

Lars-Peter Kamolz · David Benjamin Lumenta *Editors*

# Dermal Replacements in General, Burn, and Plastic Surgery

Tissue Engineering  
in Clinical Practice

 Springer

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Lars-Peter Kamolz • David Benjamin Lumenta  
Editors

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Tissue Engineering in Clinical Practice

 Springer

*Editors*

Lars-Peter Kamolz, MD, MSc  
Division of Plastic  
Aesthetic and Reconstructive Surgery  
Research Unit for Tissue Regeneration  
Repair and Reconstruction  
Department of Surgery  
Medical University of Graz  
Graz  
Austria

David Benjamin Lumenta, MD  
Division of Plastic  
Aesthetic and Reconstructive Surgery  
Research Unit for Tissue Regeneration  
Repair and Reconstruction  
Department of Surgery  
Medical University of Graz  
Graz  
Austria

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Gerd G. Gauglitz and Jürgen Schauber

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## 1.1 Introduction

The skin is the largest organ of the human body. It covers approximately 1–2 m<sup>2</sup> of surface area and accounts for around 12–16 % of an adult's body weight. In direct contact with the outside environment, the skin provides the following essential functions:

- Retention of moisture and prevention of loss of other molecules
- Barrier function against harmful external influences
- Immune function and protection of the body from microbes
- Sensory function (e.g. heat, cold, touch, pressure, vibration, tissue injury)
- Endocrine function (e.g. vitamin D production)
- Regulation of body temperature

To understand cutaneous biology, wound healing and skin diseases, it is critical to be aware of the defined structures and functions of normal human skin. Human skin consists of a stratified, cellular epidermis and an underlying dermis of connective tissue which together form the cutis (Fig. 1.1).

The dermal–epidermal junction is undulating and ridges of the epidermis, known as rete ridges, project into the dermis. The junction provides mechanical support for the epidermis and acts as a partial barrier against larger molecules.

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G.G. Gauglitz, MD, MMS (✉)

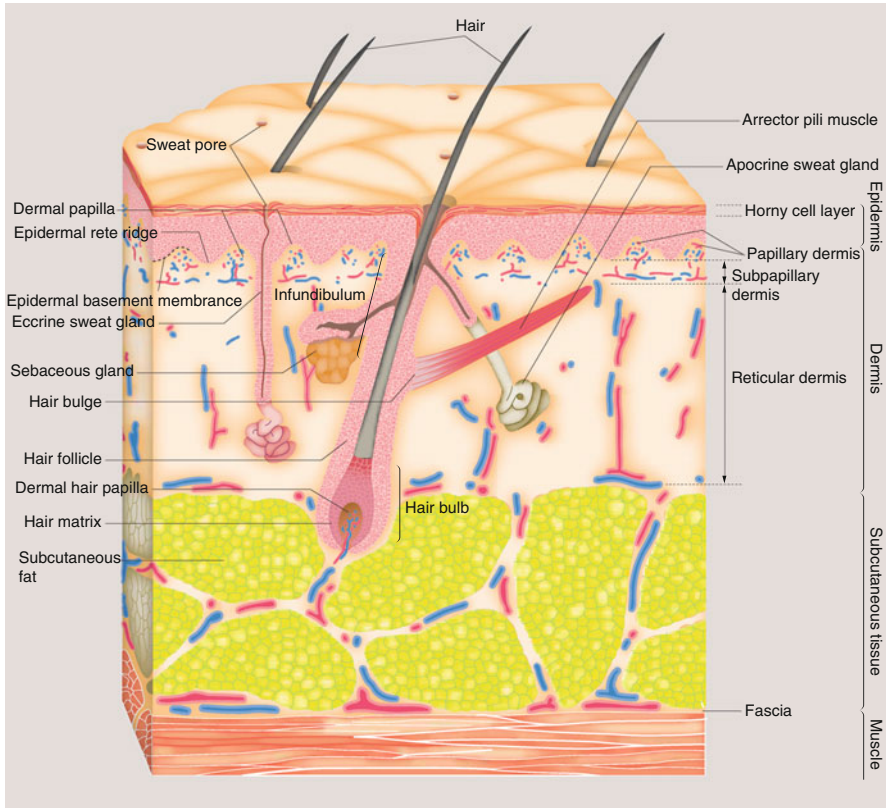
Department of Dermatology and Allergy, Ludwig Maximilian University,  
Munich, Germany

Scar Clinic, Department of Aesthetics, Department of Infectious and  
Sexual Transmitted Diseases, Department of Dermatology and Allergy,  
Ludwig Maximilian University,  
Frauenlobstr. 9-11, Munich 80337, Germany  
e-mail: gerd.gauglitz@med.uni-muenchen.de

J. Schauber, MD

Department of Dermatology and Allergy, Ludwig Maximilian University,  
Munich, Germany





**Fig. 1.1** Structure of the skin (From Nakagawa 2001)

Below the dermis, a fatty layer, the panniculus adiposus, exists, usually designated as ‘subcutaneous fat’. It is separated from the rest of the body by a vestigial layer of striated muscle, the panniculus carnosus (Fig. 1.1).

Two variants of human skin can be differentiated: Glabrous skin (non-hairy skin), found on the palms and soles, is grooved and its surface shows individually unique configurations by continuously alternating ridges and sulci, known as dermatoglyphics. It is characterised by a thick epidermis divided into several well-marked layers including a compact stratum corneum, by the presence of encapsulated sense organs within the dermis and by a lack of hair follicles and sebaceous glands. Hair-bearing skin, on the other hand, has both hair follicles and sebaceous glands but lacks encapsulated sense organs. Hair-bearing skin shows a wide variation between different body sites.

The motor innervation of the skin is autonomic and includes a cholinergic component to the eccrine sweat glands and adrenergic components to both the eccrine and apocrine glands, to the smooth muscle and the arterioles and to the arrector pili muscle. Several sensory nerves are found in skin: Some show free endings, some terminate in hair follicles and others have expanded tips. Only in glabrous skin some nerve endings are encapsulated.

## 1.2 Epidermis

The epidermis is the outer layer of the skin and acts as the body's major barrier against a hostile environment. The epidermis is a multilayered epithelium composed of 4–5 layers depending on the respective region of skin. In humans, it is thinnest on the eyelids and thickest on the palms and soles. The layers in ascending order are the basal/germinal layer (stratum basale/germinativum), the spinous layer (stratum spinosum), the granular layer (stratum granulosum), the clear/translucent layer (stratum lucidum) and the cornified layer (stratum corneum).

The epidermis is aneural and avascular, nourished by diffusion from the dermis, and contains keratinocytes, melanocytes, Langerhans cells, Merkel cells and inflammatory cells. Keratinocytes are the major resident cells constituting 95 % of the epidermis.

### 1.2.1 Stratum Germinativum or Basal Layer

The innermost layer of the epidermis which lies adjacent to the dermis comprises mainly dividing and non-dividing keratinocytes which are attached to the basement membrane by hemidesmosomes. As keratinocytes divide and differentiate, they move from this deeper layer to the surface. Melanin-producing melanocytes make up a small proportion of the basal cell population. These cells are characterised by dendritic processes which stretch between relatively large numbers of neighbouring keratinocytes (Bensouilah and Buck 2006). Merkel cells are also found in the basal layer predominantly in touch-sensitive sites such as the fingertips and lips. They are closely associated with cutaneous nerves and seem to be involved in light touch sensation.

### 1.2.2 Stratum Spinosum

As basal cells reproduce and mature, they move towards the outer layer of skin, initially forming the stratum spinosum. Intercellular bridges, the desmosomes, which appear as 'prickle' at a microscopic level, connect the cells. Langerhans cells are dendritic, immunologically active cells derived from the bone marrow and are found on all epidermal surfaces but are mainly located in the middle of this layer. They play an important role in immune reactions of the skin, acting as antigen-presenting cells (Bensouilah and Buck 2006).

### 1.2.3 Stratum Granulosum

Continuing their transition to the surface the cells continue to flatten, lose their nuclei and their cytoplasm appears granular at this level.

### 1.2.4 Stratum Lucidum

The stratum lucidum represents a layer of 3–4 rows of dead flat transparent cells and is only found in the skin of palms and soles.

### 1.2.5 Stratum Corneum

The final result of keratinocyte maturation is found in the stratum corneum which is made up of layers of hexagonal-shaped, non-viable cornified cells also known as corneocytes. In most areas of the skin, there are >10 layers of stacked corneocytes with the palms and soles having the thickest stratum corneum. Each corneocyte is surrounded by a protein envelope and is filled with water-retaining keratin proteins. The cellular shape and orientation of the keratin proteins adds strength to the stratum corneum (Bensouilah and Buck 2006). Surrounding the cells in the extracellular space are stacked layers of lipid bilayers. The resulting structure provides the natural physical and water-retaining barrier of the skin.

The different epidermal compartments undergo constant cellular turnover to replace dead or damaged cells. This homeostatic process is thought to involve several types of stem cells each located in a specific epidermal region and contributing to the maintenance of a discrete compartment of the skin (Blanpain and Fuchs 2009). In addition to their self-renewing capacity and multipotency, these cells are quiescent with a low tendency to divide, but upon injury are characterised by an extensive and sustained self-renewal capacity (Tiede et al. 2007).

The epidermal turnover time is about 1 month. The keratinocytes reach the interface of the granular layer and the stratum corneum after about 2 weeks. Another 2 weeks are normally required for the corneocytes to reach the surface of the stratum corneum and be shed into the environment. In psoriasis, this turnover time is dramatically decreased. Derangements in the process of keratinisation are responsible for a wide variety of disorders, including ichthyoses and palmoplantar keratoderma.

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## 1.3 Intercellular Junctions

Several types of cellular junctions link adjacent keratinocytes and are responsible for mechanical biochemical and signalling interactions between cells. These include desmosomes, adherens junctions, gap junctions and tight junctions (McGrath et al. 2010).

*Desmosomes* represent the major adhesion complex in the epidermis, anchoring keratin intermediate filaments (IFs) to the cell membrane and bridging adjacent keratinocytes, thus allowing cells to withstand trauma. The main components of desmosomes consist of the products of three gene superfamilies: the desmosomal cadherins, the armadillo family of nuclear and junctional proteins and the plakins (Breathnach 1971).

*Adherens junctions* are electron dense transmembrane structures that associate with the actin skeleton, part of the keratinocyte filament network concerned with cell motility, changes in cell shape and cell interactions. The transmembrane component of adherens junctions is E-cadherin, which forms calcium-dependent homophilic adhesive interactions with E-cadherin on opposing cells (McGrath et al. 2010).

*Gap junctions* comprise clusters of intercellular channels, also known as connexons, that directly form connections between the cytoplasm of adjacent keratinocytes (and other cells) (Kelsell et al. 2001a, b).

*Intercellular (tight) junctions* are the major regulators of permeability in simple epithelia, but they are also present in skin, with a key role in skin barrier integrity (Furuse et al. 2002). Tight junctions, including those linking keratinocytes, are composed of transmembrane and intracellular molecules that include occludin, junction adhesion molecule and claudins (Sato et al. 1977). Next to controlling permeability, tight junctions also play a role in maintaining cell polarity. Claudins may regulate epidermal permeability either through formation of tight junctions or via direct binding to certain transcription factors (McGrath et al. 2010).

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## 1.4 Dermis

The dermis is a layer of skin between the epidermis and subcutaneous tissues and is composed of two layers, the papillary and reticular dermis. The dermis is responsible for the bulk of the skin, as it is far thicker than the epidermis. It also significantly varies in thickness, ranging from 0.6 mm on the eyelids to 3 mm on the back, palms and soles (Bensouilah and Buck 2006).

The basis of the dermis is a supporting matrix or ground substance in which polysaccharides and protein are linked to produce macromolecules with a remarkable capacity for retaining water. Within and associated with this matrix are two kinds of protein fibre: collagen, which has great tensile strength and forms the major constituent of the dermis, and elastin, which makes up only a small proportion of the bulk. The papillary dermis contains mostly thin loosely arranged collagen fibres. Thicker bundles of collagen run parallel to the skin surface in the deeper reticular layer, which extends from the base of the papillary layer to the subcutaneous tissue. The cellular constituents of the dermis include fibroblasts, which produce collagen, elastin and structural proteoglycans, together with immunocompetent histiocytes (monocyte/macrophages). Embedded within the fibrous tissue of the dermis are the dermal vasculature, lymph vessels, nerve cells and fibres, sweat glands, hair roots and small quantities of striated muscle.

The vessels serve as the site of many inflammatory reactions, as the host immune cells migrate into the dermis and encounter invading pathogens, whether organisms, foreign antigens or other factors. The dermis has a very rich blood supply, although no vessels pass through the dermal–epidermal junction.

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## 1.5 Epidermal–Dermal Junction

The epidermis and dermis are held together by a very complex region known as the epidermal–dermal junction (Braun-Falco et al. 2005). Ultrastructurally, the epidermal–dermal junction is composed of four component areas: (1) the basal cell plasma membrane with its specialised attachment devices or hemidesmosomes; (2) an electron-lucent area, the lamina lucida; (3) the basal lamina; and (4) the sub-basal lamina fibrous components, including anchoring fibrils, dermal microfibril bundles and collagen fibres. The light microscopic ‘basement membrane’ comprises only the sub-basal lamina fibrous zone. Other cell types, including melanocytes and Merkel cells, are also found at the epidermal–dermal junction. Structures at the junction derive from the epidermis and dermis: The basal lamina is primarily of epidermal origin, the anchoring fibrils of dermal origin. The junction serves the following functions: (1) epidermal–dermal adherence, (2) mechanical support for the epidermis and (3) a barrier to the exchange of cells and of some large molecules across the junction (Briggaman and Wheeler 1975). The structure is highly irregular, with dermal papillae from the papillary dermis projecting perpendicular to the skin surface. Congenital lack of some of the elements leads to fragile skin; epidermolysis bullosa or bullous skin diseases results. In addition, in adults, there may be damage to the epidermal–dermal junction as a result of the production of autoantibodies against various component structures or because of intense inflammation in the region, as can be seen with lichen planus or lupus erythematosus. The epidermal–dermal junction also flattens during ageing which accounts in part for some of the visual signs of ageing skin.

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## 1.6 Subcutis

The subcutis is made up of loose connective tissue and fat which can be up to 3 cm thick on the abdomen. The subcutaneous fat serves as protective padding and a reservoir of energy. Types of cells that are found in the subcutis are fibroblasts, adipose cells and macrophages. To date, adipose tissue has been identified as a source of multipotent cells which have characteristics similar to bone marrow-derived stem cells (BM-MSCs) (Kim et al. 2007) and have the capacity to differentiate to cells of adipogenic, chondrogenic, myogenic and osteogenic lineages when cultured with the appropriate lineage-specific stimuli (Zuk et al. 2001, 2002; Kim et al. 2006). Adipose-derived stem cells (ADSCs) can be obtained from the processing of either liposuctioned or excised fat. Given their convenient isolation compared with BM-MSCs and extensive proliferative capacities *ex vivo*, ADSCs hold great promise for the usage in wound repair and regeneration.

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## 1.7 Blood and Lymphatic Vessels

The dermis has a rich blood supply. A superficial artery plexus is formed at the papillary and reticular dermal boundary by branches of the subcutis artery. Branches from this plexus form capillary loops in the papillae of the dermis, each with a single

loop of capillary vessels, one arterial and one venous. The veins drain into mid-dermal and subcutaneous venous networks. Dilatation or constriction of these capillary loops plays a direct role in thermoregulation of the skin. Lymphatic drainage of the skin occurs through abundant lymphatic meshes that originate in the papillae and feed into larger lymphatic vessels that drain into regional lymph nodes.

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## 1.8 Nerve Supply

The skin has a rich innervation with the hands, face and genitalia having the highest density of nerves.

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## 1.9 Derivative Structures of the Skin

### 1.9.1 Hair Follicles

The hair follicles comprise pockets of epithelium that are continuous with the superficial epidermis. They undergo intermittent activity throughout life. During the active phase, the follicle forms a small papilla of dermis at its base (McGrath et al. 2010). Each hair follicle is lined by germinative cells which produce keratin and melanocytes which synthesise pigment. The hair shaft consists of an outer cuticle, a cortex of keratinocytes and an inner medulla. The root sheath, which surrounds the hair bulb, is composed of an outer and inner layer (Bensouilah and Buck 2006). An erector pili muscle is associated with the hair shaft and contracts with cold temperatures, fear and emotion to pull the hair erect, giving the skin ‘goose bumps’. Above the insertion, the holocrine sebaceous gland opens by a short neck into the pilary canal, and some follicles in certain areas of the body, notably the axilla, have, in addition, an apocrine gland (McGrath et al. 2010). Also derived from the epidermis, and opening directly to the skin surface, are the eccrine sweat glands, present in every region of the body in densities of 100–600/cm<sup>2</sup> (McGrath et al. 2010).

The hair follicle becomes quite complex, acquiring multiple layers with delicate epithelial–mesenchymal interactions controlling the cyclic pattern of hair growth. They change from infantile lanugo hairs to vellus and terminal hairs, such as those on the scalp. Later, under primarily hormonal influence, terminal hairs develop in other regions, such as axilla, the groin, the male beard region and on the trunk and limbs, exceptions being on the palms, soles and glans penis.

The first evidence that skin stem cells can differentiate into interfollicular epidermis, sebaceous gland and hair follicle lineages came from transplantation of bulge stem cells – a cell population located at the base of hair follicles (Blanpain 2010). Using transplantation of the murine bulge region, Oshima et al. (2001) demonstrated that bulge cells could repopulate the epidermis, sebaceous glands and the epithelial layers of the hair follicle. Roh and Lyle (2006) showed that stem cells extracted from the human bulge region can be induced to exhibit hair follicle differentiation and form epidermal and sebaceous cells in vitro as well as form an epidermis and sebaceous cells in vitro, thus supporting the multipotential capacity of human epidermal stem cells.

### 1.9.2 Nails

Nails and claws of mammals are specialised epidermal derivatives which protect the delicate tip of fingers and toes against trauma and act as tools or weapons. Human fingernail gross anatomy consists of three structures. Starting from the outer structure, there are the nail plate, the nail bed and the nail matrix. The nail plate is a thin (0.25–0.6 mm for fingernails and up to 1.3 mm for toenails), hard, yet slightly elastic, translucent, convex structure and is made up of approximately 25 layers of dead keratinised, flattened cells. They are tightly bound to one another via numerous intercellular links, membrane-coating granules and desmosomes, which are cell structures specialised for cell-to-cell adhesion and randomly arranged on the lateral sides of plasma membranes. Fingernails grow at 0.1 mm/day, the toenails more slowly (Braun-Falco et al. 2005).

### 1.9.3 Sebaceous Glands

The sebaceous glands are large at birth, then involute but become prominent once again in puberty. The main function of the sebaceous glands is to provide lipids in order to maintain a lipid film on the skin surface.

These glands are derived from epidermal cells and are closely associated with hair follicles, especially those of the scalp, face, chest and back; they are not found in hairless areas. They are small in children, enlarging and becoming active at puberty, being sensitive to androgens. In adolescence, the sebaceous follicles are the site of inflammation in acne.

### 1.9.4 Eccrine Glands

Eccrine glands are found all over the skin, especially on the palms, soles, axillae and forehead. They are primarily temperature-controlling glands. They are under extensive neural regulation and excrete sweat, during physical activity, excess temperature or emotional stress. Wetting the skin surface also improves one's grip, as often needed in sports or manual labour. Some medications are concentrated in eccrine sweat and reach the skin through this route, in a sprinkler effect.

### 1.9.5 Apocrine Glands

Apocrine glands are larger, the ducts of which empty out into the hair follicles. In humans, apocrine glands are concentrated in the axillae, perianal region and genitalia and are under thermal control. They become active at puberty, producing an odourless protein-rich secretion which when processed by skin bacteria produces a characteristic odour. These glands are under the control of sympathetic (adrenergic) nerve fibres.

## 1.10 Skin Functions

The skin is a complex metabolically active organ, which provides the following important physiological functions:

### 1.10.1 Skin Barrier Function

The epidermis functions as the primary defence layer to the external environment and includes epidermal cells and a cornified layer. Most of the barrier function is provided by the stratum corneum and the tight junction which can be found at the level of the stratum granulosum. Extracellular stratum corneum lipids also play a key role in limiting the diffusion of compounds through the stratum corneum. Lipids can be found in bilamellar structures in the stratum corneum extracellular space, each layer separated from the other by a thin water sheet associated with the polar head group of the lipids. The intercellular lipids are organised into lamellae, which run parallel to the skin surface. Intercellular lipid is required for a competent skin barrier and forms the only continuous domain in the stratum corneum.

### 1.10.2 UV Protection

Melanocytes, located in the basal layer, and melanin have important roles in the skin's barrier function by preventing damage by UV radiation. Melanin is also the main determinant of skin colour, distinguishing between skin types I–VI in whites, orientals and blacks. Melanocytes produce melanin and transfer it to adjacent keratinocytes where it remains in granules. In the inner layers of the epidermis, melanin granules form a protective shield over the nuclei of the keratinocytes; in the outer layers, the granules are more evenly distributed. Melanin absorbs UV radiation, thus protecting the cell's nuclei from DNA (deoxyribonucleic acid) damage.

Chronic exposure to light increases the ratio of melanocytes to keratinocytes, so more are found in facial skin compared to the lower back and a greater number on the distal arm compared to proximal arm. The number of melanocytes is the same in equivalent body sites in white and black skin but the distribution and rate of production of melanin is different. Intrinsic ageing diminishes the melanocyte population. Some individuals lack enzymes needed to produce melanin and thus have pale skin; these individuals are known as albinos. In other individuals, melanocytes are either absent at birth or later disappear from the area of skin, causing depigmentation (a condition called vitiligo).

### 1.10.3 Thermoregulation

The skin plays an important role in maintaining a constant body temperature through changes in blood flow in the cutaneous vascular system and evaporation of sweat from the surface (Bensouilah and Buck 2006).



### 1.10.4 Immunological Surveillance

Skin also plays an important immunological role. It normally contains all elements of innate and adaptive immunity, with the exception of B cells.

### 1.10.5 Sense and Neurological Functions in Skin

The skin contains a variety of nerve endings that jump to heat and cold, touch, pressure, vibration and tissue injury. All cutaneous nerves have their cell bodies in the dorsal root ganglia, and both myelinated and non-myelinated fibres are found. Free sensory nerve endings lie in the dermis where they detect pain, itch and temperature. Specialised corpuscular receptors also lie in the dermis allowing sensations of touch (perceived by Meissner's corpuscles) and pressure and vibration (by Pacinian corpuscles). The autonomic nervous system supplies the motor innervation of the skin: Adrenergic fibres innervate blood vessels, hair erector muscles and apocrine glands, while cholinergic fibres innervate eccrine sweat glands. The endocrine system regulates the sebaceous glands which are not innervated by autonomic fibres.

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Maïke Keck, David Benjamin Lumenta,  
and Lars-Peter Kamolz

The skin is the largest organ in the body making up 16 % of body weight, with a surface area of 1.8 m<sup>2</sup>. Because it interfaces with the environment, skin primarily serves as a protective barrier allowing and limiting the inward and outward passage of water, electrolytes and various substances while providing protection against microorganisms, ultraviolet radiation, toxic agents and mechanical insults. An injury of large portions of skin, as in severely burned patients, may result in significant disability or even death. Limitations in the use of autografts and local and free flaps in patients with skin and soft tissue loss have led to the development of tissue-engineered skin. The term tissue engineering came up in 1987 as a result of combining knowledge from the field of engineering and biology to create bioartificial tissue for regenerative medicine.

The development of a bioartificial skin would facilitate the treatment of patients with deep burns and various skin-related disorders, but this approach also has to meet high demands with respect to the interaction of the bioartificial devices and the natural tissues of the human body. A multidisciplinary approach including experts in biomaterial development, cell–matrix interaction, angiogenesis, tissue engineering, simulation, design and fabrication methods is mandatory to succeed in this complicated field.

Adult skin consists of three layers: a keratinised, stratified epidermis, an underlying layer of collagen-rich dermal connective tissue, and subcutaneous adipose tissue (hypodermis). Appendages such as hair and glands are derived from the epidermis,

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M. Keck, MD (✉)  
Division of Plastic and Reconstructive Surgery,  
Department of Surgery, Medical University Vienna,  
Vienna, Austria  
e-mail: maïke.keck@meduniwien.ac.at

D.B. Lumenta, MD • L.-P. Kamolz, MD, MSc  
Division of Plastic, Aesthetic and Reconstructive Surgery,  
Research Unit for Tissue Regeneration, Repair and Reconstruction,  
Department of Surgery, Medical University of Graz,  
Graz, Austria

but they project deep into the dermal layer. The epidermis is thin and totally cellular. The epidermis is also exposed to ultraviolet radiation, and the resulting damage is one of the factors contributing to the constant sloughing of cells from the stratum corneum, which are replaced by migrating cells from the basal layers (Alonso and Fuchs 2003).

The dermis consists of collagen with some elastin and glycosaminoglycans. Fibroblasts present the major cell type of the dermis and play a major role in wound healing since they are capable of producing remodelling enzymes such as proteases and collagenases. The hypodermis is composed mainly of fat and loose connective tissue and contributes mostly to mechanical as well as thermoregulatory properties of the skin.

Great efforts have been made to develop dermal and epidermal replacements in order to overcome poor skin quality and subsequent scarring. Far less attention has been paid to the reconstruction of the hypodermis. So far, there are no established models of artificial skin constructs that entirely replicate normal uninjured skin (Priya et al. 2008). Skin substitute products that are currently on the market differ in their five main characteristics: their anatomical structure (epidermal, dermal, composite), the duration of coverage (temporary, permanent), the type of biomaterial (natural, synthetic), their use for *in vitro* or *in vivo* tissue engineering and if the product is cellular or acellular. Conventionally, tissue-engineered skin exists when cells cultured *in vitro* are subsequently seeded onto a scaffold which is then placed *in vivo* at the site of injury.

The three essential components of skin tissue engineering are:

1. Scaffold
2. Type of applied cells
3. Environmental conditions

In conventional approaches of skin tissue engineering, these three factors are constantly being modified.

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## 2.1 Scaffold

In the context of skin tissue engineering, cells can be seeded onto scaffolds in order to avoid “cell slipping” and stabilise their adherence for future grafting. Especially collagen scaffolds can also mimic the dermal layer of the artificial skin. The use of carriers also allows for a better determination of cell survival, cell differentiation, extracellular matrix generation, overall host integration and scaffold vascularisation (Stillaert et al. 2008; Hemmrich et al. 2006). Besides biocompatibility and mechanical stability, an adequate porous structure of the matrix is crucial for cell differentiation and successful integration. An ideal matrix should be completely degradable and ultimately be replaced by host tissue with close to normal properties. Therefore, a three-dimensional (3-D) scaffold in the shape of the tissue that needs to be replaced is an ideal approach (Stock and Vacanti 2001). Long-term function of 3-D tissue constructs depends on adequate vascularisation after implantation. To achieve vascularisation, modification of biomaterial properties of the scaffold is crucial by

creating microvascular networks within 3-D tissue constructs *in vitro* before implantation (Laschke et al. 2006).

Materials for a scaffold could be naturally occurring substances such as collagen or could be prepared from biodegradable polymers. Resorption, along with adequate cell adhesion onto the matrix surface, gives the biological materials an attractive potential in tissue regeneration (Metcalf and Ferguson 2007). To mimic the quality of autologous skin, a number of natural materials are widely preferred for tissue-engineering applications. Examples of natural materials include polypeptides, hydroxyapatites, hyaluronan, glycosaminoglycans, fibronectin, collagen, chitosan and alginates. Such materials have the advantage that they have low toxicity and a low chronic inflammatory response. Examples of synthetic materials include polyglycolide, polylactide and polylactide coglycolide, polytetrafluoroethylene and polyethylene terephthalate. Synthetic polymers generally show better mechanical stability and slower degradation than natural materials. A major challenge creating the ideal scaffold for skin tissue engineering is to define the shape and porous structure that can direct tissue (Griffith and Schwartz 2006). New technologies such as three-dimensional printing (Mironov et al. 2003; Seitz et al. 2005) and electrospinning are aiming to create a defined architecture of the constructs (Luu et al. 2003). At the same time, these new polymers must be free of any toxic or immunogenic potential. A major task to overcome in the use of synthetic materials is the lack of cell-recognition signals. Incorporating cell-adhesion peptides into synthetic scaffolds could lead to better cellular interactions.

Collagen and fibrin are the most common natural materials used for skin tissue engineering. Collagen naturally exists in the skin where it is produced mainly by fibroblasts. It makes up approximately 70 % of the dry weight of skin (Jin et al. 2011). There are 20 different types of collagen-forming fibrils (I, II, III, V, XI) or networks (IV). Collagen has frequently been used for skin tissue engineering. Hudo et al. (2003) and Tremblay et al. (2005) were even able to create a model of endothelialised, reconstructed dermis that promotes the spontaneous formation of a human capillary-like network (Hudo et al. 2003; Tremblay et al. 2005). Butler and Orgill (2005) describe a tissue-engineering technique that combines disaggregated autologous keratinocytes and a highly porous, acellular collagen–glycosaminoglycan matrix that has been shown in a porcine model to regenerate dermis and epidermis *in vivo* (Butler and Orgill 2005). Simultaneous growth of preadipocytes and keratinocytes on a three-dimensional collagen matrix could also shown to be successful (Keck et al. 2011). Electrospun collagen promotes cell growth and penetration of cells into the engineered matrix (Matthews et al. 2002). The concept of using an electrospinning array to form multicomponent nanofibrous membranes is what tissue engineers are working on at the moment. These novel scaffolds for tissue-engineering applications could offer much better opportunities for the ingrowth of cells of different lineages such as preadipocytes, endothelial cells, fibroblasts and keratinocytes.

Fibrin is the other natural material frequently used in skin tissue engineering. During the initial phase of wound healing, endogenous fibrin is known to form a provisional matrix to promote angiogenesis. Growth factors such as vascular

endothelial growth factor (VEGF) increase in wounds to stimulate angiogenesis. This secretion of VEGF could also be shown when fibrin was used as a dermal substrate for cultured skin substitutes (Hojo et al. 2003). Fibrin, associated with fibronectin, has been shown to support keratinocyte and fibroblast growth both in vitro and in vivo and may enhance cellular motility in wounds (Currie et al. 2001). When used as a delivery system for cultured keratinocytes and fibroblasts, fibrin glue may provide similar advantages to those proven with conventional skin grafts (Currie et al. 2001; Kamolz et al. 2005).

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## 2.2 Cells

Sort and origin of cells that are being used for generating tissue comply with the structure that needs to be replaced. The cells need to show sufficient ability to proliferate and differentiate, to be readily available and to be obtained by relatively simple techniques (Keck et al. 2009).

### 2.2.1 Keratinocytes

A vital precondition for tissue-engineered skin was the establishment of an efficient cultivation protocol for keratinocytes. In principle, keratinocytes can be obtained either from skin biopsies (interfollicular keratinocytes) or from hair of the external epithelial hair follicle sheath (outer root sheath keratinocytes). As it became possible to cultivate human keratinocytes serially in vitro (Rheinwald and Green 1975) and to rapidly expand the number of patient keratinocytes ex vivo, this technology was rapidly transferred into clinical applications (Gallico et al. 1984).

At present, we are able to create cultured epidermal autografts or allografts (CEA) large enough to cover the entire surface of the body in 3–4 weeks from only a 3 cm<sup>2</sup> skin biopsy (Chester et al. 2004) and even faster when using a fibrin matrix as a support for keratinocytes (Ronfard et al. 2000).

For the cultivation of keratinocytes, skin biopsies are taken from healthy donors or, in the autologous approach, from the patient at the time of arrival in the hospital.

The epidermis is separated from the dermis and keratinocytes are released by exposure to trypsin. These keratinocytes then start to form colonies in the presence of mitotically inactivated mouse fibroblasts or fibroblasts from human donors and special culture medium containing fetal calf serum and supplements. It is also possible to expand keratinocytes in xenogeneic-free conditions (Notara et al. 2007) with the disadvantage of a reduced proliferative lifespan of the cultured cells though (Ronfard et al. 2000; Papini et al. 2003). In order to form a skin substitute, keratinocytes are even able to form a stratified epithelium in culture when they are exposed to an air interface (Kivinen et al. 1999).

With cultured autologous or allogenic keratinocytes, large wound areas, especially in burn patients, can be covered. However, the value of cultured keratinocytes

remains controversially discussed since the quality of the resulting skin is not comparable to that after split thickness skin grafting.

Incomplete adherence to the wound bed often tends to blister formations, especially when the new skin is exposed to shear forces. One of the explanations for this phenomenon is based on the assumption that the junctional attachment with fibrils of the basement membrane gets lost during cell cultivation, and additional damage is caused by the enzymatic detachment from the culture flask before transplantation (Boyce et al. 2000; Koller et al. 2002). Another problem is the lack of dermal components when covering full-thickness burns with CEAs.

This is why experiments were conducted to develop dermal equivalents from cultivated dermal fibroblasts, skin matrix components or dermo-epidermal skin equivalents. During the 1990s, most researchers working in cell culture laboratories wanted to turn various technologies into commercial products. Kamolz et al. were able to show that commercially available fibrin gels enriched with human fibroblasts could act as a dermal substitute for keratinocyte culture (Kamolz et al. 2005).

The first approved products, Dermagraft, Apligraf, and cultured epidermal autograft (Epicel), are still being marketed (Ehrenreich and Ruszczak 2006). At present, there are a number of different engineered skin substitutes available for clinical use, but none of them fulfils the criteria for fully functional skin. So far, most of the actual tissue-engineered products can cover wounds and provide an environment that stimulates their repair. In patients with large burns, they serve as temporary biologically active dressings, donors of cytokines and structural molecules while the patient's own skin regenerates to be used for serial autografting (Shevchenko et al. 2010). Graftskin (Apligraf) is the most advanced bioengineered skin product. High cost due to its complex and elaborate production leads to the fact that most commercially available products are used for chronic wounds rather than for the treatment of large burn wounds.

### 2.2.2 Fibroblasts

Fibroblasts are the most common type of cells found in connective tissue. Their main function is to maintain the structural integrity of connective tissues by continuously secreting precursors of the extracellular matrix. Fibroblasts from different anatomical locations show an array of common phenotypic attributes and are capable of expressing multiple regulatory molecules, including cytokines, growth factors, chemokines, cell surface antigens and adhesion molecules, and low molecular weight substances (Chang et al. 2002). This enables them to influence their microenvironment and also to respond to environmental cues in a complex manner.

Fibroblasts can be readily cultured in the laboratory. Mouse embryonic fibroblasts (MEFs) are often used as "feeder cells" for keratinocyte cultures. But also fibroblasts obtained from human foreskin are suitable for the same purpose (Kamolz et al. 2005). Fibroblasts have been incorporated into various tissue-engineered products such as

Dermagraft® and Apligraf® and used for a variety of clinical applications, including the treatment of burns, chronic venous ulcers and several other clinical applications in plastic surgery (Wong et al. 2007).

### 2.2.3 Human Embryonic Stem Cells

The reason for the huge scientific interest in stem cells is because of their ability to either split endlessly when in an undifferentiated state (potential to proliferate) or to differentiate into any type of cell of three blastodermic layers. Depending on the different streams of differentiated cell types originating from one stem cell, they are called unipotent (ability to differentiate into one type of cell), multipotent (ability to differentiate into more types of cells of one blastodermic layer) or pluripotent (ability to differentiate into types of cells of more blastodermic layers) (Riedel et al. 2008).

Basically, embryonic stem cells derived from the inner part of the blastocyst can be distinguished from adult stem cells. Embryonic stem cells are omni- or totipotent and can be used to develop any known form of basic tissue. After isolating embryonic stem cells from the inner part of the blastocyst, the cells are cultivated in a culture medium. By adding embryonic mouse fibroblasts, differentiation of the human embryonic stem cells is inhibited. As soon as human embryonic stem cells become adherent, they differentiate spontaneously. Human embryonic stem cells are pluripotent and have a big potential for the application in tissue engineering. However, there remain problems to be solved before they can be used in a clinical setting. They are also problematic from an ethical point of view.

In Germany, the isolation and use of human embryonic germline cells for scientific purposes is only permitted when they are harvested from aborted or spontaneously aborted fetuses. Isolating human embryonic stem cells from in vitro fertilised blastocysts is prohibited in Germany. Since July 2002, after application for a special permission, however, it is possible to import and use human embryonic stem cell lines, which were cultivated before January 1, 2002. For this purpose, it must be guaranteed that isolation of these stem cell lines was carried out in accordance with the legal situation in the country of origin (Riedel et al. 2008). And for this reason, there are only used autologous human stem cells in clinical settings (De Bari et al. 2004; Goessler et al. 2005; Toma et al. 2001; Zuk et al. 2001, 2002).

### 2.2.4 Human Adult Stem Cells and Preadipocytes

Contrary to embryonic stem cells, adult or mesenchymal stem cells derive from differentiated tissue of the postnatal organism. It could be observed that a pool of little differentiated cells stayed in the postnatal organism, which spread to the organ tissue and seemed to serve physiologic tissue generation. These multipotent stem cells



are able to differentiate to adipocytes, fibroblasts, osteoblasts and chondroblasts and seem to be very promising for appliances in tissue engineering (Zuk et al. 2001, 2002; Cao et al. 2005; Choi et al. 2005; Brayfield et al. 2010). Adult stem cells can be isolated from bone marrow and adipose tissue. For the patient though, harvesting bone marrow is by far more invasive and an extremely unpleasant intervention (De Ugarte et al. 2003) in contrast to liposuction.

Adult stem cells are positive for CD9, CD10, CD13, CD29, CD44, CD49d, CD49e, CD54, CD55, CD59, CD73, CD90, CD105, CD106, CD146, CD166, HLA 1, fibronectin, endomucin, smooth muscle cell-specific alpha actin, vimentin and collagen I. In contrast to haematopoietic stem cells, they do not express CD11b, CD14, CD19, CD31, CD34, CD45, CD79  $\alpha$ , CD80, CD117, CD133, CD144, HLA-DR, c-kit, MyD88, STRO-1, Lin and HLA II (Schaffler and Buchler 2007; Fraser et al. 2006).

Isolation of the cells from adipose tissue is achieved by digestion supported by a collagen solution, repeated filtration and centrifugation. The cell fraction in the remaining cell pellet is subsequently cultivated, and then proliferation or differentiation is stimulated (Rubin et al. 2007; Schipper et al. 2008; Wei et al. 2006). It must be considered though that depending on age, sex and localisation, adipose tissue can show different endocrinologic activity, which can affect the proliferation and differentiation performance of the harvested stem cells (Giorgino et al. 2005; Tholpady et al. 2006).

### 2.2.5 Preadipocytes

At present, there exists no clear distinction between preadipocytes and adult mesenchymal stem cells. Preadipocyte factor 1 (Pref-1) is a transmembrane protein and specific marker for preadipocytes. Pref-1 is highly expressed in preadipocytes but is extinguished during adipocyte differentiation. Therefore, Pref-1 serves as an excellent marker for preadipocytes (Keck et al. 2011).

