers of both arterial and venous etiology. Principle: Compression therapy is useful in venous ulcers; however, in arterial wounds and/or post revascularization surgery, mild compression is recommended. • Guideline: Autografts and allografts can act as biological dressings and improve wound healing after revascularization, as well as extracellular matrix dressings. Principle: Success of any graft is dependent on appropriate wound bed preparation and perfusion.
Dressings
• Guideline: Dressings that maintain a moist wound-healing environment are recommended for perfused wounds; dry gangrene or eschar is best left dry until revascularization is successful. Principle: Moist wound care accelerates wound healing; dry dressings are used only on intact skin as they cause desiccation of the wound. • Guidelines: Dressings that can be changed once a day or less often, based on the wound etiology and characteristics, are preferable whenever possible. Principle: Moist saline dressings are not considered the most cost efficient because of time and healing outcome as compared to advanced dressings.
Adjuvant therapy
• Ultrasound therapy, electrical stimulation, spinal cord stimulation, negative pressure wound therapy, intermittent pneumatic leg compression, and hyperbaric oxygen therapy are sometimes useful but need more RCTs for conclusive evidence. • Pentoxifylline and prostaglandins are not useful in the treatment of arterial wounds. • Pain control should address the cause and use local, regional, and/or systemic measures. • Stem cell therapy is promising but needs more research. • Gene therapy with VEGF may be helpful in healing arterial ulcers. • Further study is required to clarify benefits of topical oxygen therapy.

Adapted from Hopf HW, Ueno C, Aslam R, Burnand K, Fife C, Grant L, Holloway A, Iafrati MD, Mani R, Misare B, Rosen N, Shapshak D, Slade JB, West J, Barbul A. Guidelines for the treatment of arterial insufficiency ulcers. *Wound Rep Reg.* 2006;14:693–710

2.14 Edema

Chronic venous insufficiency is a well-defined and frequently diagnosed condition that can cause venous leg ulcers. However, the effect of edema on the healing of wounds caused by other etiologies is less recognized and sometimes neglected as an integral part of treating lower extremity or surgical wounds. Any patient with a non-healing lower extremity wound deserves a thorough vascular screening for arterial insufficiency; however, a thorough evaluation for edema is also advised, especially if the patient has a history of ankle injury with decreased ankle range of motion or weakness of the gastrocsoleus muscle. Both of these conditions can reduce the effectiveness of the venous pump in returning fluid from the distal extremities to the central vascular system with subsequent chronic edema which in turn reduces oxygenation of the skin and impairs wound healing. Some scenarios where chronic edema can occur are history of ankle fractures, spinal cord injury, hemiplegia, rheumatoid arthritis, hip or knee surgery, or lower extremity trauma. Lower extremity wounds on these patients will heal more rapidly if the edema is managed adequately.

The edema is sometimes difficult to detect visually if there is gastrocsoleus atrophy. Applying pressure to the extremity with the finger for 5 s and assessing the ability of the tissue to return to normal after release is a good way to test for pitting edema. (Figs. 16, 17, and 18) Girth measurements are also recommended to detect possible edema and to measure outcomes of treatment.

The effect of edema on tissue oxygenation and wound healing is well-documented, and compression is the accepted standard of care for edema. The parameters for treating edema, especially in patients with arterial disease (termed combined arterial and venous disease) or with diabetes, are not so specific. Extensive research on types of compression and sub-bandage pressure has been reported by Partsch [76]. Mosti and Partsch [77] have reported that higher applied pressures using inelastic compression systems apply higher pressures at the ankle and limb during ambulation and lower resting pressures during inactivity (e.g., sleeping). This type of compression is usually more comfortable for the patient than elastic compression and avoids complications, especially in the presence of mild to moderate arterial insufficiency. For patients who have good arterial flow, standard high-pressure compression aims to apply at least 40 mmHg of compression at the ankle, decreasing to 30 mmHg at the calf, and is best obtained with multilayer compression systems.

In addition, a thorough medical history is necessary to determine any other causes of lower extremity edema, including medications, congestive heart failure, and renal or kidney disease. Generally speaking, bilateral edema is usually systemic, whereas unilateral edema is usually vascular or traumatic. Another indicator of edema NOT due to venous insufficiency is the absence of skin changes associated with that disorder (e.g., hemosiderosis, eczema, atrophie blanche, lipodermatosclerosis, mild leg heaviness, achiness) (Fig. 19) [78]. Patients who have sudden onset of severe, hard unilateral edema that does not respond to compression are suspicious of having groin or abdominal lymph

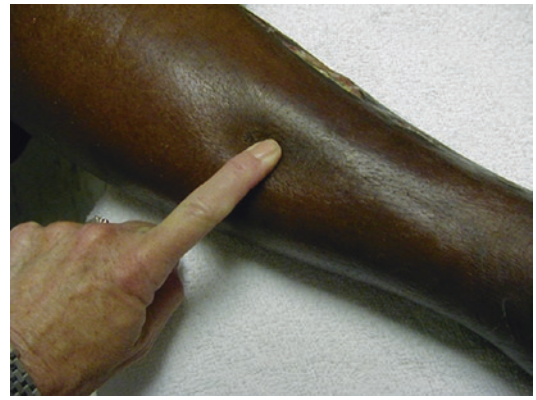


Fig. 16 Applying pressure on the lower extremity to assess for pitting edema

Fig. 17 Pitting in the skin illustrates edema that has not yet become fibrotic due to chronicity

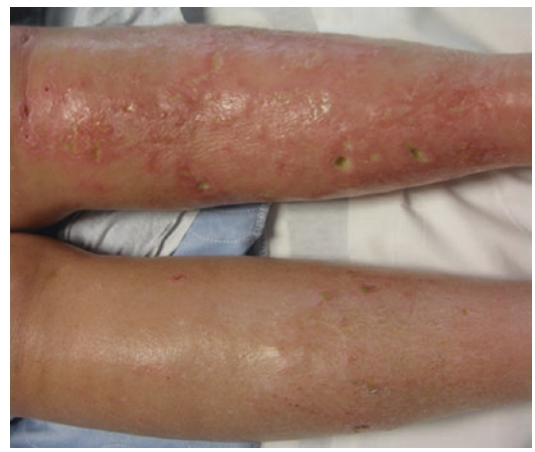


Fig. 18 Chronic edema results in fibrotic subcutaneous tissue and orange skin like appearance of the skin, termed peau d'orange. This type of edema frequently is associated with secondary lymphedema

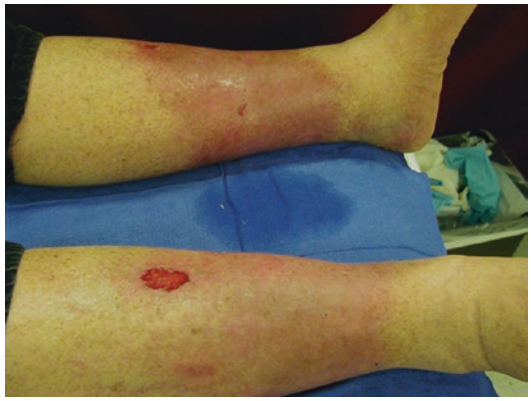


Fig. 19 Patient with bilateral lower extremity edema and wounding secondary to congestive heart failure. Note that the wounds are more proximal than typical CVI wounds, another indication that something else is causing the edema

node obstruction due to an undiagnosed malignancy and should have a thorough medical evaluation.

For patients with moderate arterial insufficiency, the goal is 20–25 mmHg of compression with less at the calf [79]. It is the differential between the ankle and calf pressures, along with the pumping action of the gastrocsoleus muscle pushing on the bandage during exercise or ambulation that facilitates the removal of fluid from the tissue and thereby increases tissue oxygenation [80]. In addition, patients with combined arterial and venous insufficiency may have improved wound healing with the use of pentoxifylline, a theophylline derivative that is an antioxidant, vasodilator, and rheologic agent that improves the microcirculation and increases oxygen delivery to injured tissue [81].

A specific protocol for applying compression to edematous lower extremities was reported by Dr. Marston as follows:

1. Patients with an ABI between 0.6 and 0.9 or toe pressure between 50 and 80 mmHg can be safely treated with reduced amount of sub-bandage pressure.
2. Patients with symptoms of severe arterial disease (severe claudication or rest pain) would require revascularization.
3. Patients with an ABI < 0.5, an ankle pressure < 70 mmHg, or a toe pressure < 50 mmHg should be evaluated for revascularization and compression avoided [78].

The protocols for treatment of lower extremity edema used by the author are summarized in Tables 10 and 11.

Patients who have decreased ankle range of motion and/or gastrocsoleus weakness can also improve venous return by performing the following exercise routine on a regular basis:

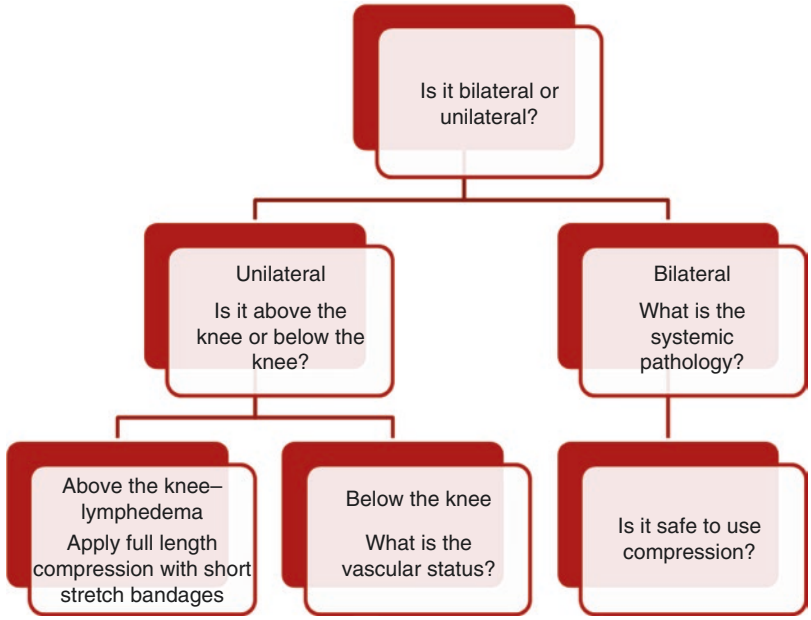
1. Gastrocsoleus stretches to optimize ankle range of motion
2. Ankle pumps and circumduction
3. Heel/toe raises in both sitting and standing positions
4. Ankle rocker board exercises
5. Step-over a 3–4-in. obstacle using a heel strike in front, toe push-off in back
6. Exaggerated heel/toe sequence during ambulation
7. Walking or bicycling for fun

In summary, successful management of any patient with a lower extremity wound complicated by edema requires accurate diagnosis of the edema etiology, vascular screening for arterial insufficiency, and appropriate compression and exercise to decrease the fluid causing tissue congestion and impaired wound healing.

2.15 Cardiac Disease

Cardiac disease is commonly associated with several other disorders that impair wound healing – diabetes, peripheral arterial disease, obesity, and increased risk of cancer. Sometimes, however, poor cardiac output or dysregulated inflammation of cardiac tissue may be overlooked as a factor in poor healing of lower extremity wounds of other etiologies. If a wound is not responding to standard care, e.g., a pressure ulcer on the malleoli and/or chronic venous wound on a patient with revascularized PAD,

Table 10 Algorithm for deciding WHAT to wrap when treating a patient with a venous wound



Selecting the compression intervention begins with a careful assessment of the edema. If the patient has bilateral lower extremity edema, systemic disorders such as congestive heart failure, kidney failure, or liver disease must be ruled out, as well as carefully reviewing the medications. If the patient has acute congestive heart failure, compression may need to be deferred until the patient is diuresed and there is no risk of overloading the heart. Any systemic issue must be addressed in order for local treatment to be effective

If the edema extends above the knee, there is probably secondary lymphedema which would be treated with manual lymphatic drainage, exercise, and compression applied from toe to upper thigh. If the edema is limited to below the knee, the next step is to evaluate the vascular status to determine the type of material that is best for the individual patient

Adapted from Sigman M, Ochoa C, Rowe V. Vascular wounds. In Hamm RL (Ed). Text and Atlas of Wound Diagnosis and Treatment. 2015. New York: McGraw-Hill Education. pp. 99–141. Used with permission

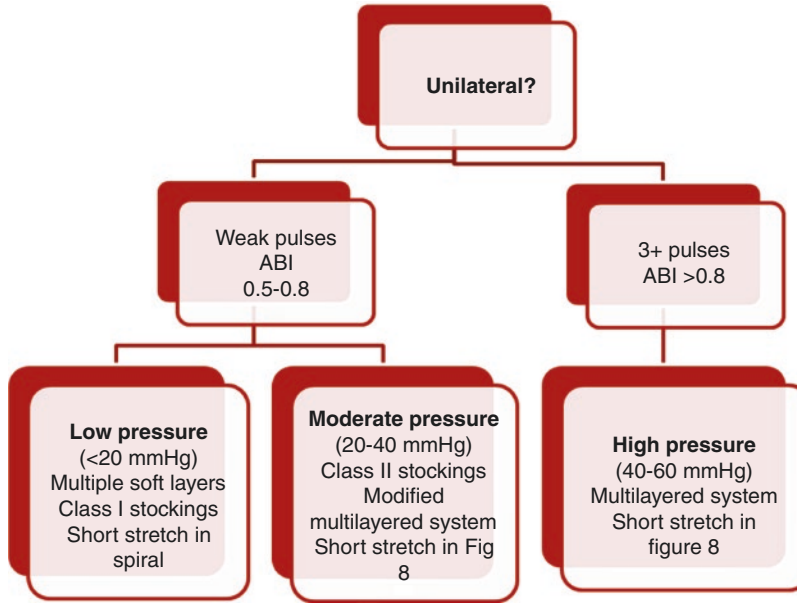
cardiac function studies may provide insight into delayed or impaired healing [52].

A study by Meyborg et al. [82] found that patients with reduced ABIs who had open-heart surgery had more wound healing difficulties and higher long-term mortality compared to those with normal ABIs. Although reduced ABIs were not associated with increased peripheral risks in open-heart surgery, they advised that "ABI may be helpful in selecting the site for saphenectomy to potentially avoid delayed healing of related wounds in legs with severely impaired arterial perfusion." Sites of saphenous vein harvesting may also have delayed healing if resulting edema is not adequately managed, and ABIs are the best indicators for the appropriate compression.

2.16 Sickle Cell Disease

Chronic, recurrent ulcers on the distal lower extremities are a common problem in patients with sickle cell disease. The mechanism of ulceration is a combination of vaso-occlusion of the microvasculature by the entrapment of sickled red blood cells, vasculopathy, and a chronic inflammatory state that results in a wound much like that of chronic venous insufficiency [83]. The first ulceration usually occurs between 10 and 25 years of age and is frequently the result of minor trauma or pruritus that provokes scratching and skin tears [84]. They are usually located on the peri-malleolar area and less frequently on the gaiter area, foot, or toes.

Table 11 Algorithm for selection of appropriate compression therapy



Selection of the appropriate compression therapy is based on vascular examination and patient comfort and tolerance. Every garment, compression system, elastic or nonelastic wrap has a tension that determines the amount of pressure when properly applied. Manufacturer's guidelines should be followed carefully to avoid complications that can occur with inappropriate compression therapy. Compression of any type is generally contraindicated if the ABI is less than 0.5; however, if the patient can tolerate multiple layers of soft gauze wrapping, it may be beneficial in activating the lymphatics as well as anchoring the appropriate primary dressing.

Adapted from Sigman M, Ochoa C, Rowe V. Vascular wounds. In Hamm RL (Ed). *Text and Atlas of Wound Diagnosis and Treatment.* 2015. New York: McGraw-Hill Education. pp. 99–141. Used with permission

Chronic ulcerations due to congenital hemolytic anemia have also been reported for patients with thalassemia intermedia, hereditary spherocytosis, pyruvate kinase deficiency, and congenital dyserythropoietic anemia. The common factor in these diseases appears to be chronic anemia and hemolysis [85].

One of the most significant differences between SCD wounds and other chronic edema wounds is the exquisite pain, which may also lead to gait disorders, decreased ankle motion, and inadequate venous pump, all of which further impede wound healing. Other factors that impede the healing of SCD wounds include poor nutrition, thrombophilia, hyper-inflammatory response, and poor socioeconomic status [85].

The affected extremity is often edematous, and surrounding skin may be hypo- or hyperpigmented with decreased hair follicles and muscle atrophy. The wound bed is usually fibrinous with moderate to copious drainage, and the edges are even, much like an arterial wound. Wounds that heal have decreased tensile strength making them vulnerable to recurrent ulcerations, especially during periods of stress. Function as measured by the 6-min walk test will be diminished due to pain and resulting gait impairments [85]. Minniti and Kato [85] provide an excellent review of laboratory tests, microscopic analysis of skin biopsies, and imaging.

Treatment for patients with SCD wounds is multifactorial and multidisciplinary, beginning with management of the SCD by a hematologist. Interventions may consist of transfusions to elevate the hemoglobin levels with concurrent infusion of desferrioxamine to manage the iron overload because high iron levels may also interfere with the wound healing [83]. Wound care

consists of debridement (initially enzymatic may be preferred due to intense pain levels), absorbent dressings, identification and treatment of infection, and compression therapy (e.g., multilayer compression systems). In addition, low-frequency non-contact ultrasound may help mitigate the pain and facilitate wound healing [83]. Physical therapy to increase ankle range of motion, gastrocsoleus strength, gait quality and endurance, as well as cardiopulmonary therapy will benefit both the wound healing and the patient quality of life. Because of the early onset of SCD ulcers, multidisciplinary care is essential for long-term management, optimal outcomes, and ultimately for patient survival.

2.17 Infections

2.17.1 Bacterial

The skin is known to have a certain amount of flora present at all times; however, when pathogens (defined as any microorganism that can cause disease in its host) invade a wound, they can adhere to and colonize in the wound tissue and thereby evade the host innate and adaptive immune system. The presence of infection per se is determined by the number of colony-forming units (CFUs), the type of bacteria, and the health of the host immune system. The amount of bacteria on a wound is classified as contamination, colonization, critical colonization, and infection (Table 12). Colonization and critical colonization may respond to topical antimicrobials; infection and sepsis require that the patient receive systemic antibiotics specific to the invading microbe [52].

Pathogenic bacteria use a variety of strategies to avoid destruction by the immediate immune system, including covering themselves with a thick exopolymeric matrix (composed of polysaccharides, proteins, and DNA synthesized by the bacteria) that adheres to the wound bed, termed biofilm. The adhered layer provides the bacteria an environment for replication, and because of its stickiness, the biofilm is not easily removed by mechanical cleansing (Fig. 20)

Identification of an infected wound is not always easy for even the most astute clinician.

Table 12 Terms defining the presence of bacteria on a wound

- **Contamination**—presence of nonreplicating bacteria on the wound surface without any effect upon the wound healing process
- **Colonization**—presence of replicating bacteria attached to the wound surface with no harm to the host and no effect on the wound healing process
- **Critical colonization**—presence of replicating bacteria on the wound surface with sufficient numbers to visibly affect the wound healing process
- **Infection**—presence of replicating bacteria that have invaded the surrounding tissue with visible effects in the wound healing process and in the periwound tissues. Clinical infection for most bacteria is defined as 10^5 CFUs/g of tissue
- **Sepsis**—presence of replicating bacteria that produces a whole-body inflammatory state termed systemic inflammatory response syndrome (SIRS)

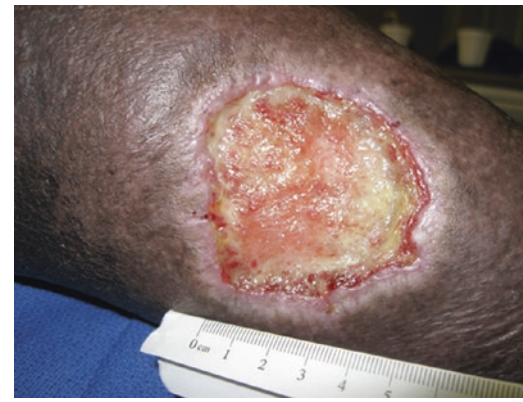


Fig. 20 Biofilm on a chronic wound

The most common signs are persistent periwound erythema, drainage, epidermal sloughing, odor, and failure to respond to standard care. Friable granulation tissue in a previously progressing wound bed is another sign of infection. Specific to the diabetic foot ulcer, erythema that extends more than 2 cm from the edge of the wound is highly correlated with infection; and if the wound can be probed to the bone, there is a strong probability of osteomyelitis [86]. Another indicator is skin temperature which is normally 92–96 ° F on the trunk and 75–80 ° F on the extremities. An increase of more than 4° higher than the contralateral side or an adjacent area on the same

extremity is indicative of some type of pathology, either inflammation, infection, or acute Charcot foot fracture.

In order to distinguish superficial and deep infection, Sibbald et al. [87] developed the mnemonics NERDS© and STONES©. NERDS is used to describe superficial infection:

1. **N**on-healing wounds
2. **E**xudative wounds
3. **R**ed and bleeding wound surface granulation tissue
4. **D**ebris (yellow or black necrotic tissue) on the wound surface
5. **S**mell or unpleasant odor from the wound

STONES is used to describe deeper infection:

1. **S**ize is bigger.
2. **T**emperature is increased.
3. **O**s probe to or exposed bone.
4. **N**ew or satellite areas of breakdown.
5. **E**xudate, erythema, edema.
6. **S**mell.

The superficial bacterial burden may respond to topical antimicrobial dressings; the deep infection usually requires systemic antibiotics. The diagnosis of infection is made clinically; however, a bacterial swab can identify the microorganisms and guide the selection of appropriate antibiotics, especially in the case of drug resistant bacteria.

2.17.2 Fungal

Fungal skin infections, termed mycoses, are caused by dermatophytes, defined as organisms that obtain their nutrients from keratin in the stratum corneum, hair, and nails. Superficial fungal infections, or tinea, are classified according to the location: tinea (t) capitis (scalp, eyelashes, eyebrows), t. barbae (beard), t. pedis (foot), t. unguium or onychomycosis (nails), t. cruris (groin), t. manus (hand), and t. corporis (skin not covered by other nomenclature, also called ringworm) [88]. Fungal infections seen most frequently in conjunction with wound care

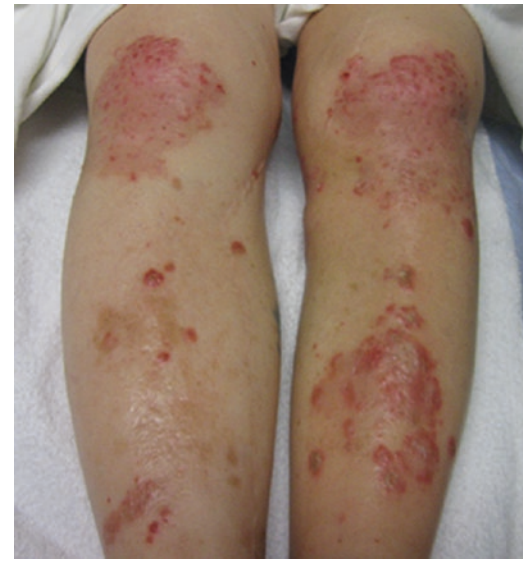


Fig. 21 Fungal infection that occurred on moist skin underneath compression bandages

patients are onychomycosis (common on the diabetic foot), t. corporis (occurring under compression bandages if the skin tends to be moist) (Fig. 21), and candidiasis (a yeast-like fungus caused by *Candida albicans*) that infects the mucous membranes, the gastrointestinal tract, and the vagina (frequently seen on the skin of patients with urinary infections). A systematic review of skin conditions in the aged found that the most prevalent skin disease in the elderly was fungal infections [89].

Predisposing factors for acquiring mycotic infections include a warm moist skin, prolonged use of antibiotics, immunosuppression (by disease or drugs), diabetes mellitus, impaired circulation, poor hygiene, poor nutrition, trauma, and tropical climates. Additionally, people who perform work or hobbies that result in prolonged wet feet or hands are susceptible to acquiring mycotic infections. Tinea on the skin usually presents as well-demarcated, scaling, and inflamed lesions which are accompanied by itching and burning sensations. In the case of tinea pedis, maceration and vesiculation may also be present [87]. Clinical symptoms of onychomycosis are thick nails due to hyperkeratosis of the undersurface of the nail, yellow or chalky-white discoloration,

longitudinal folds in the nail bed, accumulation of debris under the nail causing the nail to separate from the nail bed, crumbly distortion of the nail, and possible loss of the nail, resulting in loss of fine motor skills if a finger is involved or pain and altered gait if the toes are involved [90].

Dermatology referrals are recommended for t. pedis, t. capitis, and onychomycosis. The mainstay of treatment for fungal infections is twice-daily topical application of an antifungal cream, e.g., imidazoles, triazoles, and allylamines. Systemic treatment with antifungal agents such as fluconazole, terbinafine, or itraconazole is reserved for severe cases only [31, 91]. Non-pharmacologic therapies that have been reviewed for onychomycosis on patients who have contraindications for receiving oral antifungal medications are laser therapy [92] and photodynamic therapy [93].