The characteristic of preadipocytes is that they differentiate spontaneously into adipocytes and are not able to differentiate into other cells (e.g. chondrocytes or osteoblasts). Primary animal and human preadipocytes have been isolated from adipose tissue since the 1970s and have also been utilised for studying adipogenesis in vitro (Billings and May 1989). Preadipocytes are immature precursor cells located in adipose tissue that mainly consists of mature adipocytes (Smahel 1989). These cells can serve as an ideal autologous cell donor for adipose tissue-engineering approaches, since they are more resistant to mechanical damage and ischemia than mature adipocytes (von Heimburg et al. 2005). In contrast to adipocytes, they are still able to proliferate and to differentiate.

Preadipocytes lose their ability to differentiate though after some cycles in vitro. And despite various promising research projects in this field, it is still unknown if their ability to proliferate in vitro is sufficient to generate enough tissue for large soft tissue defects (Coleman and Saboeiro 2007; Patrick et al. 2008).

## 2.3 Growth Factors

Regeneration is characterised by a constantly changing environment in which cells are exposed to a complex pattern of molecular cues and signals, which impart positional information necessary for correct development (Metcalfe and Ferguson 2007).

Applying sufficient growth factors can support the transformation of the cell–matrix construct to skin tissue since they are major components of early developmental pathways for cell specification. There are two different methods for applying growth factors: *in vitro* and *in vivo*. In the *in vitro* method, it is necessary to add certain growth factors like, for example, glucocorticoids and insulin, to achieve a differentiation of the mesenchymal stem cells. In the *in vivo* method, their application is optional and might accelerate the transformation of the cell–matrix construct to the desired tissue. It could be shown that glucocorticoids, thyroid hormones, transforming growth factor- $\beta$ , epidermal growth factor and platelet-derived growth factor can have positive effects on adipogenesis (Sarkanen et al. 2011). In the *in vitro* method, adding the aforementioned growth factors in a medium accelerates the differentiation of preadipocytes into adipocytes. In the *in vivo* method, they can have favourable effects on the transformation of the tissue constructs to mature to adipose tissue (Croissant et al. 2002; Katz et al. 1999; Mandrup and Lane 1997; Yuksel et al. 2000).

Apart from adipogenesis, growth factors can be used to support angiogenesis. A decisive factor for the vitality of an *ex vivo*-generated bioimplant is a rapid adhesion to the tissue on the grafted site. To support angiogenesis and to maintain a rapid vascularisation of the bioimplant, growth factors such as VEGF can be used. Vascular endothelial growth factor (VEGF) is induced during the initial phase of skin grafting, where endogenous fibrin clots are known to form a provisional matrix and to promote angiogenesis. Frank et al. (1995) could show in a study on mice that failed to produce VEGF that wound healing was significantly impaired (Frank et al. 1995). Fibrin glue has been used for skin grafting for some time in clinical practice; nevertheless, it could not be clarified if VEGF is induced when fibrin is used as a dermal substrate for cultured skin substitutes (Currie et al. 2001).

Keratinocytes and fibroblasts can also be stimulated by a number of growth factors.

*Hepatocyte growth factor (HGF)*, for example, is a pleiotropic growth factor produced by cells of mesenchymal origin and known to stimulate keratinocyte migration and proliferation *in vitro* (Sato et al. 1995). HGF has been implicated in enhancing the cutaneous wound healing processes of reepithelialisation, neovascularisation and granulation tissue formation (Bevan et al. 2004).

*Transforming growth factor- $\beta$  (TGF- $\beta$ )* is the most potent growth factor in terms of wound healing (Ferguson and O’Kane 2004) differentiation and ECM formation (Roberts et al. 1990). In humans, there are three isoforms, TGF $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. In particular, in relation to wound healing in the skin, TGF- $\beta$ 1 and TGF- $\beta$ 2 are implicated in cutaneous scarring, whereas TGF- $\beta$ 3 is known to have an anti-scarring effect (Ferguson and O’Kane 2004).

*Platelet-derived growth factor (PDGF)* is an activator for cells of mesenchymal origin. PDGF stimulates stimulator chemotaxis and proliferation in fibroblasts and

accelerates ECM deposition (Shure et al. 1992). It has also been suggested that reduced levels of PDGF may play a role in the mechanism of scarless cutaneous repair (Ferguson and O'Kane 2004).

*Fibroblast growth factors (FGFs)* are a family of 21 isoforms with a wide range of effects just as cell migration, proliferation and differentiation. FGF-7 and FGF-10 are secreted by fibroblasts but stimulate keratinocytes to migrate and proliferate during wound healing (Tagashira et al. 1997).

*Epidermal growth factor (EGF)* was originally isolated from mouse salivary gland extract as a factor accelerating the corneal wound healing (Cohen and Elliott 1963), but it was soon recognised that it is indeed a general growth factor exerting various actions including cell migration and proliferation on a wide variety of cells (Carpenter and Cohen 1990; Wells et al. 1999). Soluble EGF was also shown to increase paracrine secretions including VEGF and HGF from MSC (Tamama et al. 2010).

Another interesting aspect in the use of growth factors could be their role in culture media. Current in vitro expansion strategies generally rely on the use of fetal bovine serum, which, due to its disease risk, makes its use in clinical practice critical.

A combination of transforming growth factor- $\beta$  (TGF- $\beta$ ), platelet-derived growth factor (PDGF) and basic fibroblast growth factors (bFGF) was shown to replace serum component in cell culture medium to expand human mesenchymal stem cells ex vivo without compromising differentiation potentials, at least up to five passages (Ng et al. 2008). Subsequently, serum and animal component-free MSC culture media became available on the market (Tamama et al. 2010)

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## 2.4 Outlook

At present, only approaches that create a very simple skin substitute have been successful. All of these tissues are missing a sufficient hypodermis. Not only for mechanical reasons but also for the aesthetic appearance, the presence of a hypodermis would be of great value. Skin appendages such as sweat glands and sebaceous glands along with nerves and blood vessels are probably the most challenging structures since even with skin autografts these structures are neither restored nor regenerated (Metcalf and Ferguson 2007). A recent study by Huang et al. indicates the feasibility of constituting an engineered skin construct incorporating sweat glands in vitro for improving the quality of skin repair and maintaining homeostasis during skin regeneration and wound healing processes (Huang et al. 2010).

Up to now, various approaches for the generation of bioartificial tissues have not succeeded due to insufficient nutrition and oxygen supply. Therefore, current tissue-engineered products have only been realised for non-vascularised tissues such as cartilage and heart valves. A bioartificial vascularised skin would be of great value in a variety of clinical settings as well as for research purposes. This is why the actual main research focuses on generating vascularised artificial skin tissues that will hopefully allow us to design tissue replacements with optimum properties in the future.

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# Use of Novel Biomaterial Design and Stem Cell Therapy in Cutaneous Wound Healing

# 3

T. Hodgkinson and Ardeshir Bayat

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## 3.1 Introduction

The spontaneous regenerative capacity of skin is dependent on the depth and area of cutaneous damage. This is the case as it dictates the extent of destruction of reparative basal and stem cell populations. Minor epidermal injury, where basement membrane and basal keratinocyte populations remain intact, results in rapid and complete cutaneous regeneration. However, partial-thickness (papillary dermal damage) and particularly full-thickness (papillary and reticular dermal damage) defects often heal through debilitating scar formation and contraction. Indeed, without surgical intervention the damage to physiological homeostasis resulting from large full-thickness wounds can be so acute that death may result. The rapid closure of such wounds is essential to restore the barrier functions of the skin and reduce scar formation (Cubison et al. 2006).

The clinical gold standard for the treatment of full-thickness wounds is the application of autologous split-thickness skin grafts, the well-known side effects of which include lack of donor site availability in severely injured patients and resulting

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T. Hodgkinson, BSc (Hons), MSc  
Plastic and Reconstructive Surgery Research, Dermatological Sciences,  
Manchester Interdisciplinary Biocentre, University of Manchester,  
131 Princess Street, Manchester M1 7DN, UK

School of Chemical Engineering and Analytical Science,  
Manchester Interdisciplinary Biocentre, University of Manchester,  
131 Princess St, Manchester, UK

A. Bayat, BSc (Hons), MBBS, MRCS, PhD(✉)  
Plastic and Reconstructive Surgery Research, Dermatological Sciences,  
Manchester Interdisciplinary Biocentre, University of Manchester,  
131 Princess Street, Manchester M1 7DN, UK

Department of Plastic and Reconstructive Surgery,  
South Manchester University Hospital Foundation Trust, Manchester, UK  
e-mail: ardeshir.bayat@manchester.ac.uk



donor site morbidity (MacNeil 2007; Groeber et al. 2011). Clearly this solution, although it remains an important life-saving tool, is far from ideal. This fact has resulted in the development and increasing implementation of engineered artificial skin substitutes. These substitutes exist in a diverse array of materials and designs ranging from inert synthetic polymer lattices (e.g. Biobrane™, Dow B. Hickam, Inc., USA) to biological matrices containing live cellular material (e.g. Orcel® (Ortec International, USA)). These products, although often successful in achieving wound closure, fall short in terms of resulting wound functionality, mechanical strength, aesthetics and cellular complexity and organisation (Kemp 2006). This is due in part to the top-down approach that arises when applying a model ‘mature’ tissue rather than constructing tissue *de novo* from progenitor cells, as in nature. Accordingly, recent research has focused on the local application, direction and manipulation of stem and progenitor cell populations to enhance the natural wound-healing response and direct cutaneous regeneration. This, it has been proposed, can be achieved in a number of ways (Discher et al. 2009). Acellular scaffolds could mobilise and attract resident adult stem cell populations through the release of growth factors/cytokines and presentation of cell attachment motifs in artificial niche environments (Discher et al. 2009). Once stem cells have been recruited to the wound site, cell fate decisions could be manipulated by presentation of dermis-specific cell attachment sites, further growth factor presentation and mechano-physical matrix properties (Discher et al. 2009). Alternatively, stem cells could be directly delivered to the wound. It is hypothesised that stem cells can augment cutaneous healing via (1) the enhancement of endogenous cell regenerative capacity through paracrine signalling, (2) direct cellular contribution through transdifferentiation and/or (3) cell fusion (Prockop et al. 2003; Spees et al. 2003; Stoff et al. 2009). Aside from safety concerns including tumour formation and disease transmission, the direct transplantation of stem cells is currently limited by the poor survival rates and low level of persistence of viable cells at transplant sites (Kolokol’chikova et al. 2001; Navsaria et al. 2004). At present, less than 5% of cells survive initial engraftment (Discher et al. 2009), which is almost certainly due to harsh necrotic environment of the cutaneous wound milieu and the destruction of suitable stem cell niches. It is clear for stem cell therapy to be effective; the mode of cell delivery must confer some level of protection to maintain viability whilst allowing direct contact with, and migration into, the wound environment.

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### 3.2 Stem Cell Populations for Cutaneous Wound Healing

Stem cells are defined by their ability to self-replicate and produce more specialised progeny (Schofield 1978; Lajtha 1979). Several populations of stem cells have been identified that hold potential for therapeutic use in cutaneous wound healing including embryonic stem cells, adult stem cells (in particular bone marrow-derived mesenchymal stem cells (BM-MSCs), bone marrow-derived hematopoietic stem cells (BM-HSCs), adipose-derived stem cells (Ad-SCs) and epidermis and hair follicle-derived stem cells (HF-SCs)) and induced pluripotent stem cells (iPSCs).

Each source has specific characteristics, markers and advantages, although all must overcome similar obstacles to become therapeutically successful, including safety concerns such as tumour formation and disease transmission as well as technical issues such as controlling cell fate decisions (Table 3.1).

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### 3.3 Embryonic Stem Cells

Human embryonic stem cells can be derived from the inner cell mass of blastocysts, 5-day-old embryos or 4–8-day-old morula and are pluripotent (Thomson et al. 1998a). Uniquely, embryonic stem cells are capable of indefinite undifferentiated in vitro propagation (Beddington and Robertson 1989). Due to their source, the use of ESCs is surrounded by well-known public controversy and governed by stringent, complex legislation and regulations. Additionally, teratoma formation when using this stem cell source remains a serious safety concern (Yao et al. 2006). As such, stem cells alternatively sourced stem cell populations are favoured by researchers for regenerative medicine and wound-healing applications.

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### 3.4 Adult Stem Cell Populations

Adult stem cells populations have been identified in almost all tissues in the human body, where their function is to maintain cell turnover and replace damaged cells (Hodgkinson et al. 2009). Within these tissues, stem cells reside in the basal layers of tissue in protective microenvironments termed ‘stem cell niches’. The stem cell population within a tissue niche is maintained in a stable naïve state by cell–cell and cell–matrix binding, cell–matrix mechanical interaction and soluble factor gradients (Watt 2000). It is the recreation of these niche environments, and the signals, which establish and maintain them, which currently is a main research focus of tissue engineering and regenerative medicine (Fig. 3.1).

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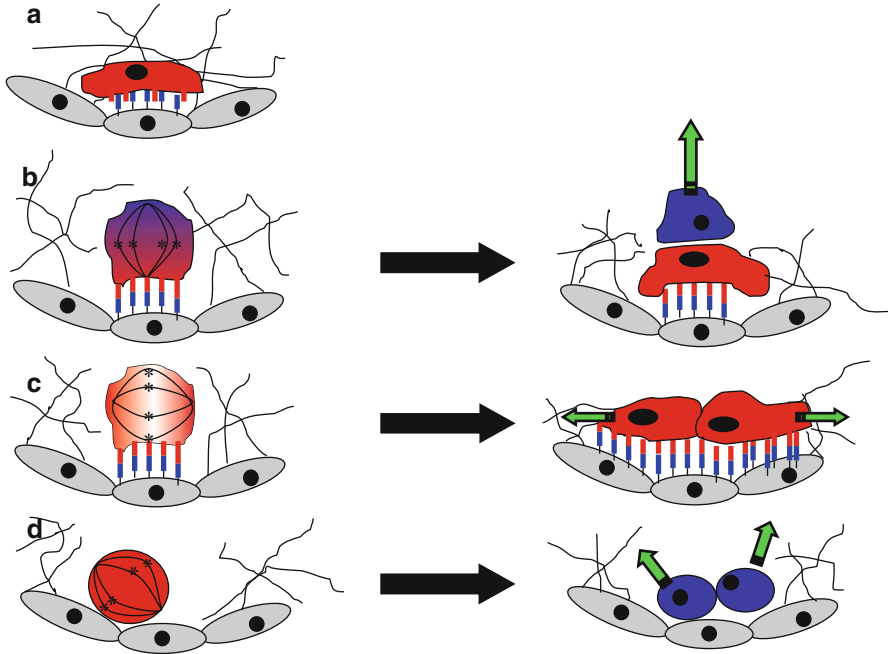
### 3.5 Bone Marrow-Derived Stem Cells

Bone marrow is a well-characterised reservoir of multipotent mesenchymal and haematopoietic stem cells (Crisan et al. 2008; Kränkel et al. 2011). Under physiological conditions, small numbers of stem cells migrate from the bone marrow, enter tissues and their niches maintaining a dynamic equilibrium (Kränkel et al. 2011). Bone marrow collection is an invasive procedure and the collected cells contain less than 0.05 % stem cells (Kita et al. 2010; Pittenger et al. 1999). Haematopoietic stem cell therapy was the first available stem cell therapy (Wagner and Gluckman 2010). The local and systemic delivery of BM-MSCs are potentially effective treatments to heal acute and particularly chronic wounds (Fu et al. 2006; Badiavas and Falanga 2003; Falanga et al. 2007). Proof of principle applications of BM-MSCs to cutaneous defects have demonstrated significant wound closure acceleration in

**Table 3.1** Selected stem cell sources, selected markers and advantages/disadvantages for therapeutic use in cutaneous wound healing

Stem cell population location	Selected cell markers	Advantages	Disadvantages	References
<i>Embryonic</i>	<p>Positive for SSEA-4, TRA-1-60, TRA-1-82, AC133, c-kit (CD117), flt3 (CD135), CD9, Oct3/4, Sox2, Nanog</p>	<p>Pluripotent Can be propagated indefinitely Some demonstrated immunoprivileged properties Open to genetic manipulation</p>	<p>Teratoma formation Legal/ethical restrictions Currently allogeneic only – immunosuppressive therapy required Difficult to isolate</p>	<p>Thomson et al. (1998b), Knoepfler (2009), Schlüter and Kaur (2009), Carpenter et al. (2004, 2009), Andrews et al. (1984), Kaufman et al. (2001), Hoffman and Carpenter (2005)</p>
<i>Bone marrow</i>				
Mesenchymal	<p>Positive for CD73, CD90, CD105, CD106, Stro-1, CD29, CD166, CD146, CD44 Negative for CD34, CD45, CD14</p>	<p>Multipotent  Demonstrate immunoprivileged properties – no immunosuppressive therapy required Can be autologous Recruited in natural wound healing</p>	<p>Careful control of differentiation needed  Extraction can be painful and low yield  Allogeneic transplantation risks disease transmission</p>	<p>Togel and Westenfelder (2007), Arthur et al. (2009), Dennis et al. (2002), Osawa et al. (1996), Larochele et al. (1996), Kawashima et al. (1996), Michallet et al. (2000), Goodell (1996), Uchida et al. (2001)</p>
Hematopoietic	<p>Positive for CD133, Bcrp-1, CD34 (variable) Negative for CD38, Lin</p>			

<i>Adipose</i>	Mesenchymal	Positive for CD13, CD29, CD44, CD49e, CD54, CD55, CD63, CD73, CD90, CD105, CD144, CD146, CD166. Negative for: CD11b, CD14, CD19, CD31 (variable), CD34 (variable), CD45, Stro-1 (variable), CD3, CD117, CD62L, CD95L	Available in large quantities and easily harvested	Body site source and age-dependent properties	Pittenger et al. (1999), Deans and Moseley (2000), Gronthos et al. (2001)
<i>Hair follicle</i>	Epithelial (bulge)	Positive for CK15, CK19, CD200, PHLDA1, follistatin, Frizzled homolog 1	Most abundant tissue source of adult stem cells	Allogeneic transplantation risks disease transmission	Kloepper et al. (2008), Hoogduijn et al. (2006), Tiede et al. (2007)
	Mesenchymal	Negative for CD34, CD24, CD71, CD146, connexin43 and nestin	Several populations identified	May require in vitro expansion to obtain sufficient cell quantities	
<i>Induced pluripotent stem cells</i>		Positive for CD44, CD73, CD90, CD34, Lef-1	Easily extracted	Long-term safety unknown	Takahashi and Yamanaka (2006), Takahashi et al. (2007), Nakagawa et al. (2008)
		Positive for SSEA-4, TRA-1-60, TRA-1-82, AC133, c-kit (CD117), flt3 (CD135), CD9, Oct3/4, Sox2, Nanog	Autologous pluripotent cells generated from somatic cells	In vitro expansion required	No standard practices or procedures in place



**Fig. 3.1** Schematic mechanisms of stem cell niche maintenance. (a) Stem cells maintained in relative quiescence. (b) Asymmetric cell division can be controlled by cell–cell and cell–matrix binding, which can segregate cell fate factors in the cytoplasm. The orientation of the spindle results in only one of the progenitor cells remaining in contact with the niche cells, whilst the other daughter cell is differentiated and moves out of the niche. (c) When the niche is in need of repopulation, both progenitor cells are able to maintain contact with the niche cells and are exposed to the same microenvironment. In this case both cells retain stemness and the stem cell pool is expanded. (d) When the niche signalling environment is disrupted or when contact with niche cells is lost, symmetric cell division resulting in two differentiated cells can occur (Modified from Lutolf and Blau 2009)

mice/rats (Falanga et al. 2007; Wu et al. 2007; Amann et al. 2009; Chen et al. 2008; McFarlin et al. 2006) and in humans (Badiavas and Falanga 2003; Falanga et al. 2007; Ichioka et al. 2005) increased epithelialisation and angiogenesis (Wu et al. 2007; Sasaki et al. 2008; Javazon et al. 2007). Despite the lack of blinded studies in the literature, therapeutic potential is evident. Whilst others have identified limitations, for example, in the treatment of severe burns where sulphadiazine toxicity (Gamelli et al. 1993) and sepsis (Gamelli et al. 1995) significantly suppress response to BM-MSCs.

### 3.6 Adipose-Derived Stem Cells

Adipose-derived stem cells that possess characteristics which are similar to MSCs represent the most abundant adult tissue population (Kim et al. 2007). Liposuction surgery offers opportunity to obtain volumes of anywhere from 100 mL to >3 L

of adipose tissue in the form of lipoaspirate, which is normally discarded (Katz et al. 1999; Bunnell et al. 2008). The stem cell yield from such a procedure is typically around 400,000 viable cells per ml extracted (Zuk et al. 2002; Guilak et al. 2006). Importantly, it is thought that in vitro expansion of Ad-SCs can yield 100–1,000 times more progenitor cells than isolation from bone marrow (Utsunomiya et al. 2011). Recently, Ad-SCs have been reported to promote human dermal fibroblast proliferation through both direct cell–cell contact signalling and secretory paracrine activation, which in turn accelerated wound re-epithelialisation (Kim et al. 2007). It is likely that physiologically Ad-SCs play a crucial role in the healing of full-thickness skin damage through direct migration from subcutaneous adipose tissue. Additionally, adipose tissue acts as an endocrine organ, secreting numerous hormones, growth factors and cytokines such as leptin; epidermal growth factor (EGF); tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ); basic fibroblast growth factor (bFGF); keratinocyte growth factor (KGF); transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1); vascular endothelial growth factor (VEGF); hepatocyte growth factor (HGF); interleukins (IL)-6, IL-7, IL-8, IL-11 and IL-12; macrophage-colony stimulation factor; and platelet-derived growth factor (PDGF) (Utsunomiya et al. 2011; Witkowska-Zimny and Walenko 2011).

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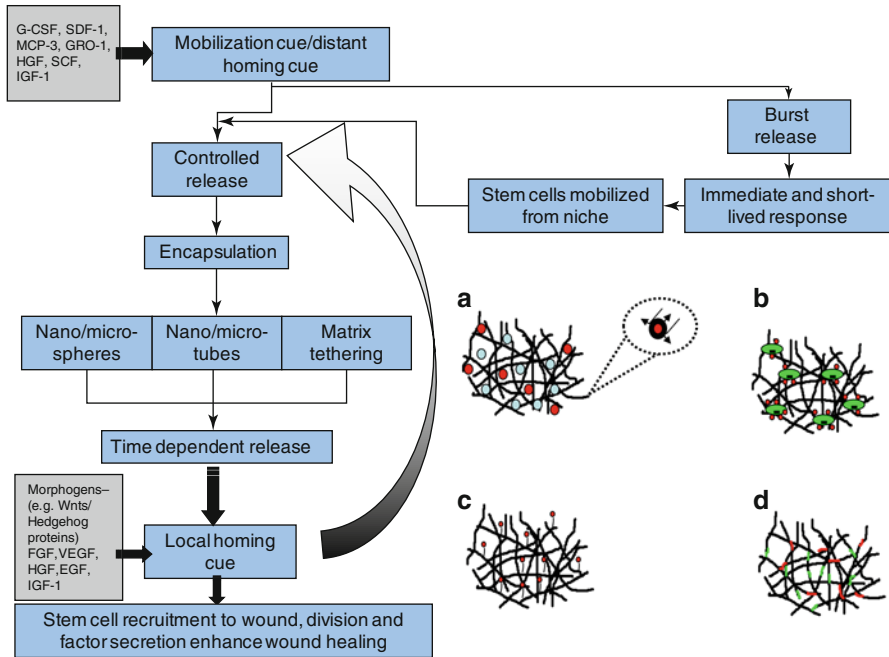
### 3.7 Hair Follicle-Derived Stem Cells

Due to their ease of access and demonstrated multi-(and even possibly pluri-) potency, HF-SCs could be a promising population for application in cutaneous wound healing. Utilising HF-SCs, located mainly in the hair follicle bulge, has the added advantage of being naturally involved in healing both epidermal and dermal injuries (Ito et al. 2005; Jahoda and Reynolds 2001). Following cutaneous injury HF-SCs are involved in the secretion of HGF, EGF and in particular heparin-binding (HB)-EGF, FGF-7 and FGF10, which activate the STAT3 and AP1 signalling pathways, promoting re-epithelialisation (Gurtner et al. 2008).

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### 3.8 Induced Pluripotent Stem Cells

The research interest in iPSCs since the original breakthrough by Takahashi and Yamanaka has been intensive (Takahashi and Yamanaka 2006). Their initial reprogramming of mouse embryonic fibroblasts through the enforced expression of four transcription factor genes (*OCT3/4*, *Sox2*, *Klf4*, and *c-Myc* (Takahashi and Yamanaka 2006; Müller et al. 2009)) has since led to the generation of human iPSCs epigenetically and developmentally indistinguishable from ES cells (Maherali et al. 2007; Okita et al. 2007; Park et al. 2007; Wernig et al. 2007; Liu et al. 2008). The generation method has been refined by the dispensing of *c-Myc* which has led to a reduction in malignant transformation of iPSC derivatives (Müller et al. 2009). To address safety fears of retroviral gene transfer-induced mutagenesis, alternative viral-free methods for the induction of pluripotency have been developed. This has been achieved through the repeated addition of



**Fig. 3.2** Flow chart showing series of events to recruit endogenous stem cells to wound. (a) Protective encapsulation allows controlled release through diffusion and/or enzymatic degradation (*inset*) nanotube encapsulation. (b) Ionic binding and sequestering of factors, localising factors to the matrix. (c) Covalently tethered factors, released through enzymatic cleavage. (d) Cell adhesion sites and niche-specific matrix molecules (e.g. laminin, heparan sulphate)

polyarginine protein transduction-tagged recombinant proteins (Zhou et al. 2009; Kim et al. 2009) and with highly basic HIV-TAT-derived basic peptide sequences (Kim et al. 2009). Several serious safety concerns, such as tumour formation potential, remain to be addressed prior to clinical application of iPSCs. Despite this, it is likely that stem cells obtained through pluripotent induction in some form are likely to be therapeutically important in the future.

### 3.9 Biomaterial Transplantation-Induced Homing of Endogenous Stem Cell Populations for Cutaneous Repair

The implantation of an acellular biomaterial that possesses the ability to mobilise, attract and manipulate endogenous stem cell populations offers significant potential benefits over *in vivo* delivery of viable stem cells. Such cell-homing devices would be less costly and complex to produce, have longer shelf life and increased safety whilst avoiding the need for allogeneic transplant or autologous tissue harvest (Fig. 3.2).

### 3.10 Mobilisation and Navigation Cues

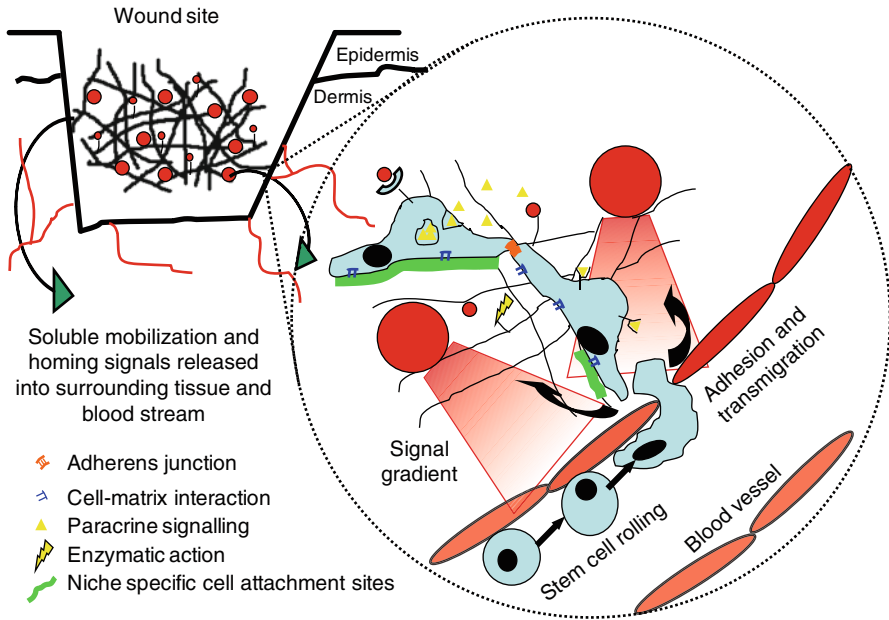
On implantation into the wound site, the scaffold must release a stimulatory mobilisation signal which when received by ASCs causes them to exit their niche, whether this is in the uninjured surrounding tissue or nonlocal niches, e.g. bone marrow. Physiologically, following wound healing, such stem cell mobilisation naturally occurs both locally and from bone marrow-derived stem cells, which then go on to contribute to the healing wound (Wu et al. 2007; Badiavas et al. 2003). This relies on growth factor, cytokine release and cellular expression of the relevant receptors; however, the precise regulatory mechanisms that co-ordinate this response are incompletely understood (Yoshikawa et al. 2008; Wang et al. 2007; Sordi 2009). In an effort to enhance this natural signal, a plethora of growth factors and cytokines have been trialled in preclinical and clinical studies. At present, granulocyte colony-stimulating factor (G-CSF) is the most widely utilised stem cell mobilisation agent in the clinic (Chen et al. 2011). This growth factor also serves as a good example of the cautionary approach that must accompany the administration of growth factors as it is also linked with inflammatory cell mobilisation and promotion of atherosclerosis (Kränkel et al. 2011; Chen et al. 2011). Aside from G-CSF, it appears that a degree of specificity in recruitment targets can be conferred by the choice of growth factors. Stromal-derived factor-1 (SDF-1) recruits cells expressing its receptor CXCR4 (cytokine receptor type 4) such as haematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), cardiac stem cells (CSCs) and MSCs (Askari et al. 2003; Tang et al. 2005; Schenk et al. 2007; Hohensinner et al. 2009; Haider et al. 2008; Unzek et al. 2007; Lapidot 2001). Monocyte chemoattractant protein-3 (MCP-3) mobilises MSCs (Schenk et al. 2007), growth-related oncogene-1 (GRO-1) attracts bone marrow-derived EPCs (Kocher et al. 2006), hepatocyte growth factor (HGF) targets myoblasts (O'Blenes et al. 2010), and stem cell factor (SCF) and insulin growth factor-1 (IGF-1) activate cardiac stem cells (CSCs) (Kuang et al. 2008; Guo et al. 2009; Hohenstein et al. 2010). SDF-1 $\alpha$ /CXCL12 appears to be pivotal in the mobilisation following injury in a number of tissues.

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### 3.11 Local Navigation Cues and Cell Binding: Designing the Artificial Stem Cell Niche

Locally to the wound site, further signalling molecules are required to direct the recruited stem cells through the implanted scaffold and within the wound. This could be created through localised cytokine/growth factor gradients, matrix-tethered factors or incorporation of specific cell attachment motifs. The list of soluble local navigation candidate molecules is long and includes morphogens like Wnts and hedgehog proteins alongside growth factors like FGF (Lutolf and Blau 2009). Importantly for biomaterial design, soluble growth factors in the physiological niche are electrostatically bound to heparan sulphate proteoglycans (e.g. heparin), localising their action to the niche and serving as a protective reservoir, preventing proteolytic degradation (Lutolf and Blau 2009; Ramirez and Rifkin 2003).





**Fig. 3.3** Schematic representation of stem cell migration stimulated by biomaterial factor release. Stem cells recruited migrate through vascular system adjacent to implanted factor releasing biomaterial. Upon signal-receptor activation cell adhere and transmigrate into biomaterial along signalling gradients. Cells adhere to matrix to niche-specific matrix and to each other through adherens junctions. Cells secrete paracrine and autocrine factors, enzymatically release tethered matrix factors and directly contribute to wound healing through asymmetric cell division

Mimicking such associations through incorporation of relevant proteoglycans and glycosaminoglycans (GAGs) into biomaterials may provide a simple and effective way of delivering tissue-specific localised stem cell cues (Fig. 3.3).

Problematically, the required signalling factors have short half-lives *in vivo* and consequently they must be protected from degradation and released in a controlled way to sustain effect. To achieve this several strategies have been proposed. The majority of those involve the protective encapsulation of growth factors, which are then released either passively through diffusion or polymer breakdown or through active enzymatic degradation. Modes of encapsulation are mainly in the form of polymer spheres or polymer fibres on the micro-nano scale. By controlling the encapsulated ‘payload’ of bioactive factor and the degradation profile/diffusion properties of the encapsulating polymer, the delivery of the factor can be controlled. Delivering a cocktail of growth factors/cytokines with differing release profiles and concentrations could make the effective local control of recruited cells possible.

In the case of micro-nanofibre encapsulation, the bioactive soluble factors are contained within the supporting matrix itself. One promising method of fibre production on this scale is electrospinning. This process involves the application of a high-voltage electrical current to a droplet of polymer solution or melt. When the repulsive

charge generated inside the droplet is sufficient to overcome the surface tension of the fluid, a liquid jet is expelled from the tip of the droplet towards a grounded collector plate. In transit to the ground electrode, the fluid jet is stretched by inertial and electrostatic forces and the solvent evaporates. A dry uniform fibre, typically in the nanometre range, can be collected (Ayres et al. 2010). This scale corresponds to that of natural ECM fibres and has been reported to increase cell–cell and cell–matrix interactions (Li et al. 2002; He et al. 2007). Bioactive molecules can be blended with spinning solutions prior to deposition, depending on spinning parameters and solvent systems used, and have been shown to maintain their action both *in vitro* and *in vivo* (Fu et al. 2008). Alternatively, core–shell fibres can be obtained by the use of a concentric spinneret and two-fluid spinning system (Jiang et al. 2005) allowing greater control of encapsulation and release of desired bioactive factors.

The electrospinning technique could be easily adapted for the creation of a sequentially layered scaffold (Ayres et al. 2010), mimicking the layered structure of the skin. This could be achieved by the adjustment of spinning process parameters, the spinning solution used and directing fibre alignment. Interestingly, Yang and co-workers electrospun viable human dermal fibroblasts and keratinocytes (Yang et al. 2009), although further investigation is needed to confirm cells were unaltered by the process.

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### 3.12 Cell Adhesion Sites and Mimicking the Spatial Complexity of Signalling in the Niche

Establishment of signalling gradient polarity in a complementary but spatially discrete manner is essential for the maintenance of engineered niche environments. To date, attempts to engineer these structures have centred on 2D systems or simplified simulated 3D environments. Biomaterial technologies such as atomic force dip-pen lithography (Piner et al. 1999), stencil lithography surface patterning (Kim et al. 2003) and inkjet and microcontact printers (Roth et al. 2004; Théry et al. 2007) hold promise for the precise spatial patterning of factors. Inkjet printing has successfully been used to create 2D artificial niches by the patterning of immobilised BMP-2 which inducted mesenchymal progenitors to differentiate osteogenically, where in off-pattern sections myogenic differentiation was observed (Phillippi et al. 2008). In particular, inkjet systems have received particular attention but several limitations in the current methodology exist. Importantly, the concentrations of ECM-based ‘bioinks’ is limited by constraints on solution viscosities, surface tensions and densities required by printing set-ups (Mironov et al. 2006). In turn, this restricts the mechanical properties of 3D printed materials where bioinks must provide support to the layers deposited above them. It has been suggested that to improve the structural support of 3D bioprinted scaffolds, a combined materials approach is required where bioink is deposited onto a bio-paper and sequentially layered (Nakamura et al. 2005; Mironov et al. 2009). This bio-paper could, for example, be in the form of a nanofibrous electrospun polymer mat, which itself could be engineered to provide important biological cues.

## Conclusions and Future Perspectives

The activation, homing and control of endogenous stem cells through novel biomaterial design have become significant goals of tissue engineering and regenerative medicine. Several material processing techniques now exist that allow the biomimicry of important biological structures. Electrospinning, most prominently amongst other fibre formation techniques, allows the creation of fibres on the same scale as natural ECM (50–500 nm). Micro-nanosphere factor encapsulation allows the controlled and sustained release of soluble factors, which can be used for local and systemic recruitment and homing of stem cells. The identification of exactly which factors should be delivered and when remains to be seen and could be variable depending on the type of cutaneous wound that requires treatment, e.g. a chronic wound environment may be different from a burn. High-throughput techniques will allow the screening of the identified factors, and others, in different combinations to maximise their effect. The ability to engineer biomaterials with increasingly accurate cell signalling capabilities will continue, with a feasible endpoint being the precise delivery of only the required factors only where they are needed, only when they are needed.

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Nicole Lindenblatt and Alicia D. Knapik

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## 4.1 Introduction

Today split- and full-thickness skin grafting represent standard procedures to close skin defects, i.e. in burn injuries and other acute or chronic wounds (leg ulcers, traumatic skin losses, defects after tumour resections, etc.). Nevertheless, the exact process of skin graft revascularisation remains elusive. A better understanding of this process becomes crucial for the increasingly popular research field of tissue engineering (Paquet et al. 2010). A variety of skin substitutes have been developed in the last decade and become commercially available. However, in particular, full-thickness substitutes still lack complete successful incorporation into the human body (Llames et al. 2006). A better knowledge of the mechanisms of the taking of skin grafts and their acquisition blood supply would aid substantially in their fabrication. By this the composition of demo-epidermal substitutes could be tailored according to the physiologic process of revascularisation.

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## 4.2 Previous Studies

The free autologous transplantation of skin was introduced by Reverdin at the end of the nineteenth century (Reverdin 1869). The time period between grafting and reperfusion has been traditionally referred to as plasmatic imbibition and is said to last 48 h. Between 48 and 72 h, a direct connection between bed and graft vessels (inosculation) is assumed (Garrè 1888; Hübscher 1890). This is supposed to be followed by some kind of neo-vascularisation involving the sprouting wound bed vessels (angiogenesis) into the graft (Goretsky et al. 1995).

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N. Lindenblatt (✉) • A.D. Knapik  
Division of Plastic and Reconstructive Surgery,  
Department of Surgery, University Hospital Zurich,  
Raemistraße 100, Zurich 8091, Switzerland  
e-mail: niclindenblatt@hotmail.com; alicia.knapik@usz.ch



More recent histological studies reported revascularisation starting at day 3 as a combination of vessel growth of the wound bed, vessel regression of the graft and inosculation from the periphery to the centre (Capla et al. 2006). In contrast to this, another histological evaluation showed that vessel anastomoses can be detected within the centre of the graft already at day 2 (O’Ceallaigh et al. 2006). Thus, it is obvious that still great discrepancies in the results of *ex vivo* studies exist. Especially, the estimation of the formation of microvessels, which grow and arrange as a three-dimensional network, appears to be difficult. *In vivo* studies concerning this issue hardly exist, are more than 35 years old and therefore were not able to apply modern imaging methods (Medawar 1944; Birch et al. 1969; Zarem 1965).