2.18 Medications

Medications can be miracle workers in some diseases, greatly improving both longevity and quality of life; however, there can also be side effects that require a balancing act for the physician and the patient. This is especially true for some medications that are known to interfere with wound healing, e.g., steroids, NSAIDs, antirejection medications, and anticoagulants [94, 95]. The wound care specialist and the prescribing physician need to work together to determine the optimal dosing and possible alternative medications, at least until the wound is healed.

2.18.1 Steroids

Steroids, including corticosteroids, are known to inhibit wound healing by delaying the migration of inflammatory cells and fibroblasts to the wound site, decreasing deposition of collagen and ground substances that compose extracellular matrix, inhibiting cytokine release and chemostasis, and inhibiting angiogenesis, wound contraction, and reepithelialization [52, 96]. These delayed effects appear to be largely due to the downregulation of TGF-ß and ILGF1 and are apparent in all phases of wound healing. Besides a delay in the inflammatory response, there is reduced autolysis of necrotic tissue and pathogens secondary to delayed migration of macrophages, which in return leads to increased risk of tissue infection. During proliferation, there is decreased production of the components of granulation tissue (extracellular matrix and capillaries), and the fibroblasts do not differentiate into myofibroblasts; thus, there is less wound contraction and decreased wound tensile strength [52].

Numerous studies have looked at the results of orthopedic surgery on patients (specifically with rheumatoid arthritis) who are on anti-inflammatory medications, either steroidal or disease-modifying antirheumatic drugs (DMARDs) such as methotrexate. A study on adverse events with craniovertebral junction fusion concluded that prednisone dosages <7.5 mg and/or methotrexate were safe with no effect on outcomes, whereas daily prednisone dosages >7.5 mg may impact clinical outcomes, as measured by the Nurick score [97]. Three studies on postoperative complications (surgical site infections and delayed wound healing) on patients taking DMARDs found no statistically significant difference in wound healing in those taking the medications [98–100].

In their review of perioperative use of DMARDs on patients undergoing plastic surgery, Tsai and Borah [101] suggest that in younger patients who have been placed on the medications recently, "it is reasonable to withhold therapy based on 3-5 half-lives of the specific agent," whereas in older patients with more advanced disease, discontinuing therapy must be carefully considered by the patient and the rheumatologist. Two specific guidelines were issued by the British Society for Rheumatology: (1) methotrexate is unlikely to increase the risk of surgical complications if continued [102], and (2) anti-TNFα drugs (infliximab, etanercept, adalimumab) "should be withheld for 2 to 4 weeks prior to major surgical procedures" and resumed when wound healing is satisfactory [103].

2.18.2 Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are commonly used to reduce pain and inflammation in patients with acute injuries and arthritis and have been reported to interfere with wound healing, especially when given long-term and in higher doses [95]. The effects of NSAIDs can occur at all phases of wound healing, beginning with a prolonged hemostasis phase and delayed clotting as a result of decreased production of thromboxane A_2 which decreases platelet aggregation and increases the risk for bleeding and hematoma formation, especially postoperatively [52, 104]. Similarly, the reported effects of NSAIDs on anastomotic leaks due to poor healing after gastrointestinal surgery suggest an increased risk of leakage [105].

Decreased neutrophil migration to the wound site during inflammation may result in increased risk of infection, and during proliferation, inhibition of hyaluronic acid production with decreased granulation formation may occur [52]. Other reported effects include decreased numbers of fibroblasts, decreased tensile strength of scar tissue, decreased wound contraction, and delayed angiogenesis [60].

More specific studies on the effects of different classifications of NSAIDs reveal somewhat more specific conclusions. Fairweather et al. [106] studied the effects of cyclooxygenase-2 (COX-2) on full-thickness wound in mice and found a significant delay in wound healing, indicating that a downstream production of PGE2 "modulates the activity of multiple essential functions of the inflammatory stroma, including epithelial proliferation, angiogenesis, and ECM production." Ren et al. [107] investigated the effect of COX-2 inhibitors on random skin flap survival in rats and found significantly larger amounts of necrosis on the flaps of subjects who received parecoxib. They also found significantly less VEGF protein in the tissue, concluding that the low levels of VEGF caused suppression of neovascularization. A human study of the use of antiplatelet and/or anticoagulant medications (aspirin, clopidogrel bisulphate, or warfarin sodium) before facial plastic surgical procedures found no increase in the complication rates as compared to those in a matched control group, concluding that patients undergoing this type of surgery could continue anticoagulation therapy during the perioperative period safely with minimal complications [108]. However, a review of drug safety in patients with rheumatoid arthritis suggested that patients who used NSAIDs may have increased risk of bleeding after orthopedic surgery [109].

The need for more research on the effects of NSAIDs on the healing cascade is reflected in the research reported on healing of bone fractures. Several studies, on both animals and humans, have suggested that the use of NSAIDs potentially impairs bone healing [110–114]; however, some reviews state that there is no firm evidence and more robust studies are needed to determine the exact mechanism by which NSAIDs could interfere with bone healing [115–117].

In summary, the studies on the effects of NSAIDs on wound healing are inconsistent, and although some studies recommend that NSAIDs be discontinued 1 to 4 weeks before surgery and withheld until after the surgical wound is healed, the patient's pain, disease progression, and other risks need to be considered [52, 118]. The exception may be cardiac patients who are taking low-dose aspirin and would be at risk for a cardiac event without the medication [60]. If a patient with a nonsurgical chronic would does not respond to standard care and shows signs of bleeding easily, poor granulation tissue, failure to reepithelialize, and vulnerability to break down during the remodeling phase, a review of the medications for NSAID use, especially long-term and high doses, may be beneficial, and if the reason for taking the medication is not related to specific disease progression, cessation of the NSAIDs may be helpful (Fig. 22).

2.18.3 Anticoagulants

Anticoagulants (e.g., warfarin, apixaban, rivaroxaban, and dabigatran) inhibit the coagulation

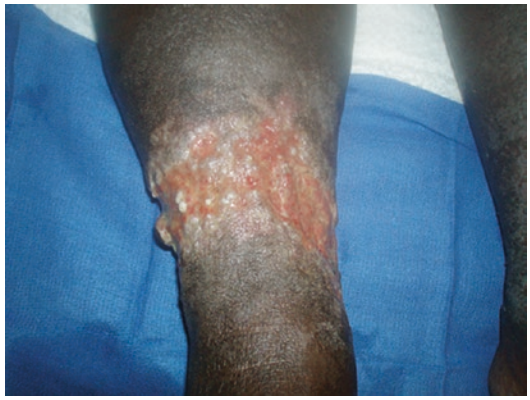


Fig. 22 Wound on the lower extremity of a patient with chronic venous insufficiency who was also taking 800 mg of NSAIDs per day. Compression therapy and discontinuing medication resulted in full closure of wounds. In addition to the edema, note the poor quality of granulation tissue and senescent edges, typical of wound healing impeded by NSAIDs

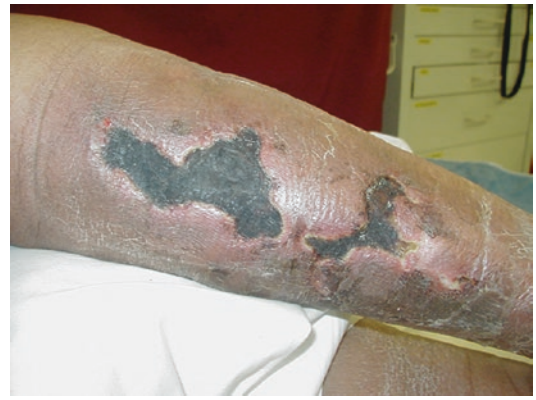


Fig. 23 Patient with warfarin-induced skin necrosis. Treatment included discontinuing warfarin, sharp debridement, and moist wound healing

cascade and thereby may prevent fibrin deposition and delay the healing process [94, 95]. Two patient populations that were found to have significantly higher rates of postoperative wound and bleeding complications were women who received anticoagulation during pregnancy and required Cesarean delivery [119] and patients who were female and/or received oral anticoagulation and had lower extremity bypass surgery for critical limb ischemia [120]. This does not undermine the importance of continuing anticoagulation therapy for those patients who require it for cardiac or thrombosis reasons. A study by Nelms et al. [121] found that anticoagulation with warfarin could be safely continued on patients who had minor soft tissue procedures without wound complications. Similar results were found for patients undergoing hand and wrist surgery [122]. For any patient undergoing surgery and on anticoagulation therapy, the decision to continue medication during the perioperative period requires careful consultation between the surgeon and the prescribing physician to consider benefits and potential complications.

Cutaneous reactions to anticoagulation that have been reported are heparin-induced bullous hemorrhagic dermatosis, hematomas, ecchymoses, erythematous plaques, nodules, contact dermatitis, and urticaria [123], as well as warfarin-induced skin necrosis (Fig. 23) Although these conditions are rare, they are differential diagnoses to be ruled out in patients who develop skin necrosis, especially within 2 weeks of initiating therapy [124].

2.18.4 Antirejection Medications

Antirejection medications (Table 13) are an integral part of postoperative care for patients who receive organ transplants; however, they can also result in delayed healing or dehiscence of the surgical incision [125]. The action of these medications includes inhibition of cytokine transcription, inhibition of nucleotide synthesis, inhibition of growth factor signally, inhibition of the stimulation of T-cell interleukin-2 receptor sites, and diminished chemotaxis, all part of the inflammatory healing phase [126]. This combined with the corticosteroid effects on healing make these patients high risk for wound complications and infections.

Two long-term complications that can result from antirejection medications are drug-induced diabetes and squamous cell carcinoma, a malignant skin cancer linked to the increased number of senescent cells and an oxidative environment [52]. Careful monitoring of glucose values and skin inspections are advised for this patient population for early detection and intervention.

Table 13 Antirejection medications used after organ transplants

Azathioprine (AZA, *Imuran*)
Corticosteroids
Primary endogenous glucocorticoid
Cortisol
Prednisone
Methylprednisolone
Cyclosporine (CsA)
Tacrolimus (Prograf)
Mycophenolate mofetil (Cellcept)
Sirolimus (SRL, *Rapamune*)
Everolimus (Afinitor)
Polyclonal antibodies
Thymoglobulin
Atgam
Monoclonal antibodies (OKT3)
Daclizumab (Xanapax)
Basiliximab (Simulect)
Calcineurin inhibitor (CI)
CsA (*Sandimmune, Neoral, and SangCya)*
Mycophenolate mofetil (MMF)

Adapted from: Lake DF, Briggs AD, Akporiaye ET. Chapter 55. Immunopharmacology. In: Katzung BG, Masters SB, Trevor AJ, eds. *Basic & Clinical Pharmacology*. 12th ed. New York: McGraw-Hill; 2012 http://www.accessmedicine.com/content.aspx?aID=55831418. Accessed May 3, 2013

Table 14 Calorie and protein requirements for healthy wound healing

	Calories[1]	Protein[2]
Normal (at rest)	20–25 kcal/kg/day (age dependent)	0.8 g/kg/day 60–70 g
Postoperative/ ill/injured	30–50% above normal	1.2–2 g/kg/day
Large open wounds, burns	30–35 kcal/kg/day	2–2.5 g/kg/day
Malnourished	50% above normal	1.5 g/kg/day plus anabolic agent[1]

[1]Demling RH, Nutrition, anabolism, and the wound healing process: an overview. *ePlasty.* 2009;9:65–94

[2]Huckleberry Y. Nutritional support and the surgical patient. *American Journal of Health-System Pharmacy.* 2004:61:7

2.19 Nutritional Deficits

Healthy wound healing requires adequate calories, proteins, and nutrients for synthesis of new tissue (Table 14); it is a widely accepted principle of medical care for the patient with a non-healing wound [52, 60, 127]. The issues that are less definitive are how to diagnose malnutrition and how to treat the patient with supplements, especially in terms of macro- and micronutrients.

Table 15 Etiology-based definitions of malnutrition

1. Malnutrition in the context of social or environmental circumstances (Starvation-related malnutrition). May be pure starvation due to financial or social reasons or caused by anorexia nervosa.
2. Malnutrition in the context acute illness or injury, such as organ failure, pancreatic cancer, rheumatoid arthritis, or sarcopenic obesity.
3. Malnutrition in the context of chronic illness, such as major infections, burns, trauma, or closed head injury.

Historically, protein energy malnutrition (PEM) was diagnosed with laboratory values using serum levels of albumin, pre-albumin, or transferrin (normal values are listed in Table 1). Recent literature, however, discusses the limitations in using these values to determine malnutrition because the inflammatory process can affect these levels in almost all chronic conditions, terming the values "negative acute-phase reactants" and, thus, no longer valid to use alone as a basis for providing nutritional interventions [127, 128]. In 2012 the Academy of Nutrition and Dietetics (Academy) and the American Society for Parenteral and Enteral Nutrition (ASPEN) released a joint consensus statement that proposes a three-pronged etiology-based definition of malnutrition (Table 15) and the following six characteristics for the diagnosis: insufficient energy intake, weight loss, loss of muscle mass, loss of subcutaneous fat, localized or generalized fluid accumulation that may sometimes mask weight loss, and diminished functional status as measured by hand-grip strength [128]. Guidelines state that the presence of two or more of these criteria constitutes a diagnosis of malnutrition. Thus, one of the most important aspects of diagnosing malnutrition is the subjective patient history (including recent food intake, unintentional loss of body weight, medications), physical examination, and functional assessment. Laboratory values for inflammation (C-reactive protein, white blood count, and blood glucose

levels) can help determine if the malnutrition is due to starvation, chronic disease, or acute disease/injury [128].

Two other screening tools that have been published are the Canadian Nutrition Screening Tool and the MEAL Scale. The Canadian Tool asks the patient the following two questions:

1. Have you lost weight in the past 6 months without trying to lose this weight? (If the patient reports a weight loss but gained it back, consider it as no weight loss.)
2. Have you been eating less than usual for more than a week?

Two yes answers indicate malnutrition risk, and this was shown to be valid with or without considering the patient's body mass index. Validity testing of the Canadian Tool was performed on 1,015 patients admitted to 18 Canadian hospitals for more than 2 days [129].

Fulton et al. [130] looked at 18 factors thought to be associated with malnutrition and found only 4 to be statistically significant: presence of multiple wounds, eating less than usual, eating less than 3 meals per day, and a low activity level. Using this information, they developed the MEAL Scale (Table 16) for nutrition screening in the wound patient population.

The Mini Nutritional Assessment (MNA)® was developed by the Nestle Research Center and Toulouse University, France, for the older population but is also useful in other patient groups. The MNA® consists of 18 easily measurable items classified into the following 4 categories:

1. Anthropometric measurements (four questions on weight, height, and weight loss)
2. Dietary questionnaire (six questions related to number of meals, food and fluid intake, autonomy of feeding)
3. Global assessment (six questions related to lifestyle, medication, and mobility)
4. Subjective assessment (two questions on self-perception of health and nutrition)

Table 16 The MEAL Scale for nutrition screening in the wound patient population

Multiple wounds	**Do you have > 1 open wound?**
0	No
1	Yes
Eats <3 meals	**How many meals, not including snacks, do you eat in a typical day?**
0	≥3 meals
1	≤3 meals
Appetite loss	**Thinking about your normal food intake,**
	would you say you are eating about the same,
	more, or less than usual?
0	About the same or more than usual
1	Less than usual
Level of activity	**Thinking about your normal level of activity,**
	how would you consider your activity level
	over the past month?
0	Normal
0	Not quite normal, but able to do most things
1	Not feeling up to most things, in bed or chair less than half the day
1	Able to do little activity and spend most of the day in bed or chair
2	Pretty much bedridden, rarely out of bed

Total points: 0–1 not at risk; 2–4 at risk
Adapted from Fulton J, Evans B, Miller S, et al. Development of a nutrition screening tool for an outpatient wound center. Advances in Skin and Wound Care. 2016;29(3):136–140

Validation studies demonstrated the strong capacity of the MNA® to evaluate the nutritional status of older adults and had a strong correlation with biochemical parameters, especially albumin. The full tool with explanations for administering is available on their website at https://www.nestle-healthscience.com/asset-library/documents/newsroom/fact_sheet_mna_e.pdf [131].

Two questions that are helpful during the subjective examination are "What did you have for breakfast this morning?" and "What did you have for dinner last night?" The answers can give useful insight into the patient's eating habits

such as protein intake, caloric intake, and consumption of high-glycemic index foods (especially important for patients with pre- or diagnosed diabetes). Concerns that are raised as a result of the answers can be followed up with additional tests and measurements.

The macronutrients needed for wound healing include carbohydrates which stimulate insulin production and help in the anabolic process of wound healing, fats which supply additional calories, proteins which are needed for collagen synthesis and granulation formation, and fluids (particularly water) which maintains skin turgor and promotes tissue perfusion and oxygenation [127]. The micronutrients (including amino acids, vitamins, and minerals) are vital to wound healing; however, the need for supplementation is less definitive. Arginine is an amino acid used in the biosynthesis of protein, a necessary process for wound healing to occur. Benefits of arginine supplementation include increased collagen deposition, increased lymphocyte mitogenesis, increased production of growth hormone, and increased activation of T cells [132]. A study by Leigh et al. [133] on patients with pressure ulcers found that 4.5 g/day supplementation of arginine was sufficient to facilitate wound healing but must be given with adequate protein intake to be effective.

Vitamins A, C, and D all play a role in the wound healing process, and deficits have been shown to impede the progression of the healing cascade [53]. Supplementation is recommended only if there are measured deficits with the following recommendations for amounts:

1. Vitamin A – 10,000–15,000 IU/day administered in a short course of 10–14 days to prevent toxicity
2. Vitamin C – 500–1,000 mg in divided doses for wound healing and 1–2 g/day for severe wounds, e.g., extensive burns
3. Vitamin D – deficient in patients with venous ulcers and pressure ulcers; dosages not given [127]

The minerals which have been suggested as critical in wound healing, specifically in enzyme and metalloenzyme structure, include zinc, selenium, and iron. Zinc deficiency affects all phases of wound healing; however, supplementation is recommended only in the case of deficiency [53]. Recommended supplementation in the zinc-deficient patient range is either 40 mg/day or 220 mg twice a day for 10–14 days [127].

In summary, all patients with non-healing wounds need to be screened for nutritional deficiencies. Any patient found at risk by a screening tool or who has other red flags that indicate inadequate nutrition may be a factor in poor healing capacity is advised to have further testing for specific deficits, counseling by a registered dietician, and supplements when deemed appropriate.

2.20 Psychosocial Behaviors

Psychosocial behaviors that affect wound healing can be easy to detect but very difficult to manage, especially in the outpatient setting. Those behaviors include stress; tobacco, alcohol, and drug abuse; and self-defamation.

2.20.1 Stress

The effects of stress on wound healing have been studied in both animal and human models using punch biopsies, blistering, and tape stripping as injury mechanisms in order to compare healing rates in stressed and non-stressed subjects. Results have consistently shown that stress slows the healing rate [134, 135]. Psychological stress activates the hypothalamic-pituitary-adrenal and the sympathetic-adrenal-medullary pathways, resulting in increased glucocorticoid (cortisol and prolactin) and catecholamine (epinephrine and norepinephrine) production [60, 134]. The documented changes that subsequently occur at the wound site and impair wound healing include the following: significantly fewer macrophages, both fewer and lower activation of immune cells and Langerhans cells [136], and excessive

recruitment of neutrophils and the gene expression of chemokines (MIP-2 and KC) [137]. The diminished expression of cytokines at the wound site can delay and/or prolong the initial inflammatory healing phase. Lower IL-1α, IL-8, and IL-1ß levels have been reported in stressed human populations, as well as TNF-α, PDGF, MMP-2, and MMP-9 [134].

The clinical results of the cellular changes are not only delayed wound healing but also increased risk of infection due to the suppression of the immune system. Other behaviors associated with stress that may have an impact on wound healing are smoking, alcohol abuse, poor sleep patterns, depression with a decrease in appetite, decreased physical exercise, and fewer positive social interactions [60, 134, 138]. Recognizing and reducing stress prior to surgical procedures or during wound healing will help improve clinical outcomes.

2.21 Tobacco

In addition to increasing the risk of diseases such as arteriosclerosis, cardiovascular disease, stroke, many cancers, and chronic lung disorders, smoking has a significant deleterious effect on wound healing. Smoking has also been associated with faster aging of the skin, more wrinkling, and higher incidence of skin diseases such as skin cancer, psoriasis, and other inflammatory skin diseases (e.g., hidradenitis suppurativa, acne, alopecia, lupus erythematosus, polymorphous light eruption, and tobacco-associated oral lesions) [139].

The nicotine in tobacco stimulates the sympathetic nervous system resulting in the release of epinephrine and thereby causes peripheral vasoconstriction; this is turn reduces the supply of oxygen and nutrients to the wounded tissue. The nicotine also increases platelet adhesiveness and blood viscosity which further contributes to tissue hypoxia [60].

Other cellular effects of smoking on wound healing include the following: carbon monoxide binds to hemoglobin with more affinity than oxygen, thereby causing a decreased percentage of oxygenated RBCs, impaired cellular oxygen metabolism caused by hydrogen cyanide, and decreased number of monocytes and macrophages at the wound site with reduced bactericidal activity [60].

During the inflammatory phase, there is impaired white blood cell migration with fewer monocytes and macrophages at the wound site and depressed lymphocyte function, as well as decreased cytotoxicity of killer cells and depressed production of IL-1, all of which increase the risk of infection in both chronic and surgical wounds [140]. During the proliferative phase, smoking results in decreased fibroblast activity, reduced wound contraction, slower reepithelialization, decreased ECM production, and increased proteases [140].

Numerous studies have shown increased surgical complications, including infections, in surgical patients who smoke [141–143]. A literature search by Pluvy et al. [144] resulted in recommendations that in order to optimize surgical conditions, a patient should stop smoking 4 weeks preoperatively and abstain until the operative site has reached primary healing, or at least 2 weeks. In summary, although with some patients it is extremely challenging, it is worth the time and effort to assist patients who smoke in quitting in order to optimize wound healing.

2.22 Alcohol Abuse

Alcohol (or ethanol) abuse is defined as 4 drinks per day or 14 drinks per week for males and more than 3 drinks per day or 7 drinks per week for females [145]. The effects of both short- and long-term alcohol abuse have been studied, and the results show that even single alcohol exposure can affect wound healing as a result of molecular and cellular disruptions, depending on the tissue that is involved. For example, the canonical Wnt signaling pathway that is critical to bone fracture healing is significantly decreased in animal studies, resulting in both less and different cartilage and boney callus composition and longer healing times [146]. Increased infection rates, with longer hospital

stay and increased mortality, have been documented for patients with major burns [147] and postsurgery [145, 148].

Most of the studies on the effects of single-exposure, blood alcohol content on dermal healing have been performed on mice that were injected with alcohol versus saline. Changes occur in the tissue that affect each of the healing phases, including defective neutrophil function, reduction of pro-inflammatory chemokines (specifically MIP-2 and KC which is equivalent to IL-8 in humans), delayed reepithelialization, decreased angiogenesis and tissue vascularity with resulting hypoxia (due to reduced phosphorylation of the VEGF receptor), significantly less collagen with an increase in matrix proteolytic activity, and decreased fibroblast proliferation and collagen synthesis [146]. These changes create a wound bed that is more vulnerable to dehiscence and to infection.

Alcohol intake has been shown to result in higher blood glucose levels which can impair wound healing, and patients who chronically use alcohol have a higher risk for malnutrition as a result of poor eating habits. In addition, chronic alcohol use causes changes in the skin that can be an early sign of abuse, personal neglect, or resulting systemic disorders (Table 17) [149].

In summary, alcohol intake is an important issue to address in any patient's subjective history, especially in outpatient and presurgical settings. Cessation of alcohol consumption can improve healing rates, reduce risk of infection, and reduce risk of dehiscence or recurrence.

Table 17 Skin changes and other disorders associated with chronic alcohol consumption

Skin changes
Jaundice
Pruritis
Hyperpigmentation
Urticaria
Skin granulomas
Ulcerations
Recurrent infections
Vascular changes
Spider telangiectasias
Angiomas
Caput medusas
Flushing
Palmar erythema
Cancers
Skin cancer
Oropharynx cancer
Liver cancer
Breast cancer
Pancreas cancer

Adapted from Liu SW, Lien MH, Fenske NA. The effects of alcohol and drug abuse on the skin. *Clinics in Dermatology.* 2010;28(4):391–399

2.23 Drug Abuse

Drug users are at risk for soft tissue infections, cellulitis, and dermal wounds as a result of using dirty needles, skin picking, poor nutrition, and poor hygiene; however, the mechanism of action on healing and immunity have not been studied in depth. An extensive murine study by Mihu et al. [150] investigated the effects of methamphetamine (METH) on *S. aureus* skin infections and showed that METH does indeed impede wound healing and "facilitates host-mediated collagen degradation by increased expression and production of matrix metalloproteinase-2 (MMP-2)." Other specific findings included increased biofilm formation with detrimental effects on the function of phagocytic cells; apoptotic death of thymic and splenic lymphocytes; "intense inflammatory infiltrates in both the epidermal and dermal layers, along with extensive cell necrosis" [150]; decreased collagen synthesis; and diminished angiogenesis in the dermis. Their conclusion was that METH use leads to chronic, recalcitrant wounds because of a combination of bacterial persistence, drug-related immune deficiency, and deleterious social habits that include poor hygiene.

Levamisole, an adulterant used to "cut" cocaine, has been reported to cause levamisole-induced necrosis syndrome (LINES) in cocaine users. Initially LINES presents with painful, retiform purpuric lesions with some bullae that progress to full-thickness dermal sloughing. Small vessel thrombi and vasculitis are confirmed by histologic studies, possibly due to the formation of autoantibodies. In some cases, the skin

lesions progress to full-thickness necrosis, usually on the lower extremities and face [151]. Other symptoms include arthralgia and leukopenia. Diagnosis begins with urine toxicology for cocaine, preferably within 48 h of use; if positive, tests for ANCA, cryoglobulins, and antiphospholipid antibodies help to make a differential diagnosis. Treatment involves stopping the cocaine use, extensive and complete debridement of necrotic tissue, and moist wound management followed by grafting, similar to treatment of full-thickness burns [152].

2.24 Factitious Wounding

Patients with factitious disorder (FD) use false symptoms or self-injury in order to appear sick and/or to gain access to medical care. An extensive review by Yates [153] found that more than 62% of the patients reported were female and the mean age at presentation was 34.2 years. They also found a stronger correlation with depression than with personality disorders (e.g., borderline personality). Dermatological wounds due to self-picking are frequently a symptom of factitious disorder and may either cause a wound or prevent a wound from healing (Fig. 24). Skin lesions as a result of FD are generally atypical and located on areas of the body that are easily accessible with the hands (e.g., face, arms, torso, legs, but rarely on the back) [153].

Other factors that may indicate a patient presents with FD include inconsistencies between history and objective findings, vague and inconsistent details, long medical history of multiple admissions at different hospitals, multiple surgical scars or recurrent wounds, and psychological signs and symptoms [154]. Treatment of patients with factitious wounds must involve immediate psychiatric care as well as standard wound care.

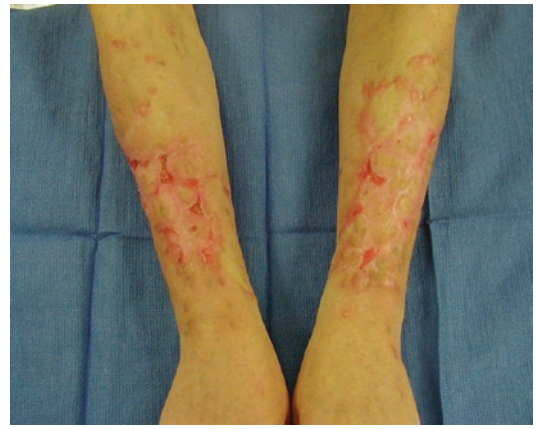


Fig. 24 Factitious wounds on the arms of a subcutaneous drug user. The initial presentation was multiple subcutaneous soft tissue infections due to vein-popping, but after the initial wounds healed, the patient continued to present to the clinic with open areas due to skin-picking

2.25 Foreign Bodies

Foreign bodies are detected by the body's immune system, thereby initiating or increasing an inflammatory state that can impede wound healing, cause granuloma formation, or increase infection risk. The foreign body may be an identifiable exogenous material or an endogenous material that has become altered in such a way that the body responds to it as a foreign body [155]. Examples include tattoo and cosmetic filler particles, biomaterials and synthetic materials associated with medical devices and prostheses [156], foam remnants associated with negative pressure wound therapy [157], suture materials [158–160], infected orthopedic hardware, and objects as a result of trauma (e.g., glass, nails, needles, metal shards) (Figs. 25, 26, and 27) The latter is especially of concern in the neuropathic foot when the wound is in an area other than a bony weight-bearing surface.

The histological sequence of a foreign body reaction and granuloma formation is composed of macrophages and foreign body giant cells and is the end-stage response of the inflammatory and wound healing response [155, 156]. Signs and symptoms of a foreign body in a wound include the following:

1. Failure of the wound to achieve full closure
2. Persistent drainage from a residual wound opening
3. Consistent epithelial breakdown of a closed wound

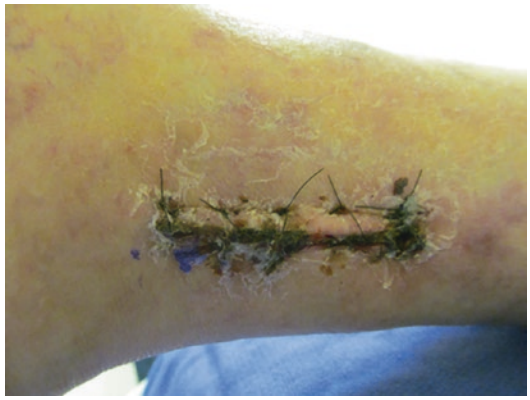


Fig. 25 Sutures not removed in a timely sequence can cause periwound inflammation and impede the healing process. Removal of the sutures and eschar is the first step in initiating the healing cascade

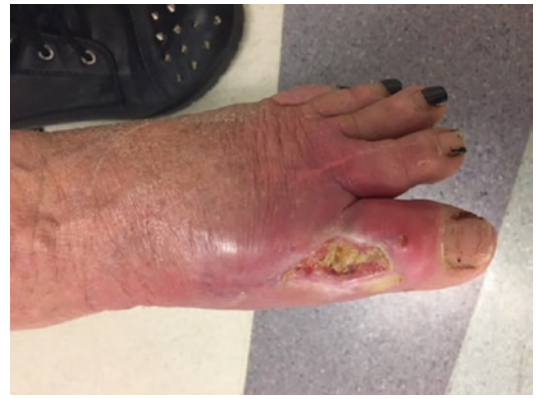


Fig. 27 Erythema, edema, pain, and dehiscence of the surgical wound are signs of the infected hardware in this patient's great toe after bunion surgery

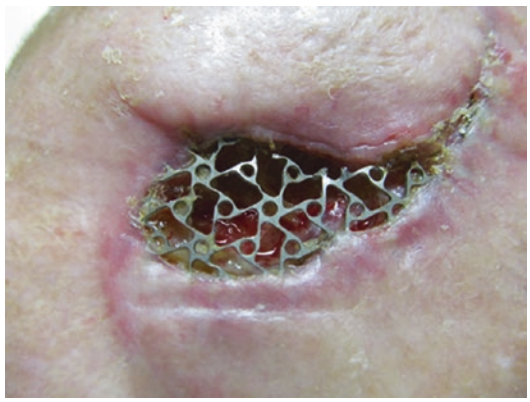


Fig. 26 Hardware placed to protect the brain after removal of a basal cell carcinoma. In order to obtain full closure by secondary intention, the hardware was removed in stages. Full closure was obtained without have a skin graft or flap for coverage

4. Subcutaneous tenderness
5. Palpable subcutaneous nodule
6. Periwound erythema and edema
7. Pain with weight-bearing (in the neuropathic foot)

Radiography can detect a metal foreign body; however, ultrasonography is recommended for a nonradiopaque foreign body [161]. Surface markers, multiple-projection radiographs, wire grids, fluoroscopy, or stereotaxic devices are also helpful in locating the embedded foreign body [162]. Removal of the foreign body is advised if possible, considering patient risk factors, followed by standard wound care to facilitate timely closure.

2.26 Clinician-Induced Factors

Reduction of bacteria is one of the integral components of wound bed preparation; however, the continued use of antimicrobials after the wound is clean can have detrimental effects on the healing process by inhibiting fibroblast synthesis of collagen. This is especially true for some of the traditional antiseptics such as Dakin's solution, povidone iodine, acetic acid, and hydrogen peroxide. Hydrogen peroxide is not an antibacterial agent and is not recommended for wound cleansing; it is, however, useful for dissolving clots. Acetic acid is effective against pseudomonas aeruginosa and is recommended only as a wash when bacteria are known to be present, not as a wet-to-dry dressing component because it can halt the proliferative process (Figs. 28 and 29). Iodine is now available in cadexomer form which releases the iodine at a concentration that is effective against microbes without damage to the healthy cells. Dakin's is advised only in a necrotic, infected wound and should be discontinued once the wound is debrided and clean [163].

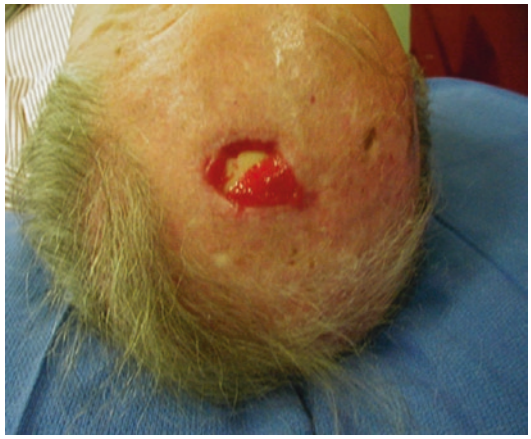


Fig. 28 Nonhealing surgical wound treated twice daily with hydrogen peroxide. After the use of hydrogen peroxide was halted, the wound granulated and reepithelialized in less than 6 weeks

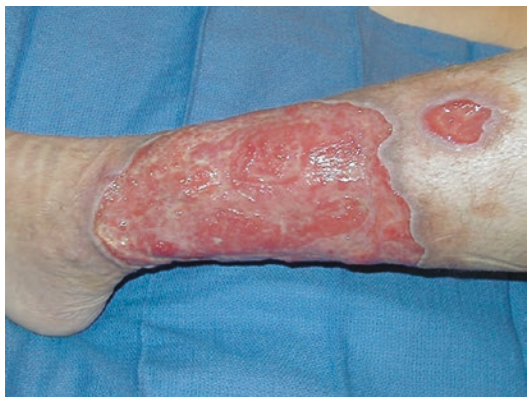


Fig. 29 Chronic venous insufficiency wound being treated by the patient at home with acetic acid twice a day (per physician orders). Cessation of acetic acid and moist wound dressings with compression resulted in immediate increased granulation and decreased pain

Conclusions

When the wound fails to progress toward full closure or shows signs of regression, further assessment of the patient's medical history, medications, and social habits can provide information to help solve the conundrum. Additionally, a close and critical evaluation of the clinician's plan of care is a necessary component of each and every patient encounter in order to obtain the optimal outcome of timely wound healing.

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Traditional and Nontraditional Evaluation of Wound Healing Process

Apichai Angspatt, Chankit Puttilerpong, Chaiyos Sirithanakorn, and Pornanong Aramwit

1 Introduction

Wound healing is considered a complicated procedure, due to several stages of the healing cascade. It is dynamic process and takes place over months to years. Food and Drug Administration (FDA) guidance defines complete wound closure as skin closure without drainage or dressing requirements [1]. The assessment of healing is crucial since further treatment, especially conclusions about a dressing's performance, will be planned according to the healing status of the wound [2] (Table 1). However, objective measurement and quantifying healing remain a considerable challenge because of the complexity of the phenomena implicated and variations in their manifestations [4]. The ideal measuring tool should produce results that are relevant, accurate, unbiased, sensitive, unidimensional, and efficient [5]. Reliability is an essential component of any measuring tool and must be established prior to clinical use. Accuracy and consistency in clinical and research applications are essential for complete documentation and for the corroboration of research data. In addition, the effects of a particular treatment can be more precisely calculated and evaluated with accurate measurements [6]. In clinical settings, any wounds should be evaluated regularly in order to increase the effectiveness of short- and long-term treatment planning. Nevertheless, a safe, practical, accurate, and effective method is needed (Table 1) [3].

Before further evaluation, the definition of "healing" should be specified. There are normally three levels of wound healing [7]:

1. An ideally healed wound is one that returned to normal anatomic structure, function, and appearance.
2. Minimally healed wound is characterized by the restoration of anatomic continuity but without a sustained functional result.
3. Acceptably healed wound is characterized by restoration of sustained function and anatomic continuity.

The clinician should decide on the level of healing needed according to the wound and patient condition, concomitant disease, etc.,

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Table 1 Wound management strategy [3]

Appearance	Cell biology cause	Treatment	Cell biology effect
Necrotic tissue	Accumulation of cell debris	Debridement	Limit infection and inflammatory stimulus
Infection	Bacteria, fungus, or other microorganism load	Antimicrobial agent (intravenous, oral, or topical)	Microorganism balance
Chronic inflammation	Increase protease Decrease growth factor activity	Protease inhibitor Agent or bioactive therapy	Eliminate inflammation Balance of growth factor
Increase exudate Decrease exudate	Maceration Decrease keratinocyte migration	Moisture management therapy	Moist wound environment

Table 2 Wound dimension assessment

Methodologies	Measurement	Instrument used
One-dimensional measurements	Depth or perimeter	Ruler, tape
Area measurement	Width and length	Ruler, tape, planimeter (area measurement of a plane surface obtained by tracing its contours) [12]
Volume measurement	Width, length, and depth	Ruler, tape, software or saline filling, gel filling
Three-dimensional wound reproducing system	Width, length, and depth (volume)	Kundin device Stereophotogrammetry

which is also related to the method of healing evaluation. Since the main goal of wound treatment would be complete wound closure, time to complete closure should also be recorded. Even though this seems simple, it can be problematic when a wound that was healed at a certain point goes on to breakdown. Therefore, stability of wound closure should be monitored for at least 3 months [8] following therapy completion or even longer for chronic wounds.

MEASURE has been used as a key concept that includes seven parameters for routine evaluation: Measure, Exudate, Appearance, Suffering, Undermining, Reevaluate, and Edge [9]. Although wound dimensions are the most frequently used parameters for healing evaluation in clinical practice, several researchers still fail to consider dimensions such as size, shape, color, or physical properties, which best reflect wound healing. However, wound dimension assessment is still the most popular procedure for wound evaluation.

2 Wound Dimension Assessment

Intact skin has been used as an outcome measure in most clinical settings. However, not all wounds can be evaluated by wound closure, and the reduction in wound area is used as a primary outcome in most situations [10]. The reduction rate chosen should consider the margin of error for the method of measurement, as well as the baseline size of the wound, particularly in the chronic type where the healing process is nonlinear. In order to evaluate the healing process by wound size reduction, width, length, area, and volume measurements are the most frequently used parameters [4]. Even though this is the most common procedure, several factors including type of device used, experience of evaluator, and wound shape still reflect the accuracy of this technique. Regardless of the tools used, wound boundary delimitation remains the main source of error in wound measurement [11].

There are several ways to evaluate wound dimension, as shown in Table 2. There are advantages, disadvantages, and also limitations for each method. One-dimensional measurement is simple and quick, but shows poor sensitivity and reliability, while area measurements are the most frequently used methods for assessing wounds in clinical and research settings [13] (Fig. 1), since this technique is quick, cheap, and more reliable than one-dimensional measurements. Linear or one-dimensional measurement using wound tracing is a straightforward and reliable technique that is sensitive to changes in wound size. For this approach, wound

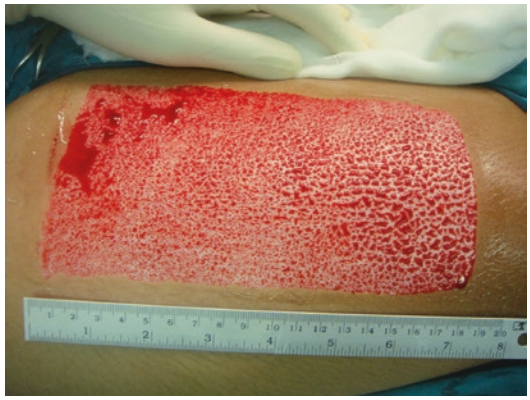

Fig. 1 Ruler-based assessment of wound size (one-dimensional measurement)

outlines are drawn onto a clear plastic sheet with a marker pen. A second clear plastic sheet is positioned between the tracing sheet and the wound itself and is discarded after use to prevent contamination. Wound tracings are placed in a patient's chart for sequential comparison. Patients can easily comprehend a successful pattern of healing when they observe a series of progressively smaller concentric circles representing successive wound tracings [14]. However, the reliability of the tracing technique normally depends on consistent and precise location of the wound edge.

For area measurement, wound area is generally calculated in square centimeters by multiplying two perpendicular linear dimensions. Again, the limitation of this approach is its potential for inaccuracy due to the assumptions regarding wound shape. If a rectangular shape is assumed, the product of length times width overestimates the true area of a circular wound by about 25% [14]. If an elliptical shape is assumed, finding the product of the major and minor diameters also overestimates the area. Due to this limitation, computerized planimetry is still the favorable method [14]. If planimetry is considered the standard, the concurrent validity of wound area calculations is 0.93 [15], which is appropriate for the clinical setting where the goal is to track relative information over time.

From wound tracings, wound area can be accurately determined by digital planimetry. This technique has excellent reported intra- and inter-rater reliability and concurrent validity compared with other methods [16]. In a study of digital planimetry, wound outlines were traced onto a digital Artpad (Model ET-0405-L1, Wacom Co., Ltd. Taiwan) with a stylus [17]. The resulting digitized outline may be visualized on ImageJ software (National Institutes of Health), which is free of charge and an open-source application for the processing of multidimensional images [18], from which areas are calculated. Two area calculations of the same tracing are averaged to determine the wound area. Using this method, intra- and inter-rater reliability have been found to be 0.99 [19], which is considered as well-accepted in clinical practice.

Volume measurements are normally used for deep ulcer or sinus assessment. In general, wound volume measurements are most reliable for deep cavities without long sinus tracts and least reliable for shallow, irregularly shaped wounds. The length of a wound tract (linear depth) can be determined using a sterile cotton swab or curette, inserted into the maximum tract depth in a particular dimension and documented on the wound tracing. Volume can be calculated as area times depth, although there are inherent errors with this method. A deep wound is not a rectangular solid but is better approximated as a spheroid [14]. More accurate methods of determining volume have been evaluated using molding materials such as calcium alginate [20] and Jeltrate dental material [21], by putting these materials into the wound to reproduce its volume. The wound volume is determined by weighing the volume displaced by the mold.

The disadvantages of the volume measurement technique are difficulty in obtaining accurate volume measurement, and trauma and pain from the technique used. Moreover, other factors such as wound debridement, patient positioning and edema can influence wound volume [4].

Photography can also be used to calculate wound area. The borders of the wound are traced from a digital photograph using a mouse or digital pen on a digital tablet. A ruler, or other accurate scales, photographed near the wound allows the user to calibrate the software to enable it to measure the distance. An advantage of photography is that it does not require contact with the wound. It provides a permanent record of not only wound size but also appearance [22]. Important considerations when using photography are photo quality,

lighting, and camera angle. Variation in camera angle can lead to an underestimation of wound area by up to 10% [23]. Given that a two-dimensional image represents a three-dimensional structure, apparent wound size discrepancy may also occur when tracing circumferential wounds, or those on curved body surfaces [24, 25]. Despite these difficulties, studies have still reported very good intra- and inter-operator reliability [26]. For analysis, ImageJ programming language can be used to evaluate wound size (Fig. 2). The wound size analysis using photography and ImageJ program shows no statistical difference in wound area measurement compared to other methods such as digital planimetry system. Moreover, the photographic method is a more appropriate technique for clean and uncontaminated wounds, as contact with the wound bed is avoided, negating the risk of wound contamination, wound bed damage, and patient discomfort [22].

For three-dimensional wound evaluation, the Kundin device (Pacific Technologies and Development Corporation, CA, United States) is another choice to consider. It is a plastic-coated, disposable gauge using a three-dimensional Cartesian coordinate system to measure wound volume in regular and irregular-shaped wounds [27]. It has three arms for length, width, and depth, each with a pre-marked scale for measuring in centimeters. A mathematical formula is used to calculate volume by multiplying length (L) × width (W) × depth (D) by a constant factor (C = 0.327) which calculates a volume of about 60% that of a spheroid. The constant factor corrects for deviations in wound shape from the implied rectangular solid; it assumes a shape somewhere between a cylinder and a sphere. This approach is convenient, inexpensive, and user-friendly and can monitor changes in wound size within a minimal amount of time [28].

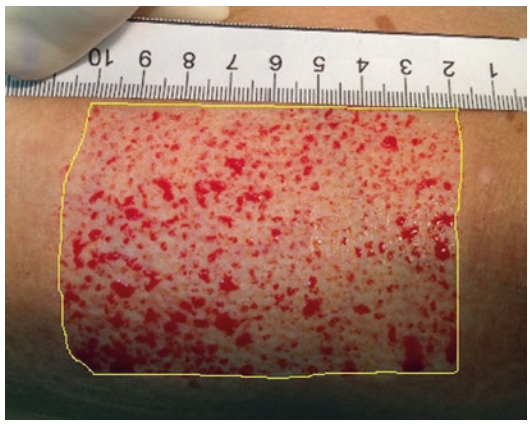

Fig. 2 Software-based system (ImageJ software)

Stereophotogrammetry, another device for three-dimensional wound evaluation, uses a video camera that is attached to a computer with wound measurement system (WMS) software (Vista Medical, Winnipeg, Manitoba, Canada) and a computer pointing device (usually a mouse). To use stereophotogrammetry for wound measurement, the clinician places a target plate in the principal plane of focus beside the wound and captures the wound images on videotape using the video camera. The computer software uses photogrammetry to analyze the target plate, determine the camera's position and orientation, and correct for distortion caused by lens curvature. The clinician then uses a cotton-tipped applicator to mark the wound depth at its deepest point, laying this in the plane of the wound for inclusion in the picture. After the image is downloaded to the computer, the clinician uses the computer screen and mouse to trace the length and width of the wound. The length of the cotton-tipped applicator is measured from the tip end to the wound depth mark, and this measurement is recorded as the depth. The wound length, width, and depth are calculated in centimeters using the same formula as used for the Kundin device ([L × W × D] × 0.327). The volume is calculated in cm^3 by the computer software. This approach provides accurate, reproducible wound measurement of irregular defects in two and three dimensions and offers a noninvasive method for measuring wound size and volume [6].

3 Granulation Tissue

Since healing is directly related to the tissue, monitoring the change of all tissues is considered a key for successful healing. There are three main

types of tissue in the wound bed: red granulation tissue or pink epithelial tissue, yellow fibrous tissue or slough, and black necrotic tissue. The granulation tissue is new connective tissue and microscopic blood vessels which form on the surfaces of a wound, normally 2–4 days after wounding, and is generally described as red tissue [29]. Granulation tissue typically grows from the base of the wound and is able to fill wounds of almost any size. The fibrous tissue or slough is a combination of white and yellowish material covering the wound bed. The necrotic tissue represents dead tissue and is generally described as black tissue. Increased slough and necrotic tissue promote wound infection and prevent normal wound healing [30]. On the other hand, increased granulation tissue formation indicates the progress of wound healing. The differentiation of granulation tissue, fibrin tissue or slough, and necrotic tissue is generally judged by experienced clinicians using visual inspection utilizing the black-yellow-red scheme or the wound healing continuum [31]. An increase in the amount of granulation tissue in the wound bed points toward healing, and the presence of healthy granulation tissue can be quantified in terms of percentage (scale or area measurement) [10].

The wound healing continuum (WHC) is an aid to understanding the type of tissue present in, and the progress of, the wound [31]. As shown in Fig. 3, the key is to determine the type of tissue in each present stage. Following the continuum from left (black) to right (pink), it correlates with the colors found in a healing wound.

In order to use the WHC, clinicians need to identify the color that is furthest to the left of the continuum. For example, if the wound contains black necrosis and yellow slough, it would be defined as a "black/yellow wound." In this case, the management plan should focus on the debridement of black tissue and removal of the yellow sloughy tissue in order to promote the red granulation tissue [32]. The wound can progress along the continuum toward the right until the "pink/healing" status is obtained.

4 Evaluation of Tissue Color

Besides wound dimensions, measuring any wound characteristics that fluctuate during healing is a potential method of wound healing assessment. Among these, wound color, which allows the assessment of early healing when no variation in wound size is noticeable [33], is the most frequently used. Since healing not only involves wound edge evolution, healing also results from a progressive central growth of the epidermis which is responsible for color variations of the whole wound (especially in such

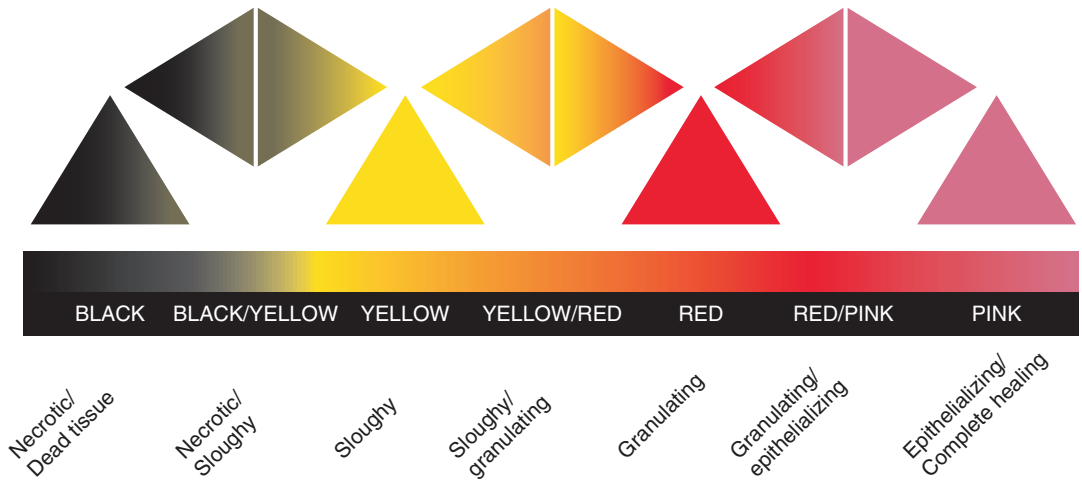

Fig. 3 The wound healing continuum (WHC) [32]

purely epidermal wounds) [34]. Wound color assessment could be better suited to assess early healing [33]. Color evaluation takes into account granulation tissue spread, which is an important sign of healing [35]. For differentiation from granulation tissues, which are normally evaluated using practitioners' experiences, the evaluation of tissue color uses advanced techniques in order to obtain a quantitative, objective parameter. In superficial wounds, color assessment can monitor central growth of the epidermis, which produces color variation without changing wound size [36]. However, there are several factors which may interfere with color evaluation, such as skin pigmentation, lighting, blood, eschar formation, and even non-uniform-colored wound.