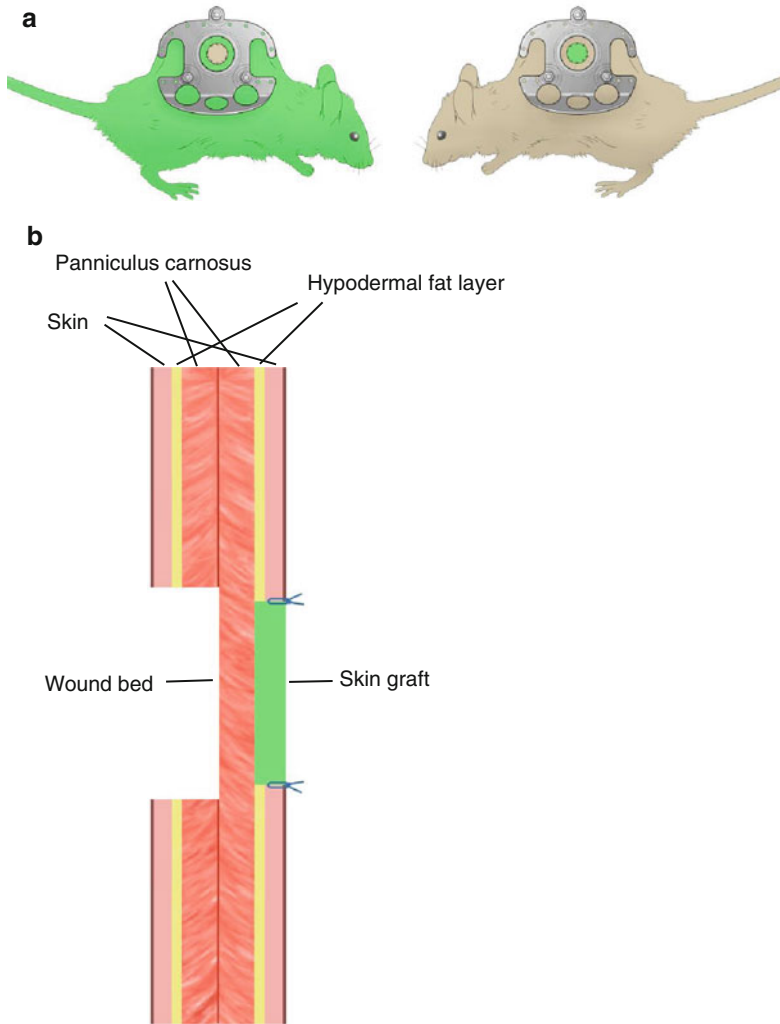
Generally, it is thinkable that vessels from the wound bed grow towards capillaries of the graft and reach existing vascular structures within the first 3 days. According to earlier studies, demonstrating dermal vessel growth at a rate of 5  $\mu\text{m}/\text{h}$  (Zarem 1965), a 300  $\mu\text{m}$ -sized skin graft may be completely invaded by angiogenic vessels within 72 h. In contrast to this stands the complete *de novo* vascularisation of the graft without connection to existing structures by vessels originating from the wound bed, which most likely involves several weeks (O’Ceallaigh et al. 2006).

The past decade brought about numerous efforts to engineer epidermal, dermal, and recently demo-epidermal skin substitutes (Auger et al. 2004; Larouche et al. 2009). Endothelial cells differentiated from circulating endothelial progenitor cells were used to promote vascularisation of tissue-engineered human skin substitutes. Tremblay et al. showed that a tissue-engineered endothelialised human skin formed capillary-like structures, to which the graft vessels anastomose leading to graft revascularisation within 4 days. These authors assumed that this most likely takes place by direct inosculation of graft and bed vessels rather than vessel formation within the wound bed and rapid ingrowth into the graft (Tremblay et al. 2005). Recently, it was shown in a histological study that in autologous *in vitro* reconstructed demo-epidermal skin containing appropriate microvascularisation, inosculation between host and graft vasculatures occurs within 4 days. Next to this angiogenesis within the wound is led to successful revascularisation of the construct (Gibot et al. 2010). Other investigators depicted that pre-vascularisation of PLGA scaffolds significantly improved and accelerated the establishment of blood perfusion when compared to untreated PLGA scaffolds (Laschke et al. 2008).

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### 4.3 The Modified Dorsal Skin Fold Chamber

With the intention to gain applicable knowledge for the fabrication of full-thickness skin substitutes, we designed a new *in vivo* mouse model to study the revascularisation of skin grafts. We based the new model on the classic dorsal skin fold chamber, which is an established model to visualise striated muscle microcirculation over several weeks (Lehr et al. 1993). We performed the preparation in C57BL/6J mice and transgenic green fluorescent protein (GFP) mice, which expressed the GFP gene under a beta-actin promoter. Procedures were carried out in intraperitoneal ketamine/xylazine anaesthesia.



**Fig. 4.1** (a) Model of the modified dorsal skin fold chamber and crossover transplantation principle between GFP mouse (*left*) and B6 wild-type mouse (*right*) for vasculature origination. (b) Cross section of modified dorsal skin fold chamber preparation (B6 host with GFP graft): skin, hypodermal fat and muscle (panniculus carnosus) are sandwiched between the titanium frames. The wound bed can be accessed from the wound bed side (*left*) and the graft side (*right*) simultaneously by intravital microscopy

### 4.3.1 Dorsal Skin Fold Chamber Preparation

Two symmetrical titanium frames were mounted on a dorsal skin fold of the animal, which includes a double layer of skin, hypodermal fat and the panniculus carnosus (Fig. 4.1a, b). The panniculus carnosus is a thin striated skin muscle covering the

entire back of rodents, similar to the platysma muscle in humans. During the skin fold chamber preparation, one skin layer is completely removed in a circular area of 15 mm in diameter, and the remaining are covered with a glass cover slip incorporated into one of the titanium frames.

### 4.3.2 Skin Graft Harvesting and Transplantation

On day 3 after the dorsal skin fold chamber preparation, skin and most parts of the hypodermal fat layer were carefully removed in a circular area of 7 mm in diameter from the back of the chamber, leaving only the panniculus carnosus as wound bed. A full-thickness skin graft of identical size was harvested from the groin of the animal and placed into the defect in the back of the chamber. By this, it is possible to view the microcirculation of the wound bed from the front of the chamber and, at the same time, of the graft in the back of the chamber (Fig. 4.1b). Additionally, this new model is suitable to study angiogenesis in many different settings (biomaterials, gels containing growth factors, organ tissues, etc.) where the object to be studied can be placed in the window within the back of the chamber.

### 4.3.3 Intravital Microscopy

Due to the visibility of both the wound bed and the skin graft through a glass window, it was now possible to follow vessel development and in particular perfusion characteristics in the living animal by multifuorescence microscopy. Repetitive intravital microscopic analyses of both the wound bed and skin graft were carried out over 10 days. Microvascular blood flow in arterioles, venules and capillaries was visualised after the application of fluorescent dyes (fluorescein isothiocyanate-labelled or rhodamine-labelled dextran) using an intravital microscope (Axiotech vario, Zeiss, Jena, Germany). The epi-illumination setup included a 100 W HBO mercury lamp with a blue filter (450–490 nm/>520 nm excitation/emission wavelength) and a green filter (530–560 nm/>580 nm). Microvascular analysis was quantified off-line by a computer-assisted image analysis system (CapImage, Zeintl Software, Heidelberg, Germany) and included the determination of vessel diameter ( $\mu\text{m}$ ), red blood cell velocity ( $\mu\text{m}/\text{s}$ ) and functional capillary density, defined as length of perfused capillaries per area of observation ( $\text{cm}/\text{cm}^2$ ). Further, bud and sprout formation was assessed and is given in number per area of observation ( $\text{n}/\text{mm}^2$ ).

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## 4.4 The Crossover GFP/Wild-Type Transplantation Model

With the intent to identify the origination of vascular structures *in vivo*, we designed a crossover GFP transplantation model between C57BL/6J wild-type (WT) mice and C57BL/6-Tg(ACTB-EGFP)10sb/J mice (“GFP” mice). The GFP mice are bred on a

B6 background and express green fluorescent protein (GFP) in all cells except erythrocytes and hair driven by a beta-actin promoter (Okabe et al. 1997). By this cells from GFP mice can be identified due to their green fluorescence under blue light in vivo. Crossover transplantation was performed between a wild-type mouse and a GFP mouse leading pairs of animals, in which each mouse carried the full-thickness graft of the other. By this we created a model in which it was possible on the one hand to track vessels coming from the GFP wound bed and on the other hand to view changes of the vasculature during engraftment within the GFP skin graft. In parallel, it was possible to view the blood flow under the green light (intravasal RITC-dextran).

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## 4.5 Immunohistochemistry and Corrosion Casting

Five days after the transplantation, cross-section tissue blocks of the modified dorsal skin fold chamber were harvested. Twenty-five  $\mu\text{m}$  sections were cut. These were fixed in acetone, dried and washed in PBS for 5 min, followed by overnight blocking with 5 % donkey serum in PBS-Tween 0.1 %. Rat anti-CD31 (1:100, Abcam, Cambridge, United Kingdom) and goat anti-desmin (1:50, Santa Cruz Biotechnologies) were used as primary antibodies, followed by incubation with the secondary antibodies as well as DAPI nuclear staining. Fluorescence microscopy was performed.

Standard methods were used for vascular corrosion casting. The chest was opened and the left ventricular chamber entered using a 23-gauge intravenous “butterfly” canula. The right atrium was incised to allow for the exsanguination of the animal while the circulatory system was flushed with 20 ml of artificial cerebrospinal fluid. The casting medium PU4ii was used as described previously (Krucker et al. 2006). Then the tissue specimen was explanted after complete resin curing after 4–6 days at room temperature. Soft tissue of the skin fold chamber was macerated in 7.5 % KOH at 50 °C overnight.

For light microscopy, untreated PU4ii casts were evaluated (Leica MZ 16A, Leica Microsystems). Lyophilised samples consisting of the casting material were mounted on metal stubs using double-sided gluing tape and colloidal silver. Samples were sputter coated with gold at 1.5 kV for 3 min and examined using a scanning electron microscope at 15–1,000 times magnification (SUPRA 50 VP scanning electron microscope, Zeiss).

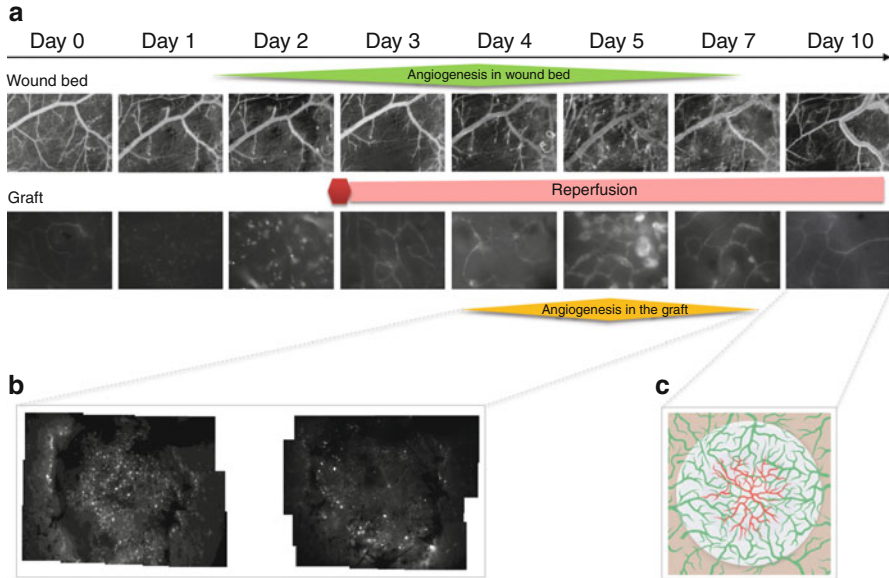
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## 4.6 Revascularisation Process of Full-Thickness Skin Grafts

### 4.6.1 Time Course of Wound Bed Angiogenesis (Fig. 4.2a)

#### 4.6.1.1 24 Hours Post-Grafting

Within the wound bed a widening of the muscle capillaries occurred. This was accompanied by small capillary protrusions, which represent precursors of bud formation within the angiogenic process. The graft did not reveal any blood perfusion but a considerable number of shedded keratinocytes.



**Fig. 4.2** (a) Intravital microscopy of wound bed (*above*) and skin graft (*below*) in the modified dorsal skin fold chamber over a time period of 10 days. Characteristic angiogenic changes were observed from day 2 to 7 in the wound bed. Reperfusion occurred between days 2 and 3 followed by a temporary angiogenic response of the graft between days 3 and 8. Magnification wound bed  $\times 25$  and skin graft  $\times 200$ . Intravasal application of FITC-dextran. (b) Temporary angiogenic response of the graft 5 days (*left*) and 7 days after grafting (*right*). Magnification  $\times 25$ . (c) Schematic view of vascular replacement in the crossover model 10 days after transplantation (wild-type graft on GFP host): The peripheral vasculature of the graft is replaced by the host vessels while in the centre still 40 % of the capillaries still are derived from the graft itself

#### 4.6.1.2 48 Hours Post-Grafting

At 48 h post-grafting, capillaries within the wound bed demonstrated typical signs of angiogenic transformation, i.e., the formation of buds. The graft did not show signs of reperfusion.

#### 4.6.1.3 72 Hours Post-Grafting

Angiogenesis in the wound bed further progressed with an increase of budding and sprouting as well as further capillary widening. At this time point, the graft showed first sign of perfusion according to the loop-like vessel pattern of the original microvasculature of the skin. Capillary diameters of the graft vessels were not increased.

#### 4.6.1.4 96 Hours Post-Grafting

After 96 h angiogenesis in the wound bed was abundant. In addition, the graft showed improved capillary perfusion with an increase in functional capillary density and a marked rise in capillary diameters. Bud and sprout formations at the capillary branchings occurred within the graft symbolising a temporary angiogenic response. Because interconnection between newly formed vessels from the wound

bed and autochthonous capillaries of the graft with reperfusion usually develop earlier, this may also represent a further ingrowth of newly formed vessels into the graft tissue.

#### **4.6.1.5 120 and 168 Hours Post-Grafting**

In the wound bed, functional capillary density and capillary diameters decreased. In contrast, capillary diameters and functional capillary density within the graft still increased as a component of the angiogenic response.

#### **4.6.1.6 240 Hours Post-Grafting**

Capillary architecture within the wound bed returned to baseline conditions. The skin graft was fully reperfused according to the original vascular pattern.

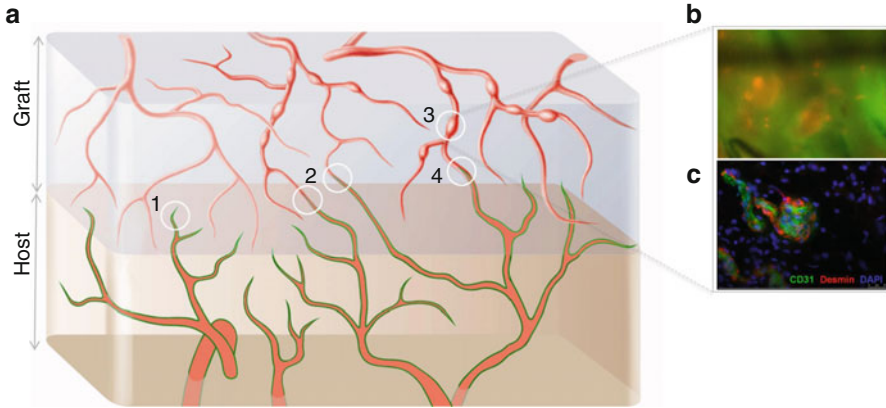
### **4.6.2 Angiogenic Reaction of the Graft Vasculature (Fig. 4.2a)**

#### **4.6.2.1 Angiogenic Response in GFP-Positive Skin Grafts**

Since we were also interested into the changes of the skin graft itself during the revascularisation process, we transplanted GFP skin grafts on wild-type mice. No changes of the morphology of the graft vasculature were seen immediately after reperfusion until day 3. Temporary angiogenic buds appeared predominantly in the outer periphery of the skin graft from day 4 to 7 (Fig. 4.2b). These structures largely expressed GFP in their walls and therefore could be identified as originating from the autochthonous graft vasculature. The buds were up to 7× wider than normal dermal capillaries and exhibited a reduced microvascular flow (Fig. 4.3b). GFP-positive bed vessels contributed to bud formation.

#### **4.6.2.2 Graft Angiogenic Response Differs from Sprouting Angiogenesis of the Wound Bed**

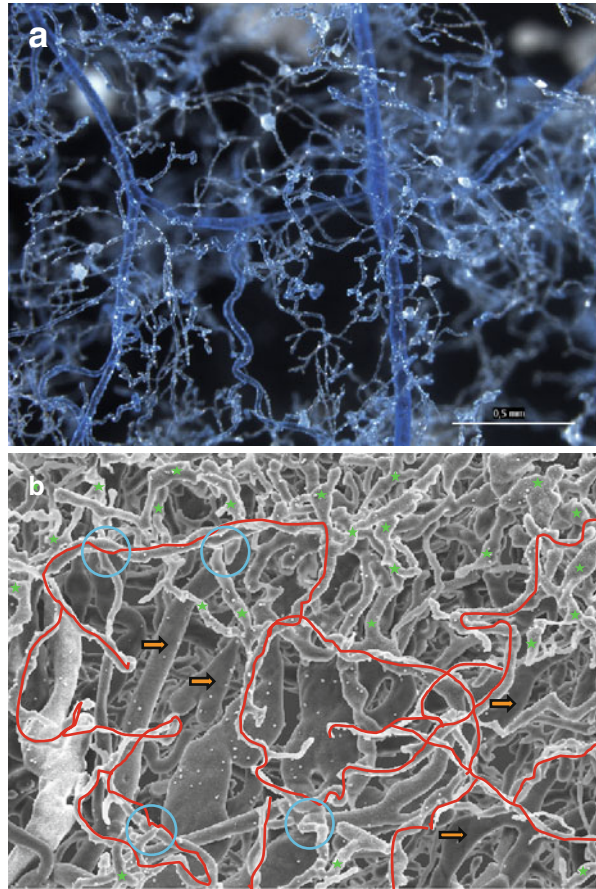
Laschke et al. have shown that tissue-engineered compounds containing a pre-engineered microvascular network are able to inosculate with the host microvasculature by outgrowth of vessels and subsequent external inosculature (Laschke et al. 2008, 2010). This may in particular be the case if the microvascular network exhibits a high sprouting activity, e.g., due to pre-vascularisation in vivo and subsequent presence of pro-angiogenic factors. In contrast to this, in skin graft revascularisation – where naturally a pre-existing vascular network within the graft is present – it has not been observed in any previous study that vessels from the skin graft actively invade into the surrounding tissue. Instead, internal inosculature takes place, i.e., angiogenic vessels from the wound bed invade the graft, connect to the existing network and grow along the pre-existing channels. Even though the graft is not able to undergo angiogenesis or produce an outgrowth of vessels during the period of tissue ischemia, it in fact reacts after reperfusion with a temporary angiogenic response. As we showed in the present study, the angiogenic changes within skin and muscular wound bed differ significantly in their timing, morphology and development. In the wound bed, angiogenic buds appearing after 24–48 h represent



**Fig. 4.3** (a) Schematic view of vascular processes during skin graft revascularisation in the modified dorsal skin fold chamber (wild-type graft on GFP host). 1 angiogenic vessel ingrowth, 2 inosulation to existing graft vasculature, 3 angiogenic response of the graft vessels after reperfusion, 4 ingrowth of wound bed vessels into graft vasculature and replacement. (b) Identification of the origination of angiogenic buds by intravital microscopy (GFP graft on wild-type host, rhodamine-dextran for staining of blood flow): GFP-positive angiogenic buds originate from the graft at day 5 after grafting. Magnification  $\times 200$ . (c) Immunohistochemistry of angiogenic buds reveal their “mature” character since they are covered with desmin-positive pericytes. Magnification  $\times 200$ . DAPI nuclear staining, rat anti-CD31, goat anti-desmin followed by Alexa 488-donkey-anti-rat, Cy3-donkey-anti-goat

starting points for capillary sprouting and massive angiogenesis. In the skin graft, the temporary angiogenic transformation starts 24–48 h after reperfusion and produces larger, somewhat deformed, bud structures, which regress after 3–4 days without further growth. From the observation that these considerably larger buds continue to grow over several days without transformation into angiogenic sprouts, it can be deduced that the angiogenic process is reversed and pruning is initiated. Pruning represents the final phase of angiogenesis and the factors involved are largely unknown (Jain 2003). Angiogenesis is a complex process, including basement membrane degradation, endothelial cell proliferation, organisation into immature vessels, and maturation and stabilisation of these vessels followed by pruning or remodelling of the vessels (Karamysheva 2008). It is now common knowledge that the critical event in the regulation of angiogenesis is the signalling cascade involving vascular endothelial growth factor (VEGF) (Gerhardt et al. 2003). During sprouting angiogenesis, some endothelial cells inside the capillary, the so-called tip cells, react to the VEGF gradient that specifies the direction of their migration and move forward of the growing capillary (Semenza 2003). Next to this, it is known that hypoxia stimulates VEGF expression via hypoxia-inducible factor (HIF-1 $\alpha$ ), stimulating endothelial cells to initiate branching angiogenesis (Jain 2003). Based on this it can be assumed in our study that the angiogenic stimulus, i.e. the VEGF production, subsides after reperfusion, most probably to the establishment of normoxia and downregulation HIF-1 alpha (Hanemaaijer et al. 1993). This supports the hypothesis that the graft undergoes angiogenesis as a consequence of reperfusion

**Fig. 4.4** (a) Light microscopy of the corrosion cast on day 5 after transplantation: a 3-dimensional microvascular network of newly formed vessels within the wound bed and existing vasculature of the skin graft. (b) Scanning electron microscopy reveals massive angiogenesis (*green asterisks*) originating from wound bed vessels (*orange arrows*). Connections (*blue circle*) between these vessels to the graft capillaries (*outlined in red*) can be identified. Magnification  $\times 50\text{--}1,000$



and the subsequent delivery of pro-angiogenic factors from the wound bed vessels. This is underlined by the fact that the *in vivo* observations did not reveal any specific function for the temporary graft microvascular buds. However, the temporary angiogenic buds were able to connect to each other and may function as connection sites for further ingrowing vessels after reperfusion.

#### 4.6.2.3 Immunohistochemistry and Corrosion Casting

Staining of the endothelium and pericytes with CD31 and desmin in thick cryo-sections and subsequent evaluation by confocal microscopy revealed that both molecules were present within the vascular wall of the spherical structures within the graft at day 5 (Fig. 4.3c).

Light microscopy showed large wound bed vessels which could be identified due to their dark blue colour, while the smaller capillaries appeared light blue and white (Fig. 4.4a). Vessels from the wound bed and the typical capillaries of the superficial and deep dermal plexus were interconnected. Evaluation by scanning electron microscopy made the two different natures of the vascular beds clear; on the one



hand, the wound bed vessels with angiogenic transformation, on the other hand the graft vascular plexus in a more superficial plane (Fig. 4.4b). The high magnification under the scanning electron microscope also stressed the angiogenic transformation of the wound bed vessels making reperfusion of the existing graft vasculature as a consequence of the connection with newly formed bed vessels highly likely.

We describe for first time the transient formation of angiogenic buds in the graft capillaries between days 3 and 8 after grafting. Additionally, confocal microscopy showed that the wall of the structures expressed CD31 and desmin, suggesting the presence of both endothelial cells and pericytes (Gerhardt and Betsholtz 2003). Presumably the angiogenic response was preceded by a docking of sprouting vessels from the wound bed and subsequently a connection to the pre-existing graft vessels. These findings contradict the generally accepted delay of several weeks of neo-vascularisation (Goretsky et al. 1995). We propose the following interpretation for the temporary angiogenic response (Fig. 4.3a): After reperfusion, the graft is flushed with blood and subsequently angiogenic factors and nutrients reach the hypoxic tissue. Since the angiogenic response does not start until 24–48 h after reperfusion, it can be deduced that during the 3-day period of ischemia, no angiogenesis takes place within the graft. This is most likely due to lack of energy as a consequence of hypoxia and also contradicts the theory that the graft itself is able to perform angiogenesis before reperfusion (Shepherd et al. 2006; Laschke et al. 2008). After reperfusion the increasing oxygen supply from the wound bed causes the still functioning graft vascular network to react. Angiogenesis starts in the centre of the graft already at day 3 implying that the hypoxic stimulus may be most prominent in this area. It then progresses to the periphery where the maximum density of buds was found on day 6. It is reasonable to argue that the hypoxic stimulus and subsequently the continuous production of pro-angiogenic molecules are terminated after successful revascularisation leading to a pruning of unnecessary vascular buds until day 8. Another theory may be that the graft vasculature itself degenerates and is subsequently replaced by ingrowing host vasculature. These results also support the theory that revascularisation of tissues with a pre-existing microvascular network like the skin is predominantly a process driven by the respective recipient bed rather than the graft itself.

### 4.6.3 Vessel Origination and Spatial Distribution

#### 4.6.3.1 Vessel Origination

Transplantation of wild-type skin on GFP mice showed the presence of GFP-positive structures within the graft vessels starting 48–72 h after transplantation. The distribution of the GFP structures was congruent with the pattern of the perfused graft capillaries and therefore these structures most likely were ingrowing vessels. The proportion of GFP vessels to perfused vessels was higher right after reperfusion at day 2–5 in the centre of the graft and approached values of 60–70 % overlap with the perfused vessels until day 10. The outer periphery had a higher proportion of up to 100 % GFP vessels, while in the centre it was as low as 60 % (Fig. 4.2c). The formation of angiogenic buds of the graft capillaries after reperfusion was

predominantly originating from the graft vasculature itself. However, also a few GFP structures in the reciprocal situation (wild-type graft on GFP host) were detected, indicating that vessels from the wound bed were partly involved in this vessel response after reperfusion.

#### **4.6.3.2 Vessel Ingrowth from the Wound Bed into the Skin**

While no GFP structures were detected in the wild-type skin graft after the first 2 days, we observed an increase of GFP structures to 60–90 % in the centre and inner periphery of the graft. Despite increasing reperfusion of the skin graft, the presence of GFP-positive vessels did not reach more than 70–80 % on average in all 3 areas of the graft. In the outer periphery, the proportion of GFP-positive vessels was considerably higher after day 7, than in the inner periphery and centre and reached almost a proportion of 100 %. This may imply that the vessels in the outer periphery were completely invaded by vessels from the host by day 10, while this was not the case in the centre of the graft, where the proportion was at maximum 60 % vessel ingrowth between days 7 and 9.

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## **4.7 Implications for Tissue Engineering of Skin**

The engineering of human tissues represents a promising strategy to replace lost organs or to at least regenerate tissues to a certain extent. Tissue engineering of skin has been pursued for the past decade yielding different epidermal, dermal and lately demo-epidermal substitutes. Substantial progress has been made in the initial treatment and survival of severely burned patients raising the demand for appropriate skin substitutes to cover extensive burn wounds. An important limitation during treatment with large 3D skin substitutes is whether they will rapidly become vascularised. Indeed, cells require oxygen and nutrients for their survival and are therefore located within 100–200  $\mu\text{m}$  of blood vessels, which is the diffusion limit for oxygen (Metcalf and Ferguson 2007). Long-term survival and function of such substitutes depend on rapid development of new blood vessels that will provide nutrients and oxygen to the cells, not only at the margin but also in the centre of the tissue grafts (Gibot et al. 2010).

Based on the results of our studies, it has to be presumed that the existence of a pre-existing vascular network within a skin substitute is of major importance for a successful revascularisation. This is based on the fact that vessels growing in from the wound bed can connect to the existing vascular channels of skin graft and lead to reperfusion of the tissue and subsequently supply of nutrients within less than 5 days. Later on these vessels are able to grow along the already existing vascular sheaths in a centripetal fashion and replace most of the vascular network with host vessels. This process appears to be driven by an oxygen gradient *in vivo*, since it stops after most of the skin graft has regained sufficient oxygen supply and hypoxia is terminated. We also observed a constant and reproducible temporary angiogenic response of the skin graft itself. This is probably due to the fact that the formation of the bud-like structures is a three-dimensional process which can only truly be estimated in an *in vivo* model. This holds equally true for the massive angiogenic

transformation within the wound bed from an early time-point on. The angiogenic reaction of the graft appears to be a reaction of angiogenic factors flushing the vascular network of the graft after reperfusion. Likewise it may symbolise the connection sites of the vessels growing in from the wound bed. As seen in the GFP transplantation series, vessels from the wound bed grow along the existing graft vascular channels which seem to provide a sheath into which the angiogenic vessels can grow and eventually replace them. There seems to be no active angiogenesis coming from the skin graft itself. Part of this may be due to the fact that the graft lacks energy due to ischemia. Hypoxia, which is known to be a strong stimulus of angiogenesis, does not produce angiogenic vessels sprouting in the ischemic graft itself but rather effects the vital and well-perfused wound bed, which in turn reacts with massive sprouting angiogenesis. The fact that the angiogenic reaction of the skin graft is transient and is pruned after 3–4 days goes in line with the pure reactive character of this phenomenon.

These findings can readily be transferred to the field of tissue engineering and would imply that full-thickness skin substitutes require a pre-existing vascular network that wound bed vessels can connect to within several days. By this nutrient supply would be restored and the substitute can survive and be incorporated into the host tissue. From the fact that vessels from the wound bed eventually partly replace the existing vascular sheaths of a skin graft, it can be derived that the required pre-existing vascular channels will not necessarily have to be fully functional vessels. This knowledge will contribute substantially to the manufacturing of fully functioning skin substitutes in the future.

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Upon approval by the local government, all experiments were carried out in accordance with the German and Swiss legislation on protection of animals.

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Jelena Rnjak-Kovacina and Anthony S. Weiss

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## 5.1 The Role of Elastin in the Function of Healthy Human Skin

Elastin makes up 2–4 % of the dry weight of the human skin dermis, yet despite its relatively low abundance, it serves major structural, mechanical, and cell-signaling roles (Fig. 5.1) (Vrhovski and Weiss 1998). The integrity of the elastic fiber network is a strong determinant of the skin's elasticity, resilience, quality, and texture (Kielty 2006; Roten et al. 1996). Its importance is particularly exemplified by the severity of skin impairment in various genetic disorders that affect elastin expression in the dermis, such as cutis laxa and pseudoxanthoma elasticum (Kielty 2006).

Elastin is an insoluble biopolymer formed through cross-linking of its precursor tropoelastin. The elastic fiber is composed of two distinct components, the predominant elastin core wrapped in a lower-abundance sheath of microfibrils. This microfibrillar sheath comprises an array of proteins including fibrillins, fibulins, and microfibril-associated glycoproteins (Ramirez 2000). Elastogenesis occurs primarily during late fetal and early neonatal periods, and healthy adult tissue undergoes little elastin turnover (Uitto and Larjava 1991).

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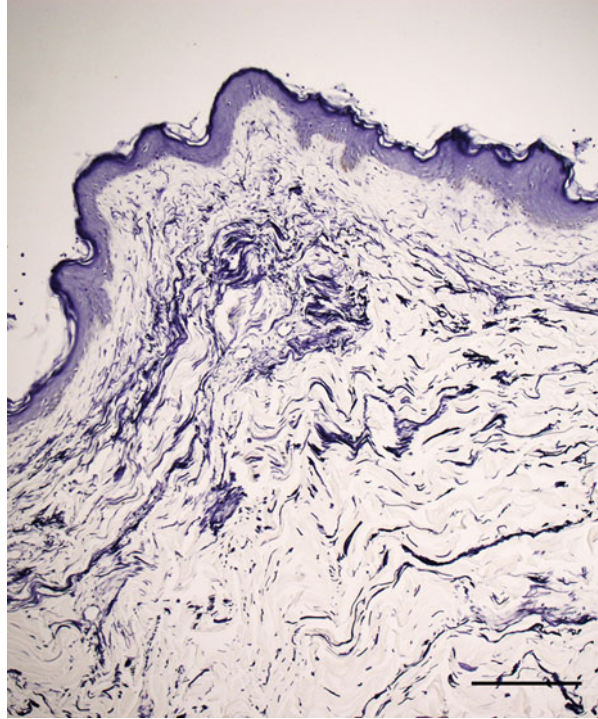
J. Rnjak-Kovacina  
Department of Biomedical Engineering, Tufts University,  
Boston, MA, USA  
e-mail: jelena.rnjak@tufts.edu

A.S. Weiss (✉)  
School of Molecular Bioscience, University of Sydney,  
Sydney, NSW, Australia

Bosch Institute, University of Sydney,  
Sydney, NSW 2006, Australia

Charles Perkins Centre, University of Sydney,  
Sydney, NSW 2006, Australia  
e-mail: anthony.weiss@sydney.edu.au

**Fig. 5.1** Histological representation of elastin fibers in the skin dermis. Elastin fibers are stained *black/blue* with Verhoeff-Van Gieson (VVG) stain. Scale bar is 200  $\mu\text{m}$



Elastin is not adequately regenerated following severe burn injuries and is functionally and spatially disorganized in scar tissue (Amadeu et al. 2004; Chen et al. 2009). The role of elastin in wound healing, contraction, and scar formation is becoming increasingly evident as clinicians and researchers recognize the need to regenerate the elastic fiber network in order to achieve functionally and aesthetically acceptable healing following severe burn injuries (Balasubramani et al. 2001; Berthod et al. 2001; Compton et al. 1989; Hafemann et al. 1999; Haslik et al. 2007a; Raghunath et al. 1996; Rnjak et al. 2011a). Elastin has been underrepresented in dermal substitute design, but its presence may be the key to improved elastic fiber regeneration following burn injuries. The increased availability of soluble animal elastin and its derivatives, and recombinant human tropoelastin, now allow dermal substitute design to be revisited in light of recent findings about the role of elastin in wound healing.

We describe three main classes of novel elastin-containing dermal substitutes:

1. Synthetic or natural polymer-based dermal substitutes where the surface is decorated with a thin coating of soluble elastin, elastin peptides, or tropoelastin
2. Composite substitutes made of collagen or other proteins/polymers in combination with elastin or tropoelastin
3. Soluble elastin or tropoelastin-based dermal substitutes

While decorating the surface of a dermal substitute with elastin has provided incremental improvements over other common dermal substitutes due to its

important cell-signaling roles (as will be detailed below), we maintain that the presence of elastin as a cell signaling and a structural component to provide important mechanical cues is of vital importance.

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## 5.2 The Presence of Elastin and Its Derivatives May Improve the Performance of Dermal Substitutes

Several key mechanical and cell-signaling features of elastin point to its utility in reducing severe skin contraction and scarring associated with burn injuries and aiding the wound-healing process. Elastin may exert these effects by modulating fibroblast differentiation into contractile myofibroblasts (Arora et al. 1999; Hinz et al. 2003; Hinz and Gabbiani 2003; Kloxin et al. 2010), promoting new elastin expression in the healing wound (Daamen et al. 2006, 2008; Hinek et al. 2005), signaling migration of fibroblasts and monocytes (Bisaccia et al. 1994; Grosso and Scott 1993; Senior et al. 1980, 1982), promoting migration and terminal differentiation of keratinocytes (Fujimoto et al. 2000), and enhancing angiogenesis (Daamen et al. 2006, 2008).

While the mechanism of wound and graft contraction is not fully elucidated, the most widely accepted hypothesis embraces the role of myofibroblasts, a contractile fibroblast phenotype with smooth muscle cell-like features, including the expression of alpha-smooth muscle actin ( $\alpha$ -SMA) (Farahani and Kloth 2008; Harrison and MacNeil 2008). This process is predominantly mediated by transforming growth factor-beta 1 (TGF- $\beta$ 1) and connective tissue growth factor, but other endogenous and secreted factors, keratinocyte cells, and the mechanical properties of the wound bed and the applied dermal graft are also implicated in this process (Arora et al. 1999; Hinz et al. 2003; Hinz and Gabbiani 2003; Kloxin et al. 2010; Farahani and Kloth 2008; Harrison and MacNeil 2008; Darby and Hewitson 2007; Desmouliere et al. 1993; Goffin et al. 2006; Hinz 2007; Leask and Abraham 2004). Cell contractile forces are influenced by the elasticity of the surrounding tissue or the substrate to which the cells are attached. Interfering with myofibroblast stress perception and transmission may therefore modulate wound and graft contraction (Hinz 2007). The level of fibroblast differentiation into the contractile phenotype is reduced on more elastic substrates, and the level of  $\alpha$ -SMA increases with increased substrate rigidity (Arora et al. 1999; Hinz et al. 2003; Hinz and Gabbiani 2003). The presence of  $\alpha$ -elastin in collagen-based scaffolds suppresses the differentiation of phenotypically proliferating fibroblasts into contractile myofibroblasts and modulates collagen contraction (Kloxin et al. 2010); it remains to be seen if this effect is a result of reduced scaffold stiffness or specific cell-signaling domains in elastin. Myofibroblast dedifferentiation has also been induced by culturing differentiated cells on more elastic substrates (De Vries et al. 1994). If this effect can be replicated in vivo, the presence of elastin in the healing wound has the potential to prevent wound contraction and scar formation.

The newly synthesized elastin fibers in scar tissue are thin, fragmented, and immature and do not reach the maturity of healthy elastin fibers even many years post-injury (Roten et al. 1996; Amadeu et al. 2004; Chen et al. 2009; Tsuji and

Sawabe 1987). Reduced elastin and fibrillin-1 expression is especially prominent in excessive hypertrophic scars, accounting for their inherent inelasticity (Amadeu et al. 2004). Elastin expression can be upregulated in cultured dermal fibroblasts of human skin explants or in the skin of nude mice *in vivo* in the presence of proteolytic digests of bovine elastin (Hinek et al. 2005). Furthermore, adding elastin to collagen-based scaffolds helps to induce elastin deposition when implanted subcutaneously in rats compared to collagen-only scaffolds (Daamen et al. 2006, 2008). On this basis, elastin-containing dermal substitutes can be expected to reduce scar tissue formation by helping to signal the upregulation of *de novo* elastic fiber synthesis.

Elastin contains specific cell-interacting domains that promote cell adhesion, spreading, proliferation, and signaling involved in wound healing and skin homeostasis (for a review, see (Almine et al. 2010) and references therein). In particular, elastin peptides act as chemoattractants for fibroblast and monocyte migration (Bisaccia et al. 1994; Grosso and Scott 1993; Senior et al. 1980, 1982) and can induce migration and terminal differentiation of epidermal keratinocytes (Fujimoto et al. 2000). Furthermore, the presence of  $\alpha$ -elastin in collagen-based scaffolds has been shown to induce angiogenesis (Daamen et al. 2006, 2008), a process essential for meeting the metabolic demands of the newly formed tissue, and to limit wound hypoxia.