Wounds can present with several colors such as black, black-yellow, yellow, yellow-red, red, pink, or even multiple colors, which also indicate the stage of healing. A black wound indicates the "unhealthiest" color. However, a black covering on the wound may be very superficial, and healthy tissue may be found below such tissue. Debridement is required before the wound can heal. Following debridement, the wound may be classified as yellow or yellow/red depending on the degree of slough present on the wound bed. Yellow wounds should be assessed to exclude the possibility of pus and therefore localized or spreading infection. Yellow also can be attributed to slough which serves as a medium, for the growth of microorganisms and need to be removed [37]. Red wounds are normally classified as healthy tissues, but not always. Since red tissue may develop unhealthy characteristics such as critical colonization, care should be given in order to avoid failing to heal and possible deterioration in the wound bed [31].

The study reported that intra- and interobserver variability was 30% for judgment of color shades of the skin in the same lesion within 6 months by 15 dermatologists [38]. For this reason, software-based computerized techniques have been developed, such as Wound Healing Analyzing Tool (W.H.A.T) and iCLR technology®. This technique is called the red-yellow-black technique because the software differentiates the different colors of wound healing. The software measures tissue composition from a digital photograph by measuring the color spectrum of the wound and calculating a percentage of tissue content [39, 40]. This software-based technique is easy to use and does not require contact with the wound, preventing infection, pain, and damage. iCLR technology® is a mobile application which is being developed for android and is more convenient than W.H.A.T. It is important to note that tendons and bones are also yellow, while dried blood and fresh bleeding are also black and red, respectively. Therefore, the wound should be carefully cleaned before taking a photograph. In addition, the shadows in deep cavities of wounds may be falsely identified as necrosis; therefore, this technique is not appropriate for wounds with deep cavities [30, 40, 41].

The color and texture of the wound bed should also be monitored. In order to report quantitative measurements, wound color can be determined by measuring the wavelengths of reflected light. According to this principle, two different methods can be distinguished: tristimulus reflectance colorimetry and narrowband spectrophotometry [42]. In tristimulus reflectance colorimetry, any color will be described by three values which are L* (the brightness), a* (redness: the amount of green or red), and b* (pigmentation: the amount of yellow or blue). For narrowband spectrophotometry, the fundamental is based on the differences in light absorption of red and green by hemoglobin and melanin [43]. If there is no barrier from opaque wound dressing, the clinician normally evaluates wound color daily using his own experience since it is one of the most important factors, which can give several details about the condition of the wound.

5 Infection

Infection is the most frequently occurring complication of nonhealing wounds [10]. Prompt diagnosis and treatment of infection promotes wound healing and minimizes the impact on patients, caregivers, and healthcare systems. The

strategic management of wound infection consists of effective management and regular reassessment. Effective management of wound infection requires a multidisciplinary team approach. The goal of therapy is to readjust the interaction between the patients and the microorganism-infected patients by optimizing host response, reducing the quantity or virulence of microorganism load, and promoting optimal environment for wound healing with general measures. Wound infection may produce different signs and symptoms associated with stages of the wound infection continuum which are summarized in Table 3 [44, 45].

The diagnosis of wound infection is principally based on the clinician's evaluation of the patient (host), the tissues around the wound (periwound), the wound itself and host responses, as well as factors likely to increase the risk and severity of infection. Comprehensive assessment for wound infection depends mainly on clinical judgment, early investigations of which can guide appropriate management and timely treatment. Stages of the wound infection continuum illustrate the gradual increase in the quantity and virulence of microbes together with the host's response (Fig. 4) [46].

Spreading infection such as rapidly increasing cellulitis can be a life-threatening condition. The principal visible clinical cue is a rapidly advancing redness (greater than 2 cm around the wound margin) which may be accompanied by other signs and symptoms, notably pain, and often includes very high exudate levels, malodor, and, in the surrounding tissues, heat, swelling, and blistering. Local infection is characterized by less than 2 cm of redness around the wound margin, sometimes with symptoms similar to spreading infection but to a lesser degree [47].

Bacterial colonization, which is a state of host manageable bioburden, is normal in a wound healing condition by secondary intention and does not hinder progression toward closure at expected rates [48]. In this case, the term "coloni-

Table 3 Signs and symptoms associated with stages of the wound infection continuum [44, 45]

Contamination	Colonization	Local infection	Spreading infection	Systemic infection
Presence of nonreplicating microorganisms in wound. Its suitable nutritive and physical conditions are not available for each microbial species, or they are not able to successfully evade host defenses, they will not multiply or persist, their presence is therefore only transient, and wound healing is not delayed	Presence of replicating microorganisms successfully grow divide and adhere to wound bed without causing cellular damage to host or initiate wound infection	Classic (overt) signs: –Erythema –Local warmth –Swelling –New or increasing pain –Purulent discharge –Delayed wound healing –Increasing malodor Subtle (covert) signs: –Hypergranulation (excessive vascular tissue) –Bleeding, friable granulation –Epithelial bridging and pocketing in granulation tissue –Wound breakdown and enlargement –Delayed wound healing –New or increasing pain –Increasing malodor	–Extending in duration +/− erythema –Lymphangitis –Crepitus –Wound breakdown/ dehiscence with or without satellite lesions –Malaise/lethargy or nonspecific general deterioration –Loss of appetite –Inflammation, swelling of lymph glands	–Severe sepsis –Septic shock –Organ failure –Death

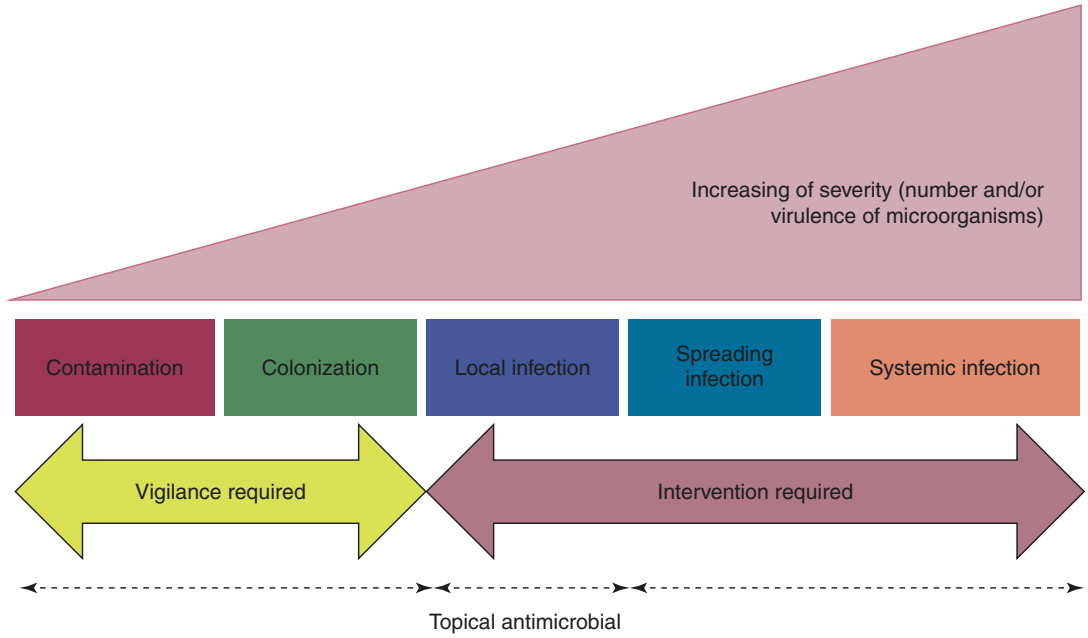

Fig. 4 Stage of the wound infection continuum (WIC) [45, 46]

zation" on the infection continuum describes a wound that is free of untoward or unexpected symptoms for a healing wound such as tenacious slough, excessive wetness, malodor, and dull granulation and is actively improving. A clearly visible reduction in the wound size over a 2-week period would suggest an acceptable level of colonization [11].

Factors associated with an increased risk of wound infection include characteristics of the host (comorbidities such as poorly controlled diabetes, prior surgery, immune system disorders, malnutrition, conditions associated with hypoxia/poor tissue perfusion, alcohol/smoking, and drug abuse), the wound (acute or chronic wound), and the wound environment (hospitalization, unhygienic environment, inadequate management of moisture, exudate, edema and pressure off-loading, poor hand hygiene and aseptic technique, repeated trauma). In most cases, the development of wound infection is multifactorial and occurs when cumulative risk factors overwhelm the host's defense system [44].

Antimicrobial therapy may be required when other methods of reducing wound microbes' load are likely to be insufficient in local infection or when it is spreading or systemic infection. In general, most healing wounds do not require the use of antimicrobial therapy. Topical antiseptics should only be used at the lowest effective concentration to minimize harm to skin and tissue cells involved in wound healing. The use of topical antibiotics, which contain a low-dose of antibiotic, is not recommended for the general management of wound infection and should only be considered under very specific conditions by experienced clinicians. Reasons for this include inadequate penetration for deep skin infections, the development of antibiotic resistance, hypersensitivity reactions, systemic absorption when applied to large wounds, and local irritant effects leading to further delay in wound healing. Using systemic antibiotics in wound infection should be reserved for the treatment of severe infection (spreading or systemic wound infection) or prophylaxis wound which is the high risk of infection (contaminated or dirty surgical incision or traumatic wounds) [44, 46, 49]. However, antibiotics must be used in combination with appropriate wound management strategies.

6 Temperature and pH

The local increase in temperature is one of the markers associated with wound infection and is extremely important to assess the efficiency of wound healing since neutrophil, fibroblast, and epithelial cell activities decrease for values below 33°C [50]. However, in order to monitor accurate temperatures in the wound, a sensor is needed. In some cases, the clinician uses body temperature as a representative for wound temperature, which should not be totally accurate. In order to monitor the healing process, exact temperature from wound is necessary to avoid other confounding factors.

pH has a critical role for the treatment of chronic and acute wounds. It affects matrix metalloproteinase (MMP) and fibroblast activity, keratinocyte proliferation and microbial proliferation, and oxygen release to the tissues and can alter the immunological response in a wound [51]. In order function as the skin's barrier, healthy human skin pH values are in a broad range from 4.0 to 6.0 [52]. This pH milieu also seems to be important for resistance to external chemicals. When wounded, the skin's acidic milieu is disturbed since the underlying tissue with the body's internal pH milieu of 7.4 becomes exposed [53]. Normally, physiological acidosis might be beneficial for the healing process since a significant decrease in cell migration and DNA synthesis occurred after an increase in the pH milieu. For this reason, an acidic milieu is more beneficial for the investigated parameters related to wound healing [54]. However, this strategy can be changed in certain circumstances, as chronic and infected wounds tend to have high bacterial load and their pH values are normally above 7.3, while acute wounds with pus or necrotic tissue or chronic wounds that progress in their healing process normally show an acidic pH [54]. Moreover, in chronic venous leg ulcers and in pressure ulcers, an increase in pH, compared with the normal surrounding skin, is a sign of infection [55]. It is noteworthy that pH value in wounds is a dynamic factor that can change rapidly with therapeutic interventions.

7 Measurement of Skin Barrier Function

Skin performs five main functions: protection, maintenance, sensation, metabolism, and communication. It is composed of three layers which are epidermis, dermis, and subcutaneous tissue. The outermost barrier of epidermis is the stratum corneum, which blocks foreign substances and traps moisture to protect skin from desiccation. Excessive water loss (increased transepidermal water loss (TEWL)) is regarded as one of the most important parameters for skin barrier function and will occur as a result of the disruption of this skin barrier function or wound. TEWL is a measure of the flux density of condensed water diffusing from the deeper highly hydrated layers of the dermis and epidermis to the skin surface; the values are affected by the state and function of the stratum corneum [56]. The increased TEWL is associated with skin barrier dysfunction, whereas normal or decreased TEWL is regarded as an indicator for intact or recovered skin barrier [57, 58]. For this reason, the TEWL value can be an indicator of wound healing [59]. Recovered skin barrier function is demonstrated by a decreased TEWL value. The TEWL measurement technique was used to study functional barrier recovery times and evaluate the effects of wound treatment in several studies [60–62]. The pattern of functional barrier recovery is an exponential decrease of the TEWL value in the first 5–7 days after wounding [60]. When reepithelialization was achieved, the TEWL value remained high. Normalization of TEWL was obtained 2–6 months after wounding, depending on the treatment [63]. However, the TEWL measurement technique is not suitable for wounds with excessive exudate, wounds applied with water-containing topical substances, or skin sweating [64].

One concern about using TEWL as an indicator is the fact that the TEWL value can be affected by age and skin area [65, 66]. Elderly patients (70.5 ± 13.8 years) show a decreased TEWL in all skin areas (except the post auricular and palm areas) [67]. Although a correlation between TEWL and age was not found [68], the TEWL value was significantly decreased in the group

aged 60–80 years, in the hand and forehead areas. The highest TEWL value was found at the axilla, and the lowest TEWL value was found at the breast [65]. Due to the variation in TEWL with age and area measured, comparing the TEWL of wound areas with healthy, adjacent skin would be appropriate to evaluate the skin barrier function and healing process.

8 Measurement of Skin Humidity

Generally, high exudate production occurs in the wound after initial injury, decreases over time, and varies depending on the wound etiology. Therefore, clinicians may consider the amount of exudate to monitor healing status. The measurement of skin impedance is a method to evaluate the degree of skin humidity—as a result of exudate production. The principle of the impedance measurement is based on differences in electrical conductivity by applying two electrodes, one on the wound and one on the adjacent normal skin [69, 70]. The common units of skin impedance are Ω or kΩ. A low skin impedance value is defined as high exudate production, and skin impedance values will reach the baseline level after the wound is healed [69, 71]. The skin impedance value can also be affected by skin area. At a frequency of 10 Hz, the highest skin impedance value was found on the upper arm (700 kΩ), while the lowest value was found on the forehead (40 kΩ) [70, 72]. The advantages of using this method are that it is noninvasive and therefore it is not necessary to remove wound dressings, which allows the wound healing process to continue undisturbed [69].

Besides the impedance measurement, the wound exudate continuum (WEC) can also be used (Fig. 5). The WEC is an aid to estimate both the volume and viscosity of wound exudate [32], which allows the wound exudate to be categorized by a score. For example, a low volume and medium viscosity would be a "low/med" category and would score 4, placing it in the low exudate portion of the continuum. To use this tool, both exudate in the wound and on the dressing should be assessed. Moreover, the number of dressing changes required over a 48-h period should also be considered. The most severe wound needing close attention would receive ten points, while any wound scoring six points would be regarded as requiring regular review [32]. Any score falling in the green zone should be considered advantageous to wound healing, and normal wound care can be applied, while wound score in the amber zone would cause concern if the previous recording was green, as this indicates deterioration of the wound healing process. The treatment situation would be changed if the wound score falls into the amber zone, but the previous recording was red; in this case, an amber reading indicates a step in the direction of healing. A score in the red

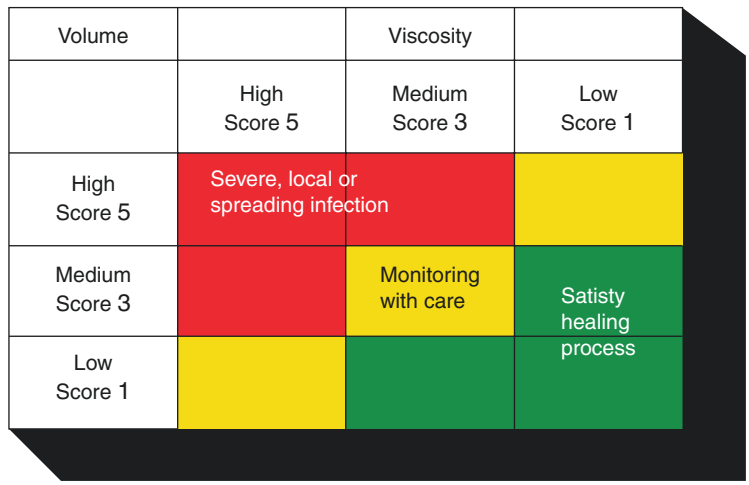

Fig. 5 The wound exudate continuum (WEC) [32]

zone should be further investigated, as this my indicate local or spreading infection.

9 Assessment of Keratinocyte Morphology

The topmost layer of human skin, the stratum corneum, is composed of corneocytes "glued" together with intercellular lipidic cement. The geometry of corneocytes is of large interest in dermatology since it can correlate with the type of skin epidermis, its age, and its health [73]. Keratinocytes are formed in the final stage of keratinocyte differentiation, are pentagonal or hexagonal in shape in normal skin, and cell size varies upon skin area and age. The size of keratinocytes in normal skin ranges from 930 (scapular area) to 1,000 (hip area) μm^2 [74]. Keratinocytes increase in size from 650 to 850 μm^2 (back of the hand) and from 600 to 1,000 μm^2 (forearm) in a young group, while keratinocytes increase in size from 800 to 1,050 μm^2 (back of the hand) and from 1,000 to 1,300 μm^2 (forearm) in an older age group [75]. Keratinocytes from inflammatory skin such as irritated skin, allergic contact dermatitis, and steroid application show an irregular shape and are 10–15% smaller than in normal skin [74, 76]. For this reason, the size and shape of keratinocytes can represent the quality of wound healing and indicate side effects of treatment. Keratinocyte morphology can be obtained either directly from the wound using a swab or indirectly from the wound dressing after peeling it off [59]. However, the limitations of this assessment are that the keratinocyte appearance under the microscope can be affected by dried blood and the process can be time consuming.

10 Measurement of Blood Flow

Blood supply is a very crucial parameter during the healing process. It is also an indicator to identify dead tissue. Fortunately, noninvasive measurement of cutaneous blood flow velocity can be evaluated by several techniques depending upon the degree of exposure of the tissue in which flow is being measured [76]. These range from the application of plethysmography and calorimetry to the intact skin to electromagnetic and ultrasonic systems for evaluation of blood blow in exposed vessels. However, there are some drawbacks of these techniques mainly due to the limitation of measure flow in large vessel and lack of ability to define regional or microcirculation flow [77]. Laser Doppler, technique based on the measurement of the Doppler frequency shift in monochromatic laser light which is backscattered from moving particles (in this case red blood cells), seems to be the most practical and has the least limitation to measure blood flow. This method used optical heterodyning which involved mixing a reference beam with light scattered from the moving target on the surface of an optical detector where they "beat" together and produced a frequency proportional to the Doppler-shifted frequency [76]. Laser Doppler system can be used for the measurement of cutaneous blood flow rests both in its accurate representation of flow through the skin and considered as noninvasive, easy-to-use technique. The effective depth of laser penetration at approximately 1–1.5 mm is also considered as an advantage of this method. Moreover, laser Doppler can also be used for evaluation of burn depth with careful clinical assessment and by experienced surgeons [78].

11 Mediators Related to Wound Healing

Since the successful repair of injured tissues requires diverse interaction between cells, biochemical mediators, and the cellular microenvironment, these biochemical mediators may be used to monitor the stage of healing. Biochemical markers in wound healing can include the biochemical components of nonhealing wound exudates, which vary considerably from those found in acute wounds. Nonhealing wound exudates contain a number of inhibitory and excitatory factors including MMPs, pro-inflammatory cytokines, and growth factors. Cytokines and growth factors play an important role in communication between cells. Cytokines are not stored in glands

Table 4 Selected crucial mediators in wound repair

Mediators	Source	Main functions
IL-1 [77–83]	Macrophages, leukocytes, fibroblasts, keratinocytes	Inflammation, reepithelialization, angiogenesis, tissue remodeling
IL-6 [84–89]	Macrophages, fibroblasts, keratinocytes, endothelial cells	Inflammation, reepithelialization, angiogenesis, collagen biosynthesis, tissue remodeling
IL-10 [90–95]	Monocytes, macrophages, keratinocytes, T cells	Tissue remodeling, reducing the scar formation
TNF-α [96–100]	Neutrophils, keratinocytes, macrophages	Inflammation, cell proliferation, collagen deposition
PDGF [101–105]	Platelets	Inflammation, reepithelialization, collagen biosynthesis, tissue remodeling
FGF [104, 106–109]	Fibroblasts, keratinocytes, endothelial cells	Granulation tissue formation, angiogenesis, tissue remodeling
EGF [110–112]	Macrophages and keratinocytes	Reepithelialization, angiogenesis
IGF [113–115]	Plasma and platelets	Cell proliferation, reepithelialization
VEGF [116, 117]	Macrophages, fibroblasts, keratinocytes, endothelial cells	Inflammation, angiogenesis
TGF-β [118–120]	Platelets, keratinocytes, macrophages, fibroblasts	Inflammation, chemotaxis, granulation tissue formation, collagen biosynthesis, tissue remodeling
Chemokines CX3CL1 [83, 121]	Macrophages, endothelial cells	Inflammation, collagen deposition, angiogenesis
Chemokines CXCL10 and CXCL11 [122, 123]	Keratinocytes and endothelial cells	Tissue remodeling, reepithelialization
MMPs [124, 125]	Macrophages, epidermal cells, endothelial cells, fibroblasts	Granulation tissue formation, tissue remodeling

as preformed molecules but are rapidly synthesized and secreted by different cells mostly after stimulation. They act on many different target cells and also affect the action of other cytokines (additive, synergistic, or antagonistic manner). Monitoring these molecules can also provide not only an understanding of the stage of healing which reflects the treatment plan but may also be key factors in future regenerative therapies. Due to the variety of biochemical modulators and cytokines involved in the healing process, crucial mediators in wound repair are summarized in Table 4. Monitoring these mediators may also be another evaluation process for wound healing. Unfortunately, a practical monitoring system of these mediators is not available. Enzyme-linked immunosorbent assays (ELISAs) or other molecular techniques are needed in order to follow up the change of these parameters, which are normally performed only in questionable wounds.

The most famous of the pro-inflammatory cytokines that mediate the wound healing process are the interleukins 1α and 1β and their receptors (IL-1α, IL-1β, and IL-1R), IL-6, IL-10, and tumor necrosis factor alpha (TNF-α). These cytokines activate not only the proliferation of keratinocytes and fibroblasts but are also involved in extracellular matrix deposition, chemotaxis, and immune response. The above cytokines are upregulated in the inflammatory phase of wound healing, triggering the activation of polymorphonuclear leukocytes and macrophages [126–128].

11.1 Interleukin 1 and Its Receptors (IL-1 and IL-1R)

The IL-1 superfamily contains 11 cytokines which play an important role in the modulation of immune response. IL-1α and IL-1β belong to this group, which is involved in the wound healing process after injury. IL-1α is constitutively expressed in keratinocytes, but IL-1β is predominantly released from monocytes during wound healing. The activity of increased IL-1 can be detected at wound environments within 72 h after injury [126, 127, 129, 130]. After IL-1 release, neutrophils, macrophages, and endothelial cells

are activated. In addition, it also stimulates collagen synthesis and the growth of fibroblasts and keratinocytes [77–80]. However, high levels of IL-1 after 1 week of wound healing are considered pathogenic and deleterious [81, 82]. Moreover, the IL-1 receptor antagonist (IL-1RA) is a member of the IL-1 superfamily and shows a conserved β-pleated sheet structure, as reported for IL-1. Thus, IL-1RA can bind with the IL-1 receptor (IL-1R) at the same site and affinity as IL-1. However, it does not associate with the accessory protein of IL-1R. Surprisingly, IL-1RA is also upregulated in the inflammatory phase of wound healing. IL-1RA-deficient mice demonstrated delayed wound healing but increased the recruitment of neutrophils through activation of nuclear factor-kappa B (NF-κB) [83, 131]. Therefore, the balance between IL-1 and its antagonist is crucial for wound healing process.

11.2 Interleukin 6 (IL-6)

IL-6 is a cytokine that has several bioactivities, including response to wound repair after tissue injury [84, 85, 132]. It was detected in wound fluid from mice and humans [133–136]. IL-6 knockout mice have been shown to delay the wound repair process, but this was rescued by overexpressing recombinant IL-6 through the intradermal injection method [86]. IL-6 is highly expressed in epidermal keratinocytes at the wound edge. It induces dermatotoxic reactions against to the pathogen infection [87]. IL-6 is also involved in many skin-related diseases such as psoriasis, scleroderma, and systemic lupus erythematosus [137–139]. It can also promote the proliferation of intestinal epithelia and wound repair [88]. Keratinocyte growth factor (KGF) and epidermal growth factor (EGF) are upregulated in fibroblasts and keratinocytes, respectively, after being induced by IL-6. In addition, IL-6 also acts as a protector by reducing the degradation of the extracellular matrix via the inactivation of superoxide anion production, which is related to chronic inflammation [140] and raised matrix metalloproteinase inhibitors [141, 142].

11.3 Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine that is generated from various cell types such as T cells, monocytes, macrophages, and keratinocytes [143]. It functions to balance with pro-inflammatory cytokines such as IL-6 and IL-8 [144]. IL-10 has been found to be a regulator for fibrogenic cytokines, for example, transforming growth factor-β (TGF-β), which mediated the tissue remodeling process [90]. The signaling pathway of IL-10 has been primarily established in monocytes through the phosphorylation cascade of the JAK/STAT3 pathway [144]. IL-10 plays an important role in wound healing of the skin, tendons, and myocardium [91, 92]. It also has an essential role for scarless wound repair in embryos [93, 145]. Furthermore, IL-10 has been shown to decrease scar formation in mouse and human models of cutaneous wounds [93, 94]. Recently, it was found that overexpressing mammalian IL-10 facilitated wound repair by limiting inflammation and scarring in a full thickness wound model [95]. Consequently, IL-10 is considered to be a potential therapeutic for wound repair by reducing the scar formation.

11.4 Tumor Necrosis Factor Alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine which is predominantly secreted from macrophages and monocytes in the first phase of wound healing and returned to the basal level when the proliferation phase is completed. The downstream signaling from TNF-α is diverse, depending on stimuli leading to cell apoptosis or necrosis [96]. Previous studies show that it is involved in various diseases such as cancer [146], endotoxic shock [147], and hematopoiesis [148]. In addition, the proliferation of fibroblasts [97, 98], prostaglandin biosynthesis [149], and collagenase gene expression [150] were induced by TNF-α, leading to stimulation of the secretion of platelet-derived growth factor (PDGF) and other growth factors, thus facilitating the functions of macrophages. Moreover, TNF-α has been shown to activate MMP expression in rat skin fibro-

blasts [150] and human lung fibroblasts [98]. MMPs are important for remodeling of the extracellular matrix (ECM), leading to collagen deposition and the biosynthesis of new fibrotic tissue [124, 125].

11.5 Matrix Metalloproteinases (MMPs)

MMPs take part in the inflammatory phase. They are a family of zinc-dependent endoproteinases that contribute to the degradation of ECM components. MMPs are also involved in the removal of devitalized tissue and are therefore believed to play an important role in normal wound healing and remodeling. During the repair phase, MMPs are necessary for angiogenesis, wound matrix contraction, the migration of fibroblasts and keratinocytes, and epithelialization [151].

11.6 Platelet-Derived Growth Factor (PDGF) Family

The PDGF family is a group of growth factors that contains different homo- or heterodimeric isoforms such as PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD [152, 153]. PDGFs were secreted from platelets, resulting in activating and recruiting macrophages, neutrophils, and fibroblasts after tissue injury [101, 102, 152]. Moreover, PDGFs can be produced by macrophages facilitating the biosynthesis of collagen and proteoglycans. It has long been known that PDGFs are essential for wound repair. Decreasing PDGF receptors delayed the wound healing process [102, 152]. In addition, a recombinant human PDGF-BB named "becaplermin" has been approved by the US FDA for the treatment of diabetic foot ulcers [103]. However, there are some limitations of the clinical studies. It might have occurred due to the half-life of the growth factor or the expression level of PDGF receptor in the residing cells. Nonetheless, a single growth factor might not be sufficient to be a therapeutic candidate due to the complexity of the wound healing process. Thus, the combination of many growth factors should be optimized for the wound healing process.

11.7 Epidermal Growth Factor (EGF) Family

The EGF family consists of several members such as EGF, heparin-binding EGF (HB-EGF), amphiregulin, and transforming growth factor-α (TGF-α), which are produced by keratinocytes and macrophages. After ligands bind to its receptors (EGFR), a signaling cascade occurs, resulting in activation of the proliferation, migration, and reepithelialization of keratinocytes, facilitating closure of the wound surface by epithelium cells [110–112, 127]. In acute wounds, EGF signaling provides mitogenic effects to endothelial cells, epithelial cells, and fibroblasts. It also stimulates various processes including fibronectin biosynthesis, angiogenesis, fibroplasia, and collagenase activity [154].

In addition, there are two transcription factors that mediate EGFR signaling, called activator protein 1 (AP1) and STAT3. The lack of AP1 [155] or STAT3 [156] impaired the healing process. Recently, decreasing EGFR on the cell surface reduces the downstream processing of EGFR signaling. Hence, EGFR could be another potential factor for the wound repair process [157].

11.8 Fibroblast Growth Factor (FGF) Family

The FGF family is a multifunctional mediator that plays an essential role in the proliferation and tissue remodeling phases of wound healing. Fibroblast growth factor-2 [FGF-2 or basic FGF (bFGF)] and keratinocyte growth factor-1 (KGF-1 also known as FGF-7) are examples that belong to this family [102, 127, 152]. It has been shown that bFGF is stored in endothelial cells, the ECM, fibroblasts, and keratinocytes and activates the proliferation and migration of fibroblasts, promoting angiogenesis [104, 106–108, 158], whereas FGF-7 targets epidermal cells by promoting skin repair in mice [109]. FGF-7 and

FGF-10 activate TGF-α secretion from keratinocytes, which indirectly promotes epithelialization [109]. In addition, the FGF receptor (FGFR) also impacts on the wound healing process. The lack of FGFR-2 IIIb reduced the proliferation and migration of keratinocytes and delayed the wound repair in the mouse model [109].

11.9 Insulin-Like Growth Factor (IGF)

Insulin-like growth factors are a group of proteins that share high similarity to insulin and mediate cell physiology. IGF-I and IGF-II are members of this family that play a crucial role in tissue repair by increasing the mitogenic effect of keratinocytes [113]. A previous study also demonstrated that the combination of IGF and EGF leads to the increased migration of keratinocytes; these two factors are required for the reepithelialization process [113]. The synergistic effect of a combination of PDGF and IGF also impaired wound healing in a diabetic mice model [105]. IGF-1 can stimulate proliferation and wound healing in human periodontal ligaments (PDLs) under inflammatory stimuli conditions [114]. According to all of the information above, the cocktail of growth factors could be a candidate therapeutic for wound healing.

11.10 Vascular Endothelial Growth Factor (VEGF) Family

The VEGF family is a group of signaling proteins that has a significant role in angiogenesis by generating a new blood vessel for restored oxygen demands due to hypoxic conditions. VEGF is secreted from various cell types including macrophages, fibroblasts, keratinocytes, and platelets, targeting endothelial cells that mediate the new tissue formation phase of wound healing [152, 159, 160]. It has been shown that the repair process of diabetic wounds was increased by the administration of VEGF by increasing epithelialization, angiogenesis, and granulation tissue formation while reducing scar formation [116].

11.11 Transforming Growth Factor Beta (TGF-β) Family

The TGF-β family is a multifunctional cytokine which belongs to the transforming growth factor superfamily. It consists of 3 isoforms including TGF-β1, TGF-β2, and TGF-β3 [152, 159, 161]. These three isoforms are expressed from different genes but they show high similarity. However, the function of each isoform is different to each other depending on the stimuli [152]. It has been observed that TGF-β1 has a predominant role in wound repair. TGF-β1 is secreted from various cell types, such as macrophages, fibroblasts, keratinocytes, and also platelets [127]. In the inflammatory phase, TGF-β1 activates heterodimerization of TGF-β receptor I and II, initiating downstream signaling through the cascade of signaling proteins called Smad2, Smad3, and Smad4 in order to form a complex. Then, this complex moves into the nucleus and regulates the expression of proinflammatory genes, resulting in the production of cytokines and the recruitment of inflammatory cells and macrophages, and has an essential role in tissue debridement [118, 161].

In addition, TGF-β1 also participates in the proliferation phase of wound healing. Its signaling proteins induce the accumulation of fibroblasts, resulting in the formation of granulation tissue, collagen biosynthesis, and angiogenesis [119, 120, 162]. Therefore, TGF-β1 has been reported to be a potential facilitating the process of wound healing [163, 164]. It has been reported that TGF-β activated the migration of keratinocytes [165], whereas it induced the inactivation of keratinocyte propagation and reepithelialization [166, 167]. Furthermore, TGF-β also plays a role in the tissue remodeling phase of wound healing. It activates MMP-9 through the Smad signaling proteins, mediating matrix formation and angiogenesis [168].

11.12 Chemokines

Chemokines are small molecules (8–10 kDa) which belong to the family of heparin-binding cytokines and contain conserved cysteine residues

depending on disulfide linkage formation. They can be categorized into four subclasses by arrangement of the first 2 of the 4 cysteine amino acids: C, CC, CXC, and CX_3C, respectively. After chemokines bind to its receptor, the downstream signaling is initiated, leading to chemotaxis effects [169]. It has long been known that chemokines have an essential role in all processes of wound healing.

During the inflammatory phase of wound healing, chemokines are crucial for leukocyte accumulation at the wound edge. CX3CL1, which belongs to the CX_3C subfamily, was found in macrophages and endothelial cells, mediating cell-cell interactions. Binding between CX3CL1 and its receptor (CX3CR1) triggers an accumulation of macrophages in the wound environment. The suppression of CX3CR1 in mice reduced macrophage accumulation and its products such as TGF-β and VEGF, leading to a delayed repair process [83, 121].

As mentioned above, angiogenesis is an important step in the new tissue formation phase of wound healing. This step is tightly regulated through the balance between angiogenic and angiostatic factors [169]. It has been established that the chemokine CXC subfamily is a potential regulator for angiogenesis. CXC chemokines which contain the ELR motif (Glu-Leu-Arg) promote angiogenesis, whereas ELR-negative chemokines inhibit this process [170, 171].

Finally, important CXC subfamily chemokines that mediate the remodeling phase of wound healing are CXCL11 and CXCL10, produced by keratinocytes and neovascular endothelium, respectively. These two chemokines can interact with the CXCR3 chemokine receptor. After CXCR3 signaling is initiated, leading to activation of the formation of collagen fibers, the proliferation and migration of endothelial cells is inhibited, suggesting that CXCR3 signaling is essential for dermal maturation [122, 123].

11.13 Protease Enzymes and the Extracellular Matrix (ECM)

Protease or proteinase is an enzyme that catalyzes proteins into small peptides or amino acids. It has been noticed that MMPs and serine proteases are major protease enzymes that are crucial for the wound repair process [172–174]. Wound-related proteases are specific depending on the substrate proteins, such as ECM proteins or connective tissue proteins, for example, collagen, gelatin, proteoglycans, and elastin. Another factor that mediates the repair process and is related to proteases is the ECM. It promotes communication between fibroblasts and keratinocytes and also stimulates the proliferation, migration, and differentiation of the surrounding cells, modulating the cellular response [175].

In the normal wound repair process, proteases digest the damage ECM and pathogens, leading to activation of the formation of new tissue and closure of the wound. However, if the level of proteases is high, the balance between tissue damage and new tissue formation is disturbed, resulting in the delayed wound healing. Thus, understanding the molecular mechanism and its regulation of wound-related proteases and ECM provides a new therapeutic target for wound healing.

Wounds normally present with an exudate, which is a fluid that contains a plethora of components including electrolytes such as sodium, urea, creatinine, fibrinogen, MMPs, and proteins such as TNF-α and C-reactive protein (CRP) [100]. Even though, currently, there is no practical instrument to monitor electrolytes at wound sites, changes in these biomedical parameters using research level sensors can be used as an indicator for wound healing. Some blood-containing agents which can be clinically monitored in routine work and are related to wound healing are:

1. Uric acid: A decrease in uric acid concentration in exudate can be associated with the risk of bacterial colonization of the wound.
2. Creatinine phosphokinase (CPK): CPK present in the exudate may act as an indicator of the severity of deep tissue injuries, which are deep muscle injuries that rapidly deteriorate to stage III and IV pressure ulcers.
3. C-reactive protein: CRP plays a major role during the inflammatory phase. In response to injuries and the presence of bacteria, the immune system triggers the synthesis of CRP, which promotes the action of macrophages to remove necrotic and apoptotic cells and bacteria [81].

Conclusions

There is no gold standard method for the qualitative and quantitative evaluation of wound healing; each technique has its own limitations. Numerous studies have attempted to develop new techniques for the measurement of wound area, tissue color and composition, skin barrier function, skin humidity, and keratinocyte morphology in order to solve the problems of traditional techniques or to assist in evaluation by clinicians. It is very important to understand the type of wound, sample size, results obtained, advantages, and limitations of each technique. However, more research is needed before these techniques can be used instead of, or alongside, traditional assessments. Further studies with different types of wounds, larger sample sizes, and strict statistical performance criteria would be useful.

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Mechanoregulation of Wound Healing and Skin Homeostasis

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1 Introduction

Skin is a multifaceted biological system which integrates different cells in the area of a tightly organized extracellular matrix. It is exposed to many external and endogenous factors which disintegrate its structure and functions. Skin has unique plasticity and regeneration ability. The reconstruction of anatomic continuity and restoring functions of damaged tissues is a complex, dynamic process coordinated in time referred to as wound healing. The process of wound healing is divided into four consecutive stages: homeostasis, inflammation, reepithelization, and tissue remodeling. These stages are tightly organized and precisely regulated by a complex of interaction between cells, signalization pathways, and extracellular matrix (ECM) [1, 2].

Immediately after wounding, the blood vessels close, fibrin aggregates are formed, growth factors (such as PDGF and EGF) are released, and cells associated with inflammation (monocytes, neutrophils) migrate into the wound. During the next 1–3 days, epidermal keratinocytes, almost damaged, migrate to the wound bed reproducing layer of the epidermis. This process, described as reepithelization, is crucial in regeneration of functional epidermis and prevents from the development of infections. Dermal fibroblasts translocate toward the wound area, start the synthesis of ECM components, and take part in ECM remodeling. Fibroblasts in the wound area transform into myofibroblasts whose contraction is responsible for tightening wound borders. In this stage, myofibroblasts strongly proliferate and synthesize components of ECM while maintaining tissue integrity and promoting its regeneration [3].

Developing a new, healthy tissue in the wounded area is dependent on cell proliferation, migration, and differentiation. Disorder of mechanisms which regulate these processes at any stadium leads to impaired wound healing. Impaired wound healing may be either slow (as in the case of diabetes, bedsores, or exposure to radiation) or accelerated (related to hypertrophy and keloid scars). In the case of accelerated healing, there is a large amount of deposition of extracellular matrix, increased cell proliferation, and wound vascularization [4].

Human skin is an organ which, in an active manner, reacts to physical forces affecting it. Skin cells react to mechanical forces, and their specific reaction is crucial to the way wounds behave in physical environment. The in vitro

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studies show that fibroblasts and keratinocytes are responsible for mechanical stimulation, and almost all aspects of cellular behavior may be subject to modulation [5]. It becomes more obvious that improper mechanotransduction is the cause of many pathological changes, including impaired wound healing and scar formation [6].

The improper process of wounds cicatrizing, following past injury, is often the cause of many functional complications and aesthetic problems, and traditional therapies often turn out to have limited effectiveness in treatment [7]. The formation of hypertrophic scars is a major problem, and therapeutic regimens as surgery, injections of corticosteroids, or radiation do not give the expected results. In contrast, high efficacy in reducing the formation of hypertrophic scars was observed in therapies based on mechanical impact on wound environment [8]. Preclinical studies have shown that excessive scarring may be limited by the equalization of mechanical forces acting on wounds and by maintaining the mechanical equilibrium of wound environment via suitable compression covers. Discharge of mechanical tensions, after surgical incisions made during abdominoplasty, limits wound scarring. Phase I trials have shown that the use of compression bandages for 8 weeks after surgery significantly affects the process of wound healing and improves the appearance of scars when compared to patients without such intervention [9].

Another form of pathological scarring is keloid changes, whose etiology is quite poorly known. The reasons for pathogens include genetic disorders, apoptosis, dysregulation of mesenchymal-epithelial signaling, and variations of mechanical tension in wound environment [10]. As a result of the mechanical tension, fibroblasts which form keloid scars exhibit a higher expression of profibrotic cytokines and increased collagen synthesis in response to activation of focal adhesion kinase (FAK). Mechanical stress and mechanical stimulation in wound healing clearly increase the likelihood of developing keloids, and controlled mechano-modulation therapies can limit the progression of these changes [11].

2 Mechanical Regulated Cell Proliferation and Differentiation

A widely accepted concept of homeostasis describes that structural damage to tissues activates the response of the organism to restore impaired mechanical equilibrium of the skin. Wounds of different etiologies and anatomic location have specific mechanical properties that affect the way of their healing [12].

Mechanotransduction enables a cell to sensing and to quickly adapting to mechanical forces and physical limitations. The way in which a cell receives mechanical stimuli, how such mechanical signals are transmitted into cells, and how those signals regulate gene expression and protein synthesis are important issues. Cell structures described as mechanosensors are a cell membrane [13], mechanosensitive ion channels [14], glycocalyx [15], focal adhesions, and proteins and intercellular complexes [16].

The essential mechanism of mechanotransduction is based on converting mechanical signal received by cell structures into intercellular signaling pathway which determine its interaction with different cofactors and target gene specificity [17]. These mechanical stimulations are then targeted into signaling pathway induced by soluble factors and consequently regulate transcriptional changes. In an alternative model, the cell itself is considered a compartmentalized mechanical body with given physical properties such as its viscosity, elasticity, or stiffness [18].

Cells are influenced by mechanical forces suitable for their environment, such as softness or rigidity of extracellular matrix (ECM), differentiated adhesion to substrate, or the tension exerted by neighboring cells. The physical properties of ECM influence micro- and nano-topography of integrins which bind ECM components. Integrins are a part of protein complexes which form focal adhesion. They are responsible for producing a direct physical connection between the components of the extracellular matrix (e.g., collagen, laminin, fibronectin, vitronectin) and the adapter

proteins of actin cytoskeleton. The process of binding of actin filaments with the participation of integrins generates tensions in cells, which, at the same time, activates actin-associated proteins that regulate polymerization of F-actin. This affects the spatial organization of actin filaments and integrins and thereby increases cell adhesion to the ECM [19].

The interaction of actin filaments and myosin is responsible for the contractile force and ultimately the creation of intracellular mechanical tension. The structure and dynamics of formation of actin-myosin complexes are regulated by RHO family GTPases.

Inhibition of RHO, ROCK kinase, and MYLK (myosin light chain kinase) or the inhibition of the polymerization of actin filaments reduces the strength of internal tension of a cell and causes a change in cell shape. A similar effect can be achieved when culturing cells on a soft substrate [20]. This effect is closely related to cell shape and intracellular tension forces [21].

The cells adjacent with large adhesive area to the substrate have the ability to proliferate in more intensive manner, whereas the cells adjacent with a small adhesive area do not proliferate and most often die [22]. Preventing cells from spreading on the substrate leads to shifts in the organization of actin filaments and activation of transcription factor SRF (serum response factor), which together with cofactor MAL is transported to the nucleus. SRF-MAL complex activates the transcription of c-FOS and JUNB elements of AP-1 complex [23]. Activation of the SRF is the result of a shift in cell shape; it is independent of the density and composition of the extracellular matrix and the assembly of focal adhesives [22]. As SRF is a downstream effecter of RHO-A and actin polymerization, it provides an important link between the cytoskeleton and gene regulation. A direct connection between SRF and various cellular responses to mechanical and biophysical stimuli has been demonstrated. In keratinocytes, myocardin-related transcription factor-A (MRTF-A) and SRF are required for shape-induced terminal differentiation, whereas, in fibroblasts, the T-cell factor family (TCF) of cofactors control the switch from proliferation to transcription following loss of adhesion [24].

Microenvironmental signals determine the fate of epidermal stem cells: the loss of contact with the basement membrane and the transfer of cells to a higher layer direct them to terminal differentiation. Keratinocytes cultured in vitro in suspension exit cell cycle and enter the differentiation pathway, regardless of binding ligands with appropriate integrins. The direction of differentiation of mesenchymal stem cells (MSCs) also determines their geometry. In the in vitro environment, the outspread MSCs, strongly adherent to the substrate, differentiate into osteoblasts, and when in culture they take on a spherical shape, they differentiate into adipocytes. Equally important determinant of differentiation of mesenchymal stem cells is the rigidity of their immediate surroundings. MSCs differentiate into osteoblasts when grown on synthetic substrate of bone-like rigidity, into myoblasts when they grow on a substrate of intermediate rigidity, and into neurons and adipocytes on a soft substrate. Cell environment, like growth factors, may affect the growth of the cell population and the direction of differentiation [19, 25].

The fate of epidermal stem cells and mesenchymal cells appears to be regulated by mechanical feedback from the extracellular matrix. Stem cells put tension on the extracellular matrix and receive in return the force of environmental influences which determines their fate. ECM with open weave does not provide stem cells with an appropriate signal, because of which it is not able to respond while fitting focal adhesion and initiating signaling to MAPK/ERK pathway. The strength of binding with extracellular matrix proteins and the density of integrins affect the shape and direction of keratinocyte differentiation [22].

It has been shown that the cytokeratin cytoskeleton has a significant impact on the mechanics of keratinocytes and signals mechanotransduction. Hemidesmosomes, like focal adhesion, also receive the mechanical forces of the extracellular matrix in the epidermis [26]. Human keratinocytes, exposed to mechanical stress by cell stretching, activate signaling

pathways dependent on calcium ions which regulate cell proliferation and differentiation [27].

Extracellular matrix in an injured tissue changes its chemical composition and stiffness, which initiates the repair process. Mechanical signals received by fibroblasts guide their transformation into myofibroblasts [28]. In a freshly damaged tissue, extracellular matrix is soft and rich in fibrin. Fibroblasts grown on the soft substrate in 3D cultures have little adhesion and poorly developed stress fibers [29]. In contrast, when cultured on a hard substrate, they form a number of focal adhesion and stress fibers, although they do not yet show the presence of myofibroblast markers such as α- smooth muscle actin (α-SM actin). The transformation of myofibroblasts occurs in cultures, in a rigid 3D collagen matrix, or during wound granulation and fibrotic tissues, but, nevertheless in all cases, it is necessary to stimulate with TGF-β1 [30].

In the process of wound healing, the contraction of the myofibroblasts increases the rigidity and mechanical tension of the extracellular matrix. In turn, the increase of ECM rigidity and mechanical signals, generated on the basis of positive feedback, further stimulates the differentiation of myofibroblasts. The role of the mechanical tension in the stimulation of the activity and differentiation of myofibroblasts was demonstrated experimentally on skin wounds in mice. The wounds were subjected to mechanical tensions by stretching and splinting, and the increasing activity of myofibroblasts showed the intensification of scarring, to a large measure resembling the human hypertrophic scars. Declining mechanical stress or the decrease of the rigidity of extracellular matrix can induce apoptosis and decrease the expression of α-SM actin and myofibroblasts' ability of contraction [31].

Mechanical signaling, received by integrins in a direct way, activates transcription of α-SM actin genes [32]. In response to mechanical signals and stimulation of TGF-β1 in myofibroblasts, the expression of genes responsible for the synthesis of collagen and extracellular matrix proteins increases [33], which results in changing of mechanical properties of the injured tissue. In the wound site, the TGF-β1 is released (from alluvial cells of connective tissue, platelets, and myofibroblasts) in a form which is inactive in a complex with LAP (latency-associated peptide). TGF-β1/LAP complex is bound to extracellular matrix proteins forming a reservoir of latent form of TGF-β1. Myofibroblasts, by receiving the tension forces from the extracellular matrix, express integrins which bind LAP, which activates TGF-β1 and enables binding with cell membrane receptors [31]. Both the increase in the mechanical tension and the contraction of the myofibroblasts can activate cells by increasing their contraction ability and their synthetic activity of extracellular matrix components. On the other hand, blocking integrins (αvβ5, α3β1, α11β1, αvβ1) involved in the activation of latent forms of TGF-β1 is an alternative pathway of regulating myofibroblasts' activities in the process of wound healing [34].

A collective, coordinated cell migration is an important part in the mechanism of wound healing [35], similarly as in embryonic development [36], and invasion of tumor cells [37]. In model systems of in vitro, the epithelial cells (MDCK), released from the micro-templates, distinguish a group of cell leaders which initiate migration and generate traction forces toward migration [38]. Adhesive junctions maintain tissue consistency, generate tension between cells, and entail the entire group of cells. The rigidity of the substrate, in return, regulates the migration of cells through the activation of myosin II [39]. Cell response to the rigidity of the substrate also depends on the specific integrins: for example, in myoepithelial cells, the differences in the kinetics of binding between the α5β1 integrin and αvβ6 determine the level of the traction force which the cell can generate in response to a predetermined rigidity of the substrate. Overexpression of these integrins' receptors was described during skin wound healing, which may point to their important mechanoreceptive role in the regulation of normal tissue repair [40].