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### 5.3 Currently Available Elastin-Containing Dermal Substitutes

Despite its obvious importance in healthy skin function, elastin-containing dermal substitutes are severely underrepresented in the literature and especially in the clinic. However, as we learn more about the role of elastin in wound healing and with the increasing availability of animal and human elastin, tropoelastin, and their derivatives on the market, this trend is likely to change. Here we describe three classes of elastin-containing dermal substitutes:

1. Matrices coated with elastin
2. Composite elastin-collagen scaffolds
3. Scaffolds composed solely of elastin or tropoelastin

Skin autografts and decellularized porcine or human dermis inherently contain elastin fibers. Decellularized human dermis, such as AlloDerm, can reduce wound contraction compared to several other dermal substitutes, including Integra (Integra LifeSciences Corp), Dermalogen (Collagenesis), and Dermagraft-TC (Advanced Tissue Sciences) in a nude mouse model (Truong et al. 2005). This dermal substitute has predominantly been used clinically in reconstructive surgery due to the somewhat high market cost of the commercial product (Yim et al. 2010) but has demonstrated good cosmetic appearance and functional results with no contractures on relatively small burn areas (<20 % TBSA) (Callcut et al. 2006) and increased elasticity compared to a split-thickness skin autograft only, when grafted in conjunction (Callcut et al. 2006; Lattari et al. 1997; Wainwright 1995). While autografts



and decellularized allografts are some of the currently most successful burn treatments, they present inherent disadvantages, including limited availability, risk of pathogen transfer, and batch-to-batch inconsistencies.

### 5.3.1 Matrices Coated with Elastin

Two types of matrices coated with elastin for dermal grafting or wound healing have been described. Alginate dressings covalently linked to an elastin peptide VGVAPG were developed a decade ago, but so far appear to have been tested only in small rabbit ear wounds (Hashimoto et al. 2004). MatriDerm, a bovine collagen-based dermal sponge coated with 3 % (w/v)  $\alpha$ -elastin derived from bovine ligamentum nuchae, has been extensively tested in animal models and in clinical settings.

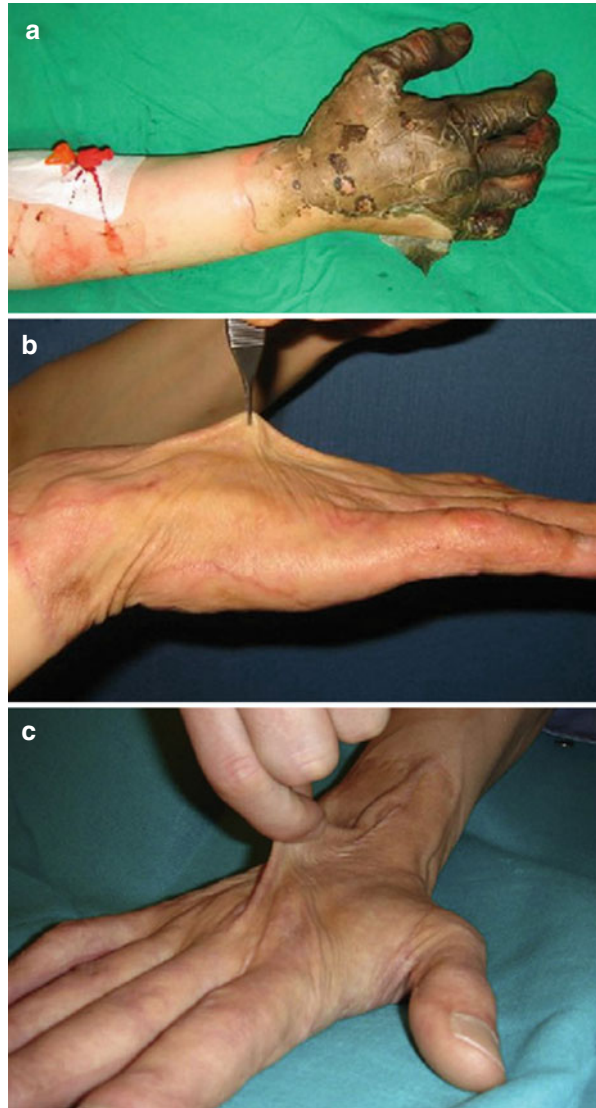
MatriDerm in combination with an autologous split-thickness mesh graft displayed excellent dermal regeneration with little wound contraction in porcine full-thickness excision wounds (De Vries et al. 1994; Lamme et al. 1996). This effect is also seen in a human punch biopsy model, where it reduces scar formation and wound contraction (De Vries et al. 1995). These positive effects are attributed to the presence of elastin in MatriDerm, as collagen sponges coated with fibronectin or hyaluronic acid do not display similar effects. MatriDerm in combination with a split-thickness mesh graft also results in improved skin pliability, elasticity, maximal extension, and immediate retraction compared to split-thickness mesh grafts alone in scar reconstruction wounds, but not in burn wounds (van Zuijlen et al. 2000). This effect may be due to the more complex environment of the burn injury wound bed compared to that of scar reconstruction wounds. However, MatriDerm has proven useful in treating burn injuries of the upper extremities. Scar and contraction-free healing of these wounds is essential, as hand injuries occur in 60–90 % of burn cases and even a small wound can cause extreme functional disability if not healed effectively. MatriDerm in combination with a sheet, but not meshed, skin autografts promotes excellent wound healing, full range of motion, excellent skin pliability, reduced scar height, and ultimately good hand function (Haslik et al. 2007a, 2010; Ryssel et al. 2008, 2010) (Fig. 5.2). The benefits of MatriDerm may be exerted by inhibiting dermal fibroblast differentiation into contractile myofibroblasts within the first 2 weeks of wound healing (Lamme et al. 1996).

While MatriDerm points to the benefits of elastin in wound healing, its effects are exerted through cell-signaling domains of elastin with no apparent contribution from the mechanical properties of the elastic fiber or scaffold elasticity.

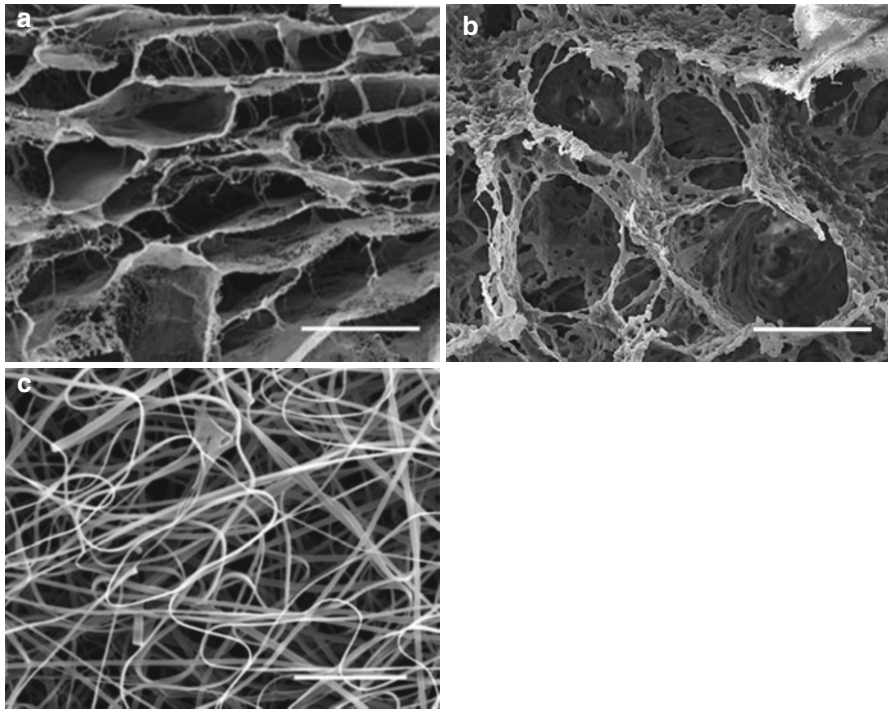
### 5.3.2 Composite $\alpha$ -Elastin and Collagen Scaffolds

Lyophilized sponges composed of soluble  $\alpha$ -elastin and collagen are constructed as highly porous, cell-interactive scaffolds that promote elastin and collagen deposition and enhance angiogenesis (Fig. 5.3a) (Daamen et al. 2008). These constructs were among the first to incorporate soluble elastin in place of insoluble elastin

**Fig. 5.2** Clinical application of MatriDerm in full-thickness burns. MatriDerm and a split-thickness autograft were applied to a hand burn. Image (a) shows the burn injury, while images (b, c) show the healed wound at 3 and 12 months post-grafting, respectively (Images are from Haslik et al. 2007b, 2010)



fibers, which caused calcification presumably due to their heterogeneous content (Daamen et al. 2006, 2008). More recently, collagen has been combined with recombinant human tropoelastin in electrospun scaffolds comprised of blended collagen-tropoelastin fibers. When the proportion of collagen was maintained at less than 50 % of total protein in these scaffolds, they displayed elasticity properties similar to those of electrospun tropoelastin-only scaffolds. In addition to these mechanical benefits, the collagen component improved the proliferation and migration of primary dermal fibroblasts, and the composite constructs are well tolerated in vivo where they support fibroblast infiltration, de novo collagen synthesis, and angiogenesis (Rnjak-Kovacina et al. 2012).



**Fig. 5.3** Potential dermal substitute scaffolds made of soluble elastin derivatives including  $\alpha$ -elastin and tropoelastin. (a) Lyophilized collagen,  $\alpha$ -elastin scaffolds (Daamen et al. 2008); (b) High-pressure  $\text{CO}_2$ -treated tropoelastin,  $\alpha$ -elastin hydrogels (Annabi et al. 2010); and (c) Electrospun tropoelastin scaffolds (Rnjak et al. 2009). Scale bars are 100  $\mu\text{m}$  in image (a) and 50  $\mu\text{m}$  in images (b, c)

### 5.3.3 $\alpha$ -Elastin- and Tropoelastin-Based Scaffolds

Highly porous, elastic hydrogels with large, interconnected pores spanning the entire scaffold can be generated from  $\alpha$ -elastin or composite  $\alpha$ -elastin and recombinant human tropoelastin in the presence of high-pressure carbon dioxide (Fig. 5.3b). Both scaffold types promote dermal fibroblast attachment, infiltration, and proliferation *in vitro*. Increased proportions of tropoelastin in these scaffolds increase scaffold elasticity (Annabi et al. 2009a, b, 2010).

Recombinant human tropoelastin has potential advantages over the use of soluble animal elastin as it displays less batch-to-batch variability, carries little risk of immune rejection and pathogen transfer, and retains a wider range of cell-signaling domains. Recombinant human tropoelastin can be electrospun into 3D fibrous scaffolds with highly interconnected pores (Fig. 5.3c) (Rnjak et al. 2009). Control over the pore size and porosity is achieved by altering the flow rate of the protein solution during the electrospinning process to generate scaffolds that support primary dermal fibroblast attachment, proliferation, and migration into the scaffold (Rnjak et al. 2009; Rnjak-Kovacina et al. 2011b) leading to ECM deposition with only minor scaffold contraction *in vitro* and are well tolerated *in vivo* (Rnjak-Kovacina et al. 2011b).

## Conclusions

Despite its fundamental role in healthy skin function and wound healing, elastin has been largely underrepresented in dermal substitute design. This has largely been the result of limited supply of sufficiently pure, soluble elastin and its derivatives. Increased availability of these materials and improved understanding of the importance of elastin in wound healing, contraction, and scar formation are increasing the use of elastin in dermal substitute design. Collagen-based scaffolds coated with  $\alpha$ -elastin are in the clinic and show benefits over collagen-only scaffolds, but utilization of a combination of elastin's cell-signaling and mechanical properties is likely to result in the greatest benefits to scar reduction and contracture-free wound healing.

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# Surface Modification by Cold Gasplasma: A Method to Optimise the Vascularisation of Biomaterials

Andrej Ring, Stefan Langer, and Jörg Hauser

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## 6.1 Tissue Engineering in Plastic and Reconstructive Surgery

Currently reconstructive techniques, such as flap surgery, represent gold standard for the treatment of extensive tissue defects. Nevertheless, tissue engineering opens up unexpected possibilities of novel therapeutic strategies to plastic and reconstructive surgery. Due to promising findings in this field of research, it is believed that in future times established surgical therapy modalities may be supplemented by the application of artificial engineered tissue.

Until now, the engineering of relatively simple tissue types such as dermis and bone still represents a technological challenge. In fact, an adequate growth of a new microvasculature represents one of the major limitations for long-term survival and successful function of large three-dimensional tissue constructs after incorporation into the host organism. The oxygen supply, which is required for the survival of cellular components of artificial tissue grafts, is limited to a diffusion distance from the next supplying blood vessel (Laschke et al. 2006).

Vascularisation of engineered tissue represents a complex dynamic process that is characterised by coordinated humoral and cellular interactions, which are regulated by various cytokines and growth factors (Carmeliet 2000).

With currently used techniques, however, the vascularisation of engineered tissue is still not fast enough to ensure an adequate transport of nutrients and oxygen in transplanted tissue constructs. To solve this problem, many potential tissue substitutes, based on biomaterials and several methods for induction and optimisation of their vascularisation, are currently under investigation. Some of these methods are summarised in this chapter.

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A. Ring (✉) • S. Langer • J. Hauser  
Department of Plastic Surgery, BG University Hospital Bergmannsheil,  
Ruhr-University Bochum,  
Bürkle-de-la-Camp Platz 1,  
Bochum, NRW 44789, Germany  
e-mail: andrej.ring@ruhr-uni-bochum.de

Below, the technique of gasplasma-assisted modification of biomaterial surfaces is described in detail by the example of dermis and bone substitutes.

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## 6.2 Vascularisation of Biomaterials and Engineered Tissue Constructs

Scaffolding biomaterials can be modified so that they retain growth factors and structural elements such as collagen. Collagen itself facilitates the cell growth because of its cell-bonding domain, which interacts with the cells via integrins or specific cell surface receptors. In scaffolds, collagen is responsible for maintaining the structural integrity and can enhance significantly the cell affinity to three-dimensional biomaterials. Besides, collagen was proven to promote the vascularisation (Ma 2008).

In the past, many factors that play a central regulatory role in the process of angiogenesis could be identified and are now available in recombinant forms to support engraftment of artificial tissue. The ingrowth of blood vessels into implanted tissue constructs might be accelerated by local application and incorporation of growth factors into scaffolds (Moon and West 2008).

Additionally, gene transfer of angiogenic growth factors to the cells incorporated into the scaffolds has been shown to stimulate the vascularisation (Riabikhin et al. 2000).

Finally, a network of newly developed microvessels may be engineered *in vitro* by seeding scaffolds with endothelial cells that develop interconnections to the mature blood vessels after implantation of pre-seeded tissues constructs (Mooney and Mikos 1999).

Another approach to induce angiogenesis and facilitate vascularisation of tissue constructs is the use of arteriovenous shunt loops (Tilkorn et al. 2010).

However, until today, there are significant unsolved problems to realise these theoretical approaches.

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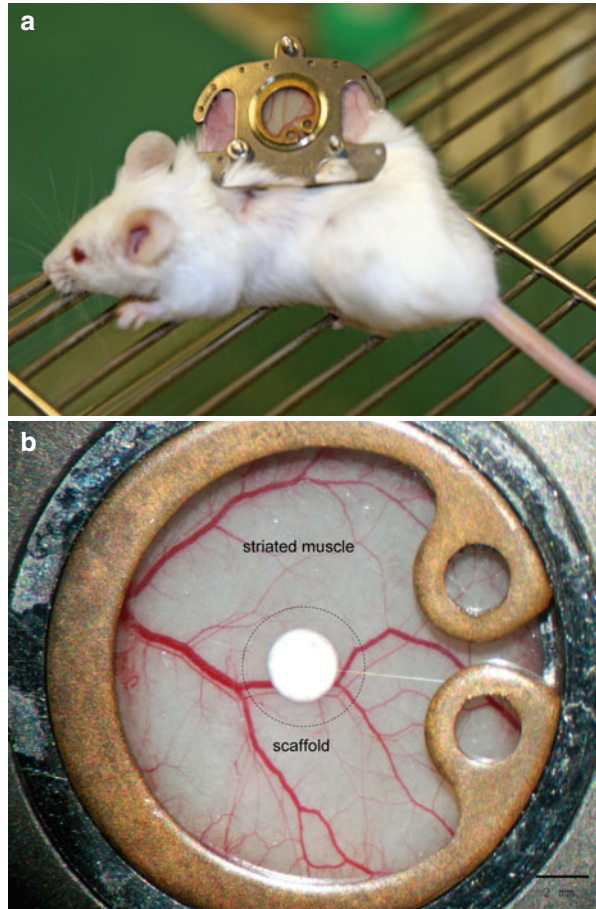
## 6.3 In Vivo Model to Study Vascularisation in Biomaterials and Tissue-Engineering Constructs

To develop new strategies for optimal vascularisation of implanted tissue constructs, experimental *in vivo* models are required.

The dorsal skinfold chamber is one of these *in vivo* models, which recently has been proven to be an extremely useful tool that enables a more detailed and long-term analysis of mechanisms of vascularisation as well as causes of transplant failure (Fig. 6.1a, b). Through combination of intravital fluorescence microscopy and computer-assisted off-line analysis techniques, this model allows a quantitative analysis of the blood vessel ingrowth in biomaterials and engineered tissue constructs *in vivo* (Fig. 6.2a, b). The observation window of the chamber allows a dynamic analysis regarding the blood vessel growth over a time period of 3–4 weeks (Menger et al. 2002; Laschke et al. 2011).



**Fig. 6.1** (a) Balb/c mouse with the dorsal skinfold chamber (weight of the titanium chamber, ~3 g). Observation window of a dorsal skinfold chamber directly after implantation of a porous PEGT/PBT block-copolymer scaffold. (b) The scaffold ( $\varnothing$  2 mm) is placed in the middle of the window onto the vascularised striated skin muscle

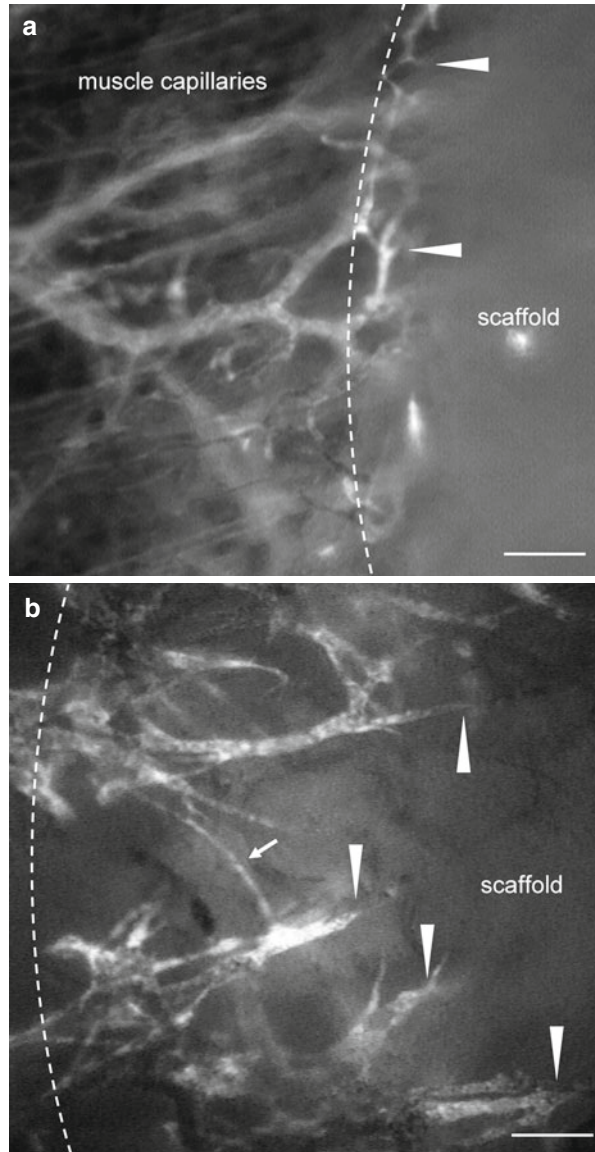


## 6.4 Structure and Surface Properties of Biomaterials

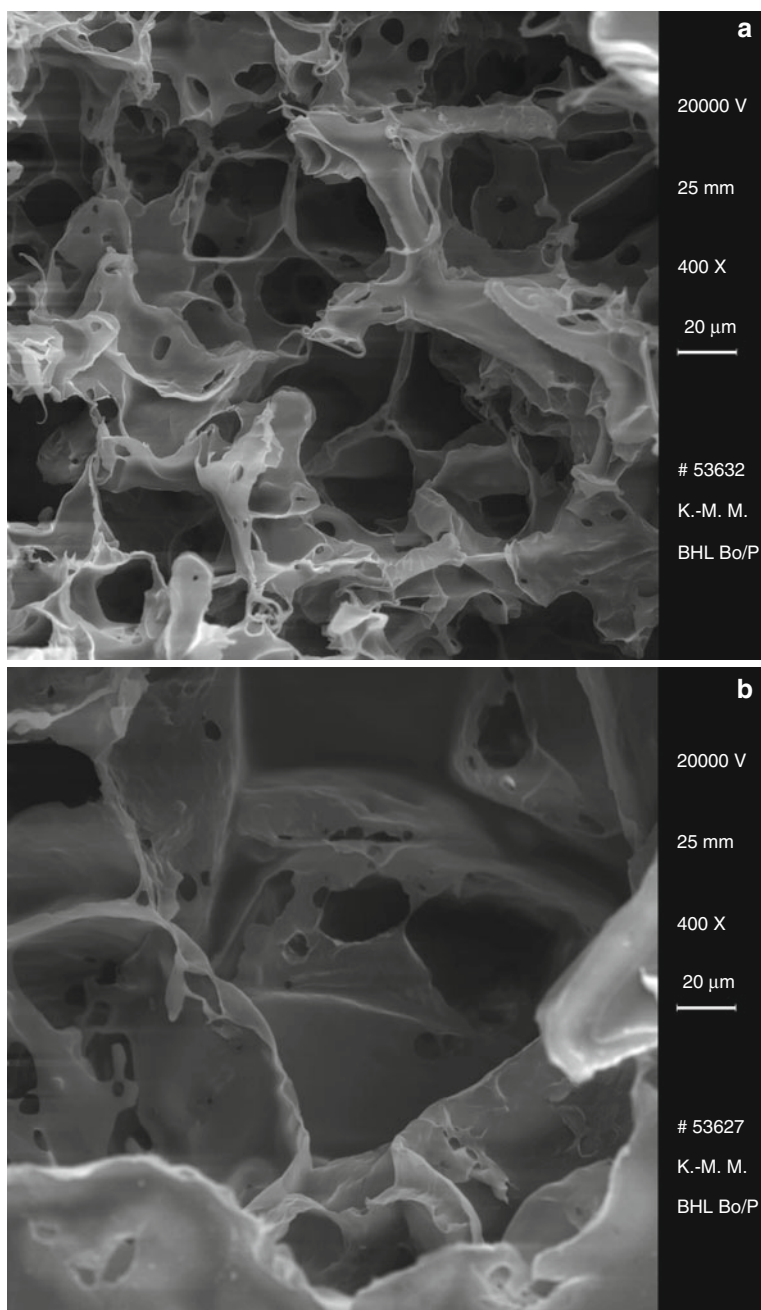
Different strategies were developed in order to promote angiogenesis and to establish microcirculation in tissue substitutes. Some of these strategies involve the use of cell pre-seeding techniques, incorporation of stem cells as well as the use of growth factors to stimulate neovascularisation. Currently, much interest has been centred on gene transfer techniques to provide locally increased concentrations of growth factors that accelerate microvascular regeneration.

Besides many biological factors that influence angiogenesis and vascularisation, the three-dimensional structure of biomaterials continues to play an important role in vascular tissue formation. This is proven by the fact that reduced vascularisation of tissue substitutes is often associated with insufficient structural architecture as determined by size and interconnection of macro- and micropores. These structural properties influence the vascular integration of the transplant (Ma 2004).

**Fig. 6.2** Intravital fluorescence microscopy after implantation of a porous PEGT/PBT block-copolymer scaffold into the dorsal skinfold chamber. Plasma contrast enhancement in microvessels is achieved by intravenous injection of 5 % FITC-labelled dextran (MW 150 kDa). Blood vessel sprouts (*arrowhead*) can be detected in the border zone (*dotted line*) of the scaffold at day 3 (**a**). Newly formed sprouts and outgrowing blood vessels (*arrowhead*) show a progressive infiltration of the porous scaffold. The vessels interconnect with each other (*arrow*) to build a new microvascular network at day 10 (**b**). Scale bars, 25  $\mu\text{m}$



Various reports underline the importance of the biomaterial architecture for the process of vascularisation. In the past, the authors used the dorsal skinfold chamber of mice in order to study vascularisation of synthetic PEGT/PBT block-copolymer scaffolds (Ring et al. 2006). The pore size of the scaffolds has been shown to be a critical determinant for blood vessel ingrowth. The data analysis revealed that the vascularisation was significantly accelerated in pores with a size  $>250 \mu\text{m}$  when compared with those  $<250 \mu\text{m}$  (Fig. 6.3a–d).



**Fig. 6.3** Scanning electron microscopy displays the microarchitecture of PEGT/PBT block-copolymer matrices with different pore sizes:  $<250\ \mu\text{m}$  (a) and  $>250\ \mu\text{m}$  (b). Cross-sectional overview of both scaffolding matrices at day 21 after implantation (c, d). Intense vascularisation of the matrix with profound ingrowth of fibrovascular tissue (*asterisks*) into the matrix pores is shown in scaffolds with the pore size  $>250\ \mu\text{m}$  (c). In contrast, the scaffolds with the pore size  $<250\ \mu\text{m}$  show a poor vascular ingrowth and an excessive fragmentation of the matrix (d)

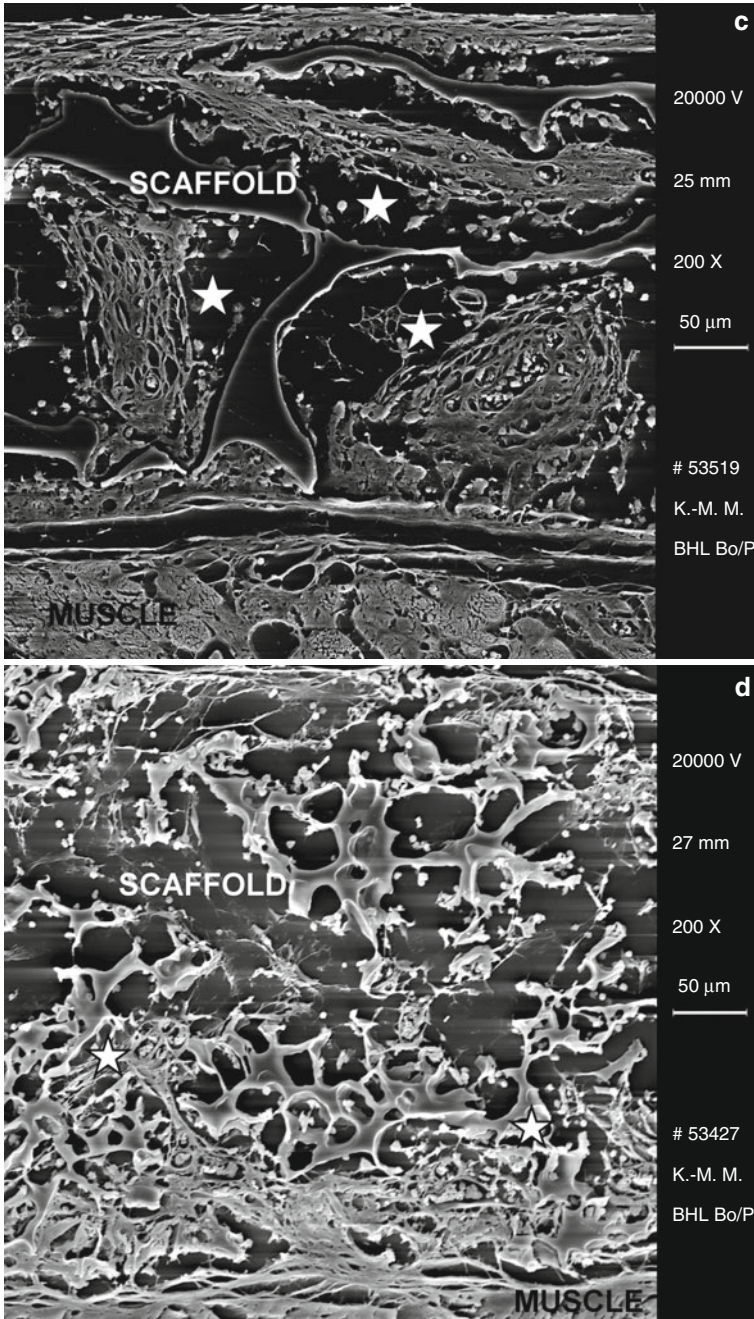
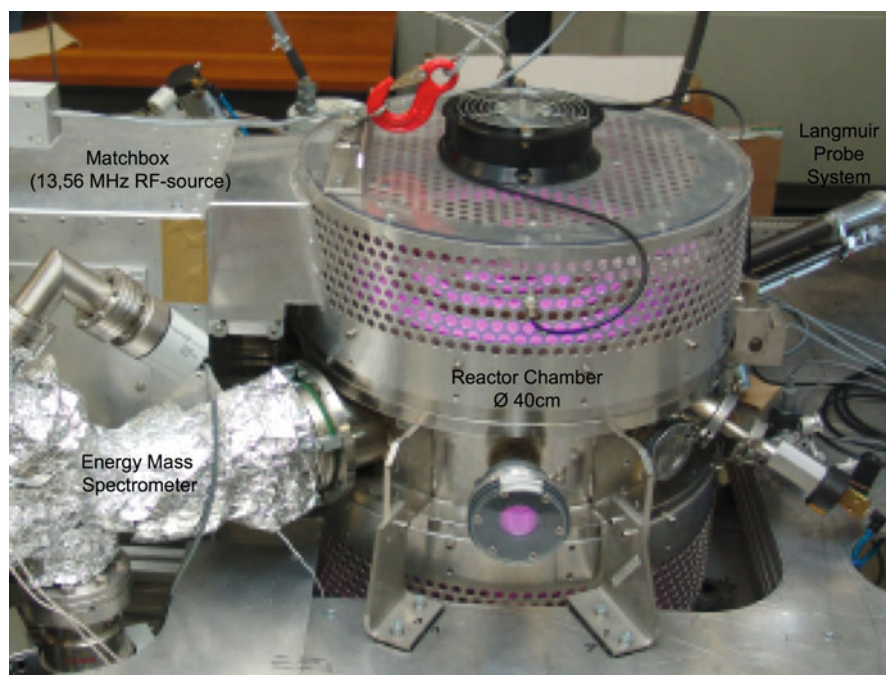


Fig. 6.3 (continued)

Scaffolds serve as three-dimensional matrices for cells to attach and to proliferate. In addition to appropriate material architecture, the surface characteristics seem also to be essential for the interaction between cells and biomaterials. The ingrowth of blood vessels into the biomaterials is a process that is accompanied by an inflammatory reaction. It is known that inflammatory cells like macrophages release angiogenic factors including VEGF, bFGF and PDGF, which induce and regulate angiogenesis. On the other hand, an excessive inflammatory reaction may lead to tissue damage and implant failure.

## 6.5 Surface Modification by Cold Low-Pressure Gasplasma

Cold low-pressure gasplasma is a partially ionised gas consisting of ions, electrons and ultraviolet photons as well as reactive neutral species (radicals, excited atoms and molecules) with sufficient energy to break covalent bonds on the material surface. The plasma is excited at low-pressure conditions at temperatures below 40 °C (Fig. 6.4).



**Fig. 6.4** Double inductively coupled plasma (DICP) reactor is used for the surface treatment of biomaterials (By kind permission of the Institute for Plasma Technology, Ruhr University Bochum). The plasma is ignited and heated by a radio-frequency source at 13.65 MHz with a forward power of 1,000 W. The gas mixture composes of argon (100 sccm) and hydrogen (5 sccm) with a pressure of 5 Pascal and temperature below 40 °C

It initiates a variety of chemical reaction pathways, which increase surface free energy. This effect is called surface activation (Hauser et al. 2008). A change of the surface polarity and the introduction of functional binding groups have been discussed as possible mechanisms of action for plasma activation of surfaces (Yasuda and Gazicki 1982). The treatment of materials with cold low-pressure gasplasma does not affect the structure but only interferes with the superficial layer and consequently avoids damage to thermolabile biomaterials (Roach et al. 2007).

Previous studies indicated that the modification of biomaterial surface by cold low-pressure gasplasma treatment improves the biocompatibility. In these studies it was shown that after treatment with gasplasma, the polarity of the biomaterial surface was significantly enhanced, thus leading to an increase of oxygen-containing functional groups on material surfaces and an improvement of adhesion characteristics. The enhanced surface hydrophilicity was found to lead to higher cell affinities (Hauser et al. 2009). Elevated protein adsorption correlating with an increase of surface energy was observed to improve cellular growth. Surface modification by gasplasma treatment was also found to increase the adhesion of proteins, thus stimulating cellular responses through the integrin-mediated mechanism (Shibata et al. 2002).

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## 6.6 Dermis Substitutes

In spite of the progress in tissue engineering, the clinical application of artificial dermis substitutes is limited. Until today, the gold standard for treatment of deep burn wounds remains the transplantation of autologous skin grafts. However, the poor quality of grafted skin is problematic, especially in functional, highly strained regions. Early studies, published by Yannas and Burke (1980), formed the basis for development of artificial dermis replacement. From then it was shown that the use of dermis substitutes optimises the clinical outcome of skin grafts in terms of elasticity, prevention of contractures and hypertrophic scarring.

Although there are a number of dermis substitutes available for clinical use, all of them fail to fulfil the criteria for rapid vascularisation of the matrix. A profound infiltration of the scaffolding matrix by vascular tissue with short ischemic time is absolutely essential to ensure the transplant survival. The poor vascularisation of dermal matrices increases the risk of infection and minimises the acceptance of co-transplanted skin grafts.

During the last years there is continuous interest in modifying dermis substitutes. Previously it was shown by the authors that surface modification by glow discharge technique does improve the vascularisation of synthetic polymer scaffolds (Ring et al. 2007). Due to these promising results, the authors were encouraged to analyse whether the glow discharge activation of biological scaffolds leads to a similar effect in terms of improved vascularisation. Using the standardised model of the dorsal skinfold chamber, quantitative data in terms of angiogenic response and neovascularisation of scaffolds could be obtained. The study showed that scaffolds consisting of a bovine, non-cross-linked collagen matrix based on collagen types I, III and V and

supplemented by an elastin hydrolysate displayed a progressive and significant increase of functional vessel density when treated with cold low-pressure plasma prior to transplantation. The results of this study indicate that cold low-pressure plasma treatment is a suitable tool for surface activation of dermis substitutes, which leads to enhanced angiogenesis and accelerated vascularisation (Ring et al. 2010).

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## 6.7 Bone Substitutes

A lack of early vascularisation of decellularised bone substitutes remains a major obstacle, both in the clinical setting as well as in tissue engineering, resulting in an early loss of transplant. The enhancement of the vascularisation is a valuable aspect to improve successful integration of bone substitutes.

Recently, the authors have shown that surface modification by cold low-pressure gasplasma treatment improves the vascularisation of allogenic bone substitute material by stimulation of angiogenesis (Ring et al. 2011). The new blood vessels formed a stable microvasculature infiltrating the bone substitutes, and the blood vessel growth in gasplasma-modified bone was enhanced.

As possible explanation for the observed enhanced vascularisation, some plasma-induced changes of chemical and physical adhesion parameters were assumed. The changes of surface chemistry of the gasplasma-treated bone have been detected by XPS analysis. The observed reduction of carbon and nitrogen atoms on the treated surfaces could well lead to an increased amount of functional binding sites influencing protein and cell adhesion.

These are important findings as they indicate that cold low-pressure gasplasma surface-modified allogenic bone may represent an adequate alternative to autologous bone grafting. Potentially, an appropriate bone substitute that vascularises significantly faster after surface modification by glow discharge gasplasma may also be used for the prefabrication of osteocutaneous tissue or osteomusculocutaneous flaps.

Experimental studies on techniques for improvement of biomaterial's vascularisation are essential for the development of novel treatment strategies and introduction of engineered tissue constructs in clinical practice. Future investigations directed towards the combination of gasplasma-mediated coatings of biomaterials with bioactive factors may produce an additional option to stimulate vascularisation.

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# Collagen Matrices with Enhanced Angiogenic and Regenerative Capabilities

# 7

Norbert Pallua, Marta Markowicz, Gerrit Grieb,  
and Guy Steffens

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## 7.1 Introduction

The application of collagen matrices in covering wound defects has been under investigation for quite some time. First applications led to the development of Integra<sup>®</sup>, a well-known product consisting of collagen and chondroitin sulfate cross-linked with glutaraldehyde and contained on a silicon foil. Application of this product requires a two-stage procedure necessitating hospitalisation of the recipient for about 2 weeks. Subsequent to vascularisation of the collagen, the foil is removed

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N. Pallua, MD, PhD (✉)

Department of Plastic Surgery, Hand Surgery, Burn Center,  
University Hospital, RWTH Aachen University,  
Pauwelsstrasse 30, 52074 Aachen, Germany  
e-mail: npallua@ukaachen.de

M. Markowicz, MD, PhD

Department of Plastic Surgery, Caritas Hospital,  
University Regensburg, Landshuterstrasse 65,  
93053 Regensburg, Germany  
e-mail: mmarkowicz@caritasstjosef.de

G. Grieb, MD

Department of Plastic Surgery, Hand Surgery, Burn Center,  
University Hospital, RWTH Aachen University,  
Pauwelsstrasse 30, 52074 Aachen, Germany

Institute of Biochemistry and Molecular Cell Biology,  
University Hospital, RWTH Aachen University,  
Pauwelsstrasse 30, 52074 Aachen, Germany  
e-mail: gerritgrieb@gmx.de

G. Steffens, PhD

Institute of Biochemistry and Molecular Cell Biology,  
University Hospital, RWTH Aachen University,  
Pauwelsstrasse 30, 52074 Aachen, Germany  
e-mail: gusteffens@ukaachen.de

and the collagen surface is covered with autologous split skin in the second stage. The long hospitalisation period creates a need for a material of improved angiogenic capabilities.

Wissink et al. (2000) proposed a new procedure for modifying collagen matrices by cross-linking heparin to the collagen with EDC, the water-soluble carbodiimide, together with *N*-hydroxysuccinimide. The heparin-binding angiogenic factor FGF1 was shown to bind physically to the incorporated heparin and to be released slowly at a rate more conducive to angiogenesis (Wissink et al. 2000, 2001). We then initiated a study for investigating whether the physical binding of VEGF, which also has a VEGF-binding domain (Ferrara et al. 2003; Nillesen et al. 2007), to heparinised collagen had a similar effect on the angiogenic properties. Collagen matrices were modified according to the procedure developed by Wissink et al. (2000). Conditions differed slightly and the following products were selected for further research: HOE0 as the unmodified matrix, HOE1 as the cross-linked only matrix and H1E1 as the cross-linked matrix with incorporated heparin (Yao et al. 2008).

In this chapter, we provide a full overview of the results of investigations performed during the last decade, demonstrating the advantages of cross-linking and heparinising collagen matrices and discussing its potentials.