In comparison with other epithelia, keratinocytes generate particularly strong intercellular connection that enables the collective migration occurring even in an environment with little or

highly dispersed ECM substrates for specific integrin receptors [41]. Junction between cells can promote wound reepithelization with limited or varied adhesion to ECM and facilitate wound closure in the absence of specific integrins. Therefore, a key area of research, that will allow to describe the precise mechanism involved in slow or accelerated wound healing, is to investigate the expression of specific integrins, components of the cytoskeleton, and target proteins involved in mechanotransduction, as well as to conduct a thorough analysis of mechanical wound environment [42].

The properties of extracellular matrix determine cell shape; thanks to which they can influence the cell cycle regardless of intercellular regulative mechanisms. The process of when cells move through to the phase of S cycle depends on critical size and shape and also on the reception of mechanical signals which indicate spatial limitations of a cell in its environment. This mechanism is related to a single cell and cell population which compose a tissue, and it results in stopping cell crowding, exclusion from tissue, and apoptosis [43].

In the in vitro models of cell kinetics, it was demonstrated that the elimination of spatial limitation causes that cells quickly move through to the phase S cycle. This mechanism is activated when the tension of the actin let down. The degree of cytoskeleton tension is a mechanical signal that determines the spatial size of a cell and that may be subject to further transduction through pathways which regulate cell proliferation, such as yes-associated protein (YAP), S-phase kinase-associated protein 2 (SKT2), or extracellular signal-regulated kinases (ERK) [44, 45]. Changes of the dynamics of the cytoskeleton, in response to changes in the mechanical environment of the cells, extend in a short period of time (faster than the average cell cycle time). Thus, open space can increase cell proliferation, but also the short duration of signal may limit the overregulation of proliferation. Functioning of this system has been demonstrated in vivo in processes related to the development and organogenesis and in pathological conditions associated with abnormal proliferation [46].

This mechanism, in a simple manner, may be observed in the model of cell regeneration and regulation of cell proliferation during wound healing. Cells do not need to register information related to wound size; they only invade the space which is available at that particular moment. Lowering mechanical tension of cells activates the process of proliferation and migration. Further cell divisions fill in the open space, and they project the primal state of spatial limitation in de novo tissue which is being formed. In consequence, cells shrink back to the size they have in an inactive state, and their further divisions are inhibited. In conclusion, it may be stated that in the processes of development, regeneration, and cancer cell invasion, the progression of cell cycle on the border of G1-S phases may be regulated by mechanosensitive control spots which receive spatial limitations of cells' environment [47].

Understanding the process of mechanotransduction requires also an answer to a question in what manner mechanical forces are transmitted to the cell nucleus. There are proofs that the direct mechanical coupling of a cytoskeleton, plus changes in the nuclear envelope and in nucleoskeleton, may be an alternative or additional way of regulating gene expression [48]. Changes in localization or structure of the cell nucleus are observable in many processes, such as cell division, migration, and differentiation. To keep a localization of the cell nucleus appropriate for the cell type is an active process, dependent on physical connection between the cytoskeleton and the structure of the cell nucleus [49].

Recently, the proteins are responsible for these connections we discovered; they complete the complex called LINC (linker of nucleoskeleton and cytoskeleton). This complex is composed of SUN proteins (Sad1p, UNC-84) and KASH proteins (Klarsicht/ANC-1/Syne homology). SUN proteins and KASH proteins bind with perinuclear space forming a bridge which connects cytoskeleton with nucleoskeleton. This bondage plays the main role in many cell processes and is responsible for maintaining correct position of the nucleus in a cell. The LINC complex participates in cell migration and intercellular transportation dependent on both

microtubules and actin filaments. The disintegration of LINC complex results in disorganization of actin skeleton and disturbs the mechanics of a cell [50, 51]. The LINC complex transmits not only the tension generated by the cytoskeleton to cell nucleus but also the mechanical tension directed at cell surface. The mechanical tension, which is received by cell surface adhesion receptors, influences the structure of nuclear envelope. These observations prove that mechanical stress may be transmitted from extracellular matrix to the cell nucleus. Mechanical cell stimulations through stretching or compression influence the shape of the nucleus and the organization of nucleoplasmatic structures [52].

The way in which the transmitted mechanical forces influence gene expression is an important issue. The mutation of the emerin gene to its phospho-resistant form influences the transcription profile of genes dependent on serum response transcription factor (SRF), which shows that mechanical forces received by nucleus influence gene expression [53]. This conception is proved by latest studies which show that laminae and emerin regulate translocation to MLK1 nucleus and transcription of genes dependent on SRF [54]. Further studies show that mutations of A-C laminae influence the reception and transduction of mechanical signals on the YAP-dependent pathway [55]. It was also stated that A-C laminae level is regulated in response to shifts in the extracellular matrix rigidity and is related to dephosphorylation and stabilization of laminae [48]. The decrease of phosphorylation of laminae may result from the processes of regulation of specific nuclear kinases or phosphatases dependent on mechanical tension. The study has shown that phosphorylation of emerin, in response to mechanical tension, strengthens connections between A-C laminae and LINC complex. Lamina and emerin in response to mechanical stress begin to interact with chromatin modifying its structure, because of which they influence gene expression [56]. In the studies related to isolation of the cell nucleus, it was observed that lamina A is subject to conformational shifts in reaction to mechanical stress. These observations prove that nuclear proteins receive and participate in the transduction of mechanical signal and bind with biochemical signalization which regulates the activities of nucleoskeleton proteins. Transferring the mechanical force onto LINC complex may cause conformational shifts of emerin and its phosphorylation regardless of the activity of specific kinases [56]. A similar mechanism of mechanotransduction was described for focal adhesion protein p130-Cas [57].

3 Transcription Factors Regulated by Mechanical Forces

3.1 Transcriptional Coactivators YAP and TAZ

YAP (yes-associated protein) and its homologue TAZ (transcriptional coactivator with transcriptional coactivator with PDZ-binding motif) are the key effectors of the Hippo signaling pathway. Their activities are inhibited by the main kinases of Hippo pathway—LATS 1/2—on the way to phosphorylation [58]. YAP and TAZ activate gene transcription through interaction with transcription factors belonging to a TEAD family (TEA domain family member) [59]. The genes induced by YAP/TAZ are involved in the regulation of cell proliferation, apoptosis avoidance, amplification of stem cells, control of the size of organs, and tissue regeneration [60].

YAP/TAZ transcription coactivators, besides being involved in signal transmission of the Hippo pathway, interact with other proteins and respond to mechanical stimulation of a cell. Many proteins which bind with YAP and TAZ molecules have the ability to bind actin and can regulate, or be regulated by, changes in the structure of the cytoskeleton [44, 61].

Many studies show that mechanical signals regulate the activities of YAP and TAZ through the pathway, which may act simultaneously with a classic cascade of Hippo pathway kinases. YAP and TAZ molecules are inactivated when F-actin is depolymerized or when the activity of RHO-GTPases is inhibited. The knockout of LATS1

and LATS2 does not rescue the activity of YAP and TAZ in the presence of actin polymerization inhibitor or in cells cultured on soft hydrogel surface. In addition, under the same conditions, the activity of mutant TAZ susceptible to LATS is permanently inhibited. The cells which grow in suspension (without contact with the components of the extracellular matrix) show no YAZ activity and undergo anoikis. The LATS knockout only partially rescues cells from death [44, 62]. It can be assumed that a spread of F-actin cells prevents the action of unknown factors inhibiting YAP and TAZ in a manner largely independent of LATS1/2.

Subcellular location and activity of YAP/TAZ is regulated by the rigidity and topography of cell substrate and remodeling of cytoskeleton [63]. The change in cell shape, into more flat and related shifts in the organization of cytoskeleton in response to integrin signaling, is an important factor which maintains YAP/TAZ active. YAP and TAZ coactivators in cells cultured on a stiff substrate are located in cell nucleus and transcript actively. However, when cells are transferred onto a soft substrate, they are removed from nucleus and thereby become functionally inactive. Similar regulation of activities of YAP and TAZ was shown in cells which grow on a micropatterned substrate. In such culture conditions, cells differ in terms of the degree of spread-out in such a way that the cells which are placed on large fibronectin islands, which enables their spread-out, have active YAP/TAZ with nuclear location. However, when cells are placed on small adhesive islands, the cytoplasmic form of YAP/TAZ is inactive. Cells with YAP/TAZ knockout proliferate extensively on high adhesive or rigid substrate, and they have a phenotype typical for cells (without knockout) cultured on a weakly adhesive or soft substrate. Activation of YAP/TAZ signaling on stiff substrates involves actomyosin-driven cytoskeletal tension but is independent of the Hippo pathway components Lats and Mst kinases [44]. Although cell-cell adhesion stimulates the Hippo pathway and inhibits YAP/TAZ, mechanical signals from the ECM can override Hippo pathway signalizations [45]. However, it remains to be precisely determined how actomyosin tension has direct effects on YAP/TAZ activity [61, 64].

Artificially forced changes in cell shape through their flattening, in a manner which does not involve integrins (with polylysine as substrate), maintain nuclear location of YAP. Moreover, inhibiting the activities of focal adhesive components, such as FAK or SRC kinases, does not affect YAP/TAZ activities. The presence of large amounts of G-actins (monomers) in cytoplasm does not affect YAP/TAZ activities. The information presented show that an important factor which maintains activity of YAP and TAZ is the change in cell shape to more flat and all shifts in cytoskeleton organization related. It seems that the activity of YAP and TAZ is dependent on the organization of actin filaments which are organized into stress fibers or which form bundles of shrinkable networks that enrich cells of outspread shape [44, 45].

The specific structure of the F-actin may, in a physical manner, sequester inhibitory molecules or may provide a platform which enables their "posttranslational modifications which block interactions with YAP and TAZ. Cells cultured on a soft substrate with limited surface of contact remodel actin skeleton in such a way that the inhibiting factors may be released or activated. There is also a possibility that actin-severing proteins regulate the activity of YAP/TAZ, exposing or covering appropriate sites that bind regulative proteins, by controlling the ability of interaction directly with YAP or TAZ or through their partners. Moreover, flattening shape of cells and consequential reorganization of actin skeleton may promote the activation of positive cofactors that enable translocation of YAP and TAZ to cell nucleus and their activation [45, 62, 64].

The activity of YAP and TAZ is also regulated by a cell-cell contact and by formation of new intercellular contacts. Many positive and negative YAP and TAZ regulators are adherens junction proteins and tight junction proteins. Angiomotins (AMOT) and zonula occludens proteins 2 (ZO-2) interact with YAP/TAZ regulating their activity [65, 66]. The component of adherens junction protein α-catenin, which binds adhesive complex with actin cytoskeleton, regu-

lates YAP activity through binding a phosphorylated complex YAP-protein 14-3-3 with the connection which binds epidermal cells [67, 68]. The next protein which is responsible for cell connections and which interacts with YAP/TAZ is zonula occludens protein 2 (ZO-2). This protein enables translocation of YAP/TAZ between the complex of tight junctions and cytoplasm and between cytoplasm and the cell nucleus. The discovery of YAP/TAZ/ZO-2 complex is extremely interesting because of the fact that proteins of tight junctions may directly affect the transcription activity of YAP/TAZ [64, 66, 69, 70].

AMOT as a partner which signals YAP/TAZ is particularly interesting because it interacts with actin filaments and adherens junction proteins [71]. AMOT in a cytoplasmic location promotes YAP phosphorylation and stops in cytoplasm by forming stable YAP/TAZ complexes. In effect, the transcription of target genes of connective tissue growth factor (CTGF) and cysteine-rich protein 61 (Cyr61) is inhibited [65]. What is crucial, similarly to YAP/TAZ, is that AMOT is phosphorylated by LATS kinases, which leads to protein dissociation from actin complex, YAP suppression through 14-3-3 enrolment, ubiquitination, degradation, and inhibition of cellular proliferation [72, 73]. In a situation when AMOT is connected with actin filaments, YAP/TAZ may be directed to the cell nucleus and take active part in transcription. AMOT plays an important role in maintaining the status of F-actin, and it participates in a competency bondage of YAP/TAZ with actin filaments. The increase of polymerization of F-actin results in a lowered degree of AMOT, which leads to the release of YAP/TAZ and its translocation to the cell nucleus and activation of genes dependent on the YAP-TEAD complex, and cell proliferation (Fig. 1) [74].

There are some conflicting data, which show models for the negative regulation of YAP/TAZ activity by the Angiomotins, which suggest a number of possibilities leading to localization of YAP/TAZ to the cytoplasm/cell junctions. It is noteworthy that a major mechanism of YAP/TAZ regulation is through exclusion from the nucleus, and it was identified a nuclear function for Amot-p130 in regulating YAP activity. Also, it was found that Amot-p130 is required for YAP function both in vivo and in vitro, which is contrasting to a YAP-inhibitory role for Angiomotins [71].

3.2 YAP and TAZ in Skin Homeostasis and Wound Healing

The process of stratification of the epidermis can be interpreted as a cell differentiation induced by the loss of cell-extracellular matrix contact. The location and activation of YAP in the epidermis also appear to be dependent on the interaction of cell-extracellular matrix. In proliferating cells of the epidermis layer, YAP is localized in the nuclei, and differentiating cells of the upper layer, it is present in the cytoplasm. The increase of YAP in nuclei increases the proliferation and inhibits cell differentiation. By contrast, inactivation of YAP leads to inhibition of proliferation and premature differentiation [75].

Overexpression of YAP active form in the basic layer of the epidermis of mice embryos leads to increased proliferation of keratinocytes, impaired stratification and hyperplasia of the epidermis, and inhibition of terminal differentiation. The lack of YAP expressions causes visible decrease of proliferation, decrease of stem cells, impaired stratification, and, in effect, decrease of epidermal thickness. A similar phenotype is observable in YAP knockout or the presence of mutated protein isoforms that are unable to interact with TEAD. Activation of MST1/2 kinases causes the inhibition of YAP activity and, in effect, the decrease of cell proliferation and initiates their differentiation. However, in the lines of human keratinocytes HaCaT, the inactivating of MST1/2 does not affect the shift of cell phenotype. Also the LATS1/2 knockout does not affect phosphorylation and YAP activity. This indicates the alterative, independent of HIPPO pathway, way of inhibiting of YAP activity in human keratinocytes [67, 75, 76].

The homeostasis of epidermis depends also on cell-cell contact. The decrease of expression of

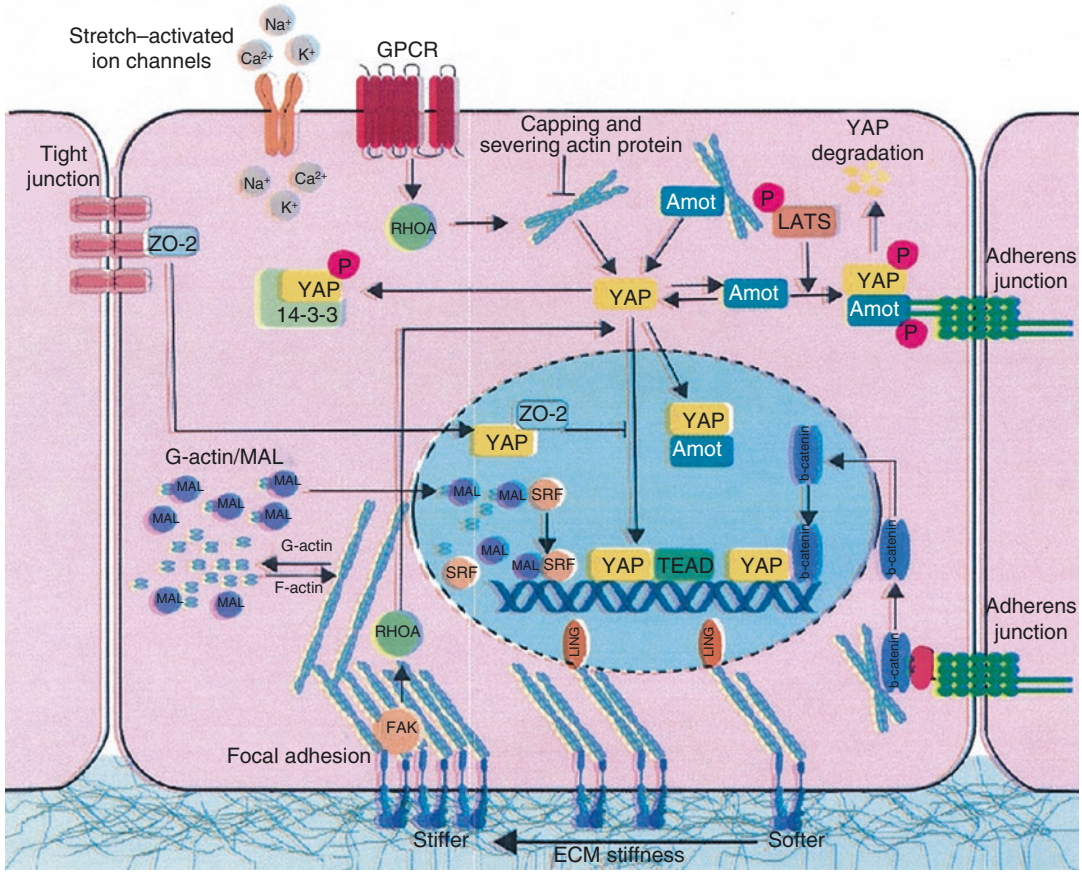

Fig. 1 Mechanotransduction in transcriptional regulation. Description: Interacting with G-actin inactivates MAL. F-actin polymerization uses up amounts of unpolymerized F-actin and removes the inhibition of MAL through G-actin and released MAL binds to the SRF. This activated SRF binds to DNA and induces transcription. YAP can be inhibited by mechanisms not connected with kinases such as AMOT. AMOT protein binds actin filaments and allows YAP to enter the nucleus. If it comes to F-actin depolymerization, AMOT dissociates from actin and retains YAP in the cytoplasm. When YAP is phosphorylated by LATS, AMOT recruits ubiquitin ligase to AMOT/YAP complex and initiates the YAP proteasome degradation. The protein bound to tight junction ZO-2, together with YAP, enters the nucleus where it inhibits the activity of YAP. The p130 isoform of AMOT acts in the opposite manner and promotes nuclear localization of YAP and acts as a transcriptional cofactor of the YAP-TEAD complex. Rho GTPases control YAP/TAZ activity through canonical GPCR-linked (G-protein-coupled receptors) manner or noncanonical activation of YAP through focal adhesion signaling and FAK kinase. It is hypothesized that the presence of F-actin and stress fiber formation (stress fibers) is crucial for the activation of YAP and TAZ. Upon translocation to the nucleus, they associate with TEAD transcription factor which drives transcription of proliferative genes. Rho GTPases and actin-associated proteins (CAP-Z Cofilin, Gelsolin) can have a stabilizing effect on the network of actin filaments and directly or indirectly regulate YAP/TAZ translocation to the nucleus. Mechanical forces generated by ECM can be directly transmitted by the cytoskeleton to the nucleus through LINC complex. Mechanical signal transduction is received by nucleoskeleton proteins (laminae, emerin) that directly or indirectly may affect gene expression. Activation of β-catenin and translocation to the nucleus in response to compressive forces. β-catenin is structural component of adherens junctions in epithelial cells, regulating cell-cell interactions. Shuttling of β-catenin between the cytoplasm and nucleus is a key step in this signaling pathway. Unphosphorylated β-catenin can enter the nucleus and activate transcription, despite the activation of the canonical Wnt pathway (adapted from Low et al. [74])

α-catenin in epidermis leads to the increase of YAP activities; however, this dependence is especially limited for the basal layer cells. In case of a correct cell-cell contact need to keep YAP active, the presences of other signals, which can include the shift of cytoskeleton dynamics in response to mechanical signals, is necessary [68].

In many studies carried out in vivo and in vitro, the crucial dependence between YAP and TAZ expression and a process of wound healing and tissue regeneration has been shown. The nuclear localization of YAP and TAZ has a main influence on the tissue regenerative abilities and feedback to outer signals during wound healing. During wound healing, the translocation to the nucleus and YAP/TAZ activation have a strong pleiotropic effect characterized by wound closing, cell proliferation, and collagen synthesis [77]. Knockdown of YAP/TAZ inhibits cutaneous wound healing, suggesting an important role for these factors in tissue regeneration as well. On the basis of the diverse functions of YAP/TAZ within the skin and as a regulator of mechanotransduction in other cell types (probably this signaling pathway also mediates epidermal mechanosensing) [24].

In normal skin, YAP is located in cell nuclei of keratinocytes of the basal layers of the epidermis and hair follicular epithelium. In healing wounds, the YAP expression highly increases within the whole area of healing tissue. What is important is the nuclear localization of YAP is visible in healing areas of the wound; however, its absence is observable in normal tissue neighboring with the wound [78]. The described topography of YAP localization suggests that in a healing skin there is the activation of signaling which participates in YAP translocation to the cell nucleus, most probably as the effect of inhibition of HIPPO pathway [76, 79]. Another explanation for shifts in YAP localization may be the fact of tissue loss and changing of mechanical environment in cell area, which may also activate YAP [67]. Immunocytochemical localization of TAZ in a normal tissue shows its presence mainly in the cytoplasm of fibroblasts. During intense wound healing, TAZ, in the majority of cells, relocates from cytoplasm to the cell nucleus. In addition, the expression of TAZ is stronger in the area of tissue which undergoes intensive regeneration [78].

Silencing YAP and TAZ on a mice model of wound healing with the use of appropriate siRNA leads to delays in wound closing in comparison to the control group. The effect of silencing YAP/TAZ in the wound area is associated with inhibition of cell proliferation and a decrease of collagen synthesis. Although silencing YAP/TAZ by siRNA is not a long-term process, the effect of this process is clearly visible [78]. In vitro conditions and in the studies on mice model of wound healing, it has been shown that silencing YAP or TAZ in fibroblasts causes a noticeable decrease level of a transforming growth factor beta 1 (TGF-β1), as a result of impairment of the sequence of processes related to appropriate wound healing. TGF-β1 is a key mediator in the process of wound healing. It is responsible for the transformation of fibroblasts to myofibroblasts, as in consequence their contraction and moving toward the wound border, for differentiation of vascular smooth muscles, and is the main simulator of collagen synthesis [80].

One of the target genes of transcriptional coactivator YAP is the gene of CTGF [59]. CTGF supports the signaling of TGF-β1/SMAD by suppression of SMAD-7 protein. This protein exhibits minimal expression in a healthy skin, and its level significantly increases in wounded skin [81]. In addition, the interaction of TAZ and TEAD is crucial to activation of the transcription of Cysteine-rich angiogenic inducer 61 gene (CYR61) [82]. CYR61 is a secreted, extracellular matrix (ECM)-associated signaling protein and participates in the regulation of a broad range of cellular activities, including cell adhesion, migration, proliferation, differentiation, and apoptosis, through interaction with integrin receptors, and is intensely synthetized by myofibroblasts during the process of wound granulation.

It has been shown that YAP and TAZ in a nuclear localization (which is dependent on the density of cells) participate in the translocation, which is induced by TGF, of SMAD to the nucleus. In contrast, when YAP and TAZ are in the cytoplasmic localization, they have an inhibiting impact on the same process [83]. TGF-β/

SMAD signaling may be also responsible for the induction of the expression of CTGF [84]. It may be assumed that, during the process of wound healing, TAZ, which directly influences the activation of TGF-β pathway, participates, in an indirect manner, in the process of control of the level and activity of SMAD-2 [85]. It seems that TAZ controls TGF-β1 signaling more effectively than YAP. TAZ knockout in a healing wound clearly reduces the transcription of SMAD-2 which is induced by TGFβ. However, YAP which influences the activity of SMAD-7 may participate in muting TGF-β1 signaling [86]. SMAD-3 and SMAD-5 interact directly with and are phosphorylated by activated TGF-β1 receptors; also SMAD-6 and SMAD-7 bind activated TGF-β1, thereby preventing phosphorylation of R-SMADs [87, 88]. These observations suggest that YAP/TAZ participate in the modulation of wound healing through the mobilization of synthesis and activation of TGF-β1, and signals related to TGF-β1 induce the activities of such factors as SMAD and CTGF. Binding the signaling pathways YAP/TAZ with TGF-β1/SMAD is an important factor which regulates a multistage process of wound healing.

3.3 Serum Response Factor (SRF)

Serum response factor (SRF) is a transcription factor which was discovered in fibroblasts treated with blood serum. The cells receive sudden exposure to serum as a signal which signifies injury and tissue damage, and in consequence, they start the healing process. Serum exposure does not only result in mitogenic activation; it is a more complex process which involves the effects on fibroblasts of epithelial cells and endothelial [89].

SRF is responsible for expression of genes which regulate proliferation and cell differentiation [90]. SRF is activated by mitogenic protein kinases or RHOA pathway, and it produces a fast transcriptional response through regulation of factors, signaling proteins, and cytoskeleton components [91].

There are approximately 300 known genes which consist of SRF response elements; among them, there are genes of early cellular response (e.g., c-FOS, cyr61), whose expression is important in the process of wound healing [92]. SRF is a key factor which induces the differentiation of fibroblasts during wound healing. Overexpression of SRF, in stem cells, in epithelial cells, as well as in fibroblasts, promotes their transformation to myofibroblasts [93].