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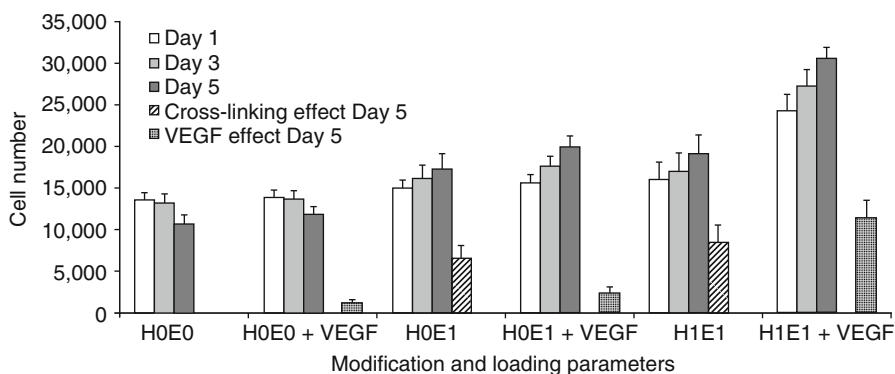
## 7.2 Methods and Materials

Collagen matrices were obtained from Suwelack Skin and Health Care GmbH, Billerbeck, Germany. The matrices consist of bovine collagen and are characterised by a pore size in the 15–30  $\mu\text{m}$  range, with an overall porosity of 98 %. Modifications were performed as described in Steffens et al. (2004). HUVECs were isolated from human umbilical cords, and only cells of up to passage 4 were used. Evaluation of proliferation was performed by counting in the Neubauer chamber. The *chorioallantoic* membrane assay was applied as in Yao et al. (2006). Subcutaneous implantation and determination of the hemeprotein contents were executed as described in Steffens et al. (2004). In vivo experiments with miniature pigs were performed as described in Markowicz et al. (2006), and full-thickness defects were covered with one of each of the three collagen matrices and grafted with autologous split skin. Wound regeneration was observed after 1, 2 and 4 weeks.

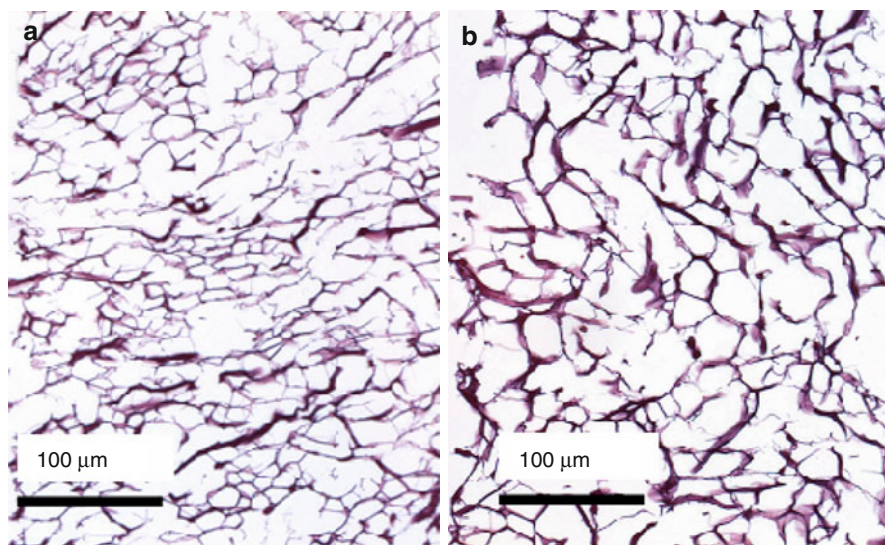
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## 7.3 Results and Discussion

In a first approach, the angiogenic properties were tested by exposing endothelial cells (HUVECs) to the three selected matrices: HOE0 as the unmodified matrix, HOE1 as the cross-linked only matrix, and H1E1 as the matrix cross-linked in the presence of heparin. Figure 7.1 shows that cell numbers increased over time for all matrices. Loading the matrices with VEGF in all cases produced an additional increase. This increase is referred to as the VEGF effect and was greatest when loading VEGF to the heparinised matrices H1E1. Also, we observed an increase in



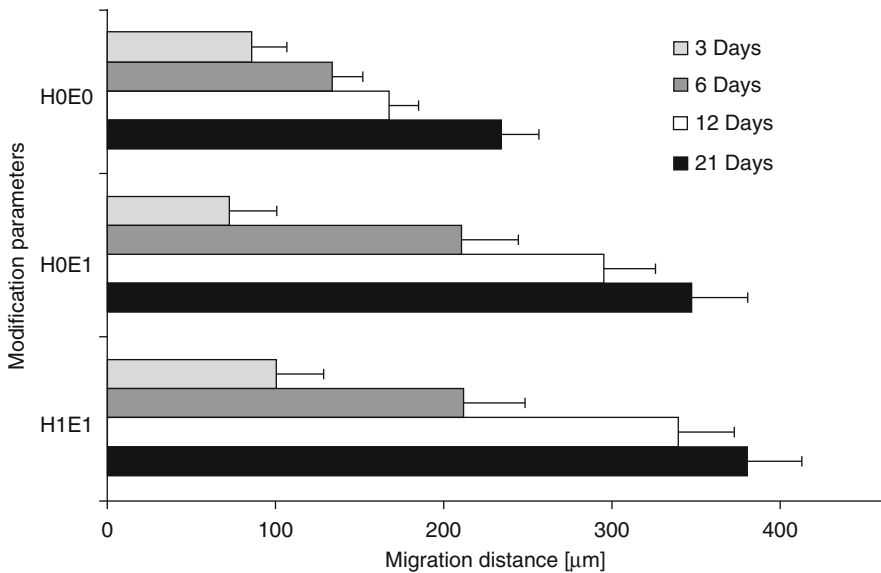
**Fig. 7.1** Angiogenic potential as determined by exposure of endothelial cells to matrices. Rate of proliferation of ECs in contact with unmodified (H0E0), cross-linked (H0E1) and heparin-loaded (H1E1) collagen matrices. Matrices were tested in absence and presence of 25 ng rhVEGF165 (+V). The proliferation rates were evaluated by counting the cell number with the Neubauer chamber after 1, 3 and 5 days. VEGF and cross-linking effects are shown for day 5



**Fig. 7.2** Pore sizes of unmodified H0E0 and cross-linked/heparinised H1E1 collagen matrices. Pore sizes were determined by microscopy of sections of (a) unmodified (H0E0) and (b) cross-linked/heparinised (H1E1) matrices after HE staining (magnification 200x)

cell numbers due to the cross-linking of the collagen. This cross-linking effect was observed to be similar in H0E1 and H1E1 (Grieb et al. 2010).

The rather surprising results due to cross-linking led us to investigate the pore size of the three selected matrices by light microscopy of HE-stained sections (Fig. 7.2). We observed that the cross-linking process, which occurs both in H0E1

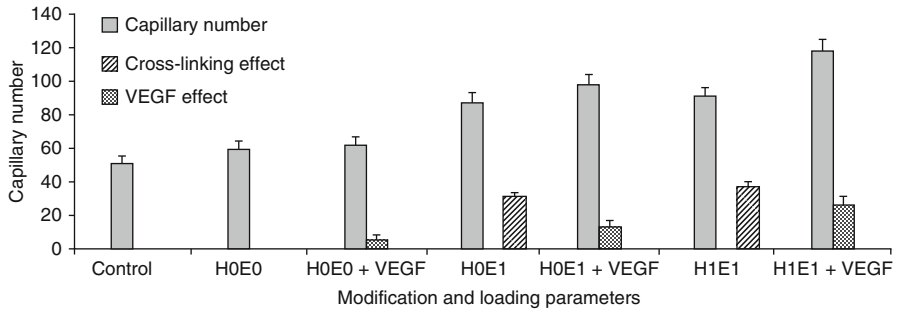


**Fig. 7.3** Migration of mouse L929-fibroblasts in unmodified (H0E0), cross-linked only (H0E1) and cross-linked/heparinised (H1E1) matrices. Fibroblasts were allowed to migrate into the three collagen matrices, with quantification of migration by microscopy after HE staining. Migration distances were determined after 3, 6, 12 and 21 days

and H1E1, led to an increase in pore size from 20–40  $\mu\text{m}$  to 50–70  $\mu\text{m}$ . Pore sizes were also determined by electron microscopy, producing similar results: the differences between the cross-linked only (H0E1) and the heparinised (H1E1) matrices were small and not significant (Lau 2005). Furthermore, we were able to show that physical stability and water-binding properties increased substantially (Yao 2003).

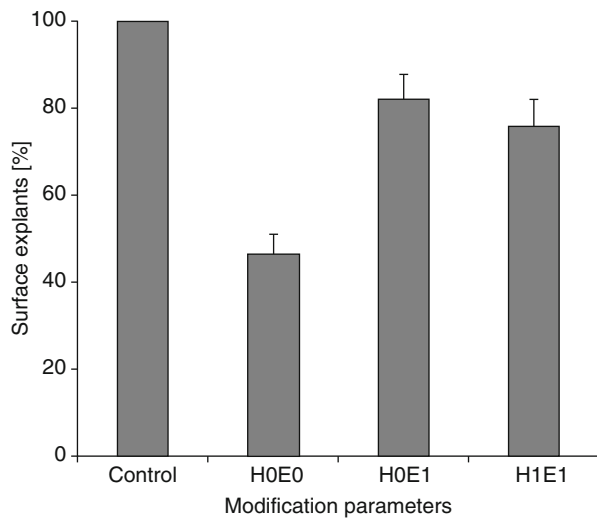
These observations prompted us to investigate whether these pore size differences would affect the migration rates of invading fibroblasts. Figure 7.3 shows quite good correlation between pore size and the migration distances of F929 fibroblasts. Invasion distances increase with migration period and the degree of cross-linking. The unmodified H0E0 matrices show the lowest invasion, while the invasion distances in H0E1 and H1E1 are approximately equal, an observation which agrees with pore size values as detected by light (Fig. 7.2) and electron microscopy (Lau 2005).

We next determined the angiogenic potential of the three selected matrices by exposing the *chorioallantois* membrane of a fertilized chicken egg to all three matrices and evaluating the angiogenic effects by counting the number of microvessels in the membrane in the vicinity of the implanted matrices. Figure 7.4 shows the results obtained with the three matrices. In the presence of an H0E0 implant, the number of microvessels slightly increases. Typically, the cross-linked H0E1 matrices showed greater increases in the number of microvessels, likewise the cross-linked/heparinised matrices H1E1. Subtracting the number of vessels induced by H0E0 matrices from the number of vessels induced by the H0E1 matrices leads to the



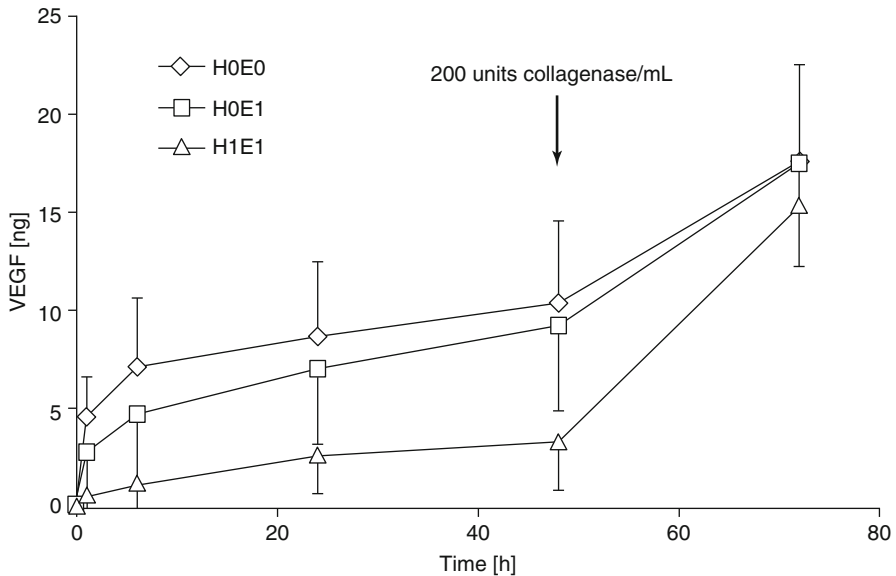
**Fig. 7.4** Angiogenic potential as determined by *chorioallantoic* assay. Evaluation of the capillary numbers in the vicinity of unmodified (H0E0), cross-linked only (H0E1) and cross-linked/heparinised (H1E1) collagen matrices after 7-day exposure on the *chorioallantois* membrane. Capillary numbers were also determined after loading of matrices with 25 ng rhVEGF<sub>165</sub>. VEGF and cross-linking effects are shown

**Fig. 7.5** Form stability and shrinkage. Surfaces of explants were determined prior to and after contact with the *chorioallantois* membrane



so-called cross-linking effect, which appeared to be similar for H0E1 and H1E1 matrices, apparently reflecting a similar degree of cross-linking and similar pore sizes. By comparing the numbers of vessels induced by the matrices loaded with 25 ng rhVEGF<sub>165</sub> to those induced by the unloaded matrices, we arrive at the VEGF effect. This effect appeared to be greatest in the case of the cross-linked/heparinised matrices, demonstrating the promotional effect of binding and stabilising VEGF to the incorporated heparin.

This technique also enabled us to compare the stability of the matrices under *in vivo* conditions. The surface areas of the explants which had been exposed to the *chorioallantois* membrane were compared to the original size (control). Figure 7.5 shows the contraction of the matrices after a 7-day exposure, the size reduction of

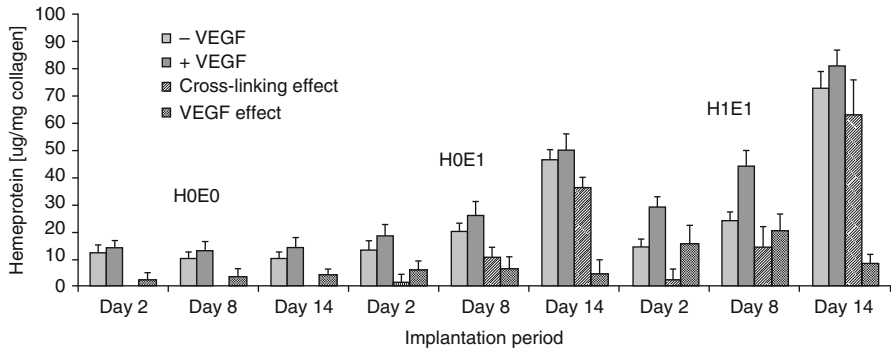


**Fig. 7.6** VEGF release from unmodified (H0E0), cross-linked (H0E1) and heparinised (H1E1) collagen matrices loaded with 25 ng rhVEGF<sub>165</sub>. VEGF concentrations were determined by ELISA. After 48 h, 200 units collagenase/ml were added

the H0E0 matrices being greater than 50 %. In contrast, size reduction for the H0E1 and H1E1 matrices is inferior to 20 and 25 %, respectively.

Our further investigation centred on the release behaviour of the selected matrices loaded with VEGF. VEGF has been shown to possess a heparin-binding domain and thus strongly binds to heparin (Ferrara et al. 2003; Nillesen et al. 2007). Figure 7.6 shows the cumulative release of the matrices loaded with VEGF. The release pattern of the unmodified matrices (H0E0) is characterised by a burst-release phase in which approx. 30–40 % of the load is released within the first hours. A similar burst phase was also observed with the cross-linked only matrices H0E1. In contrast, the heparinised matrices do not show such a burst phase. Instead, the VEGF load is released at a much slower rate. After 2 days (48 h), 200 units of collagenase were added per ml, which resulted in the accelerated release of VEGF from the heparinised matrices, demonstrating that the non-released VEGF was still present.

As a next step, we performed a series of *in vivo* experiments. Samples of the selected matrices were implanted subcutaneously in rats. Samples were implanted with and without VEGF loading. Implants were explanted after 2, 8 and 14 days. Implants were dipped in water to remove any attached blood and subsequently washed with small PBS aliquots for a period of 24 h. The aliquots were pooled and their hemoprotein content was determined spectrophotometrically. Results are shown in Fig. 7.7, where the explanted, unmodified matrices (H0E0) are characterised by a relatively low hemoprotein content, which does not increase over time. In



**Fig. 7.7** Evaluation of angiogenesis by subcutaneous implantation in Lewis rats. Implants were explanted after 2, 8 and 14 days and explants were washed to release their hemeproteins. Hemeprotein content was determined by absorption spectroscopy at 418 nm

the case of the cross-linked only matrices (H0E1), the hemeprotein content increased over time, and the effect of VEGF loading is low. In contrast, the heparinised matrices (H1E1) showed a striking increase over time, and the VEGF effects were clearly evident. These VEGF effects were observable after 2 and 8 days of implantation but subsequently decreased.

Our next investigation focused on the performance of the three collagen modifications in full-thickness wounds in miniature pigs. Figure 7.8 shows the progression for macroscopic wound healing in a number of representative cases. Healing was not optimum in wounds treated using H0E0 specimens. After 1 week, H0E0 grafts showed incomplete epithelialisation and signs of contraction. Even after 2 weeks, these wounds exhibited hypergranulation tissue and ongoing contraction. After 4 weeks, the H0E0 wounds had healed under extensive contraction with formation of scar tissue.

In the H1E1 groups, an 80–100 % graft take was observed 1-week post-excision. No infection was observed in the wounds. After 2 weeks, all wounds showed complete epithelialisation with only 20 % of all grafts becoming red in appearance. After 3 weeks, wound closure was stable and showed only slight ongoing contraction. After 4 weeks, the increase in wound contraction was insignificant.

In summary, we can conclude that the heparinised (H1E1) matrices were superior to the cross-linked only (H0E1) and unmodified (H0E0) matrices in all aspects investigated. Heparinised H1E1 matrices optimally increased the proliferation of endothelial cells, enabled the fastest migration of fibroblasts, and provided maximum induction of growth of small vessels in the *chorioallantois* membrane. Furthermore, heparinised H1E1 matrices were characterised by low shrinkage and showed the greatest vascularisation (determined by hemeprotein content), even in the absence of exogenously added VEGF. Thus, heparinisation appeared to be advantageous under all conditions, indicating that the incorporated heparin probably plays an important role in binding and stabilising endogenous growth factors. The heparinised H1E1 matrices also showed the best result in treatment of



**Fig. 7.8** Implantation of matrices (H0E0, H0E1 and H1E1) in full skin defects of miniature pigs. Following implantation of the collagen matrix, defects were covered using autologous split skin in a one-stage procedure. (a) H1E1 after 1 week. (b) H1E1 after 2 weeks. (c) H0E0 after 4 weeks. (d) H0E1 after 4 weeks. (e) H1E1 after 4 weeks



full-thickness defects in miniature pigs. Wounds regenerated faster with lower wound contraction. In the long term, i.e. after implantation periods of more than 8 days, cross-linking appeared to be the governing effect on cell ingrowth and angiogenesis. The application of cross-linked only matrices (HOE1) can be a good and relatively cost-effective alternative.

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W.J. Weninger, Lars-Peter Kamolz, and S.H. Geyer

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## 8.1 Introduction

Diagnosing skin diseases and researching the genesis of skin pathologies would substantially profit from sound topological information of the structures composing the layers of normal and pathologically transformed skin. Likewise, designing skin regeneration material, improving its quality, and evaluating the transformation it undergoes after implantation require the precise visualisation of the architecture of this material and the three-dimensional (3D) arrangement of the cells and tissues populating it. Although the last decades saw the development of a great number of potent new 2D and 3D imaging techniques (Boppart et al. 1996; Kolker et al. 2000; Smith 2001; Sharpe et al. 2002; Sharpe 2003; Johnson et al. 2006; Weninger et al. 2006; Dodt et al. 2007; Filas et al. 2007; Wanninger 2007; Cavey and Lecuit 2008; Geyer et al. 2009; Metscher 2009; Mohun and Weninger 2011), high-quality 3D visualisation of skin architecture and skin replacement materials is still a major technical challenge.

The basis for high-quality 3D visualisation is a digital volume data set. Volume data sets can be generated either by helical scanning of a specimen (as with computed tomography) or by stacking a series of digital images showing subsequent sections, which cut virtually or physically through a specimen (all other volume data generation techniques). Once a digital volume data set is generated, its content can be digitally processed and analysed three dimensionally with a broad variety of visualisation tools. Cutting user-defined virtual resection planes through the data volume is one possibility. Creating volume or surface-rendered computer models is another (Weninger and Geyer 2008).

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W.J. Weninger, MD (✉) • S.H. Geyer, MD  
IMG, Centre for Anatomy and Cell Biology, Medical University of Vienna,  
Waehringer Str. 13, A-1090 Vienna, Austria  
e-mail: wolfgang.weninger@meduniwien.ac.at

L.-P. Kamolz, MD, MSc  
Division of Plastic, Aesthetic and Reconstructive Surgery, Research Unit  
for Tissue Regeneration, Repair and Reconstruction, Department of Surgery,  
Medical University of Graz, Graz, Austria

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In principle, digital volume data can be generated with *in vivo* and *in vitro* (post-mortem) imaging techniques. Volume data generated with *in vivo* imaging techniques, such as the computed tomography (CT), magnetic resonance tomography (MR) (Hogers et al. 2000; Ahrens et al. 2006; Bain et al. 2007; Deans et al. 2008) and 3D-ultrasound (3D-US) (Turnbull 1999; Meyer-Wittkopf et al. 2001; Mittermayer et al. 2004; Phoon 2006; Avni et al. 2007; Yagel et al. 2007), *in vivo* microscopy (Visscher 2010) and optical coherence tomography (OCT) (Männer et al. 2008; Norozi et al. 2008; Happel et al. 2011) would permit *in situ* analysis of skin and implanted skin replacement materials. However, the resolution of volume data generated with CT, MR, and 3D-US is far too low to resolve the tissues of the skin. The resolution of data produced with *in vivo* microscopy and OCT is high, but these techniques only permit visualisation of very thin samples. For example, in native skin they can produce data from structures located not deeper than the papillary region of the dermis.

Not only the *in vivo* imaging techniques but also the popular *in vitro* volume data generation techniques suffer from technical limitations, which effectively prevent them from being useful for analysing skin samples. Histological section-based 3D reconstruction methods (Guest and Baldock 1995; Weninger et al. 1996; Streicher et al. 1997, 2000; Soufan et al. 2003; Kaufman and Richardson 2005) produce volume data with serious alignment errors and low spatial resolution. Furthermore data generation times are unjustifiably high. Confocal microscopy, electron tomography (Bárcena and Koster 2009; Koning and Koster 2009), and serial section electron microscopy (McEwen and Marko 1999; Shishido et al. 2000) produce high-resolution volume data but only from very small tissue cubes. Micro-magnetic resonance imaging ( $\mu$ MRI) (Dhenain et al. 2001; Schneider et al. 2003a, b; Schneider and Bhattacharya 2004; Turnbull and Mori 2007; Cleary et al. 2011) and optical projection tomography (OPT) (Davies and Armstrong 2006; Bryson-Richardson et al. 2007; Delaurier et al. 2008; Fisher et al. 2008; Sato et al. 2008) are optimised for visualising loose and watery tissues (e.g. embryos of model organisms) and produce volume data of low effective spatial resolution.

Thus, 2D *in vitro* analysis with histological sections and pseudo-3D analysis with scanning electron microscopy are still standard for analysing the microscopic anatomy of the reticular region of the dermis and of skin replacement material.

Recently, a new class of *in vitro* volume data generation techniques was developed, which bear the potential to provide meaningful topological descriptions of the deep layers of the skin. These techniques can be summarised under the term “episcopic 3D imaging techniques”. They are optimised and originally were applied for analysing morphogenetic events in early embryos of biomedical model organisms (Weninger et al. 1998; Ewald et al. 2002; Weninger and Mohun 2002; Weninger et al. 2006; Gerneke et al. 2007; Geyer et al. 2009). They create digital volume data of histologically embedded specimens, by producing stacks of inherently aligned digital images, which show the subsequently exposed surfaces of wax or resin blocks during their sectioning on a microtome. Especially one of these techniques, the high-resolution episcopic microscopy technique (HREM) (Weninger et al. 2006; Mohun and Weninger 2011), bears the potential for analysing the microanatomy of skin and skin replacement materials.

Here we present first results obtained by employing the HREM technique for visualising the microanatomy of dermal substitutes. We visualised the topology of collagen and elastin fibres composing the trabeculae of native matrix and the topology of cells growing in and on this matrix.

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## 8.2 Material

### 8.2.1 Specimens

We visualised native collagen-elastin matrix (Matriderm<sup>®</sup>, Dr. Suwelack Skin&Health Care AG, Germany) and Matriderm<sup>®</sup> populated with different kind of cells (e.g. keratinocytes). From both matrices, the native and the cell-populated one, bits of 3×3×1 mm were cut out and processed for HREM data generation.

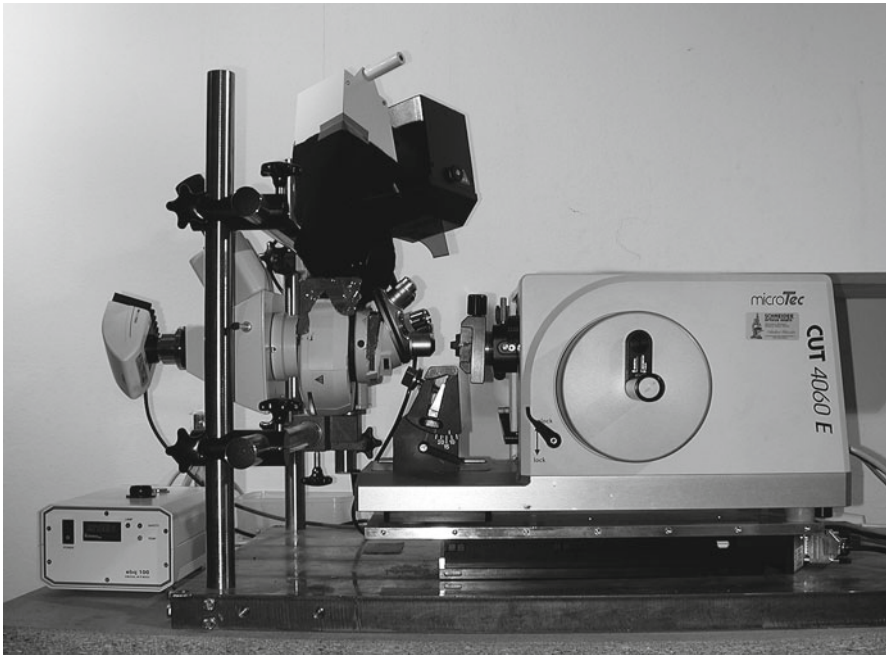
The cells, which were populating Matriderm, were immunohistochemically labelled according to following protocol: The bits of populated matrix were rinsed in PBS and TBST and blocked with 10 % goat serum for 6 h. Then they were incubated with a primary antibody (rabbit polyclonal to cytokeratin 5) for 20 h at 4 °C. Following rinsing with TBST for 2 h (four changes) at 4 °C, they were incubated with the secondary antibody GAR (goat anti-rabbit) (AP) overnight. After rinsing in TBST, NBT/BCIP was added for colour reaction. The reaction was stopped after 15 min and the specimens were processed further immediately.

### 8.2.2 HREM Apparatus

For volume data generation, we use a HREM apparatus, which was developed in our laboratory (Fig. 8.1). The apparatus comprises the following components:

1. A rotary microtome (Microtec CUT 4060E, microTec Laborgeräte GmbH), the block holder arm of which reaches its upper resting position after each section with an accuracy of approximately  $\pm 2 \mu\text{m}$
2. A motorised cross table (KTS-LC; Walter Uhl, Technische Mikroskopie GmbH & Co. KG, Aßlar)
3. The head of a fluorescence compound microscope carrying a 10× plan-apo objective (Leica, N PLAN 10× / 0.25)
4. A triple band filter cube DAPI/FITC/Texas Red (excitation filter 404/20 nm, 494/20 nm, 576/20 nm; emission filter 457/20 nm, 530/20 nm, 628/28 nm)
5. A mercury short arc lamp (Osram HXP R 120 W/45 C VIS) coupled with the microscope
6. A digital video camera (Leica DFC480)
7. A home-made device for shifting the microscope up- and downwards
8. A PC operating Windows 7 and running the camera driver and a home-made programme that drives automated image capturing

These components are arranged as follows: the microtome, a resin block mounted in its block holder, stands on the cross table. (With the aid of the cross



**Fig. 8.1** HREM apparatus

table the optical pathway of the microscope is aligned with the surface of the resin block.) In the optical path the triple band filter cube is inserted. Via a splitting mirror, which is part of the triple band filter cube, the lamp delivers light towards the block surface. Light emitted from the block surface passes through the filter cube and reaches the target of the digital camera placed on the phototube of the microscope. The camera is connected to the PC.

### **8.2.3 Visualisation Hard- and Software**

For data processing and 3D visualisation, we employed a high-end PC with Intel Xeon CPU operating at 2.66 GHz and 32 GB RAM. The PC was equipped with a graphic card with 2 GB RAM. It operated Windows XP Professional x64 Edition and ran Adobe Photoshop CS3 Version 10.0 and the 3D visualisation software Amira® 5.3.3 (Visage Imaging, Inc.)

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## **8.3 HREM Volume Data Generation and Visualisation**

### **8.3.1 HREM: Specimen Preparation**

The specimens were prepared for HREM data generation following a slightly modified HREM specimen preparation procedure (Weninger and Mohun 2007). In brief, the

specimens were dehydrated in a series of ethanols (70 %, 80 %, 90 %, 96 %, 1 h each for the cultivated Matriderm and 15 min each for native collagen-elastin matrix). Then they were transferred into an infiltration solution consisting of 100 ml JB4 embedding kit solution A, 1.25 g benzoyl peroxide, plasticized (catalyst), and 0.4 g eosin (Waldeck GmbH). The solution can be used, when all components are fully dissolved. Infiltration was performed for 3 h (two changes) at 4°C. For embedding, a fresh infiltration solution was prepared as described above, and JB4 embedding kit solution B (1 per 25 ml infiltration solution) was added directly before embedding. The specimens were embedded utilising embedding moulding cup trays and block holders provided by Polysciences. The filled moulding cup trays were covered upside down by a second moulding cup tray and sealed with adhesive tape for 2 days at room temperature to facilitate polymerisation. The polymerised blocks were removed from the moulding cup trays and transferred into an incubator for post-polymerisation at 37°C for 1–2 days.

### 8.3.2 HREM: Volume Data Generation

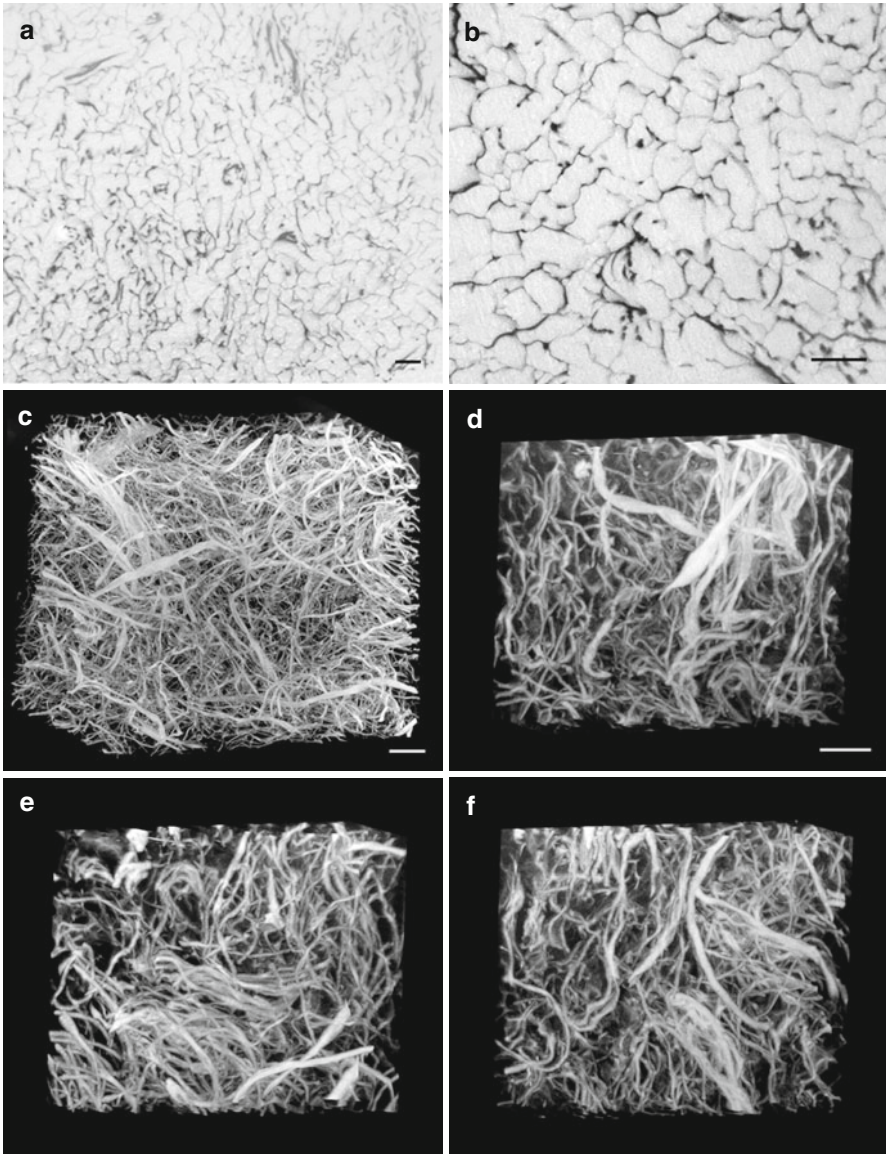
The resin blocks containing the specimens were mounted on the HREM apparatus. During sectioning of the resin block, digital images of the subsequently exposed block surfaces were captured (Fig. 8.2a, b) and stored on a hard drive. Section thickness was 1.5  $\mu\text{m}$  (cultivated Matriderm<sup>®</sup>) and 2  $\mu\text{m}$  (native Matriderm<sup>®</sup>). The sectioning and image capturing procedures were synchronised and driven by the home-made data capturing software. From each specimen, we generated a series of 500–1,500 RGB images of 8 bit colour depth. Image size was 2,560×1,920 pixel. Image resolution was 0.54×0.54 per pixel. Due to the alignment of the optics with the upper stopping position of the block holder, the images of a series were accurately aligned. Automated capturing of 1,000 images took approximately 3 h.

### 8.3.3 HREM: Data Processing

The digital images were transferred to a high-end PC. Using the batch function of Photoshop, the channels of each image were split and stored in different directories. For 3D visualisation of the native matrix, only the green channels were loaded into the Amira software. They were stacked together and converted into a volume data set (voxel size: 0.54×0.54×1.5  $\mu\text{m}^3$  and 0.54×0.54×2  $\mu\text{m}^3$ , respectively). For 3D visualisation of the stem cell-populated matrix, the green channels and the red channels were loaded into the Amira software as two separate but combined volume data sets. The volume data set stemming from the green channels was used for visualising the collagen-elastin trabeculae (Figs. 8.2 and 8.3). The red channel was used for identifying the NBT/BCIP-labelled cells (Fig. 8.3).

### 8.3.4 HREM: 3D Models

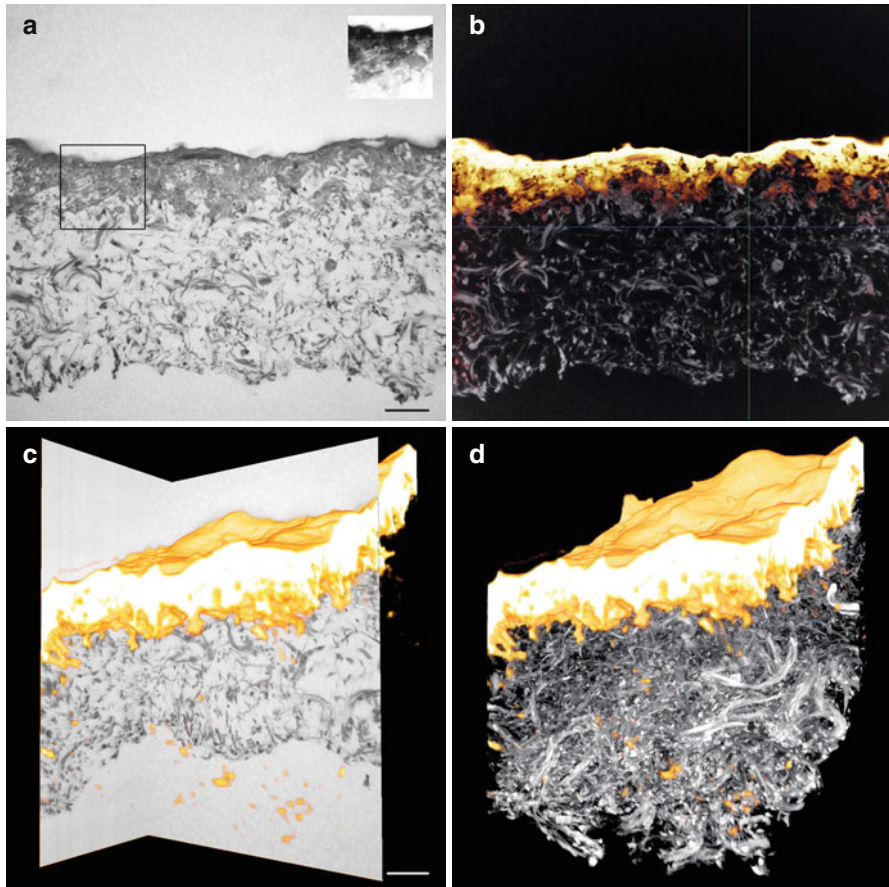
For 3D visualisation we used the volume rendering tools of the Amira software. The 2D images and 3D computer models of the native matrix show the variable thickness



**Fig. 8.2** Visualisation of native Matriderm®. (a) Original 2D block surface image. (b) Section in a in higher magnification. (c) Volume-rendered 3D model of the collagen/elastin fibres. (d–f) Virtual thick sections of 250 µm thickness. Note the different texture and thickness of the fibres. Scale bars 100 µm

and orientation of the larger collagen and elastin trabecula in different parts of the matrix (Fig. 8.2).

The 2D images and 3D computer models of the populated matrix show a continual superficial cell layer. Some cells are clustered to form cone-like structures,



**Fig. 8.3** Visualisation of cell-populated Matrigel<sup>®</sup>. (a) Green channel of the original 2D block surface image. *Inlay* shows the indicated section of the red channel of this image. Note that the red channel only highlights labelled cells. (b) Original section showing the combined *green* and *red* channels in false colours. (c) 3D model of the labelled cell clusters (*orange*) in relation to an original section. (d) 3D model of the labelled cell clusters (*orange*) in relation to the collagen/elastin fibres (*grey*). Scale bars 100  $\mu\text{m}$

which extend from this superficial layer into more profound regions. A few small clusters of isolated cells appear to be scattered deep inside the matrix (Fig. 8.3).

#### 8.4 Limitations of HREM and Future Aspects

The 3D visualisations we present in this chapter demonstrate the high potential of the HREM technique for examining native and stem cell-populated collagen-elastin matrices. However, we like to emphasise that we only present first data. We embedded the specimens following protocols, which we routinely employ for processing mouse and chick embryos (Pielers et al. 2007; Weninger et al. 2009; Geyer et al.



2012). Better results are to be expected with protocols, which are modified to meet the special demands of collagen-elastin matrices.

Preparing specimens for HREM involves dehydration in an increasing series of ethanols (Mohun and Weninger, 2010). We analysed native and cell-populated Matriderm® matrices and discovered that the specimen preparation process does neither substantially harm the cells seeded onto the matrix nor the collagen and elastin trabecula comprising it. Whether other skin replacement materials survive the specimen preparation and embedding process remains to be tested.

We visualised Matriderm® prepared for implantation. We did not visualise biopsy material stemming from already implanted Matriderm®. Visualising biopsies from native skin and implanted Matriderm with HREM is certainly possible. However, the development of decent fixation and specimen preparation protocols will be essential for the reproducible achievement of high-quality visualisations.

We think that this new technique will render new information and results concerning scaffold and tissue revascularization and nerve repair.

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Marc G. Jeschke and Ludwik Branski

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## 9.1 Introduction

Burn trauma is one of the worst injuries suffered worldwide with an incidence of approximately two million cases annually (Brigham and McLoughlin 1996). Over the past decade, progress in the treatment of severe burn injuries has significantly decreased morbidity and mortality (Pereira et al. 2004). The improvements in survival have been most notable in severely burned pediatric patients (Janzekovic 1970; Merrell et al. 1989). Four major areas of advancement in burn care have been identified:

- Fluid resuscitation and early patient management
- Control of infection
- Modulation of the hypermetabolic response
- Surgery and wound care

Because of their extensive wounds, burn patients are chronically exposed to inflammatory mediators and microorganisms. With the advent of early burn wound excision and coverage (Janzekovic 1970), the risk of serious systemic infection originating from the burn wound has been reduced (Merrell et al. 1989). The current surgical approach to burn care is based on early excision of full-thickness burn tissue followed by early wound coverage, preferably with autologous skin graft. Early excision within the first 48 h can significantly reduce blood loss and is safe and effective (Barret et al. 1999a; Herndon et al. 1989). In order to provide sufficient

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M.G. Jeschke, MD, PhD (✉)  
Ross Tilley Burn Centre, Sunnybrook Health Sciences Centre,  
University of Toronto, 2075 Bayview Avenue,  
Toronto, ON M4N 3M5, Canada  
e-mail: marc.jeschke@sunnybrook.ca

L. Branski, MD  
Klinik für Plastische, Hand- und Wiederherstellungschirurgie,  
Medizinische Hochschule Hannover,  
Carl-Neuberg-Str. 1, Hannover 30625, Germany  
e-mail: branski@web.de

temporary wound coverage in large burns, temporary coverage with allograft can provide protection for many weeks until enough donor sites are available for grafting. In addition, widely meshed autografts have been utilized with allograft overlay (sandwich technique) to provide adequate coverage. Donor sites can be utilized repeatedly following healing which typically occurs within 10–14 days (Barret et al. 1999b; Herndon et al. 1990, 1995). This approach, albeit still not implemented in many burn centers around the world, has been practiced for the last quarter century almost without change. During this time period, however, new approaches and devices have been successfully introduced or are currently being studied such as the use of fibrin sealant for autograft fixation (Dyess et al. 1995; Mittermayr et al. 2006), the use of dermal substitute Integra (Branski et al. 2007), cultured epidermal autografts (CEA) and cultured skin substitute (CSS) (Carsin et al. 2000; Herndon and Parks 1986), human amniotic membrane as stand-alone coverage or overlay (Branski et al. 2008), various biodressings (Barret et al. 2000a), and stem cell and gene therapy. This chapter introduces and discusses some of these exciting developments and gives an outlook on new ideas.

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## 9.2 Partial-Thickness Burns

Partial-thickness burns are divided into superficial and deep categories. The distinction between the two types of partial-thickness burns is based on the depth of injury. Superficial partial-thickness burns often form blisters, are painful, moist, and have normal capillary refill. These wounds spontaneously re-epithelialize from retained epidermal structures in the rete ridges, hair follicles, and sweat glands within 7–14 days. After the wound has re-epithelialized, secondary scar maturation takes place that may result in hypo- or hyperpigmentation over the long term.

Deep dermal burns into the reticular dermis appear more pale and/or mottled, have poor or no capillary refill, and are dry and less sensate. These burns can take up to 4 weeks for complete re-epithelialization from the hair follicles, often with severe scarring as a result of the loss of dermis.

Partial-thickness burns are painful secondary to exposed nerve endings. These burns are historically treated conservatively by removing the damaged epidermis after the initial injury, followed by the application of topical medications one to two times each day (Dhennin 2002; Jones et al. 2002). These procedures may cause severe pain and anxiety in patients even with the use of narcotics (Table 9.1).

### 9.2.1 Synthetic and Biosynthetic Membranes: Biobrane, AWBAT, and Suprathel

To improve patient comfort, control infection, and increase the rate of re-epithelialization, alternatives for the treatment of partial-thickness burns have been developed in the past three decades. Semiocclusive and synthetic membranes are the most important clinically applicable devices. These partly occlusive dressings allow re-epithelialization to occur beneath the dressing and eliminate the need for

**Table 9.1** Common partial-thickness burn wound dressings

Dressing agent	Active substance	Presentation	Main use	Advantages	Disadvantages
Bacitracin	Bacitracin	Ointment	Superficial burns, skin grafts	Gram (+) coverage	No G(-) or fungal coverage
Polymyxin	Polymyxin B	Ointment	Superficial burns, skin grafts	Gram (-) coverage	No G(+) or fungal coverage
Mycostatin	Nystatin	Ointment	Superficial burns, skin grafts	Good fungal coverage	No bacterial coverage
Silvadene	Silver sulfadiazine	Ointment	Deep burns	Good bacterial and fungal coverage, painless	Poor eschar penetration, sulfa moiety, leucopenia, pseudoeschar formation
Sulfamylon	Mafenide acetate	Ointment and liquid solution	Deep burns	Good bacterial coverage, good eschar penetration	Painful, poor fungal coverage, metabolic acidosis
Dakin's	Sodium hypochlorite	Liquid solution	Superficial and deep burns	Good bacterial coverage, inexpensive and readily available	Very short half-life
Silver	Silver nitrate, silver ion	Liquid solution, dressing sheets	Superficial burns	Good bacterial coverage, painless	Hyponatremia, dark staining of wounds and linens

frequent dressing changes. There are several skin substitutes that are available commercially; however, only few will be discussed in this section. It is important to note that currently there is no gold standard of care for these dressings.

Biobrane is a biosynthetic wound dressing constructed of a silicone film with a nylon fabric partially embedded into the film. The fabric presents to the wound bed a complex 3-D structure of tri-filament thread to which collagen has been chemically bound. Blood and sera clot in the nylon matrix, thereby firmly adhering the dressing to the wound until epithelialization occurs. It controls water vapor transfer, maintains a moist healing environment, and has been shown to be effective in the treatment of partial-thickness burns, particularly in pediatric patients, in numerous scientific publications since 1982 (Bishop 1995; Cassidy et al. 2005; Demling 1995; Lal et al. 2000; Lang et al. 2005; Ou et al. 1998; Whitaker et al. 2008).

A newer biosynthetic product, AWBAT, has been cleared by the FDA in 2009 and is now commercially available. Biobrane and AWBAT are comparable constructs, as both feature a thin medical grade silicone membrane, good stretchability, and pores in the silicone membrane to enable excess exudate to get from the wound surface, through the skin substitute and into a sterile outer wrap. Both have collagen peptides for the purpose of reacting with the fibrin in the wound to achieve good

acute adherence. The main difference between the two membranes is the pore size, with AWBAT about 500 % times more porous than Biobrane. The greater porosity of AWBAT is expected to result in improved transfer of exudate from the wound surface which may result in better acute adherence and shorter healing time. As of now, however, there is only limited clinical experience with this new membrane (Vandenberg 2010; Woodroof et al. 2010).

Suprathel is produced from a synthetic copolymer mainly based on DL-lactide (>70 %); the other components are trimethylenecarbonate and  $\epsilon$ -caprolactone. The monomers are polymerized by a melting procedure. The final product is a porous membrane with an interconnected structure of pores between 2 and 50  $\mu\text{m}$  and an initial porosity of over 80 %. It also boasts high plasticity and water permeability. It is applied to the wound bed with an overlay of paraffin or non-adherent gauze and peels off within approximately 2 weeks as the re-epithelialization of the wound bed progresses (Uhlig et al. 2007). Prospective randomized clinical studies in partial-thickness burns and on split-thickness donor sites showed mainly a reduction in pain, with wound healing times and long-term scar qualities comparable to other commercially available membranes (Fig. 9.1) (Uhlig et al. 2007; Schwarze et al. 2007, 2008).

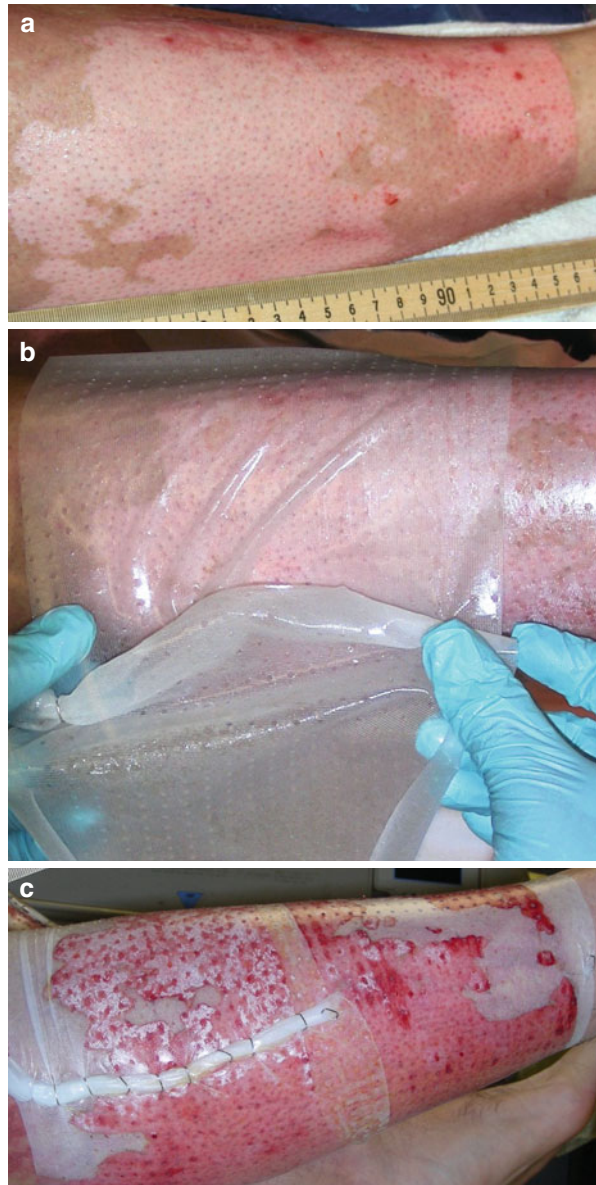
### 9.2.2 Biological Membranes: Amnion and Others

Human amniotic membrane has been used for centuries as a biological wound dressing. In western medicine, amniotic membranes have been used since the beginning of the last century. The first reported use of amnion in burn wounds was by Sabella in 1913, shortly after Davis used amniotic membrane in skin transplantations in 1910 (Maral et al. 1999). However, it soon became clear that amnion could not be used as a permanent skin transplant, but only as a temporary biological wound dressing. Many advantages of amnion as a temporary dressing have been reported, most notably alleviation of pain, the prevention of infection (Ninman and Shoemaker 1975; Robson and Krizek 1973; Robson et al. 1973; Salisbury et al. 1980), acceleration of wound healing (Maral et al. 1999; Ninman and Shoemaker 1975; Quinby et al. 1982), and good handling properties (Gajiwala and Gajiwala 2004). The first use of amnion as a *temporary* skin substitute in burn wound care has been reported by Douglas in 1952 (Douglas 1952). It has been subsequently used mainly in the treatment of partial-thickness burns (Robson et al. 1973; Quinby et al. 1982; Haberal et al. 1987; Ramakrishnan and Jayaraman 1997).

In the last 20 years, there has been an increasing body of literature addressing the use of amnion in burns. In order to make amnion a standard dressing alternative, safe and reliable production methods had to be implemented. To meet these needs, in several countries amnion banks have been established alongside tissue banks (Hennerbichler et al. 2007; Ravishanker et al. 2003; Tyszkiewicz et al. 1999).

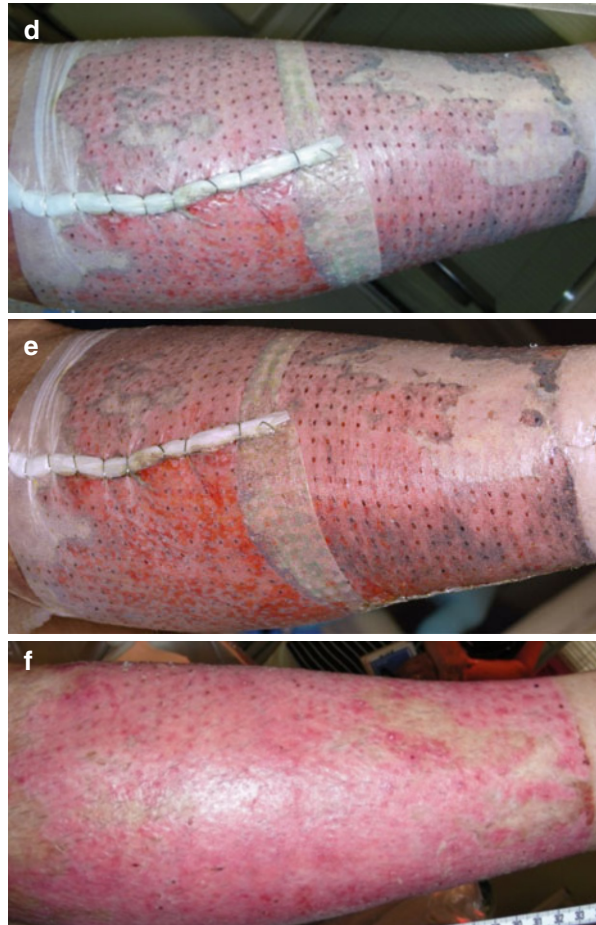
Amnion has the advantage of being thin, adhesive but not sticking, easily moldable, and removable. These qualities are of great importance, especially in the

pediatric population. In a study performed at the Shriners Hospitals for Children, Branski et al. (2008) did not observe a higher rate of infections than in the control group, achieved the same wound healing rate as with standard dressing regimens, and did not observe impaired long-term cosmetic results after treatment with amnion. The authors concluded that amniotic membranes can be used safely for



**Fig. 9.1** Temporary biosynthetic skin substitute (AWBAT®). (a) A 55-year-old male with superficial second-degree burns to both lower legs. Right calf after debridement of blisters. (b) Application of AWBAT®-S on the same day. (c) Day 3 postburn. (d) Day 6 postburn. (e) Day 8 postburn. (f) Day 13 postburn after removal of dressing. Fully re-epithelialized wound



**Fig. 9.1** (continued)

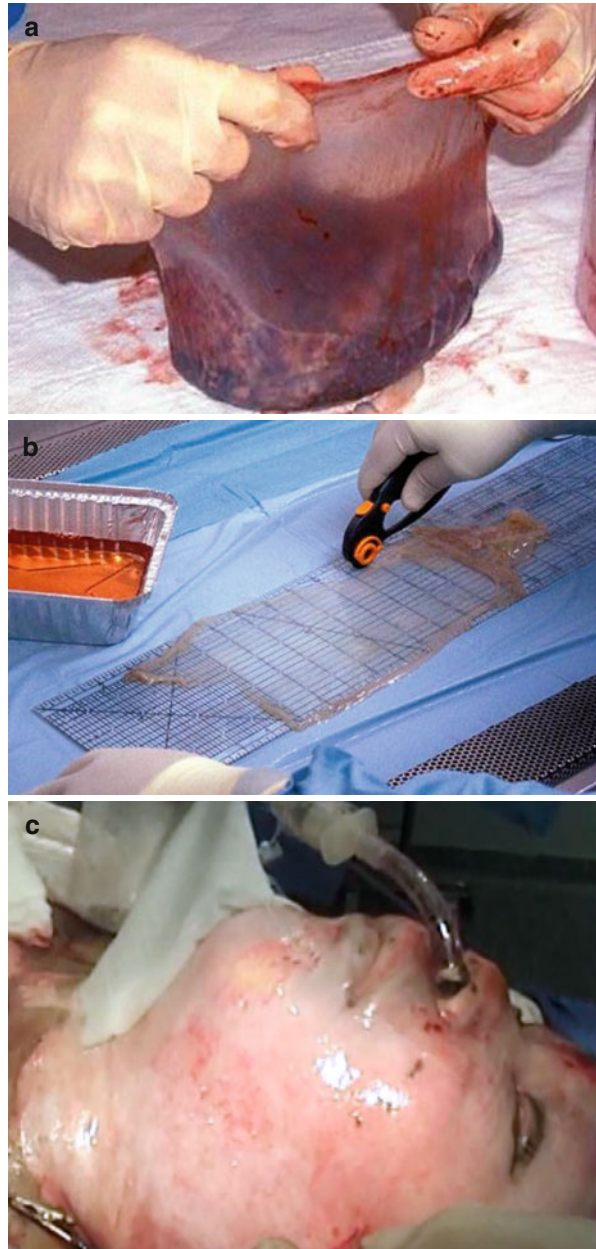
temporary wound coverage with the chief advantage of significantly less full dressing changes in the amnion group.

Recently, there has been a push towards a standardization and commercial availability of amniotic membranes. Commercially available amniotic membranes can now be found in fresh frozen (Grafix™, Osiris Therapeutics, Inc) and glycerol preserved form. However, their use is limited and has not become the standard of care (Fig. 9.2).

### 9.3 Full-Thickness Burns

Full-thickness burns (3°) or deep dermal burns which will not heal within 14–21 days are best treated by full excision and coverage with autograft. This needs to be undertaken as soon as possible. This early excision and grafting has become the gold

**Fig. 9.2** Use of human amnion. (a) Placenta with amniotic sac after delivery. (b) Sterile processing of amniotic membrane. (c) Application of processed amniotic membrane to superficial partial-thickness facial burn



standard of burn care since the 1970s. In the extensively burned patient, coverage with autograft is sometimes not possible. Therefore, one needs to consider use of homograft (allograft) and dermal substitutes which will be discussed in the following sections.

### 9.3.1 Dermal Analogs

The development of a burn wound coverage independent from autograft or homograft has been the goal of burn research around the world. The goal is to develop a fully functional composite graft that replaces dermis and epidermis and is available immediately for coverage of an excised burn. A first step in this direction were dermal analogs. Integra™ (Integra LifeSciences Corporation, Plainsboro, NJ, USA) was developed by a team led by surgeon John Burke from the Massachusetts General Hospital and by scientist Ionnas Yannas from the Massachusetts Institute of Technology. It is composed of bovine collagen and glycosaminoglycans that allow fibrovascular ingrowth. This dermal analog is placed over the wound bed after full excision of all devitalized tissue. The matrix is fully incorporated into the wound bed within 2–3 weeks, and a split-thickness autograft is placed over it. Except for a possible increased risk of infections, its use and long-term results are favorable (Branski et al. 2007; Heimbach et al. 2003). Another dermal analog available for the treatment of full-thickness burns is AlloDerm® (LifeCell Corporation, The Woodlands, TX, USA). It consists of cadaveric dermis devoid of cells and epithelial elements. Its use is very similar to that of other dermal analogs and has shown favorable results (Fig. 9.3) (Wainwright 1995; Sheridan and Choucair 1997).

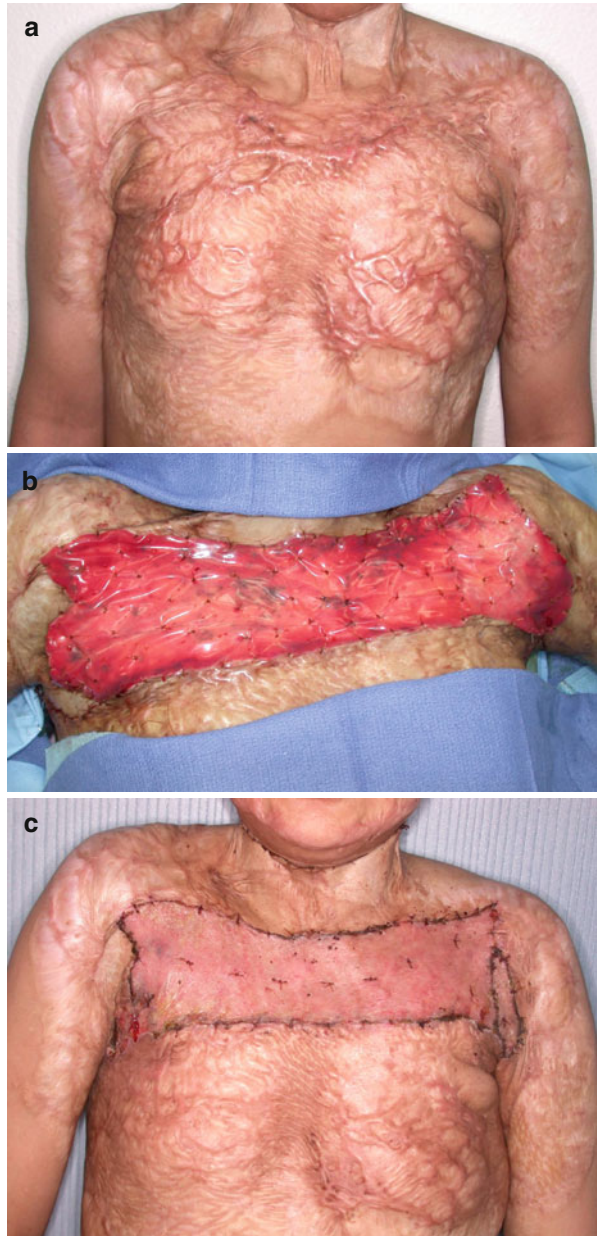
### 9.3.2 Keratinocyte Coverage

CEAs have become an important tool in the management of patients with massive burn injuries. In cases where full-thickness burns involve more than 90 % of the total body surface area (TBSA), it may be the only choice given that procurement of the uninvolved skin will not be sufficient to cover the patient's body, even when extensive expansive techniques are employed. Cultured epithelial autografting involves obtaining two 2×6 cm full-thickness specimens of unburned skin very early in the patient's course, preferably upon admission. The skin is then processed and cultured *ex vivo* in the presence of murine fibroblasts that promote growth ([http://www.genzyme.com/business/biosurgery/burn/epicel\\_package\\_insert.pdf](http://www.genzyme.com/business/biosurgery/burn/epicel_package_insert.pdf)). The final product takes approximately 3 weeks to be ready for grafting and consists of sheets of keratinocytes 5×10 cm in size, 2–8 cells thick mounted on a petrolatum gauze.

While the CEA is made available, these critically ill patients need to be excised and temporarily covered with allograft. Complications like wound infections and multiorgan failure have to be aggressively treated to increase the chances of survival and eventual graft take.

The application of CEA can be difficult because of the fragility of the grafts, which has been described as having the consistency of wet tissue paper. CEAs applied to areas like the back, buttocks, posterior lower extremities, and other dependent areas are prone to shearing and possible loss. Once healed, the skin has a better cosmetic result when compared to healed 4:1 meshed autograft but is associated with a longer hospital stay and more reconstructive procedures (Barret et al. 2000b). Recent studies have shown very variable results of CEA application.

**Fig. 9.3** Dermal substitute (Integra®) for burn scar reconstruction. (a) A 12-year-old boy with severe thoracic scar contractures 4 years after flame burn. (b) Excision of scar leads to large defect, which is covered by Integra®. (c) Wound 4 weeks after initial operation. Three weeks after the initial scar excision, the silicone layer was removed and the wound covered by a non-meshed sheet of thick split-thickness skin graft



A single-center retrospective cohort study with over 30 severely burned patients with burn sizes exceeding 75 % TBSA showed an excellent survival and permanent coverage, although no control group was provided (Carsin et al. 2000). CEA used in conjunction with an allograft base was reported to result in a graft take of over 72 % (Sood et al. 2010).

### 9.3.3 Keratinocyte Suspension

A review of the literature by Wood et al. (2006) in the use of CEA for extensive burns concluded that its application ranges from useful to non-beneficial given its difficult handling and fragility, as well as lack of standardized application at burn center. Because of these limitations, a technique consisting of a keratinocyte suspension delivered to the wound through an aerosol spray has been described.

In a porcine model by Reid et al. (2007), wounds treated with a split-thickness skin graft compared to wounds treated with this method plus the application of sprayed keratinocytes showed a significant decrease in wound contraction after healing took place. James et al. (2010) later showed in a clinical trial that the addition of sprayed cultured autologous keratinocytes may help to clinically reduce the contraction of meshed autografts and reduce healing time. Also, the use of sprayed keratinocytes proved to be a versatile procedure that overcomes some of the limitations of the CEA sheets. In this series, a split-thickness skin was obtained from unburned areas, submerged in a 2 % dispase solution and placed in an orbital shaking incubator to separate the epidermal layer. This layer was then placed in an incubator under a trypsin solution to generate a cell suspension which was cultured in growth medium. The cells were expanded during a 3-week period to a concentration of  $10^7$  cells/ml and subsequently aerosolized to the wound at a density of  $5 \times 10^5$  cells/cm<sup>2</sup>.

One of the major drawbacks of this technique is the delay of application while the cell expansion takes place. Zweifel et al. (2008) described a 3-patient case report in which non-cultured autologous keratinocyte suspension was delivered to split- and full-thickness burn wounds in an aerosol spray 2 days after admission. The results suggest a decrease in healing time and hypertrophic scarring.

### 9.3.4 Facial Transplantation

Severe facial burns can cause significant deformities that are technically challenging to treat, and traditional approach with conventional treatment modalities is insufficient to address the esthetic and functional outcome. Following the lead set by the team in Amiens, France, in 2005 (Devauchelle et al. 2006), several other groups in Europe, China, and the USA have been able to meet this complex clinical challenge with the use of composite tissue allotransplantation (CTA), which uses healthy facial tissue transplanted from donors for reconstruction thus allowing for the best possible functional and esthetic outcome. The techniques required to perform this procedure have been developed over many years and are used routinely in reconstructive surgery. The immunosuppressive regimens necessary to prevent rejection have been previously developed for and used successfully in solid organ transplantation for many years (Pomahac et al. 2011a, b). The psychosocial and ethical issues associated with this new treatment have some unique challenges, which need to be addressed by a dedicated team (O'Neill and Godden 2009; Soni et al. 2010).

The conventional treatment modalities for severe facial burns offer little improvement in function and appearance and often leave patients significantly debilitated. These patients often become socially and personally isolated, and many suffer from psychological disorders and phobias. These patients also tend to require multiple reconstructive procedures, in a setting, which there is minimal normal tissue (secondary to burns in other areas). Facial transplantation in such patients can offer the possibility of improved quality of life.

Since the initial face transplant in 2005, there have been further 12 transplant procedures that will eventually reduce the knowledge gap, as the teams performing these new reconstructive procedures share the details responsible for their successes and failures. Facial transplantation can improve the lives of those suffering with severe facial burn. Facial transplantation poses significant challenges, which when overcome provide a promising treatment modality for the severe facial burn injury (Pushpakumar et al. 2010).

### 9.3.5 Tissue Engineering and Stem Cells

Human skin performs an important protective function from the environment that is critical for survival. Despite the success of wound healing and skin grafting, the resulting skin contains scar and may lack the flexibility and elasticity of normal skin. This has led to the pursuit of a skin substitute that more closely resembles the dermal and epidermal structure of normal skin. A breakthrough in the use of dermal analogs and cultured epidermal autograft has been the development of combined dermal and epidermal skin replacements (Boyce et al. 1995). For its preparation, fibroblasts and keratinocytes are obtained from the patient and cultured *ex vivo*. These cells are then inoculated onto collagen-glycosaminoglycan substrates (Boyce 1998; Hansbrough et al. 1989). Further culture and processing at an air-liquid interface provides liquid nutrient medium to the dermal substitute and air contact to the epidermal substitute, resulting in stratification and cornification of the keratinocyte layer (Boyce and Williams 1993; Prunieras et al. 1983). In the dermal layer, fibroblasts proliferate into the collagen substrate, degrade it, and generate new autologous dermal matrix. At the dermal-epidermal junction, collagen and basement membrane formation takes place *in vitro* (Boyce et al. 2002). This increases the strength of the dermal-epidermal junction and decreases the development of epidermolysis and blistering frequently encountered with CEA application and split-thickness grafting.

New techniques have been employed to further improve these engineered skin replacements. The addition of melanocytes can decrease hypopigmentation and achieve better appearance and color matching (Swope et al. 1997). Vascular endothelial growth factors and angiogenic cytokines have also been introduced to counteract the absence of a vascular plexus, shorten healing time, and prevent graft loss (Table 9.2) (Supp and Boyce 2002; Supp et al. 2000).

Stem cells represent a new hope in the management of burns. Several mechanisms have been described by which these cells play an important role in wound

**Table 9.2** Engineered skin substitutes

Model	Description	Indications
<i>Acellular</i>		
Biobrane (Bertek Pharmaceuticals, Morgantown, WV)	Very thin semipermeable silicone membrane bonded to nylon fabric	Temporary adherent wound covering for partial-thickness excised burns and donor sites
Integra (Integra LifeSciences, Plainsboro, NJ)	Bilayer structure, biodegradable dermal layer made of porous bovine collagen-chondroitin-6-sulfate matrix, temporary epidermal layer made of synthetic silicone polymer	Grafting of deep partial- or full-thickness burns, epidermal layer removed when donor sites available for autografting
AlloDerm (LifeCell Corporation, Branchburg, NJ)	Structurally intact allogeneic acellular dermis, freeze-dried after cells were removed with detergent treatment, rehydrated before grafting	Dermal template for grafting to burns and other wounds, repair of soft tissue defects
Matriderm (Dr. Suwelack Skin & Health Care AG, Germany)	Non-cross-linked bovine collagen and elastin matrix that allows cellular ingrowth and neovascularization	Template for dermal reconstruction in the treatment of full-thickness burns
<i>Cellular allogeneic</i>		
Dermagraft (Advanced Biohealing, Westport, CT)	Cryopreserved allogeneic neonatal foreskin fibroblasts seeded on bioabsorbable polyglactin mesh scaffold; cells are metabolically active at grafting	Treatment of full-thickness chronic diabetic foot ulcers
Apligraf (Organogenesis/Novartis, Canton, MA)	Bilayer, allogeneic neonatal foreskin fibroblasts and keratinocytes in bovine collagen gel	Treatment of chronic foot ulcers and venous leg ulcers, also used for burn wounds and EB
OrCel (Forticell Bioscience, Englewood Cliffs, NJ)	Bilayer, allogeneic neonatal foreskin fibroblasts and keratinocytes cultured in bovine collagen sponge	Treatment of split-thickness donor sites in patients with burn and surgical wounds in EB
<i>Cellular autologous</i>		
Epicel (Genzyme Biosurgery, Cambridge, MA)	Autologous keratinocytes cultured from patient skin biopsy, transplanted as epidermal sheet using petrolatum gauze support	Permanent wound closure in patients with burn with greater than 30 % TBSA injury and in patients with congenital nevus
Epidex (Modex Therapeutiques, Lausanne, Switzerland)	Autologous keratinocytes isolated from outer root sheath of scalp hair follicles, supplied as epidermal sheet discs with a silicone membrane support	Treatment of chronic leg ulcers
TranCell (CellTran Limited, Sheffield, UK)	Autologous keratinocytes cultured from patient skin biopsy, grown on acrylic acid polymer-coated surface, transplanted as epidermal sheets	Treatment of chronic diabetic foot ulcers
Cultured skin substitute (University of Cincinnati/Shriners Hospitals, Cincinnati, OH)	Bilayer, autologous keratinocytes and fibroblasts cultured from patient skin biopsy, combined with degradable bovine collagen matrix	Permanent wound closure in patients with burn with greater than 50 % TBSA injury, also used in patients with congenital nevus and chronic wound

Adapted from Supp et al. (2000)

**Table 9.3** Review of stem cell nomenclature

Cell	Source	Potency	Advantages	Disadvantages	Examples of utility
Embryonic stem cells	Inner cell mass of blastocyst	Pluripotent	Pluripotent Clonogenic	Teratogenic ethical controversy	Knockout mouse
Umbilical cord blood stem cells	Umbilical cord blood	Pluripotent	Pluripotent Non-immunogenic clonogenic	Limited supply with low yield	Bone marrow transplantation
Mesenchymal stem cells	Bone marrow stroma, blood	Multipotent	Autologous  Accessible Clonogenic	Require time to culture  Harvest invasive limited supply	Parkinson's, myocardial remodeling, wound healing
Adipose-derived stem cells	Adipose tissue	Multipotent	Non-immunogenic abundant supply accessible Clonogenic	Processing required	Wound healing, tissue engineering
Resident progenitor cells	Numerous tissues/organs	Unipotent	Accessible Potential for transdifferentiation	Limited potency and clonogenicity	Re-epithelialization of wounds from hair follicular cells

With permission from Butler et al. (2010)

healing process, both locally and systemically. In humans, stem cells can be found in the bone marrow, adipose tissue, umbilical cord blood, and in the blastocystic mass of embryos (Butler et al. 2010). Even though obtaining embryonic stem cells can involve the destruction of the human embryo and raise ethical questions, the ability to obtain these cells from other tissues without affecting the source has facilitated research in the field.

The promising characteristics of stem cells are plentiful. Given the clonicity and pluripotency these cells have, they can be used to regenerate dermis and expedite re-epithelialization (Wu et al. 2007). Another important characteristic is that stem cells lack immunogenicity and can be transplanted with relative ease (Burd et al. 2007).

It has been described that stem cells present in the bone marrow migrate to affected tissue after injury and aid in the healing and regeneration process (Abe et al. 2001; Korbling et al. 2003; Mansilla et al. 2006). During this process, while the cells are blood-borne and after they reach the affected tissue, these cells have the ability to control inflammation by decreasing pro-inflammatory cytokine release and upregulating anti-inflammatory cytokines like IL-10 (Weil et al. 2009).

Embryonic human stem cells can be differentiated into keratinocytes in vitro and then stratified into an epithelium that resembles human epidermis (Guenou et al. 2009). This graft can then be applied to open wounds on burn patients as a temporary skin substitute while autograft or other permanent coverage means become available. This application, however, is still in its early stages of experimental clinical application (Table 9.3).



### 9.3.6 Gene Therapy and Growth Factors

Gene therapy, defined as the insertion of a gene into recipient cells, was initially considered only as a treatment option for patients with a congenital defect of a metabolic function or late-stage malignancy (Hernandez and Evers 1999). More recently, skin has become an important target of gene therapy research. Skin is easily accessible and the effects of therapy can be repeatedly monitored, thus allowing for gene transfer testing *in vitro* and the use of skin cells as vehicles in gene transfer (Liechty et al. 1999).

Gene transfer, using viral vectors, relies on the ability of viruses to carry and express their genes into host cells. Gene therapy vectors are developed by the modification of different types of viruses. Retroviruses and lentiviruses are non-lytic replicators produced from the cellular membrane of an infected cell, which leaves the host cell relatively intact. The lytic replication method involves the release of virions with the collapse of the host cell after infection. Human adenoviruses, adeno-associated viruses, and herpes simplex viruses are examples of lytic replicators. A large body of literature is now available which describes success and pitfalls in viral transfection of skin and wounds (Badillo et al. 2007; Bett et al. 1993; Carretero et al. 2004; Chen et al. 2005; Deodato et al. 2002; Eming et al. 1995, 1996, 1998, 1999; Galeano et al. 2003; Kozarsky and Wilson 1993; Liechty et al. 1999; Lu et al. 1997; Morgan et al. 1987; Silman and Fooks 2000).

To summarize, viral vectors are the original and most established technology for gene delivery. A wide range of applications have been developed, and many virus-mediated gene transfer models are successful. The production of viral vectors, however, is time-consuming and costly, transfection efficacy is variable, and the risk of local or systemic infections leading to fatal outcomes remains a concern.

In 1995, Hengge et al. (1995) first described the direct injection of DNA coding for interleukin-8 genes. By injecting naked genes into the skin, they found a significant recruitment of dermal neutrophils. However, the injection of naked DNA into the skin has been proven to have a low transfection efficacy and a high rate of initial degradation even before the injectate reaches the cytosol. Naked DNA constructs are not likely to penetrate the cells due to their fragility in the extracellular environment, large size, and electrical charge (Vogel 2000).

Eriksson et al. (1998) in 1998 modified the direct injection technique, termed “micro-seeding,” which delivers naked DNA directly into target cells via solid needles mounted on a modified tattooing machine. Elevated levels of transferred DNA could be maintained for 1–2 weeks, but transfection was only observed in the superficial layers of skin with minimal penetration into deeper tissue. Another technique used to penetrate the cellular membrane employs the “gene gun.” In this approach, 1–5  $\mu\text{m}$  gold or tungsten-coated particles carrying DNA plasmids are propelled into skin cells (Eming et al. 1999). Gene transfer is mostly transient and reaches its highest expression between the first and the third day after injection (Eming et al. 1996, 1998). *In vivo* transfection with epithelial growth factor (EGF) cDNA in porcine partial-thickness wounds has demonstrated an increase in the rate of wound healing and re-epithelialization (Nanney et al. 2000). Recent studies in the

rat model indicate that gene gun particle-mediated transfection of different PDGF isomers significantly improved wound healing by increasing its tensile strength (Eming et al. 1999). Differing results have been reported on the depth of transfection, with one study showing the gene gun technique primarily delivering particles no deeper than the epidermis with transfection rates of up to 10 % (Yang and Sun 1995). Dileo et al. (2003) had higher levels of gene expression in the epidermis and dermis by using a modified gene gun which delivered plasmids at higher discharge speed. Gene expression in skin and muscle reached its peak 24 h after application and remained detectable for at least 1 week with little tissue damage.

The technique of electroporation has been successfully used to accelerate the closure of diabetic and chronic wounds (Baker et al. 1997; Gardner et al. 1999). Lee et al. (2004) describe the synergistic use of electroporation, where an electric field is applied to tissue, in combination with tissue growth factor- $\beta$ 1 (TGF- $\beta$ 1) cDNA in a diabetic mouse model. In the group which received electroporation and gene transfer, the wound bed showed an increased rate of re-epithelialization, angiogenesis, and collagen synthesis. Apart from a transitional effect between the second and fourth day after wounding, the wound healing process itself was not significantly accelerated by the combined use of electric stimulation and gene transfer (Lee et al. 2004).