The activity of SRF may be regulated by many independent pathways. In relaxed smooth muscle cells, the relationship of SMAD-7 (inhibitor of TGF-β signaling) with the activity of SRF has been shown. The increase of TGF-β level weakens this interaction [94]. It has also been shown that the activation of integrin-linked kinases (ILK) and the SRF phosphorylation is linked to TGF-β1 signaling [95]. Moreover, phosphorylation of serine at position 103 increases the ability to link SRF to α-actin of smooth muscles in proportion to the ILK activity, and it also increases protein stability. However, the ILK inactivation decreases the half-length of SRF [96].

It has been proved that one of the factors which affect SRF activity is the dynamics of cytoskeleton and the activity of cofactor G-actin MAL (Megakaryoblastic Leukemia 1) [97]. The monomeric actin (G-actin) while binding with MAL closes protein pathway to the nucleus and its functional inhibition. As a result of serum stimulation and RHO activation, the polymerization of actin increases and the level of actin monomers decreases. In such conditions, MAL is released to the nucleus where it starts the transcription of independent genes. Other studies show that the activity of SRF-MAL may be also regulated by forces connected with cell migration. It has been shown that the interaction between MAL and G-actin in cellular nucleus blocks MAL binding with SRF. The factors which induce polymerization of F-actin decrease the pool of free G-actin, and, at the same time, they increase the availability of MAL, which may lead to SRF activation [98].

The model of in vitro differentiation of epidermal stem cells by mechanotransduction, with the use of micro-samples of matrix islands, showed that cells growing on the larger islands form a dense network of actin filaments and stress fibers,

thereby reducing the pool of free G-actin and increasing the availability of MAL. Availability of MAL induces the activation of SRF and genes dependent on it, such as JUNB or FOS. SRF activation through mechanical signals is an alternative or parallel way to the activity of growth factors on epidermal cells [23].

3.4 SKP2 (S-Phase Kinase-Associated Protein 2)

SKP2 (S-phase kinase-associated protein 2) for the first time was identified as an important element of Cyclin A-CDK kinase/S-phase complex [99]. In further studies, it was described as a protein which is bound to SKP1, with which it composes a ligase ubiquitin type SCF complex (Skp1-cullin-F-box) that takes part in the regulation of cell cycle through proteolysis dependent on ubiquitin. The SKP2 is a recognizing subunit of SCF complex and substrate is p27 protein. Ubiquitination and the proteasome degradation of p27 enable the transition S-phase cell cycle and promote cell proliferation [100].

SKP2 expression and the promotion of proliferation are the result of cooperation between the signalization of growth factors and mechanical forces which affect a cell. In the studies carried out on smooth muscles and fibroblasts, it was shown that the growth factors regulate the level of SKP2 on the level of protein stabilization; in contrast, the increase of mechanical tension of cells causes the increase of protein expression on the level of transcription. Cell adhesion to substrate and mechanical tension of cells are conditional to maintain the transcription of SKP2 [101].

The activation of SKP2 promotor depends on a bind of transcription factor NFAT1 (nuclear factor of activated T cells). NFAT1 belongs to a family of four transcription factors which are activated by the level of calcium ions in the cytoplasm. Calcium ions, through the mechanism dependent on calmodulin, activate the phosphatase of calcineurin. Dephosphorylation of serines in NFAT induces conformational shifts which expose nuclear localization signal (NLS) and which cover the nuclear export signal (NES). A full dephosphorylation of NFAT1 leads to conformational shifts, which activates such protein functions as translocation to the nucleus, binding with the DNA and activation of transcription [102].

The transcription factor NFAT1 is activated in response to the increase of mechanical tension of a cell, which leads to the increase of expression of SKP2 protein. Conformational shifts of NFTA1, as a result of dephosphorylation, are tightly related to cell adhesion and the formation of mechanical forces dependent on the cell adhesion surface. This point supports the studies which show that the level of mRNA SKP2 in adherent cells may be regulated by the change in their shape. Results of many studies point that Skp2 is regulated by the influence of mechanical forces onto a cell. Shifts in mechanical tension of cells regulate mRNA level in bladder and vascular smooth muscle cells and skin fibroblasts. This leads to the assumption that the regulation of SKP2 transcription through actions of mechanical forces is an element of many, if not all, physiological and pathological processes dependent on the regulation of intensification of cell divisions, such as morphogenesis, tissue regeneration, and wound healing [103].

4 Development Prospects and Clinical Implications

Basic research of cell and tissue mechanobiology and clinical studies point to the importance of mechanical forces in the process of skin regeneration and wound healing. More important questions to be answered are how these molecules in specific pathways interact with each other in response to mechanical force and what controls target gene activity and what mechanosensing perturbed in skin regenerations and wound healing.

The outcome of these studies is the development of new therapies which use mechanical forces that support proper healing. It may be observed that the development of therapies based on the use of mechanical forces, or of bandages with appropriate mechanical properties, prevents improper scarring.

The importance of mechanical signaling in scar formation points to the observation associated with the use of botulinum toxin type A in aesthetic medicine (used to treat local subcutaneous muscle paralysis). The observations noted decrease of scarring in the areas where botulinum toxin was used; these effects are attributed to the reduced wound tension during its remodeling. Early clinical studies also show that injection of botulinum toxin into the wound site reduces the formation of hypertrophic scars [104].

Wounds auxiliary therapy treatment which uses devices that generate negative pressure (NPWT—negative pressure vacuum-assisted closure technology) is an effective method that supports extensive and rapid healing of chronic wounds. Functioning of NPWT facilitates the approximation of wound edges and stabilizes the environment, which reduces edema and ascites and also reduces micromechanical forces [105].

Another beneficial therapy, which is deemed as effective physical modality for soft tissue wounds and which probably induces mechanisms of mechanotransduction and immunomodulation, are high-energy acoustic waves (ESWT—extracorporeal shock wave therapy) [106]. Results of the current studies suggest there is strong evidence documenting that ESWT application is safe and effective for the treatment of different etiologically soft tissue wounds, both acute and chronic. Clinical efficiency of ESWT shows a wide range of positive results, such as completed wound closure and reepithelialization, enhanced tissue granulation, reduced necrotic fibrin tissue, improved blood flow perfusion and angiogenesis, reduced period of total wound treatment, and decreased necessity of antibiotic treatment [107].

It seems that the mechanism of NPWT or ESWT, functioning as a technique for supporting wound healing, is based on mechanotransduction, and further researches are focused on the assessment of the optimal therapeutic parameters and the use of additional materials supporting therapy. The results of the studies and the opinions of clinicians show the importance of the transduction of mechanical forces in the process of wound healing and scar formation. The growing importance of mechanotransduction in wound healing and scar formation will contribute, to a large measure, to designing new clinical therapies and surgical procedures.

A better understanding of mechanobiology will enable the design of biomaterials, with appropriate physical and chemical properties, which will be used to treat improperly healing wounds. In addition, it will allow to develop devices which will precisely control the mechanics of the wound and to individualize the therapy depending on the type, size, and anatomical location of the wound in certain patients, which will increase the efficiency of clinical therapy. Linking mechanobiology with the science of biomaterials and nanotechnology will enable in the near future a precise interference in abnormal cell signaling responsible for the proliferation, differentiation and cell death, and the restoration of biological balance.

In addition, knowledge of the mechanisms of mechanical signal transduction and its involvement in the activation of certain genes opens up new ways for combination therapies that use mechanical and drug therapy. This can increase the effectiveness of treatment.

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Impact of Amnion-Derived Mesenchymal Stem Cells on Wound Healing

Alexandru-Cristian Tuca and Ingrid Lang-Olip

1 Introduction

Mesenchymal stem cells, also termed multipotent mesenchymal stromal cells (MSCs), are present in fetal and many adult tissues. They are able to differentiate into all three lineages, i.e., mesoderm, endoderm, and ectoderm, and have remarkable regenerative properties [1]. According to the International Society for Cellular Therapy, MSCs are defined by the following minimum criteria [2]:

1. Adherence to plastic
2. Expression of a certain set of cell surface markers, i.e., CD73, CD90, and CD105, and lack expression of CD14, CD34, CD45, and human leukocyte antigen-DR
3. Adipogenic, chondrogenic, and osteogenic differentiation potential in vitro

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MSCs are usually isolated from bone marrow but also from other adult tissues like adipose tissue. However, the proliferation and differentiation potential of MSCs varies depending on the origin of the MSCs and the age and morbidity of the donor [3]. Therefore, MSCs derived from neonatal tissues, such as the umbilical cord (Wharton's jelly), placenta, and amnion, may represent a valuable alternative for therapeutic approaches [4–6]. They have superior proliferation and differentiation abilities and could be harvested by noninvasive isolation methods with low ethical problems, as the human full-term placenta is often considered as medical waste after birth [3].

The placenta is a fetal organ which connects the fetus to the uterine wall and has different functions like nutrition, thermoregulation, and gas exchange. The amnion as the innermost layer of the fetal membranes covers the placenta and encloses the fetus and the amniotic fluid in the amniotic cavity. More than 100 years ago, the regenerative properties of the human placental amnion were known and used for wound healing purposes. Meanwhile, the amnion membrane is clinically applied in skin transplantations, as a dressing for burns and chronic ulcers and many other wound healing processes [7, 8]. The amnion serves as a rich source for amnion-derived MSCs (hAMSCs), which are available at large supply shortly after isolation (Fig. 1).

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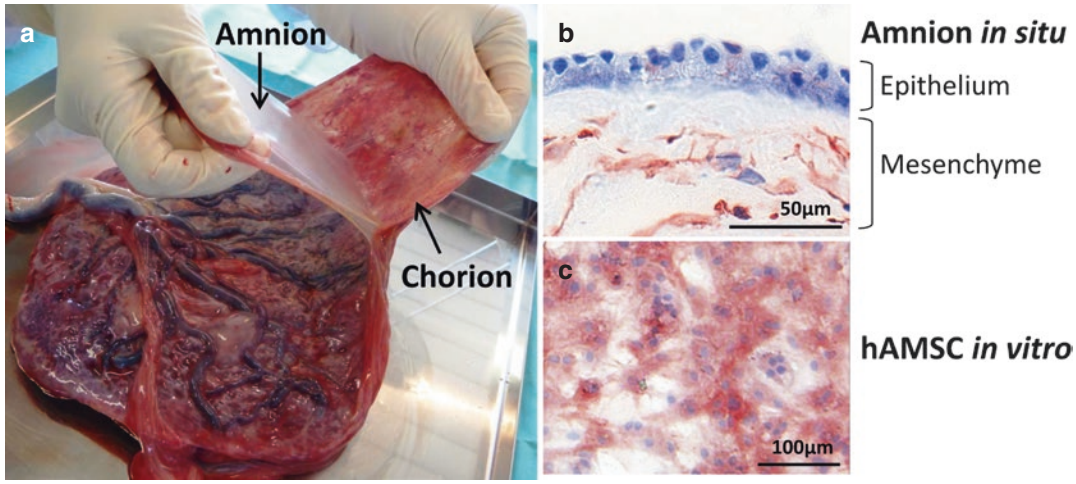

Fig. 1 (**a**)The human full-term placenta and the fetal membranes. The amniotic membrane is passively attached to the placenta and the chorion laeve. (**b**) Microscopic analysis shows that the amnion consists of an epithelial layer and a thin layer of mesenchymal tissue. Mesenchymal stromal cells were visualized by anti-vimentin staining (brown color). (**c**) Isolated amnion-derived mesenchymal stem cells (hAMSCs) in culture were stained by anti-CD90

2 MSCs-Related Mechanisms on Tissue Regeneration and Wound Healing

During the last decade, MSCs have been the subject of several investigations for understanding their mechanisms through which they promote cell repair and tissue regeneration. MSCs play a major role in all stages of wound healing (i.e., inflammatory, proliferation, and remodeling phase). They help to resolve the inflammatory phase and to advance to the proliferation phase [3].

Earlier, the theory was that MSCs would engraft around the wound area and differentiate into local cells to replace the destroyed ones. However, recent investigations showed that the cells did not long-term engraft and were not detectable at sufficient numbers that could explain the observed regenerative effects [9]. Thus, it becomes more and more evident that the regenerative effects of MSCs are rather associated with paracrine mechanisms than with cell differentiation and cell engraftment [8–10]. Especially the amnion acts as a reservoir for various soluble factors, such as growth factors and cytokines which have already been identified during the different phases of wound healing [8], such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), keratinocyte growth factor, vascular endothelial growth factor- α (VEGF- α), hypoxia-inducible factor 1a (HIF1a), and many others. These soluble factors are able to stimulate angiogenesis and reepithelialization as key processes during wound healing [3, 11, 12]. Further, literature also suggests enhanced neuronal regeneration and protection by expression neurotrophic factors [13].

3 Immunomodulatory Properties of hAMSCs

The inflammatory response after tissue injury is induced by several overlapping cellular and molecular mechanisms. Immune cells such as T-cells, B-cells, macrophages, and neutrophils are recruited from the vasculature and then guided and controlled by chemokines and cytokines to the injury site. The inflammation can be categorized into an acute response, a rapid response and a chronic one. Depending on the type of tissue damage, i.e. induced viral, bacterial, traumatic or toxic, the immune response can

vary in its secretion of cytokines and other inflammatory mediators. Resolving this inflammatory response and its cascade is the first step toward regeneration [8, 14]. hAMSCs have the ability to attenuate and modulate the immune and inflammatory response. Studies revealed the impact of hAMSCs on the migration and proliferation of different cell types, such as fibroblasts and inflammatory and endothelial cells. remodeling and production of extracellular matrix, and inhibition of apoptosis. hAMSCs are able to stop the synthesis and to decrease the levels of pro-inflammatory cytokines like tumor necrosis factor α, C-X-C motif chemokine ligand (CXCL) 9, CXCL 10, and chemokine C-C motif ligand 5 [8]. hAMSCs also express anti-inflammatory proteins, prevent proliferation and activation of T-cells, and inhibit proteinase activity to enhance wound healing [13, 15]. hAMSCs exert anti-allergic properties by suppressing (1) the proliferation of activated fibroblasts as well as (2) the synthesis of TGFß1, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) by activated fibroblasts [8]. These findings show that hAMSCs can be of high importance for regenerative medicine, because anti-inflammatory properties and immunogenicity remain crucial factors in successful transplantation for regenerative wound treatment.

4 Preclinical Applications of hAMSCs in Wound Healing

In recent in vitro studies, hAMSCs were shown to exert angiogenic properties, although they resist to differentiate into endothelial cells [16]. They promoted the survival of endothelial cells and stabilized endothelial networks [17]. Subcutaneously injected hAMSCs promoted neovascularization in a mouse model in vivo [18]. hAMSCs and MSCs from other fetal tissues are superior in their proliferation, differentiation, and angiogenic capacities. They showed improved therapeutic functionality and potency and might be more effective than patient-derived and potential dysfunctional MSCs derived from adult tissues [19].

Although the amniotic membrane has already found applications in a variety of pathologies and also in clinical wound healing settings like chronic ulcers of different origins [4, 8], there is still a lack of experience for using hAMSCs in clinical applications. Placental-derived MSCs have been proven to be safe in vivo. They have been tested or are currently under study in patients with pulmonary fibrosis, acute lung injury, graft versus host disease, and type II diabetes. Amnion-derived cellular cytokine solution containing relevant factors for wound healing is currently tested in patients with dermatitis and burns. Amniotic membrane extract showed promising results for reducing epithelial defects [4].

To our knowledge, wound healing effects of hAMSCs have been only tested in animal studies. In a mouse wound model, Liu et al. [20] compared the therapeutic potential of MSCs derived from the amnion, bone marrow, and adipose tissue by injecting them intradermally around cutaneous wounds. They described a most pronounced wound healing capacity of MSCs derived from adipose tissue. Kim et al. [19] injected hAMSCs around full-thickness wounds of diabetic mice, which in general means negative implications for wound healing, and achieved significantly enhanced wound healing, reflected by high engraftment of hAMSCs, expression of keratinocyte-specific proteins, and increased reepithelialization [19]. In our study we used Matriderm (a dermal bovine-derived collagen-elastin matrix) as carrier for the topical application of the hAMSCs onto mouse skin wounds. Although hAMSCs did not attach to the Matriderm, and despite most transplanted cells have vanished, they significantly promoted neovascularization and enhanced wound closure [21].

As the delivery and application method of hAMSCs into, onto, or around the wound might also influence the beneficial effects on wound healing, the methods for application of MSCs should be investigated and compared more deeply in future studies to improve the healing outcome. Literature still shows a lack of studies and also inconclusive data concerning this question [12, 19, 20].

Conclusions

During the last decade, breakthrough discoveries of the mechanisms of hAMSCs have made them as a promising source for future cell therapy in regenerative medicine and wound healing due to their high differentiation ability, proliferation rate, the ability to drive angiogenesis, and their capacity of immune modulation. Data suggests that the beneficial therapeutic potential of hAMSCs on wound healing is predominantly ascribed to the secretion of different growth factors. Nevertheless, this research field is in its beginning, and there are still open questions concerning the specific mechanisms on wound healing or the application methods which need to be answered. Thus, further investigations have to be done before serious clinical applications with hAMSCs can be considered.

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Kinin Receptors in Skin Wound Healing

Bruna S. Soley, Cintia S.D. Horinouchi, Priscila L. Pawloski, Michel F. Otuki, and Daniela A. Cabrini

1 Introduction

Kinins are a family of peptides derived from kininogens related to the kallikrein-kinin system. As reviewed by many [1–20], their history starts with the almost simultaneous research of E.K. Frey, Heinrich Krant, and Eugene Werle in Germany (from 1950 to 1983) and M. Rocha e Silva et al. (1949) [21] in Brazil. Since their discovery, the main polypeptides bradykinin (BK) and kallidin (Lys-BK) and their active metabolites (des-Arg^9-BK and Lys-des-Arg^9-BK) have been described as products of tissue and plasma kallikreins that cleave high- and low-molecular-weight kininogen.

The biological effects of kinins involve the activation of specific G protein-coupled receptors named B1 and B2 [2]. The B2 receptor was the first to be characterized and is activated by the primary kinins BK and Lys-BK. They are widely distributed through the body and constitutively expressed. BK B1 receptors are expressed at very low levels in healthy tissues but are induced after noxious stimuli. The ligants for B1 receptors are the active metabolites des-Arg, derived from the action of carboxypeptidases [22].

Kininases, like the angiotensin-converting enzyme (ACE), are the enzymes that hydrolyze and inactivate kinins. ACE is the same enzyme that is the target of antihypertensive drugs ACE inhibitors, being these drugs able to reduce the vasoconstrictor angiotensin II and increase vasorelaxant kinins [23].

Thus, in more than 60 years since their breakthrough, the kinin system has been extensively studied and shown to modulate many physiological cellular functions, some of which are linked to human diseases from inflammation trough carcinogenesis [19]. Despite the proven presence of these peptides and their receptors in different diseases, so far, the kinin system is only targeted for the treatment of hereditary angioedema with C1-inhibitor deficiency [24]. A BK B2 receptor antagonist icatibant (HOE 140) is one of the few self-administered available agents for treating acute attacks of angioedema [25].

Kinins modulate different phases of the inflammatory processes. It would not be different for wound healing. Thus, we will address the participation of kinins in the different stages of the healing process, presenting evidence and speculations regarding the undeniable influence of kinins and their prominence as a possible new target for therapies that involve deficiency in skin healing.

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2 Kinin Modulation of Wound Healing Inflammatory Phase

After a lesion, a cascade of events is initiated in order to mediate tissue repair [26]. The inflammation is considered the first of many processes that are involved in cicatrization [26, 27]. It is a phase of extreme importance, since it is considered an instrument to supply the tissue of growth factors and cytokines that signal the cellular and tissue movements necessary for the tissue repair [28, 29]. Kinins are important inflammatory mediators that accumulate at sites of injury and are involved in the modulation of events observed during inflammation, including vasodilation, increased vascular permeability, and pain [30, 31]. In addition, evidence indicates that cells present at the site of inflammation are more responsive to kinins than normal cells [32].

It is well known that kinins are active vasoactive peptides capable of promoting vasodilation and increased vascular permeability, which may facilitate the recruitment of immune cells [33, 34]. There are numerous studies that point out the involvement of kinins and their receptors as components of primary action in inflammation [6]. In this way, the kinin system participates in the modulation of vascular and cellular events present in the inflammatory phase of the tissue repair process (Fig. 1) [30, 31, 33, 34].

Kinins have the ability to promote vasodilation after activation of kininergic receptors, since this activation promotes the release of vasodilator prostaglandins [35]. These, in turn, promote local vasodilation, increased blood flow, and vascular permeability, all of which are capable of facilitating leukocyte infiltration. Similarly, nitric oxide (NO) production by endothelial cells seems to be related to the vasodilatory capacity of kinins, since inhibitors of NO synthesis can attenuate des-Arg^9-BK-induced increase in diameter and vascular permeability, in pleurisy model [35].

Through these vascular effects, the kinins have the ability to facilitate the recruitment of inflammatory cells to the injury site (Fig. 1) [35]. For example, the activation of kininergic receptors promotes the formation of inter-endothelial gaps and protein extravasation and facilitates the migration of cells that make up the immune system [36]. In addition, several studies show that the activation of kinin receptors can induce the expression of adhesion molecules in endothelial cells and leukocytes and evidence the regulatory effect of kinins on the adhesion of leukocytes to the endothelial wall [31, 36–38]. Guevara-Lora et al. [39] demonstrated that polymorphonuclear leukocytes stimulated with BK showed greater adhesion to endothelial cells. This effect may be a result of the ability of kinins to increase the expression of adhesion molecules, such as Mac-1, ICAM-1, or P-selectin.

In tissue repair, infiltrated leukocytes are the major cellular components of the inflammatory response. They are not only effective immune cells against the invasion of possible pathogens but are also involved in the anabolic phase of tissue degradation by producing proteases and intermediate reactive oxygen species and in the catabolic phase of tissue formation by producing growth factors and cytokines [26, 27]. There are numerous studies that point out the involvement of kinins and their receptors as components of primary action in inflammation [6]. Kinin receptors are expressed in several cells of the immune system, which are directly associated with the activation, differentiation, proliferation and, especially, migration of immune cells [40, 41]. In this context, kinins play an important role in the activation of immune cells during the inflammatory process, as represented in Fig. 1. This activation is directly or indirectly modulated by the release of other inflammatory mediators, such as cytokines, prostaglandins, leukotrienes, and reactive oxygen species [42, 43]. In this way, kinins can induce the migration of immune components indirectly and directly, by activating kininergic receptors present in these cell types. This is a process of extreme importance during tissue repair, since the infiltrate of inflammatory cells is directly involved with the injured tissue protection and release of growth factors that will induce the activation and proliferation of cells, such as fibroblasts, helping in the formation of granulation tissue [44, 45].

Neutrophils represent the main polymorphonuclear leukocyte observed during the

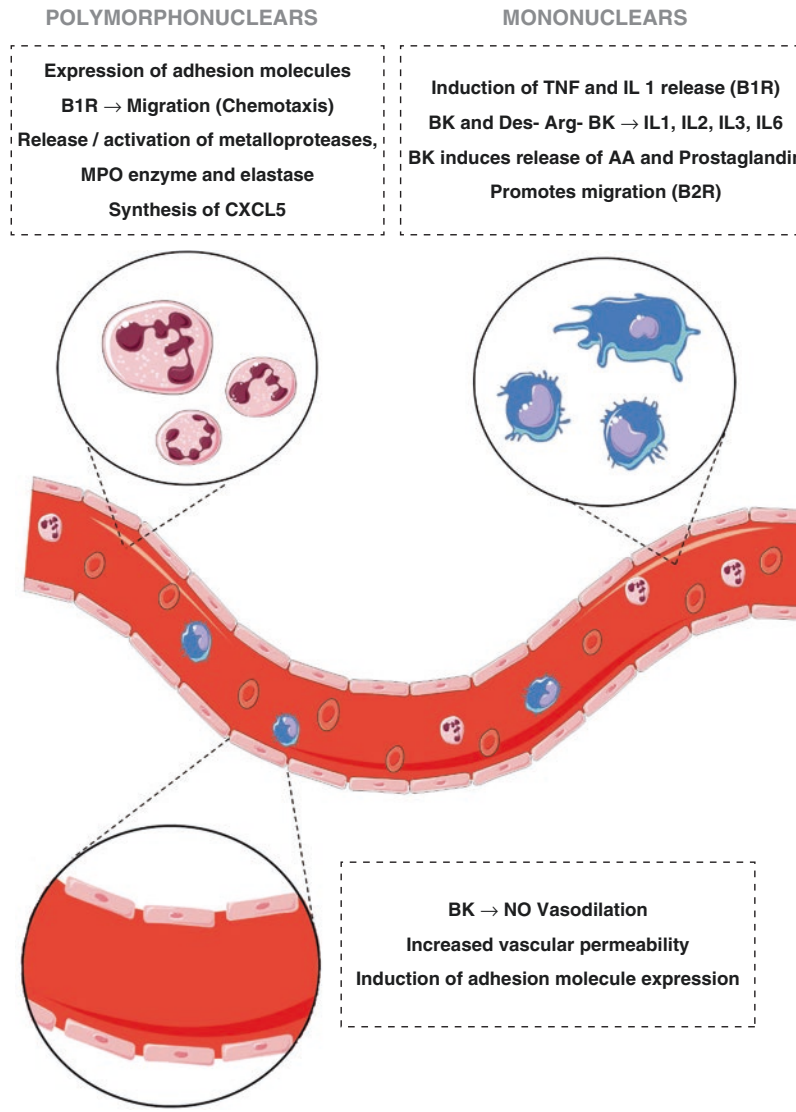

Fig. 1 The involvement of the kinin system in the inflammatory process, recruiting cells and causing vasodilation

inflammatory phase, where they are responsible for phagocytosis of possible pathogens and tissue "cleaning," removing extracellular and cellular matrix fragments [35, 46]. The presence of tissue kallikrein in human neutrophils was reported for the first time in 1989, and subsequent studies have shown high- or low-molecular-weight kininogens present in the polymorphonuclear membrane [47, 48]. Neutrophils, therefore, have the ability to promote local formation of kinins, which in turn can promote autocrine and paracrine effects by binding to their respective receptors [47].