Marti et al. (2004) showed that electroporation and simultaneous administration of keratinocytes growth factor (KGF) plasmid DNA increased wound healing when compared to controls receiving no treatment (92 % vs. 40 % of the area healed). No significant improvement in comparison to administration of KGF plasmid DNA alone was observed. Taking into account these inconclusive results, the benefit of this concept remains in question.

Another method receiving particular attention as a reliable and highly efficient therapy is the cutaneous gene delivery with cationic liposomes. Cationic liposomes (CL) are synthetically prepared vesicles with positively charged surfaces that form loose complexes with negatively charged DNA to protect it from degradation in the wound environment. The net positive charge of the complex binds readily to negatively charged cell surfaces to facilitate uptake via endocytosis (Felgner and Ringold 1989; Jeschke et al. 2000). Genes encapsulated in CL can be applied either topically or by direct injection (Felgner and Ringold 1989; Slama et al. 2001). Alexander and Akhurst (1995) used topical application of CL constructs containing the Lac Z-gene to induce transfection and expression in the epidermis, dermis, and hair follicles in shaved 4-week-old mice. Expression was observed as early as 6 h after topical application, which persisted at high levels for 48 h, and was detectable for 7 days (Alexander and Akhurst 1995). Several studies have been performed to determine CL gene transfer for growth factors (Eming et al. 1999; Jeschke et al. 1999; Sun et al. 1997). Sun et al. (1997) administered fibroblast growth factor-1 (FGF-1) by topical application and subcutaneous injection to the injured skin of diabetic mice. Transfection with FGF was found to increase tensile strength. In a preliminary study of IGF-I cDNA constructs applied to thermally injured rat skin, Jeschke et al. (2004) detected a transfection rate of 70–90 % in myofibroblasts, endothelial cells, and macrophages, including multinucleate giant cells. In an *in vivo*

approach, thermally injured rats treated with liposomal IGF-I cDNA significantly improved body weight and increased muscle protein when compared to burn controls. An accelerated rate of re-epithelialization of nearly 15 % was observed when compared to naked IGF-I protein and IGF-I protein encapsulated in liposomes (Jeschke et al. 2004). Furthermore, no evidence was found that the dermal injection of IGF-I cDNA-complexes led to transfection or increase in  $\beta$ -galactosidase or IGF-I expression in blood, liver, spleen, or kidney; thus, gene transfection and production of growth factors remain localized (Jeschke et al. 2000). Animals transfected with IGF-I cDNA increased their basal skin cell proliferation, suggesting that myofibroblasts, endothelial cells, and macrophages, identified as transfected, produce biologically active IGF-I (Jeschke et al. 2004; Jeschke and Klein 2004). Branski et al. (2010) performed the transfection of PDGF-cDNA in a large animal burn model via the liposomal vector. Gene transfer of liposomal PDGF-cDNA resulted in increased PDGF-mRNA and protein expression on days 2 and 4 post injection, accelerated wound re-epithelialization, as well as graft adhesion on day 9. The authors concluded that liposomal cDNA gene transfer is possible in a porcine wound model, and by using PDGF-cDNA dermal and epidermal regeneration can be improved.

A potential problem of single growth factor gene therapy is that simply increasing the concentration may not promote all phases of wound healing. A single growth factor cannot counteract all the deficiencies of a burn wound nor control the complexities of chronic wound healing. Lynch et al. (1987) demonstrated in a partial-thickness wound healing model that the combination of PDGF and IGF-I was more effective than growth factor alone, while Sprugel et al. (1987) found that a combination of PDGF and FGF-2 increased the DNA content of wounds in the rat better than any single growth factor. Jeschke et al. investigated the efficacy of KGF cDNA in combination with IGF-I cDNA compared to the same genes individually (Jeschke and Herndon 2007). It was noted that this combination accelerated re-epithelialization, increased proliferation, and decreased skin cell apoptosis compared to the single construct alone. The re-epithelialization in the burn model was over twice that of the untreated control with a significant improvement in cell survival (Jeschke and Herndon 2007). Applying genes at strategic time points of wound healing (sequential growth factor therapy) is therefore the next logical step in augmenting wound healing. Multiple groups worldwide are currently working towards this goal.

Other delivery routes including biomaterials (Shea et al. 1999), calcium phosphate transfection (Fu et al. 2005), diethylaminoethyl-dextran (Eriksson 2000), and microbubble-enhanced ultrasound (Lawrie et al. 2000) have been investigated. Slow-release matrices (Chandler et al. 2000) and gene-delivering gel matrices (Voigt et al. 1999) are used for prolonged transgenic expression. The concept of a genetic switch is another exciting development, where transgenic expression in target cells can be switched “on” or “off,” depending on the presence or absence of a stimulator such as tetracycline (Gossen and Bujard 1992). Biotechnological refinements, such as wound chamber technique (Breuing et al. 1992), may also improve the efficacy of gene delivery to wounds. These new techniques need further studies to define their

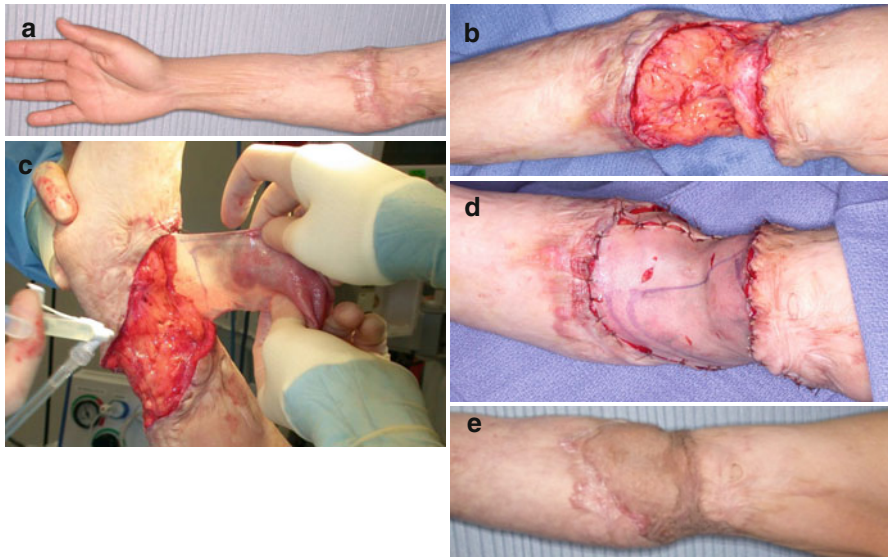
efficacy and clinical applicability. More studies are also needed to define growth factor levels in different phases of wound healing and to elucidate the precise timing of gene expression or downregulation required to better augment wound healing and control of scar formation.

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## 9.4 Fibrin Sealant for Graft Fixation

Over the past decades, new techniques in burn surgery have improved outcome and significantly reduced mortality (Pereira et al. 2004, 2006; Muller and Herndon 2007). The generally accepted approach in burn surgery consists of early excision followed by appropriate coverage to prevent hypothermia, protein and fluid loss, and risk of exogenous infection. The standard treatment for full-thickness wounds after excision is coverage by meshed split-thickness skin grafts (Janzekovic 1970; Herndon and Parks 1986; Muller and Herndon 2001; Thompson et al. 1987). As far as graft fixation is concerned, autograft skin is usually affixed to the wound bed by sutures or skin staples. These methods, however, are either time-consuming (in case of sutures) or associated with the need of additional anesthesia and lengthy removal procedures (Kulber et al. 1997; Zederfeldt 1994). In addition, it is often difficult to achieve full contact of graft and underlying wound surface, especially in severely burned patients with large grafted wound areas.

Fibrin sealant has emerged as an alternative fixation method for autografts (Furst et al. 2007; Buchta et al. 2005). It consists of thrombin and fibrinogen, is biologically degradable and nontoxic to the human tissue, and was primarily developed as a fast-clotting agent for hemostatic purposes. A novel application method is a slow-clotting version (Artiss®, Baxter Inc) with a smaller amount of thrombin, applied to the wound in a thin layer using a spray applicator. It has been shown effective for autograft fixation in both human (O’Grady et al. 2000; Gibran et al. 2007; Redl 2004) and porcine (Mittermayr et al. 2006) studies. A major advantage of this method is the fact that it prevents seroma and hematoma formation by providing complete contact between wound bed and graft. It also acts as a scaffold for collagen-producing fibroblasts, provides a matrix for vascularization, and possibly creates a barrier against infection (Currie et al. 2001; Jabs et al. 1992). After a short and steep learning curve, the use of fibrin sealant also significantly decreases the length of surgical procedures, especially in cases when grafts have to be applied to the face or areas where standard fixation methods are difficult to use. Most surgeons also find that bolsters can be omitted after fibrin sealant use. Since graft adherence and revascularization in the early postoperative phases translate into an accelerated wound closure in the late phase, it is likely that formation of hypertrophic scarring may also be reduced, although there is no controlled data as of yet. Rare side effects are allergic reaction to the fibrin sealant protein and air or gas embolism after application. Also, the cost of fibrin sealant is considerable, which reduces its applicability for most burn surgeons to a few carefully selected indications (Fig. 9.4).



**Fig. 9.4** Use of fibrin sealant (Artiss®) for graft fixation. (a) A 12-year-old girl with severe circumferential burn scar contracture of the right elbow. (b) Large defect after circumferential scar incision. (c) Application of Artiss® onto the wound bed using a Tissomat® spray device and immediate coverage with a non-meshed split-thickness skin graft. (d) First postoperative day. (e) One year after grafting

### Conclusion

The progress in the acute treatment of burn patients within last decades has been a success story, leading to a significant decrease in ICU mortality and the long-term survival of severely burned patients. This development, however, has led to a new set of challenges for burn researchers – reduction of scarring, improvement of skin graft quality, and the creation of a pluristratified dermal and epidermal constructs for the coverage of an excised burn wound. Therefore, a continuous and critical re-evaluation of all aforementioned aspects of temporary and definitive burn wound coverage is imperative. The design of new molecular methodologies and animal models for the studies of underlying pathophysiological mechanisms can lead to the ability to manipulate the disease pathology with the goal of improved patient outcome. The success integration of future technology into clinically applicable products will be based on the conduction of tightly controlled multicenter clinical studies with the use of new skin constructs and most importantly an integration of all these efforts with the multidisciplinary stem cell research.

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# Collagen Implants in Hernia Repair and Abdominal Wall Surgery

# 10

Alexander Petter-Puchner and Herwig Pokorny

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## 10.1 Introduction

Collagen implants started to attract attention in abdominal wall surgery as late as only one decade ago, whereas they have been used routinely in other surgical fields much earlier (de la Torre and Goldsmith 1990; Wasserman et al. 1989; Ansaloni et al. 2003). Within 2 years, more than a dozen of collagen implants, then still called “biological meshes,” were introduced to the market and promoted to be used especially in challenging hernia repair and the treatment of large abdominal wall defects. The following chapter shall briefly describe the different types of implants, referring to proprietary processing methods of the collagen matrices, and provide a short overview of the current experimental and clinical experiences. Finally, an outlook on future perspectives of indication finding and new developments, e.g., the role of collagen implants in tissue engineering, will be provided.

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## 10.2 Types of Collagen Implants in General Surgery

Even for the expert, the huge variety of collagen implants which are now commercially available has exceeded beyond an updated and complete overview. Existing products are continuously replaced by modified versions and implants, specifically designed for special indications (e.g., stoma, hiatus), are presented almost weekly (Wijeyekoon et al. 2010; Wassenaar et al. 2012). Currently, it is estimated that

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A. Petter-Puchner, MD (✉)  
Department of General-, Visceral and Oncological Surgery,  
Wilhelminenspital, Vienna, Austria  
e-mail: alexander.petter@trauma.lbg.ac.at

H. Pokorny, MD  
Department of General and Vascular Surgery, State Hospital Wiener Neustadt,  
Wiener Neustadt, Austria  
e-mail: herwig.pokorny@sportlerleiste.com

**Table 10.1** Collagen implants in hernia repair and abdominal wall surgery

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 An overview of matrices in use
 

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 Human cadaveric skin: AlloDerm™, AlloMax™, FlexHD™
 

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Animal collagen implants	Porcine	Cross-linked: Collamend™, PermaCol™*
		Non-cross-linked: Strattice™, Surgisis™*, Xenmatrix™
	Bovine	Cross-linked: currently none available
		Non-cross-linked: Veritas™, Tutomesh™, SurgiMend™
	Other (equine)	

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The stars indicate that clinical trials (in hernia research or other indications) had to be stopped with these materials

around 60 different collagen implants are in competition and that manufacturers offer about 100 different versions of their basis matrices. In this context, it is reassuring that only a few biological sources exist, which can be divided in human and animal origin. In general, collagen implants are derived from human or animal dermis, mucosa, or pericardium (Table 10.1).

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### 10.3 Human Allogenic Collagen Implants

Cadaveric tissues processed for allogenic implantation have a long history in surgical medicine. Human-decellularized dura mater was commonly used in neurosurgery among other examples. In abdominal wall surgery collagen grafts derived from cadaveric skin could earn a good reputation built on favorable experimental and clinical trials (Dasenbrock et al. 2011; Campbell et al. 2011; Brewer et al. 2011; Stanwix et al. 2011; Momoh et al. 2010). However, human collagen is unlikely to play a major role in the future. The main reasons are the costly manufacturing process which can hardly be maintained on a profitable level in large scales. The obstacles of constant high-quality donor availability and related factors such as ethical concerns as well as safety and quality regulations have already forced several companies to look for more cost-effective animal-derived alternatives. This background should be kept in mind when the possible medical advantages of allogenic or xenogenic implants maybe reevaluated and outbalanced at a later time point (Shah et al. 2011).

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### 10.4 Porcine Collagen Implants

By far the biggest group among collagen implants is formed by products of porcine origin. Porcine collagen can be harvested in virtually unlimited quantities and can be processed to meet specific requirements, such as mechanical endurance, different rates of degradation depending on the preserved microstructure, and cross-linking techniques involved. Skin, small intestine submucosa, and fascia are the connective tissues most frequently used for porcine implants. These differences in implant design and material processing methods are reflected by very heterogenous results

in literature. Currently, the impact of cross-linking is controversially discussed and is considered by many authors to be responsible for severe side effects, e.g., slow integration and encapsulation (Shah et al. 2011; Deeken et al. 2011; Melman et al. 2011; Sailes et al. 2011; Petter-Puchner et al. 2011). Furthermore, it has been demonstrated that decellularized biologic implants can still contain traces of DNA (Gilbert et al. 2009). The immunological consequences are not fully understood, yet, and ethical and even religious questions come into play as well. Porcine DNA in implants could be unacceptable for the use in Muslim recipients (the same problem occurs with bovine implants and Hindu populations). Nevertheless, porcine collagen implants have opened the door for xenogenic implantology in abdominal wall surgery.

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## 10.5 Bovine Collagen Implants

Bovine collagen implants are usually derived of pericardium (Burger et al. 2006). The major companies promoting bovine collagen implants have gained their experiences with this matrix in the production of organic heart valves. It is a common feature of biologic implants that they are gamma radiated and decellularized. As mentioned above, this will not always result in tissue samples, free of traces of DNA (Gilbert et al. 2009). In this context it should be emphasized that the removal of pathogenic vectors, such as prions, responsible for Jakob-Creutzfeldt disease is assumed but has not been specifically investigated by independent scientists in biologic collagen implants for abdominal wall surgery (Beekes 2010).

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## 10.6 Other Animal Sources

In contrast to heart or vascular surgery, equine collagen is an uncommon material for biologic implants in abdominal wall surgery. However, there are equine biologics on the market (Brown 2009).

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## 10.7 Cross-Linking of Collagen Implants

Native collagen is prone to rapid degradation due to enzymatic attack in the course of physiologic wound healing or inflammation. In order to overcome this problem, different strategies are applied by the manufacturers of collagen implants. Multilayer design or tissue engineering of the three-dimensional microstructure of various collagen matrices forms mechanical obstacles to degradation. This group of implants is called “non-cross-linked.” More often, chemical processes are applied in order to cross-link the collagen fibers and make them resistant to collagenases. It must be underlined that specifications of cross-linking techniques or other measures taken to prevent fast degradation are usually not provided by the companies. Unfortunately, glutaraldehyde is still used for cross-linking. Glutaraldehyde is

cytotoxic and contributes to the calcification of the implanted collagen implant. Alternatives which obviate the use of foreign agents are established, e.g., the transformation of free carboxyl groups on collagen into acyl azide groups, which react with free amino groups on adjacent side chains based on a thermal reaction (Reiser et al. 1992; Haugh et al. 2011).

Currently, the impact of cross-linking on tissue integration of collagen implants is controversially discussed (Deeken et al. 2011; Melman et al. 2011; Petter-Puchner et al. 2011). Cross-linked implants are accused of poor integration and a tendency to become encapsulated. Non-cross-linked implants are refused by many surgeons who seek mechanical endurance persisting over extended periods (Jenkins et al. 2011).

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## 10.8 Experimental Literature

Sophisticated models have been developed in experimental hernia research in order to adequately mimic inguinal, ventral, and hiatal repair procedures. Although the translational value is often disputed, animal trials in hernia research are outstandingly precise in predicting or confirming the behavior of biologic or synthetic implants when brought in contact with living tissue (Hernández-Gascón et al. 2011; Hernández et al. 2011). Experimental research is the only way to obtain second look data and histology. Currently, no noninvasive imaging technique can provide reliable information about the fate of the implant once exposed to the organism. Key parameters, such as tissue integration and foreign body reaction, can only be assessed by histology. Even before taking biologic implants to in vivo models, in vitro investigations offer useful information about biocompatibility (as discussed in the Sect. 10.7), the susceptibility to bacterial infection, and rates of shrinkage. Rauth et al. reported shrinkage rates of small intestine submucosa implants of up to 40 % in less than 8 weeks in an in vitro study (Rauth et al. 2007). Interestingly enough, this finding presented no obstacle to commercialization as hernia and vascular patches. The following paragraph will highlight more recent studies with emphasis on problematic aspects which are relevant today.

First, the issue of cross-linking has led to some confusion. It is justified to state that cross-linked collagen implants have often been related to complications in animal trials. It was demonstrated that cross-linking impaired the tissue integration and led to encapsulation and foreign body reaction. There are only few indications that cross-linked collagen implants integrate well in an acute time frame. Nevertheless, non-cross-linked implants do not seem to outperform cross-linked competitors in animal trials. In studies which at first glance may seem contradictory, Deeken et al. from Missouri, USA, and Petter-Puchner from Vienna, Austria, both demonstrated slow integration of both cross-linked and non-cross-linked collagen implants (Deeken et al. 2011; Melman et al. 2011; Petter-Puchner et al. 2011). Although integration may occur faster with non-cross-linked collagen implants, it is invariably slower than with synthetic meshes even in clean, non-contaminated wound fields. The Deeken group observed Yucatan minipigs as long as a year and found almost

complete resorption of non-cross-linked samples. The difference of conclusion between the two authors is based on the question if a slow integration and a rapid resorption is really a desirable clinical option.

Second, it is important to point out that biologic implants do not perform especially well in challenging repair. Although, experimental research has elucidated the susceptibility of collagen implants to bacterial contamination, it has become increasingly clear that best results can only be obtained in elective procedures (Medberry et al. 2012; Milburn et al. 2008; Bellows et al. 2011). High granulocyte and enzymatic activity which is present in contaminated wounds leads to an enhanced breakdown and degradation specifically of non-cross-linked implants. Even in clean wounds some non-cross-linked biologics will degrade at a critically fast rate before physiological wound healing is terminated, and recurrences are likely. The commercial argument that challenging repair was a distinctive advantage of biologic implants over synthetic meshes is simply not supported by the recent body of evidence. Second, biologic does not signify improved biocompatible. The opposite seems to be true when findings about specific antibody formation to collagen implants and prolonged inflammatory reactions are considered.

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## 10.9 Clinical Literature

These insights from experimental research are reflected by a shift of paradigms when companies now advice surgeons to implant biologic under optimized conditions in patients. In consequence there is no indication for biologic collagen implants in abdominal wall surgery, given the standards of evidence-based medicine. After it has become clear that optimistic promises on the efficacy in contaminated wound fields cannot be kept, it is currently unclear if there will be elective indications where biologics are truly superior to synthetic meshes (Bellows et al. 2011). Multicenter randomized controlled trials which could show such an improved standard of care of are missing. Several clinical trials on collagen implants had to be terminated prematurely due to slow recruitment and/or side effects not only in the field of abdominal wall surgery. Among these, the LapSis® trial was probably the most notable, because of the substantial effort put in the investigation of small intestine submucosa for endoscopic groin hernia repair. The study had to be terminated in autumn 2010 without yielding new insights. Complications with this matrix have also been reported in vascular surgery and resulted in abandoning a clinical trial (McCready et al. 2005).

In this context, it is astounding that collagen implants could gain wide popularity and general acceptance for ventral and hiatal hernia repair in the USA and Canada as well as in many parts of the EU. Great hopes have been linked to the use of collagen implants for reinforcement of the gastroesophageal junction (Wassenaar et al. 2012). Many surgeons were reluctant to use synthetic meshes because of feared mesh erosion. However, Stadlhuber et al. recently published a complication case series of hiatal meshes which showed that biologic matrices do dislocate and shrink in this indication too (Stadlhuber et al. 2009). Sadly, it is a common prob-



**Table 10.2** Clinical trials which had to be stopped with collagen implants

	Surgisis: hernia: Lapsis (Neugebauer 2009)	
	Surgisis: vascular surgery (McCrary 2005)	
Permacol	[	Plastic surgery (Cheung 2004)
		Hand surgery (Belcher 2001)
		Ophthalmology (AlWitry 2006)

lem of many studies that the patient cohorts are small, that the observation periods are short, and that operation techniques are sometimes modified for the sake of the biologic implant and not of the patient (Fortelny et al. 2010; Griffith et al. 2008). One example is the performance of an inguinal intraperitoneal onlay mesh plasty (IPOM) instead of the well-established transabdominal preperitoneal patch plasty (TAPP) technique because of the poor laparoscopic handling of the biologic implant used. An IPOM repair is obsolete in the groin because of the great likelihood of nerve injuries by blind tacking through the peritoneum and recurrences due to the lack of the required preparation of the preperitoneal space. A second example is the application of collagen implants in open interlay position in ventral hernia repair. Based on the commercial hypothesis that biologics could excel in challenging repair procedures, this technique was resuscitated because it is fast and easy to perform. However, interlay has already been abandoned with synthetic meshes because of poor results in terms of recurrences and aggravation of the local wound situation (Table 10.2) (Schumpelick et al. 2004).

## 10.10 Perspective

Despite controversial experiences in the past, it becomes increasingly clear that the clinical situation with collagen implants for abdominal wall surgery is approaching a “reset” status. There is a growing consensus that biologic implants cannot reinvent the wheel and overcome the problems and obstacles found in challenging, maybe contaminated, defects. Collagen implants are not superior to synthetic meshes in terms of biocompatibility, efficacy, or safety when basic principles of surgery and implantology are neglected. Good results can only be achieved when the best conditions of the wound field are provided. Otherwise, foreign body reaction, encapsulation, and in consequence recurrences and high failure rates are predictable. Unfortunately, high expectations on biologics have been created over years, pretending that collagen implants could excel under disastrous circumstances. The strategy to position collagen implants as alternative to synthetic meshes for especially difficult indications has failed and now presents a severe burden to any attempt to establish evidence-based recommendations for their safe use. Today, no indications for biologics for elective repairs are in sight, and it will take years before high-quality RCTs can provide the necessary data. This is a pity because the potential of elaborated biologic matrices for hernia repair and future tissue engi-

neering purposes is undeniable. In summary, the authors recommend the use of collagen implants for elective indications only and, if possible, in the setting of an RCT. The participation in workshops for the safe application and the training by surgeons experienced in the use of collagen implants seems mandatory to avoid complications.

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Gerd G. Gauglitz

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## 11.1 Introduction

Skin acts as an essential barrier, protecting organisms from their environment. Loss of the integrity of large portions of the skin as a result of injury may lead to major disability or even death. Adult skin consists of two tissue layers: a keratinized, stratified epidermis and an underlying thick layer of collagen-rich dermal connective tissue providing support and nourishment. Appendages such as hair and glands are derived from the epidermis, but they project deep into the dermal layer. Thus, skin replacement has been and remains a challenging task for surgeons ever since the introduction of skin grafts by Reverdin in 1871.

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## 11.2 Auto-, Allo-, and Xenografts

Various biological and synthetic substrates have been employed to replace the injured skin. Autografts from uninjured skin remain the mainstay of treatment for many patients. Autologous split skin grafts (SSGs) are harvested with a dermatome that detaches the epidermis and a superficial part of the dermis. Remaining epidermal cells in the residual dermis of the SSG donor site will regrow an epidermis. After application of an SSG to a full-thickness wound, its capillaries merge with the capillary network in the excised wound. This “graft take” is essential for a proper supply of nutrients and ensures graft survival (Andreassi et al. 2005; Converse et al. 1975). However, in the case of a more extensive injury, donor sites are extremely limited and might leave the

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G.G. Gauglitz, MD, MMS  
Scar Clinic, Department of Aesthetics,  
Department of Infectiology and Sexual Transmitted Diseases,  
Department of Dermatology and Allergology,  
Ludwig Maximilians University,  
Frauenlobstr. 9-11, Munich 80337, Germany  
e-mail: gerd.gauglitz@med.uni-muenchen.de

patient with too little undamaged skin to harvest enough autologous SSGs. Since early wound closure using autograft may be difficult when full-thickness burns exceed 40 % total body surface area (TBSA), allografts (cadaver skin) frequently serve as skin substitute in severely burned patients. While this approach is still commonly used in burn centers throughout the world, it bears considerable risks, including antigenicity, cross-infection, as well as limited availability (Blome-Eberwein et al. 2002). Xenografts have been used for hundreds of years as temporary replacement for skin loss. Even though these grafts provide a biologically active dermal matrix, the immunologic disparities prevent engraftment and predetermine rejection over time (Garfein et al. 2003). However, both xenografts and allografts are only a mean of temporary burn wound cover. True closure can only be achieved with living autografts or isografts.

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### 11.3 Epidermal Substitutes

Autologous epithelial cells grown from a single full-thickness skin biopsy have been available for nearly two decades. These cultured epithelial autografts (CEA) have shown to decrease mortality in massively burned patients in a prospective, controlled trial (Munster 1996). However, widespread use of cultured epithelial autografts has been primarily hampered by poor long term clinical results, exorbitant costs, and fragility and difficult handling of these grafts that have been consistently reported by different burn units treating deep burns, even when cells were applied on properly prepared wound beds (Garfein et al. 2003; Bannasch et al. 2003; Pellegrini et al. 1999). Currently commercially available autologous epidermal substitutes for clinical use include CellSpray (Clinical Cell Culture (C<sup>3</sup>), Perth, Australia), Epicel (Genzyme Biosurgery, Cambridge, MA, USA), EpiDex (Modex Therapeutiques, Lausanne, Switzerland), Bioseed-S (BioTissue Technologies GmbH, Freiburg, Germany), etc.

Epicel (Genzyme Biosurgery; Cambridge, MA), an epidermal graft, is cultured from healthy human skin and shipped back to the physician, with a 1-day half-life. EpiDex (Isotis Orthobiologics; Irvine, CA) is cultivated from pluripotent stem cells from hair follicles. In dermatology, successful treatment of pyoderma gangrenosum (Limova and Mauro 1994) and epidermolysis bullosa (Wollina et al. 2001) has been reported with epidermal autografts, and we are using EpiDex for the treatment of treatment resistant ulcers with good results. There have been several studies testing epithelial allografts like Celaderm (Khachemoune et al. 2002; Alvarez-Diaz et al. 2000; Bolivar-Flores and Kuri-Harcuch 1999); however, controlled clinical studies confirming the effectiveness and safety of these products are needed.

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### 11.4 Dermal Substitutes

Alternatively, dermal analogs that mimic human skin have been made available for clinical use in recent years (MacNeil 2007). In contrast to cultured epidermal sheets, engineered dermal constructs can prevent wound contraction and they provide a greater mechanical stability. To date, a wide variety of marketed dermal constructs is available. These skin substitutes can promote the healing of acute and chronic

wounds (Ponec 2002) by secreting extracellular matrix (ECM) proteins, a variety of growth factors and cytokines, into the wound until they undergo normal apoptosis a few weeks post-implantation (Groeber et al. 2011). An overview summarizing commercially available dermal constructs for clinical use gives Table 11.1.

Some of these substitutes are chemically treated allografts (e.g., Alloderm®), lacking the cellular elements that are responsible for the immunogenic rejection (Wainwright 1995). Dermagraft® (Advanced Biohealing; La Jolla, CA, USA), in contrast, consists of human foreskin fibroblasts, cultured in a biodegradable polygly-

**Table 11.1** Commercially available dermal constructs for clinical use

Brand name	Manufacturer	Cell-free	Cell-based	Cell-seeded scaffold (TE)
AlloDerm	LifeCell Corporation, Branchburg, NJ, USA	×		–
Karoderm	Karocell Tissue Engineering AB, Karolinska University Hospital, Stockholm, Sweden	×		–
SureDerm	HANS BIOMED Corporation, Seoul, Korea	×		–
GraftJacket	Wright Medical Technology, Inc., Arlington, TN, USA	×		–
Matriderm	Dr. Suwelack Skin and HealthCare AG, Billerbeck, Germany	×		–
Permacol Surgical Implant	Tissue Science Laboratories plc, Aldershot, UK	×		–
OASIS Wound Matrix	Cook Biotech Inc, West Lafayette, IN, USA	×		–
EZ Derm	Brennen Medical, Inc., MN, USA	×		–
Integra Dermal Regeneration Template	Integra NeuroSciences, Plainsboro, NJ, USA	×		–
Terudermis	Olympus Terumo Biomaterial Corp., Tokyo, Japan	×		–
Pelnac Standard/Pelnac Fortified	Gunze Ltd, Medical Materials Center, Kyoto, Japan	×		–
Biobrane/Biobrane-L	UDL Laboratories, Inc., Rockford, IL, USA	×		–
Hyalomatrix PA	Fidia Advanced Biopolymers, Abano Terme, Italy	×		–
TransCyte (DermagraftTC)	Advanced BioHealing, Inc., New York, NY and La Jolla, CA, USA			Neonatal allogeneic fibroblasts
Dermagraft	Advanced BioHealing, Inc., New York, NY and La Jolla, CA, USA			Neonatal allogeneic fibroblasts
Hyalograft 3D	Fidia Advanced Biopolymers, Abano Terme, Italy			Autologous fibroblasts

Modified from Groeber et al. (2011)

ctin mesh (Supp and Boyce 2005; Kolokol'chikova et al. 2001). It stimulates ingrowth of fibrovascular tissue and epithelialization. The frozen product offers an advantage but unfortunately requires storage at  $-75^{\circ}\text{C}$ . It is thawed in sterile saline and then applied to a clean, well-debrided wound. It has a 6-month shelf life and was approved by the FDA in 2001 for full-thickness diabetic foot ulcers of more than 6 weeks' duration, extending through the dermis, but without exposed underlying structures. It has found value in healing complex surgical wounds with secondary closure.

Integra was developed in 1981 and approved by the FDA in 2002. It is a bilaminar skin equivalent composed of porous matrix of cross-linked bovine collagen and shark-derived glycosaminoglycan, attached to a semipermeable silicone layer that serves as an epidermis. The membrane helps prevent water loss and provides a flexible wound covering, while the scaffolding promotes neovascularization and new dermal growth. Cells migrate into the matrix while the bovine collagen is absorbed and replaced by the patient's dermal elements. Rebuilding of the scaffolding occurs within 2–3 weeks, at which time the silicone layer is removed, allowing re-epithelialization from the wound edge. Complete wound closure takes approximately 30 days. Indications for Integra include pressure, diabetic, chronic vascular and venous ulcers, as well as surgical wounds and has been successfully utilized in immediate and delayed closure of full-thickness burns, leading to reduction in length of hospital stay, favorable cosmetics, and improved functional outcome in a prospective and controlled clinical study (Tompkins and Burke 1990; Burke et al. 1981; Yannas et al. 1981, 1982). Our group recently conducted a randomized clinical trial utilizing Integra in the management of severe full-thickness burns of  $\geq 50\%$  TBSA in a pediatric patient population comparing it to standard autograft–allograft technique and found Integra to be associated with improved resting energy expenditure and improved aesthetic outcome post-burn (Branski et al. 2007). It has been also found to inhibit scar formation and wound contraction (Clayman et al. 2006). This quality supports its use in keloid repair.

Biobrane<sup>®</sup>, a temporary synthetic dressing composed of nylon mesh bonded to a silicone membrane, helps control water loss and re-epithelialization (Junkins-Hopkins 2011).

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## 11.5 Epidermal/Dermal Substitutes

To date, the most advanced and sophisticated constructs that are available for clinical use represent substitutes that mimic both epidermal as well as dermal layers of the skin. Currently available epidermal/dermal substitutes that are in clinical use include Apligraf (Organogenesis Inc., Canton, Massachusetts, CA, USA), OrCel (Ortec International, Inc., New York, NY, USA), PolyActive (HC Implants BV, Leiden, The Netherlands), and TissueTech Autograft System (Laserskin and Hyalograf 3D; Fidia Advanced Biopolymers, Abano Terme, Italy). These constructs are composed of autologous and allogeneic skin cells (keratinocytes and fibroblasts), which are incorporated into scaffolds.

Apligraf was the first commercially available composite tissue analog on the market. This medical device containing living allogeneic cells was approved by the



US Food and Drug Administration (FDA) in 1998 for the treatment of venous ulcers of 1-month duration that have not responded to conventional therapy. It was approved in 2000 for neuropathic diabetic ulcers of more than 3 weeks' duration (Zaulyanov and Kirsner 2007). The epidermal component of this bilayer skin construct consists of neonatal foreskin keratinocytes seeded on a dermal component comprised of neonatal foreskin fibroblasts within a matrix of bovine type I collagen. This 0.75 cm disc has a 10-day shelf life and requires storage at 68–73 °F. It is secured to the prepared wound bed with sutures or a dressing and is changed weekly. Apligraf was shown to achieve significantly better results in healing large, deep venous ulcers of more than 1-year duration when compared to compression (Falanga et al. 1998). Apligraf has been also successfully used in acute surgical wounds (Zaulyanov and Kirsner 2007) and may result in a more pliable and less vascular scar when used in wounds that would otherwise be allowed to heal with secondary intention (Gohari et al. 2002).

Orocel, the first biologic cellular matrix, was initially developed in 1971 as a treatment for dystrophic epidermolysis bullosa (Eisenberg and Llewelyn 1998). Similar to Apligraf, neonatal foreskin epidermal keratinocytes and dermal fibroblasts are cultured onto a preformed porous sponge. However, it is produced in a cryopreserved format, in contrast to the fresh product of Apligraf.

Importantly, although mimicking the histoarchitecture of normal skin, epidermal/dermal skin substitutes should be considered as temporary biologically active wound dressings (Supp and Boyce 2005). Composite skin substitutes have been shown to provide growth factors, cytokines, and ECM for host cells, and by that initiating and regulating wound healing. Nevertheless, these skin substitutes are accompanied by high manufacturing costs and repeatedly fail to close the wound permanently due to tissue rejection (Groeber et al. 2011). While the immunogenic tolerance of a host towards allogeneic fibroblasts is still controversially discussed, allogeneic keratinocytes are usually rejected by the host (Clark et al. 2007; Strande et al. 1997). Thus, only autologous keratinocytes are adequate for the generation of a permanent epidermal/dermal skin substitute. TissueTech Autograft System designed by Fidia Advanced Biopolymers is currently the only commercially available product that allows permanent wound closure. It is based on autologous fibroblasts and keratinocytes, grown on microperforated hyaluronic acid membranes (Campoccia et al. 1998; Caravaggi et al. 2003), and is comprised of Hyalograft® as a dermal substitute and Laserskin as the epidermal substitute (Myers et al. 1997). However, since it combines these two independent biomaterials, which need to be applied consecutively to the wound, it cannot be considered a “true” dermal–epidermal skin substitute. A promising new construct that is not yet commercially available represents the Cincinnati Shriners Skin Substitute or PermaDerm™. This three-dimensionally (3D) reconstructed skin graft, which has been designed by Boyce and colleagues (Myers et al. 1997), is based on a collagen sponge that is seeded with autologous fibroblasts and keratinocytes. It provides permanent wound closure and can be described as a true dermal–epidermal skin substitute (Groeber et al. 2011).

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## Conclusions

Despite all efforts, an off-the-shelf, full-thickness skin replacement is not yet available. A future prospective is to incorporate cellular growth-enhancing substances or additional cell types, besides keratinocytes and fibroblasts, in the bioengineered skin substitutes to obtain constructs with improved function and higher resemblance to native skin. The development of gene transfer technology in this context appears to be a promising means to overcome the limitations associated with the (topical) application of recombinant proteins by delivering the respective growth factor directly to the wound bed. Thus, several novel technologies are under active development to aid cutaneous wound repair in order to enhance and accelerate the treatment of acute and chronic wounds.

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Stephan Spendel, Gerlinde Weigel, and Lars-Peter Kamolz

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## 12.1 Introduction

New generations of bioengineered dermal substitutes are being developed in the use for the treatment of burns (deep partial and full thickness). Other applications such as soft tissue replacement or coverage of chronic wounds with dermal substitutes were established (Jones et al. 2002). Recently these materials were used as suspensory materials or interpositional grafts in breast surgery.

Although there is a wide range of materials being used in dermal substitutes, some general requirements can be formulated for proper functioning.

The materials should be stable enough to function as a provisional matrix and should not elicit immunogenic reactions. Composition, pore size and degradability of the substitute should support cell migration and function (van der Veen et al. 2011, Anderson et al. 2008).

One of the common procedures performed by today's plastic surgeon is postmastectomy breast reconstruction after breast cancer.

Due to inherited predisposition genes, many young women at high risk of breast cancer are choosing bilateral prophylactic mastectomy (Breuing and Warren 2005).

Mastectomy followed by immediate breast reconstruction is oncologically safe, and it improves patients' psychosocial health (Breuing and Warren 2005). Many of these women are unwilling to endure the donor site morbidity, prolonged recovery and muscle weakness following autologous reconstruction (Breuing and Warren 2005). The ideal candidate for reconstruction with prosthetic implants is a thin patient requiring a bilateral reconstruction or a thin patient with normal, nonptotic

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S. Spendel, MD (✉) • G. Weigel, MD • L.-P. Kamolz, MD, MSc  
Division of Plastic, Aesthetic and Reconstructive Surgery,  
Research Unit for Tissue Regeneration, Repair and Reconstruction,  
Medical University of Graz, Auenbruggerplatz 29, 8036 Graz, Austria  
e-mail: stephan.spendel@medunigraz.at; gerlinde.weigel@medunigraz.at;  
lars.kamolz@medunigraz.at

breast who requires unilateral reconstruction (Haddock and Levine 2010). Currently staged postmastectomy prosthetic breast reconstruction is performed by placing a tissue expander deep to the pectoralis major muscle in the majority of cases. The expander is replaced with a permanent silicone or saline implant after the desired expansion has been obtained. The subpectoral position provides muscular coverage to the superior 2/3 of the implant.