The first description of the kinin B1 receptor chemoattraction capacity dates to 1996, where it was shown that, following IL-1β treatment, the release of des-Arg^{9}-BKcould induce a pronounced neutrophil migration in murine air pouch model. In addition, blocking of this effect has been shown to be achieved by administration of both B1 selective antagonists des-Arg^{9}-[Leu^{8}]-BK and des-Arg^{10}-Hoe140 [37, 48]. Similar results were observed in vitro, where the activation of the B1 receptors was able to promote neutrophil chemotaxis [47]. These results were corroborated by animal knockout studies for the

B1 kinin receptor, where deletion of this gene was shown to be related to a deficiency in neutrophil accumulation in inflamed tissues [37, 42].

In addition, B1 receptor activation in human neutrophils induces chemotaxis and stimulates the release/activation of metalloproteinases, myeloperoxidase (MPO), and elastase enzymes (Fig. 1) [42, 49]. Similarly, Campos et al. [50] demonstrated that increase in MPO enzyme activity was reversed by administration of the antagonist des-Arg^{9}-[Leu^{8}]-BK. However, in addition to this direct effect, recent studies suggest that activation of the B1 receptor is also capable of facilitating indirect neutrophil migration, mainly through the induction of CXCL5 synthesis [35], highlighting that this chemokine has an important role in neutrophil adhesion, increasing the bearing of these cell types [35]. Corroborating these data, knockout animals for kinin B1 receptors had the CXCL5 synthesis practically abolished, suggesting that this chemokine mediates the B1 receptor effects and this could be one of the ways in which kinins promote polymorphonuclear cell migration during inflammatory processes [35, 49]. Thus, the participation of the B1 receptor in the modulation of polymorphonuclear migration is undeniable. It is worth mentioning that this kininergic receptor subtype is upregulated in these cell types, predominantly during inflammatory processes [49].

It is believed that the kinins' chemotactic potential is amplified during inflammatory processes, since it has been demonstrated that peripheral neutrophils have an increase in kininergic receptor expression [48]. Kinins and interleukin-1β (IL-1β) present at the site of inflammation may be responsible for this upregulation of kinin receptors. It is known that IL-1β may be involved in an increase in the expression of kinin receptors in human synovial cells [48]. In this way, cytokines and kinins can cooperatively participate in the induction of neutrophil migration during inflammatory processes (Fig. 1), since IL-1β is able to induce polymorphonuclear cell migration leading to an upregulation of the TNF-α-dependent B1 receptor, which in turn could amplify the influx of cells in order to perpetuate the inflammatory response [50].

However, the B2 receptor is constitutively expressed and also the predominant subtype in neutrophils [48]. In this context, some studies aimed to verify the involvement of this kininergic receptor subtype in the neutrophil migration. Therefore, in vitro studies performed with human neutrophils showed that both kininergic receptors are capable of inducing the migration of these cell types [51]. Similar results were observed in vivo, where it was demonstrated that BK is able to modulate neutrophil migration through the activation of the B2 receptors in rats [42, 52]. In addition, since polymorphonuclear migration was significantly reversed by the B2 antagonist HOE 140, there is evidence that kinins also present chemotactic effects through activation of the B2 receptors [48].

The presence of the polymorphonuclear infiltrate also facilitates monocyte recruitment to the injured tissue, since the presence of apoptotic neutrophils represents an attraction sign [46]. After recruitment, circulating monocytes differentiate into macrophages, which play a central role in all the different phases of the tissue repair process. These cells are responsible for the synthesis and release of various cytokines and chemokines, which stimulate the formation of new vessels and the collagen synthesis during tissue repair. The number of macrophages increases during the inflammatory phase, with their peak during the proliferation and reduction phase during the phase of tissue remodeling [53]. Similarly, to polymorphonuclear leukocytes, kinins at the site of injury can also modulate mononuclear activity. Both kinin receptors are present in macrophages. The B2 receptors are constitutively expressed alone or together with the B1 receptor in macrophages of many tissues [48]. Studies show that the kinins are able to stimulate macrophages to synthesize and release several inflammatory mediators [41, 48]. Kinins are able to induce tumor necrosis factor (TNF) and IL-1β release in murine macrophages of the P388-D1 and RAW264 cell line. Likewise, the production of these cytokines is inhibited by the antagonist

des-Arg^9-[Leu^8]-BK, indicating the B1 receptor participation in these events [41, 48].

However, Bockmann et al. [48] showed that BK and des-Arg^9-BK could induce the release of several cytokines, such as IL-1, IL-2, IL-3, and IL-6, in cells isolated from the spleen of mice. This stimulus was inhibited by B2 receptor antagonists, suggesting that this receptor subtype is also capable of modulating the synthesis and release of cytokine macrophages and/or mouse T cells. Subsequently, BK was able to induce the arachidonic acid and prostaglandin E2 (PGE2) release, in guinea pig macrophages through activation of the B2 receptor. Thus, B2 receptor activation in monocytes regulates cytokines expression, as well as leukocyte recruitment (Fig. 1) [40, 43]. In addition, the stimulation of B2 receptors favors monocytes migration through the activation of phospholipase C and the consequent increase in the intracellular calcium concentration, reaffirming the involvement of this receptor in the migration of these cell types [47].

Sometimes discrepancies in the effects of kinins can be explained due to the difference in expression of the kininergic receptors in monocytes and macrophages [31, 48]. Constitutive expression of B2 receptor was observed in undifferentiated cells, whereas B1 expression was very low. In this way, it is suggested that the maturation of those cells leads to an increase in the expression of kinin receptors, mainly the B2 receptor [31].

These evidences show how the kininergic system is able to modulate the activity of several cell types essential to the inflammatory phase of tissue repair process. This importance was corroborated in a skin healing study, where the absence of the kinins B1 and B2 receptors promoted significant reductions in the cellular infiltrate and, consequently, delays in the resolution of the tissue repair process [54]. Thus, excessive or reduced influx of leukocytes at the tissue injury site may interfere with the migration, proliferation, and differentiation of other cells, such as fibroblasts and keratinocytes, interfering with the quality of tissue repair [27].

3 Kinin Modulation of Wound Healing Proliferative and Remodeling Phase

The proliferative phase is the second stage of the wound healing process and is triggered by signals coming from the inflammatory phase. In brief, proliferative phase is marked by re-epithelialization, new extracellular matrix formation, and neovascularization [55].

Angiogenesis is a critical factor for the success of wound healing and involves complex synchronized interactions of many different cell types, tissues, and biochemical mediators [56]. It is regulated through the activity of growth factors, cytokines, and inhibitors, these being able to stimulate the rearrangement of endothelial cells [57].

The kallikrein-kinin system is an important organization involved in the angiogenesis during pathological situations [58]. Kinins are recognized pro-inflammatory peptides that can modulate keratinocyte differentiation and proliferation/migration of endothelial cells (Fig. 2) [59]. One of the first studies showing an involvement of the kallikrein-kinin system in this process of neovascularization was published by Hu and Fan [60] and showed that BK increases angiogenesis in a sponge implantation model through the B1 receptor generation. Previous studies involving angiotensin II and ACE inhibitors showed increase vessel density in several animal models. These findings were better understood after the confirmation that the main action was related to the stabilization of kinins and not by the inhibition of angiotensin II generation [61–63].

Kinins trigger both receptors for the formation of new vessels, either on endothelial or other epidermal cells, as represented in Fig. 2. On endothelial cells, activation of B1 receptors induces the expression of vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) and promotes phosphorylation activating the VEGFR2. Nevertheless, it is believed that B2 receptor increases endothelial nitric oxide synthase (eNOS) expression (also known as NOS3)

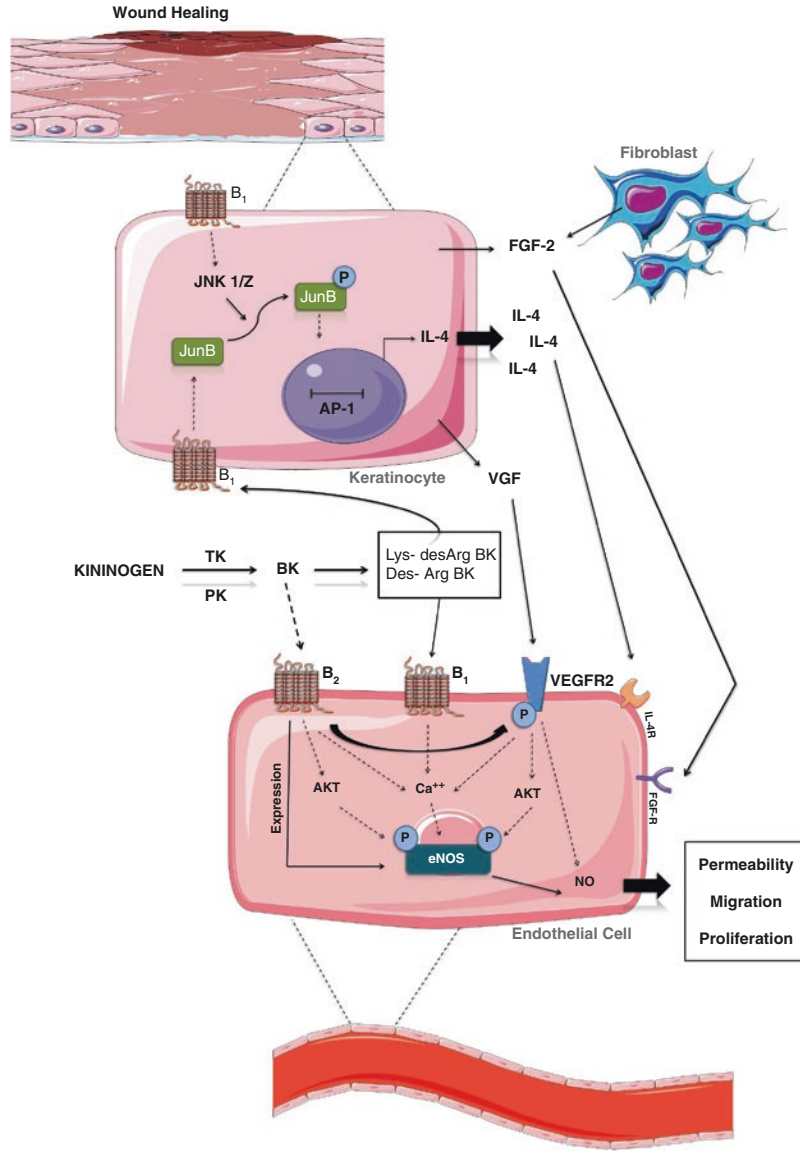

Fig. 2 The participation of kinins in the proliferation and remodeling phases, acting directly on keratinocytes, fibroblasts, and endothelial cells

and the expression of VEGF and VEGFR2 [59, 64]. All three receptors (B1, B2, and VEGFR2) can activate eNOS by increasing intracellular concentrations of calcium and changing the phosphorylation state of the enzyme mediated by phosphoinositide 3-kinase, Akt (protein kinase B), and calcineurin. On the other hand, the B1 receptor-induced NO synthesis was shown to increase the expression of fibroblast growth factor-2 (FGF-2), which is proangiogenic via its receptor FGFR1 [58, 65]. Interestedly, B1 agonists are ineffective in B2-deficient mice, showing that the cross talk between the two receptors may be important for these actions [66].

On the epithelial cells specifically, BK appears to behave through B1 receptors producing indirect effects on angiogenesis. The activation of B1 receptors by the agonist Lys-des-Arg^9-BKon keratinocytes starts signaling pathways and the cross talk between endothelial cells and fibroblasts. Evidences showed that stimulation of B1 receptors results in phosphorylation of the transcription factor JunB that translocates into the nucleus to bind AP-1 sites and

activates IL-4 cytokine synthesis. Some studies have described that IL-4 induces capillary-like formation in 2D cultures [67, 68]. Actually, IL-4 and VEGF from keratinocytes induce angiogenesis stimulating receptors specifically present on the endothelial cell surface. Indeed, in course of the inflammation, B1 receptors may be more expressed in many cells like endothelial cells by the generated cytokines, and their stimulation by the des-Arg active metabolites may intensify the release of VEGF and IL-4, thus stimulating angiogenesis [59].

Keratinocytes are the cells responsible for the re-epithelialization, which will guarantee the wound closure and the reestablishment of skin barrier function [69]. Meanwhile, fibroblasts are responsible by synthetizing and releasing the components of the ECM, which fulfill the wound gap constituting the granulation tissue that is a provisional tissue. In addition to ECM production, fibroblasts also can differentiate in myofibroblasts, a contractile phenotype that helps wound contraction [70].

Although kinin importance on wound healing response is far to be completely understood, literature evidences point to kinin ability to modulate not only inflammatory and proliferative phases but also in skin cells during the proliferative phase, as schematically represented in Fig. 2. As expected, given the complexity of wound healing process, the evidences are sometimes contradictory and still scarce. One of the first evidences of kinin system involvement on proliferative phase of skin wound healing comes from a study developed in 1971 which had the purpose of delineating the contractile phenotype of fibroblasts from granulation tissues. In this study, the authors demonstrated that BK could induce contraction of a granulation tissue extracted from rat skin wounds when tested in an isolated organ system [71]. Later other studies made clear that the kinin system is somehow involved in the modulation of skin cells behavior during the regeneration process. For instance, tissue kallikrein is known to promote skin wound healing in rats in a mechanism, which involves keratinocytes stimulation. However, tissue kallikrein effect on rat skin regeneration seems to be just partially related to kinin receptors activation but is dependent on EGFR activation [72]. In this section we will stick to what is known so far about the effects of kinins on fibroblasts and keratinocytes mediated by the activation of the receptors B1 and B2.

It is well known that the normal skin expresses the components of kallikrein-kinin system. The kinin receptor B2 is expressed in a basal level in fibroblasts and keratinocytes, and the receptor B1 appears to be enhanced after skin injuries [73]. On keratinocytes, B2R activation triggers cytosolic calcium mobilization through phospholipase C and inositol 1,4,5-trisphosphate ($InsP_3$) production besides to induce MAPK cascade activation and NF-kB phosphorylation [74, 75]. Similar mechanism is activated on fibroblasts after kinin receptor stimulation. The evoked mechanism involves calcium mobilization; phospholipases A, C, and D activation; and PKC translocation to the plasma membrane [74, 76].

Concerning the keratinocytes role, a study recently published by our group showed that the lack of kinin receptor B2 in genetic modified mice promoted the reduction of the re-epithelialization in an excision skin wound model. On this study, while B2 receptors seem to be closely related to keratinocytes activity, kinin receptor B1 knockout mice did not differ from control mice on re-epithelialization response [54]. Desposito et al. (2016) found distinct results and collaborators in a study using an excision wound model on diabetic and nondiabetic mice. According to their results, B2 receptor agonist reduced wound closure rate on diabetic and nondiabetic mice, while a B2 receptor antagonist improved wound healing on diabetic mice but did not change healing response on normal mice. Histological analysis showed that B2R agonist increased epidermal thickness on normal mice, while on diabetic mice where epidermal thickness is already increased, the antagonism of B2R promoted reduction of this parameter. In vitro experiments using cell lines of human keratinocytes demonstrated that B2 receptor agonist stimulated migration and proliferation of keratinocytes in a mechanism dependent of ERK1/2 phosphorylation [77]. Although controversial, both studies

have shown modulation of keratinocytes behavior trough B2 receptor activation. It seems like B2R is important for physiological wound healing response, but on unusual situations such as hyperglycemic condition when B2 receptor is overexpressed, the hyperstimulation of the receptor pathway triggers deleterious effects to the wound healing process.

Indeed the modulation of keratinocytes behavior by kinins has been suggested by few other studies, which basically show that kinin receptors induce migration and differentiation of keratinocytes but do not change cell proliferation. The migratory activity seems to be stimulated by B1 receptor as demonstrated in cultured keratinocytes where BK treatment promoted actin network rearrangement and induced cell migration in a mechanism involving AA metabolites and tyrosine kinase activity [78]. Matus and colleagues also have showed that the B1 receptor agonist Lys-des-Arg^9-BK induced keratinocyte migration in vitro without changing cell proliferative activity. In the same study, the treatment of mice skin excisions with the B1 receptor agonist reduced the wound area when compared with control groups. B1 receptor stimulation also promoted expression of the gelatinases MMP-2 and MMP-9 on keratinocytes in culture [79]. MMP-2 is constitutively expressed in normal keratinocytes and is related to cell survival, while MMP-9 is usually upregulated in inflammatory situations and is involved in keratinocytes migration since it degrades collagen IV from basement membrane and so contributes to the migratory activity during the re-epithelialization process [69]. According to these studies, Lys-des-Arg^9-BK effects were connected with a transactivation of EGF receptors consequent to the B1 receptor activation [79]. There is less information about kinin-induced keratinocytes differentiation, but B2 receptors stimulation by Lys-BK on primary human keratinocytes triggered differentiation response as observed by the increase of the expression of markers such as profilaggrin [75]. Also, B1 receptor activation on primary keratinocytes induces expression of filaggrin, CK10, and involucrin [79]. Filaggrin and CK10 are early differentiation markers which presence indicates activated keratinocytes trying to recover the outer layers of the epidermis in order to guarantee the barrier function. Chronic non-healing wounds are frequently characterized by reduced levels of these two markers [80]. So, the modulation of keratinocyte differentiation and induction of keratinocyte migration support the important role played by kinins in the re-epithelialization process.

Fibroblasts are also target of kinins during proliferative phase of wound healing responses. Kinins are known to modulate fibrogenic activity in different manners according with the system, organ, or situation involved. There is no doubt that kinins can influence matrix extracellular production and fibroblasts differentiation; however, their effects are controversial and can end up in either positive or negative control of fibrogenic responses. In the airways, for instance, kinins participate in the pathophysiology of several obstructive diseases by stimulating lung fibroblasts to produce connective tissue. On human lung fibroblasts, the treatment with BK induces an increase in alpha-SMA expression suggesting the induction of cell differentiation into the cell contractile phenotype myofibroblast [28, 81]. This cellular transformation is confirmed by the contraction of collagen matrix gels played by fibroblasts after treatment with BK [81, 82]. Besides, kinins induce collagen synthesis and cell proliferation on lung cells [28, 83, 84], and both kinin receptors seem to participate of these responses [82, 84]. B1 receptor is known to be overexpressed in fibrotic and chronic inflammatory disorders. In lung fibroblasts B1 receptor is responsible not only to induce collagen production but also to increase connective tissue growth factor (CTGF) mRNA levels [84]. Another interesting feature on airways fibrosis process is that prostaglandins seem to be a counterbalance to the fibrogenic effect of kinins, and these two systems work together mediating connective tissue maintenance [83]. While acting as fibrogenic agents on respiratory system, kinins play exactly the opposite effect on the kidneys preventing from extracellular matrix expansion commonly seen on chronic renal diseases. Actually, when applied to normal and not stimulated mesangial cells, BK

is able to induce proliferation and collagen secretion. However, in fibrotic situations when mesangial cells are already actively working toward fibrogenesis, BK shows protective anti-fibrotic effect. For instance, platelet-derived growth factor (PDGF) is known to induce proliferation and ECM production and contribute to the fibrosis in glomerulosclerosis. When applied together with PDGF, BK inhibits the increase of cell proliferation and collagen expression stimulated by this growth factor in rat immortalized mesangial cells [85]. On primary rat mesangial cells, BK reduces collagen types I and IV production which is induced by different stimulus such as high glucose, EGF, or TGF-beta. Besides, in vivo experiments on diabetic rats showed that the absence of B2R aggravated mesangial matrix expansion, and this event was prevented by treatment with the angiotensin I-converting enzyme inhibitor (ACEi) ramipril, in a mechanism involving ERK 1/2 phosphorylation and Akt pathway activation [86]. Similar results were found with primary human mesangial cells, where the ACE omapatrilat demonstrated anti-fibrotic effects reducing the expression of fibronectin and increasing the expression of MMP-9 suggesting that kinin system could also have a participation on the remodeling phase of wound healing response [87]. On cardiovascular system the role of kinins is even more controversial, albeit most of the evidences show that kinin stimulation of cardiac fibroblasts promotes the reduction of ECM production. Activation of kinin receptors on cardiac fibroblasts and myofibroblasts reduces the synthesis of collagen types I and III and fibronectin without changing the proliferative state of the cells [88–91]. This anti-fibrotic effect seems to be mediated mainly by B2 receptors in a mechanism related to NO, cGMP, and prostaglandins production [89, 91]. In cardiac fibroblasts from spontaneously hypertensive rats, treatment with BK after IL-1b stimulation attenuates the greater expression of pro-collagen [90]. It sounds like that in situations where the tissue is already under a basal level of stimulation by inflammatory and/or fibrogenic molecules, as in cases of hypertension; kinins can protect the tissue from remodeling and fibrosis. However, kinins are likely to be exerting positive influence on the fibrosis and scarring responses that follow tissue injuries. In a rat model of wound healing after myocardium infarction, HOE 140, an antagonist of B2 receptors, reduced the number of myofibroblasts and collagen accumulation. This effect could be explained by the well-known inflammatory effect of kinins. So, in this injury situation, kinins would be stimulating early inflammatory cells such as mast cells, and the activation of these cells are known to induce fibrotic responses [92]. Therefore, on cardiovascular system, kinins can be considered protective agents against chronic fibrotic diseases, while they seem to be fibrogenic in the early development of fibrotic tissue following tissue injuries.

During the proliferative phase, the expected function from fibroblasts is exactly to start the replacement of the dermal tissue, which in the beginning implicates mainly in the production of ECM components such as collagen and fibronectin. Although the evidences on the literature make clear the ability of kinins to influence fibroblasts ECM production and differentiation, few studies were performed in skin fibroblasts. In the study of Soley and colleagues, they verified that the healing of excisional wounds on mice lacking B2 receptors was characterized by the reduction of hydroxyproline contents, cell proliferation, and myofibroblast differentiation. Once more these results confirm that kinin has fibrogenic activity after tissue injuries. It means that in regeneration responses when the fibroblasts need to produce a new tissue, kinins seem to be necessary as positive modulators for the correct production of ECM, proliferation, and activation of fibroblasts [54]. There are not many other studies with skin fibroblasts to corroborate these finds, and when comparing with fibroblasts from other tissues, the facts can be contradictory. Indeed, some in vitro tests have been performed to investigate the direct effect of kinins on fibroblasts proliferation. On murine 3T3 fibroblasts, B2 receptor antagonist did not change cell proliferation, while B2 receptor agonist reduced fibroblasts proliferation without altering cellular migratory capacity [77]. On human gingival fibroblasts, BK did not change proliferation when exclusively applied;

however it inhibited the proliferation induced by EGF or PDGF. This effect was mediated by an increase in the PGE2 levels. In this case, it seems like BK levels at wound site act also as a negative modulator of the early proliferation in order to balance with the positive modulators broadly released in the beginning of the healing process [93]. On the other hand, BK-induced proliferation of primary fibroblasts isolated from the nasal mucosa of patients with chronic rhinosinusitis [94]. Kinin proliferative effect was also observed in corneal fibroblasts where BK induces proliferation in a well-delineated mechanism, which involves PKC, Akt, and p42/p44 MAPK activation [95]. Although scarce and many times confusing, most of the evidences follow the idea that when applied after a fibrogenic or inflammatory stimulus, kinins seem to prevent from fibrosis. However, if analyzing the role of kinins after an injury, they show fibrogenic activity during the regeneration responses in most of the tissues.

Conclusions

Kinin signaling has a positive effect in different stages of wound healing. There are many indications that activation of B1 and B2 receptors causes vasodilation and influences the recruitment of neutrophils and macrophages to the injured tissue. These events are related to the release of other mediators such as prostaglandins, cytokines, and the expression of adhesion molecules. Concerning angiogenesis, kinins stimulate the release of VEGF acting on epithelial and endothelial cells, promoting the maturation of the epidermis and restoration of an efficient barrier. In addition, on fibroblasts, kinins increase ECM production and differentiation into myofibroblasts. On keratinocytes, kinins induce migration and release of MMP and so contribute for the closure of the wound. Thus, the kinin system may be an interesting new target for the modulation of problems in skin healing, such as in diabetes and immunosuppressed patients. However, new preclinical and clinical studies should be performed in order to confirm its clinical importance.

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