There are various options for coverage of the remainder implant, one which includes the elevation and use of the serratus anterior superior muscle and a part of the external oblique fascia. Another option is to leave the lower pole of the implant in the subcutaneous position (Bindingavele et al. 2007).

Problems like rippling, bottoming out, implant malposition and symmastia due to insufficient capsular support can occur (Baxter 2003).

The third method for coverage consists of elevating the pectoralis major muscle for coverage of the superior aspect of the implant, while the remainder of the implant is covered by acellular cadaveric dermis (Bindingavele et al. 2007).

The use of the acellular cadaveric dermis will lower the chance of lateral, inferior or posterior migration of the implant.

Also a single-stage procedure without the use of tissue expanders is possible (Salzberg 2006).

This overview will discuss different currently available acellular dermal products in the use for breast reconstruction. The safety and efficacy of this technique have been widely reported. A recent 12-year series has demonstrated the excellent and safe outcomes achievable even in the setting of infection and previously irradiated breasts (Sbitany and Serletti 2011).

Natural biological materials such as human cadaver or porcine skin can be used as dermal substitutes (Gamboa-Bobadilla 2006). They provide a structurally intact 3D extracellular matrix of collagen and elastin. Adherence, outgrowth and differentiation of keratinocytes rely on the presence of basement membrane proteins, especially laminin and collagen IV (Moiemen et al. 2001, van der Veen et al. 2011). Thus the main advantage of biological materials is that it is highly similar to native dermis and that parts of the basement membrane may be conserved (van der Veen et al. 2009).

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## 12.2 Xenograft

Xenografts are tissues from one species used as a graft on another species. Porcine products are the most commonly used xenograft in today's market.

### 12.2.1 Permacol™<sup>a</sup>

Tissue Science Laboratories, Andover, MA, USA

Thickness 0.4 or 1.5 mm (van der Veen et al. 2009)

It is derived from porcine dermis that has undergone a proprietary manufacturing process to create an acellular collagen matrix. At the moment there are no clinical studies available (van der Veen et al. 2009).

### 12.2.2 Strattice<sup>TMa</sup>

Life Cell, Branchburg, NJ, USA

Acellular porcine dermis ( $\alpha$ -Gal removed)

Thickness 1.5–2.0 mm (van der Veen et al. 2009)

Strattice<sup>TMa</sup> reconstruction is a new technique of implant-based reconstruction which allows the use of a permanent implant to fill the breast envelope in one operation avoiding the need for tissue expansion. It works especially well for A, B or small C cup breast.

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## 12.3 Allograft

For breast reconstruction also acellular dermal allografts are used, that consist of de-epithelialised/de-epidermised (DED) taken from human cadaveric donors (Zienowicz and Karacaoglu 2007). The grafts are cryopreserved, lyophilized and glycerolized in preparation to remove donor cellular, infectious and antigenic materials. This structure serves as a template for ingrowth of host fibroblasts and capillaries until it is replaced by host tissue. The dermis is available in varying thicknesses (Shores et al. 2007).

### 12.3.1 AlloDerm<sup>®</sup>

Life Cell, Branchburg, NJ, USA

Thickness 0.79–2.03 and 2.06–3.30 mm (van der Veen et al. 2009)

It is one of the oldest and most used commercially available acellular human dermis. There is a wide range of varied applications such as coverage of implanted prostheses and coverage of partial and full thickness (burn) wounds (Parikh et al. 2006). It has also been used in breast implant revisions as a form of capsule reinforcement (Becker et al. 2009, Haddock and Levine 2010). Donor skin is treated with NaCl-SDS, which results in the retention of the basement membrane and in good immunogenic properties. The use of AlloDerm<sup>®</sup> results in the complete incorporation into the wound site without rejection (van der Veen et al. 2011). AlloDerm<sup>®</sup> requires no special refrigeration or freezing for storage and has a shelf life of 2 years. The grafts are prepared by hydration in saline baths according to the manufacturer's instructions. The dermal side of the graft (the less shiny surface) is orientated toward the capsule surface and is placed in an onlay fashion (Baxter 2003).

### 12.3.2 NeoForm®

Mentor Corporation, Santa Barbara, CA, USA

It is a human cadaveric dermal product that is solvent dehydrated and gamma irradiated in its manufacturing process. Its main indication is to provide soft tissue coverage for implanted breast prosthesis in breast reconstruction following mastectomy.

### 12.3.3 DermaMatrix Acellular Matrix

Synthes, Inc., West Chester, PA, USA

Thickness 0.2–0.4, 0.4–0.8, and 0.8–1.7, 1.7 > mm (van der Veen et al. 2009)

It is another acellular allogenic dermal product which uses the same raw cadaveric dermal material but is processed using a combination of detergent and acid washes and is then freeze-dried and packaged terminally sterile. Its uses include breast reconstruction. But there are – at the moment – no published studies that support the efficacy.

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## 12.4 Surgical Technique

Preoperatively the inframammary fold, breast meridian and breast perimeter is marked.

After a skin-sparing mastectomy performed by the surgical oncologist, the pectoralis major muscle is elevated from lateral to medial. At the inferomedial border, the muscle fibres are disinserted. Depending on the remaining skin envelope and the size and wishes of the patient's desire regarding postoperative size, an appropriate acellular dermal matrix is used. It is sutured to the defined inframammary fold region, to the elevated pectoralis major muscle and medially to the chest wall and rectus abdominis fascia (Haddock and Levine 2010).

Sutures are placed around the periphery of the dermal matrix, typically 1 cm apart. Irrigation with a isotonic saline solution is then performed, followed by implant insertion.

A closed drainage system is placed along the lateral and inferior aspects, and the skin envelope is closed in a multilayer fashion (Haddock and Levine 2010). If an expander is used, the expansion period is approximately 10 months with 3–4 expansions (Haddock and Levine 2010) (Figs. 12.1, 12.2, 12.3, 12.4, 12.5, and 12.6).

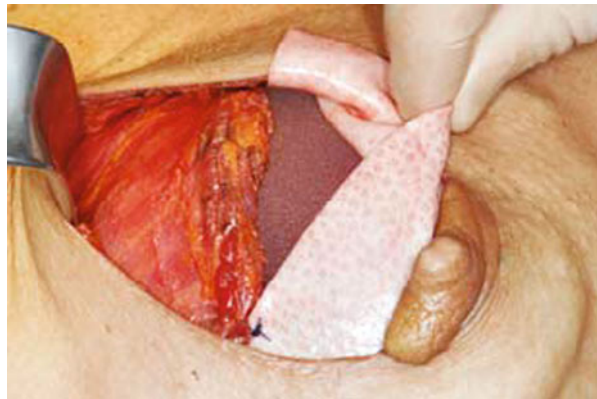
Chemotherapy can be administered during the expansion phase. Radiation treatment is commenced 1 month after exchange of the expander for a permanent implant (Preminger et al. 2008).

Postoperatively, patients are discharged from the hospital within 3 days after removal of the drain. They are instructed to return to clinic in 10 days for removal of the stitches. Oral antibiotics are given for 7 to 10 days. Shoulder abduction is limited to <90° for 1 week.

**Fig. 12.1** Fixation of biomesch (Strattice™) in the submammary fold



**Fig. 12.2** Placement of a silicone breast implant under the pectoralis muscle before suturing of Strattice™ with the muscle

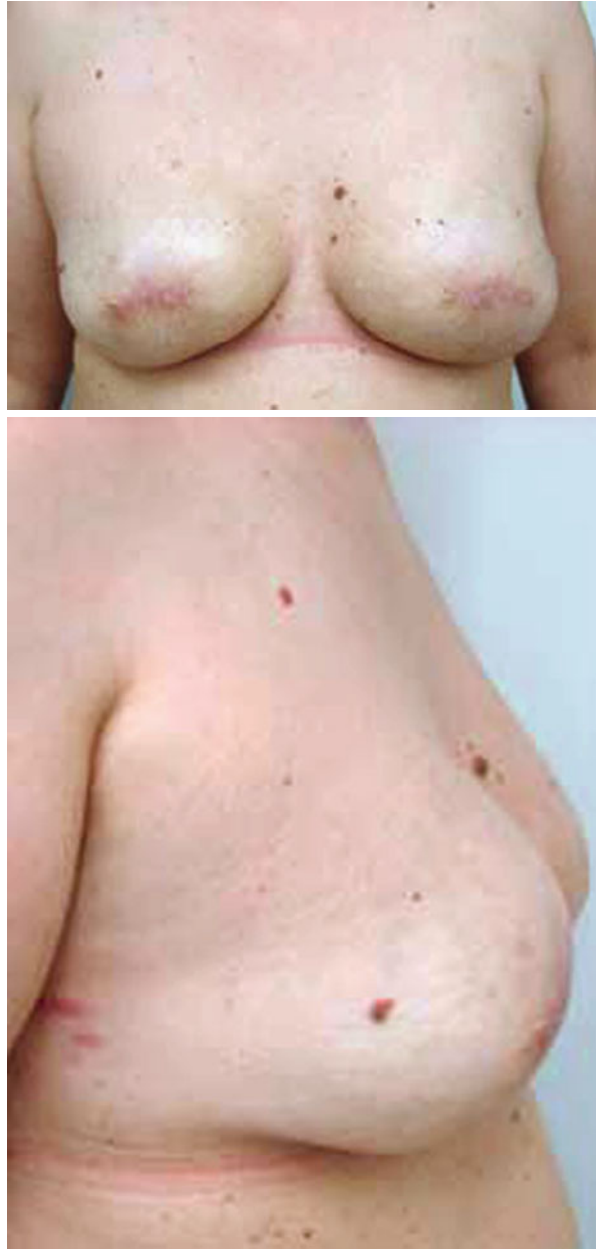


**Fig. 12.3** Fixation of the Strattice™ to the pectoralis muscle with PDS 3/0





**Figs. 12.4, 12.5, and 12.6** Postoperative result after bilateral skin-sparing mastectomy and reconstruction with silicone breast implants and biomesch (Strattice<sup>TMa</sup>)



## 12.5 Discussion

In general, dermal substitutes provide reconstructive solutions because they require less vascularised wound bed, increase the dermal component of the healing wound, reduce the inflammatory response and provide rapid and safe coverage (Shores et al. 2007).

**Figs. 12.4, 12.5, and 12.6** (continued)



The important characteristics of the ideal dermal substitute are as follows (Shores et al. 2007):

- Able to resist infection
- Able to withstand wound hypoxia
- Cost-efficient
- Easy to prepare – to store – to use
- Flexible in thickness
- Lack of antigenicity
- Offers long-term wound stability
- Provides permanent wound coverage
- Recreates dermal components
- Able to resist shear forces
- Widely available

The use of acellular dermal matrix in breast reconstruction is a technique gaining increased acceptance among reconstructive breast surgeons. The clinical benefits of this technique are multiple: (1) an increased ability for the surgeon to define placement of both the inframammary fold and expander/implant position, (2) an increased layer of protection between the prosthetic implant and potentially devascularised mastectomy skin, (3) a large initial submuscular pocket leading to improved use of

native mastectomy flaps and (4) more rapid expansion and less time to complete reconstruction. In addition it helps to reduce the formation of breast capsule (Rnjak et al. 2011, Sbitany and Serletti 2011).

This biological scaffold becomes due to recellularisation and revascularisation integrated with the surrounding tissue while resisting erosion and extrusion (Bindingavele et al. 2007). Furthermore, it has been shown to have remarkably low rates of rejection, absorption and scar formation (Bindingavele et al. 2007).

The efficacy and safety of procedures using acellular dermal matrix are in large part equivalent to those using full submuscular coverage as Sbitany showed (Sbitany and Serletti 2011). Despite the higher incidence of seroma in the acellular dermal matrix cohort, the rate of infection requiring explanation was found to be comparable between the two groups (Sbitany and Serletti 2011).

Other minor complications have occurred such as hematoma and cellulites (Preminger et al. 2008).

Two-stage expander/implant breast reconstruction remains the most commonly practised method for postmastectomy reconstruction (Sbitany and Serletti 2011).

Although a single-stage method is certainly one approach to breast reconstruction, a tissue expansion is still necessary if there is a deficiency of the soft tissue envelope (Bindingavele et al. 2007).

The use of acellular dermal matrix has multiple advantages and minimal complications, and allows for a pleasing cosmetic outcome (Bindingavele et al. 2007).

Using acellular dermal matrix sling helped to prevent from wrinkling, scalloping, stark contours and visible implant folds and is strong enough to prevent from bottoming out (Haddock and Levine 2010).

The combination of dermal substitute with pectoralis major muscle for breast implant reconstruction is an excellent addition to the reconstructive surgeon's armamentarium (Haddock and Levine 2010)

Another important point is that patient satisfaction with this technique seems to be improved because the result is immediately apparent (Haddock and Levine 2010).

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B. De Angelis, L. Brinci, D. Spallone, L. Palla,  
L. Lucarini, and V. Cervelli

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## 13.1 Introduction

A wound is a breach of the skin that can lead to infection and sepsis. Fast wound closure and minimal scar contracture must be taken into account as primary end points. Wound healing is an evolutionarily conserved complex multicellular process that aims to restore a skin barrier and consists of four stages:

- Coagulation
- Inflammation
- Cell proliferation and repair of the matrix
- Epithelialisation and remodelling of the scar tissue

These stages overlap and the entire process can last for several weeks and months. This process involves coordinated efforts of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. This complex process is executed and regulated by an equally complex signalling network involving numerous growth factors, cytokines and chemokines (Myers et al. 1995; Van Zuijlen et al. 2000). Schultz et al. (2003) demonstrated that acute wound fluid contains factors that induce cell proliferation such as platelet-derived growth factor-like peptides, interleukin-6 (IL-6), TGF- $\alpha$  and TGF- $\beta$  in contrast to chronic wound fluid, which contains lower amounts of these growth-promoting cytokines. The migration, infiltration, proliferation and differentiation of these cells will culminate in an inflammatory response, a new tissue growth and ultimately in wound closure. With this understanding of the wound healing process, principles of acute wound management were established.

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B. De Angelis, MD • L. Brinci, MD (✉) • D. Spallone, MD  
L. Palla, MD • L. Lucarini, MD • V. Cervelli, MD  
Department of Plastic and Reconstructive Surgery,  
University of Rome "Tor Vergata",  
Via Casilina 1049, Rome 00169, Italy  
e-mail: lorenzobrincimd@gmail.com

The treatment of full-thickness posttraumatic wounds is often a multidisciplinary approach: orthopaedic, microvascular and finally plastic surgery. In recent years, there have been many exciting developments in products designed to assist wound healing, such as tissue engineering and the use of dermal substitute. Debridement and appropriate dressings are often used to accelerate healing. The plastic surgery approach for coverage of these wounds is the use of autologous skin grafts, skin substitutes and free flaps. However, poor skin quality and scar contracture occur frequently and are well-known problems in split grafted areas. Yannas and Burke (1980) showed that the use of dermal substitutes are an appropriate way to minimise scar contraction and to optimise the quality of the grafted area in strained regions with loss of function and with high requirements of elasticity, pliability and stability. A cell-based wound coverage with keratinocytes and fibroblasts on the basis of a commercially available dermal substitute (MatriDerm<sup>®</sup>, Kollagen/Elastin Matrix) was generated in order to treat wide wounds as was showed recently (Golinski et al. 2009). This scaffold has already proven to be suitable for a single-stage grafting procedure as was demonstrated clearly (Haslik et al. 2007) without the disadvantage of skin grafting rejection.

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## 13.2 Material and Methods

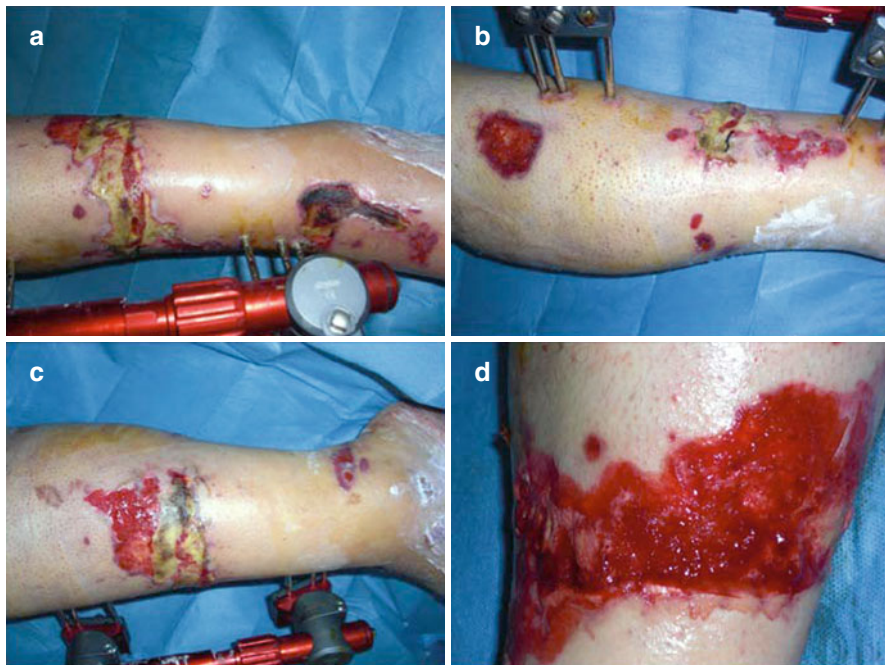
The authors have treated 60 patients with partial and full-thickness posttraumatic skin defects (Cervelli et al. 2011), and its use was previously reported by these authors in over 35 patients with diabetic ulcers (Cervelli et al. 2010). A case report of skin necrosis of scrotum due to endovascular embolisation was also published (Cervelli et al. 2012). All the posttraumatic wounds were localised on the inferior limbs (Fig. 13.1). Exclusion criteria were arterial, venous or diabetic wounds. In 12 patients, there was a tendon exposure, in six also a bone exposure. In this study, we treated 30 patients with MatriDerm<sup>®</sup> and autologous skin grafting and 30 patients with autologous skin grafting alone. The two groups were homogeneous, because the patients were randomly allocated in the treatment or control group.

Before deciding on local wound applications, it is vital to consider the possible causes of a nonhealing wound and to review and correct, if possible, patient factors that may impede healing:

- Assess and correct causes of tissue damage.
- Tissue perfusion: ensure adequate blood supply.
- Assess and monitor wound history and characteristics.

### 13.2.1 Patient Evaluation

The first step is treatment of causes and patient-centred concerns. The overall health status of a patient has a significant impact on the wound-healing process. A general medical history, including a medication record, is invaluable in identifying causes that may prevent wound healing. Systemic steroids, immunosuppressive drugs, and nonsteroidal anti-inflammatories will deter wound healing, as will rheumatoid arthritis and other autoimmune diseases such as systemic lupus, uncontrolled vasculitis or



**Fig. 13.1** Preoperative view of lower limb wound at 30 days after trauma. (a) Anterior view. (b) Lateral view. (c) Medial view. (d) Specific area of the wound

pyoderma gangrenosum. Inadequate or poor nutrition will delay healing, particularly if the patient's protein intake is low. Each patient has been examined considering the efficiency of main organs (heart, liver, kidney and lungs) and possible concomitant pathology. Our protocol was based on clinical evaluation, wound examination, swab culture, instrumental examination of the lower limbs and photographs. A laser Doppler perfusion imaging is a noninvasive method for investigating skin microvasculature. A two-dimensional flow map of specific tissues and visualisation of the spatial variation of perfusion can be created with this technique. Photographs were taken with a follow-up time at 0, 1, 2, 3 weeks and 1, 2, 6 months and 1 year.

### 13.2.2 Assess Wound History and Characteristics

If the wound is recurrent, patient education or treatment of an underlying condition may be the critical step in bringing about wound healing. The size, depth and colour of the wound base (black, yellow, red) should be recorded to provide a baseline against which healing can be assessed. The amount and type of exudate (serous, sanguous, pustular) should also be assessed: a heavy exudate may indicate uncontrolled oedema or may be an early sign of infection. The wound margin and surrounding skin should be checked for callus formation, maceration, oedema or erythema, and the causes corrected. While pain can be experienced during debridement or dressing

**Fig. 13.2** Intraoperative view. Application of MatriDerm® on wound bed



changes, continuous pain may be due to an underlying cause, local wound irritation or infection. It is important to assess continuous pain to determine whether its origin is in the wound or in the surrounding anatomical region.

In this study, the wound examination was based on wound area (cm<sup>3</sup>), bed, margins and surrounding skin. Swab culture has been performed to evaluate any microbiological infections and to find the appropriate antibiotic therapy. All procedures were performed in complete asepsis with epidural and/or loco-regional anaesthesia.

### 13.2.3 The Surgical Steps

The surgical step included debridement of damaged areas and the dermal substitute application in combination with a split-thickness autograft in one-step procedure (Fig. 13.2). This treatment was compared with the conventional treatment: a split-thickness autograft.

### 13.2.4 Wound Bed Preparation

Wound bed preparation is the management of the wound to accelerate endogenous healing or to facilitate the effectiveness of other therapeutic measures. Local management of a nonhealing wound involves:

- An ongoing debridement phase
- Management of exudate
- Resolution of bacterial imbalance



Wound healing can only take place if there is adequate tissue oxygenation. A well-vascularised wound bed provides nutrients and oxygen to sustain newly formed granulation tissue and maintain an active immunological response to microbial invasion. External factors such as hypothermia, stress or pain can all increase sympathetic tone and decrease tissue perfusion; smoking reduces microcirculatory flow, while certain medications increase it.

The optimal preparation of the wound bed requires complete debridement of devitalised tissue, bacterial balance and moisture balance. Wound bed preparation focuses on all of the critical components, including debridement, bacterial balance and management of exudate. The overall health status of the patient and how this may impinge upon the wound-healing process must be taken into account as was showed previously (Schultz et al. 2003). The ultimate aim is to ensure formation of good-quality granulation tissue leading to complete wound closure, either naturally or through skin products or grafting procedures. Debridement is widely used to clear wounds of necrotic tissue and bacteria to leave a clean surface that will heal relatively easily. Devitalised, necrotic tissue provides a focus for infection, prolongs the inflammatory phase, mechanically obstructs contraction and impedes re-epithelialisation as was illustrated previously (Baharestani 1999). Steed et al. (1996) showed that the debridement using surgical, enzymatic, autolytic or mechanical methods is often all that is required to promote the first step in the healing process. Although debridement occurs naturally, assisted debridement accelerates the wound-healing process.

### 13.2.5 MatriDerm®

MatriDerm® is a single-use three-dimensional matrix composed of native structurally intact collagen fibrils and elastin for supporting dermal regeneration. The collagen is obtained from bovine dermis and contains the dermal collagen types I, III and V. The elastin is obtained from bovine nuchal ligament by hydrolysis. MatriDerm® serves as a scaffold in the skin reconstitution and modulates scar tissue formation. Moreover, MatriDerm® has an excellent haemostatic property and thus reduces the risk of split-skin sub graft haematoma. The non-use of chemical cross-linking of the collagen results in a matrix, which is especially biocompatible as was showed previously (Bayat et al. 2003). MatriDerm®, applied using a single stage, is immediately covered with split skin through the 1 mm thick matrix by diffusion. MatriDerm® is supplied in sterile double-bagged packs, and these may only be opened under sterile conditions. Before the use, MatriDerm® must be rehydrated in ample physiological saline solution, and to avoid trapped pockets of air (air pockets can hinder the diffusion and thus jeopardise the attached graft), MatriDerm® should be laid on the surface of the water and not immerse. The matrix is ready for use as soon as the appearance of the entire surface has changed from white to translucent as was showed clearly (Bayat et al. 2003).

### 13.2.6 Skin Grafting

Classic skin grafting was performed with a dermatome using a thin split-thickness depth, meshing all grafts (1:2 ratio), and it was fixed to the wounds by 3/0 nylon sutures. A moulage compressive dressing with hyaluronic acid gauze was used to cover the surgical wound.

Postoperative follow-up consisted of four visits during the first month – one for each week – and two additional visits at the third and sixth month. During this visits, to test the effectiveness of the dermal substitute and to compare it with the current gold standard, skin grafting, we evaluated as primary end points of the study the (1) time for complete epithelisation (both treated area and biopsy site) and (2) aesthetic and functional quality of the epithelisation (colour, joint contractures). The aesthetic appearance of the epithelisation was evaluated with the help of one plastic surgeon unaware of the procedure (A.A.), according to the Manchester Scar Scale. MSS was used to assess the colour of the scars defined in perfect, slight, obvious or gross mismatch compared with the surrounding skin as was demonstrated previously (Roques and Teot 2007); the appearance of the skin in matte or shiny; the contours from flush with surrounding skin, slightly proud/indented, hypertrophic to keloid; the texture from normal, just palpable, firm to hard; the margins distinct or not. We did not include the items associated with the size and number of the scars because the wounds were all single and from 2 to 10 cm. We did not associate any other information in the Manchester scar proforma like race or ethnic background cause and symptoms of the wounds cause the groups of patients was homogeneous for these characteristics.

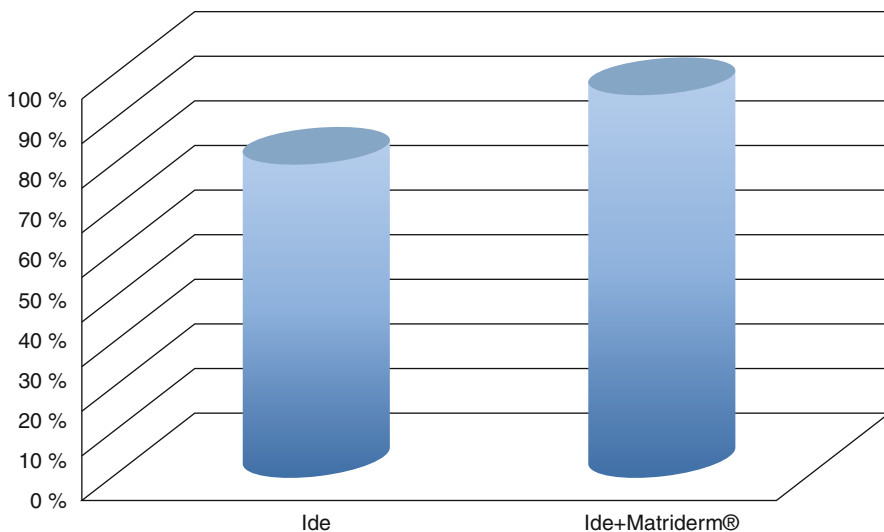
### 13.2.7 Evaluation Criteria

After 1 week of the operation, the percentage of autograft survival was observed. The patient's active and passive range of motion in a single plane was evaluated with the help of a physical therapist at 1 and 6 months from surgery. Secondary end points were the assessment of infections, inflammations or any adverse effects of the procedure, particular medications assumed and postoperative pain (evaluated with the visual-analogue scale, VAS). Mann–Whitney U test was performed for the five items of the MSS combined to the results of patient's self-estimation scale and for the re-epithelialisation percentage to test the significance between the two groups.

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## 13.3 Results

The percentage of re-epithelialisation was significantly different after 3 months from the treatment between the two groups ( $p < 0.001$ ), as clearly shown in Fig. 13.3.



**Fig. 13.3** Mean and standard deviation of the re-epithelialisation percentage for the two groups

**Table 13.1** Manchester Scar Scale Assessment combined with patient’s self-estimation

Manchester Scar Scale	Mean ± SD	Skin graft	Skin graft+ MatriDerm®	<i>p</i>
Self-estimation	4.7 ± 4.3	1.5 ± 0.5	1.5 ± 0.5	0.032
Colour	2.8 ± 1.2	1.2 ± 0.8	1.2 ± 0.8	<0.001
Contour	1.9 ± 0.8	1.1 ± 0.5	1.1 ± 0.5	0.005
Texture	3.0 ± 1.5	1.6 ± 1.2	1.6 ± 1.2	0.012
Appearance	1.8 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	<0.001
Margins	1.3 ± 0.5	1.0 ± 0.0	1.0 ± 0.0	0.038

Mean and standard deviation were reported for the two treatments, and a Mann–Whitney U test was performed for each item of the Scale to test the significance of the differences (*p*)

After the first treatment, we observed that the mean re-epithelialisation time is 15 days.

In 13 patients treated with MatriDerm® and skin grafting complete re-epithelialisation and in 17 patients epithelialisation at 80–90 % was observed. Minimal or absence of skin grafting contracture was observed in these patients.

The percentage of living tissue was evaluated in 95 % of wounds in patients treated with MatriDerm® and skin grafting, whereas it was almost 75–80 % in wounds treated with skin grafting alone. In almost all these patients treated with skin grafting alone, we observed skin grafting contracture.

All the scores associated to the items of MSS resulted significantly different between the two groups, especially colour, appearance and contour, as shown in Table 13.1.



**Fig. 13.4** Postoperative view at 30 days of follow-up. (a) Anterior view. (b) Lateral view. (c) Medial view. (d) Specific area of the wound

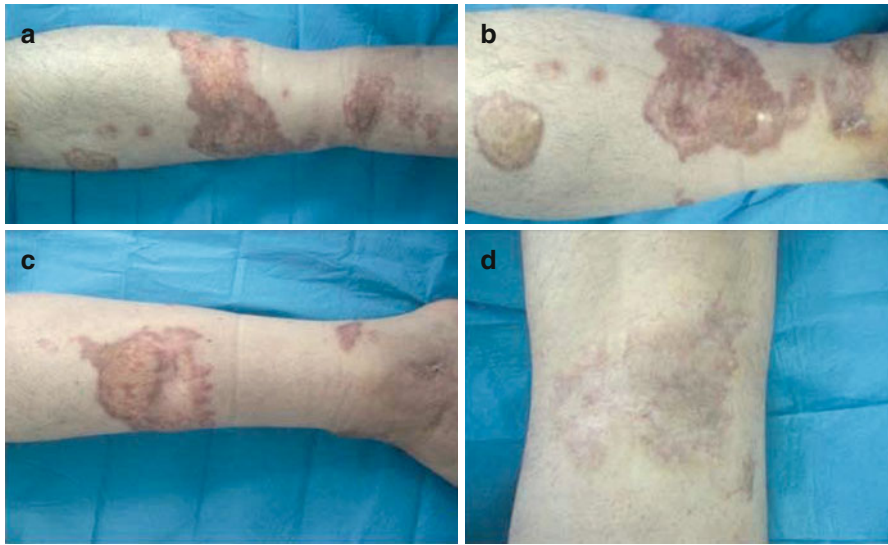
Almost all areas treated with skin graft appeared gross mismatch or obvious mismatch in colour, slightly proud/indented or hypertrophic in contour and shiny in appearance. Wounds treated with MatriDerm® had a slightly mismatch, flush with surrounding skin or slightly proud/indented contour and matte appearance. None of the 60 patients reported intraoperative or postoperative adverse effects.

The self-estimation of the patients resulted highly variable in the group treated only with skin grafting; however, also this item leads to a significant difference between the two group scores.

Dermal matrix and skin grafting integrated successfully in most of the treated sites. The resultant bio-integration enabled staged, definitive and durable soft tissue coverage. Results with MatriDerm® are presented in Figs. 13.4, 13.5 and 13.6.

## 13.4 Discussion

Wound healing is an evolutionarily conserved complex multicellular process that aims restoring skin barrier. This process involves coordinated efforts of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. The migration, infiltration, proliferation and differentiation of these cells will culminate in an inflammatory response, formation of new tissue and ultimately



**Fig. 13.5** Postoperative view at 1 year of follow-up. (a) Anterior view. (b) Lateral view. (c) Medial view. (d) Specific area of the wound



**Fig. 13.6** Posttraumatic lower limb wound. (a) Time 0. (b) 7 days of follow-up. (c) 30 days of follow-up

wound closure. Myers et al. (1995) and Van Zuijlen et al. (2000) demonstrated that this complex process is executed and regulated by an equally complex signalling network involving numerous growth factors, cytokines and chemokines.

Van Zuijlen et al. (2000) described the use of a collagen-elastin matrix for the treatment of burn wounds in a one-stage grafting model that is a highly porous, 1 mm thick membrane fully biodegradable three-dimensional composed of native bovine collagen fibre I, II and V and coated with elastin hydrolysate derived from bovine ligamentum nuchae in a concentration of three weight-to-weight ratios; the matrices were treated with gamma irradiation (1,000 Gy) and stored at room temperature and have been available in Europe since 2004 (MatriDerm<sup>®</sup>; Dr. Suwelack, Skin and Health Care AG, Billerbeck, Germany). In the literature were reported different dermal substitutes. Sinha et al. (2002) used acellular human dermal matrix (AlloDerm<sup>®</sup>), and Wax et al. (2002) used an allogenic dermis to cover the donor-site defect with prolonged healing period, ranging 8–12 weeks and 12–16 weeks, respectively. Lee et al. (2005) used successfully artificial dermis (Terudermis<sup>®</sup>) and secondary split-thickness skin graft in 13 patients, reporting minimal partial loss of the artificial dermis in two patients. Abai et al. (2004) used the Integra<sup>®</sup> for the reconstruction of defects created by excision of giant hairy nevi. This dermal substitute (Integra<sup>®</sup> LifeSciences, Plainsboro, NJ) is a bilayer membrane system for skin replacement. The dermal layer provides a matrix for migration and growth of fibroblasts and other cells involved in wound healing; the temporary epidermal layer is made of silicone.

The aim of our study was the comparison between the traditional skin autologous graft combined to dermal matrix and skin autologous graft procedure to obtain the restoration and regeneration of posttraumatic wounds.

The split-thickness autograft is used for large ulcers under general, spinal or extensive local anaesthesia in large leg ulcers; graft failure may occur because of build-up of exudates underneath the graft. Those grafts are usually successful, but on the other hand, they may contract after harvesting and they need a large donor site. In most cases, the donor site may heal slowly and cause a lot of pain. In each patient, MatriDerm<sup>®</sup> showed positive effects into accelerating the improvement of the quality and the functionality of skin reconstruction.

Dermal substitutes may act as a barrier for vascular ingrowths and hamper diffusion of nutrients by increasing the distance between wound bed and the graft; for this reason, it was postulated that survival of the overlying skin graft could be at risk after dermal substitution. Furthermore, the skin elasticity parameters of wound area treated with MatriDerm<sup>®</sup> were significantly improved in less time when sheet autografts were used as shown previously (Ryssel et al. 2008).

## Conclusions

Our study demonstrates the role of MatriDerm<sup>®</sup> and skin autologous graft in tissue regeneration and wound closure with a significant healing time reduction with faster mean re-epithelialisation time at 15 days, with differences still present after 3 months. Basing on our clinical practice, we consider MatriDerm<sup>®</sup> and skin autologous graft the key element to improve functional and aesthetic outcomes. This association guarantees a temporary barrier with multiple functions: haemostatic, reduction of contracture wound, infection, maintenance of skin elasticity and dermal architecture, and better appearance of the scar. MatriDerm<sup>®</sup>

can also be used to cover tendons and bone. Furthermore, the minimally invasive technique is well accepted by patients with a noteworthy improvement of quality of life along with cost reduction due to the fewer number of medications. In conclusion, our results show that the integration of different disciplines such as cell therapy, bioengineering and biomaterials sciences is an effective support to the surgical procedure.

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Wiltrud Meyer

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## 14.1 Implication of Subdermal Soft Tissue on Function and Three-Dimensional Form of the Integument

The integrity of the skin is a condition precedent for health. Any lesion of the integument, caused by trauma or pathologic disorder, compromises the well-being of the individual and grows fatal when large areas of skin are involved.

In recent years, numerous surgical wound closure procedures have been developed or improved. Recent plastic and reconstructive procedures include local and microsurgical free flap techniques, sophisticated skin grafting procedures with support of transient or permanent dermal substitutes, as well as optimized wound dressing and after treatment regimen. Application of these current therapeutic options facilitates restoration of the integument even in large-area or complex defects and has substantially improved functional and aesthetic postoperative outcome.

In spite of these considerable achievements, the posttraumatic loss of soft tissue and development of poor scar tissue in the subdermal layer is still a frequent observation, stigmatizing many of the affected patients for a lifetime.

Subdermal soft tissue provides the interface between skin and underlying anatomic structures, which is paramount to the mobility and pliability of the skin. Furthermore, the soft tissue padding of musculoskeletal structures represents a major parameter forming the three-dimensional appearance of the body silhouette.

Posttraumatic loss of subdermal soft tissue leads to adhesions, restricted movability and contour defects. Thus, functional and aesthetic properties of the integument are critically affected.

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W. Meyer, MD  
Clinic for Plastic, Reconstructive and Aesthetic Surgery,  
Stiftungsklinikum St. Martin,  
Johannes-Mueller-Str. 7, Koblenz 56068, Germany  
e-mail: wiltrud\_meyer@web.de

Loss of subdermal soft tissue is also a common characteristic of degenerative or age-related changes, which leads to contour defects and enhanced visibility of bony structures particularly in the face.

Consequently, successful restoration of the subdermal soft tissue interface should reduce posttraumatic adhesions as well as contour defects.

With the advent of commercially available biologic scaffold materials, the reconstruction of soft tissue emerged from laboratory and tissue engineering procedures and has reached clinical everyday routine (Badylak 2007).

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## 14.2 Biomatrices for Soft Tissue Restoration

### 14.2.1 Development of Scaffolding Materials

The visionary idea of rebuilding lost or damaged human tissue and even organs was the mainspring for the development of tissue engineering as a new discipline back in the 1980s.

WT Green, a pediatric orthopedic surgeon and a pioneer in this emerging discipline, seeded chondrocytes onto spicules of bone to generate cartilage tissue. After a series of unsuccessful efforts, he realized that only with the availability of an appropriate biologic scaffold the replacement of functional tissue and organs would be possible (Green 1977).

His prevision was proven true in the following years, when Langer, Burke, and Vacanti, among many others, investigated various scaffolding materials for their potential to support tissue regeneration (Yannas and Burke 1980; Langer and Vacanti 1993; Freed et al. 1994; Stahl et al. 2007).

Research focused on biodegradable scaffolds with a three-dimensional (3D) porous structure to support cell attachment and provide guidance for cell proliferation and differentiation (Zeltinger et al. 2001; Badylak 2007; Malafaya et al. 2007; Lawrence and Madihally 2008).

### 14.2.2 Properties of 3D Biologic Scaffolds

Scaffolding devices for tissue culture or clinical applications are based on polymers. The scaffold is either composed of isolated synthetic polymers (Hutmacher et al. 2001; Lavik et al. 2002; Pattison et al. 2005) or processed from natural polymers (Spilker et al. 2001; Taylor et al. 2002; Yang et al. 2002; Huang et al. 2005). A third group of scaffolds is derived from natural extracellular matrix (ECM) of human or animal origin (Badylak 2007).

3D scaffolding materials generated from isolated natural or synthetic polymers have the advantage to be manufactured in a highly reproducible way and with physical properties adjusted to the required settings. However, they do not provide the bioactive structures that are found in scaffolds processed from natural ECM (pECM scaffolds).

The extracellular matrix represents the native scaffolding material of any tissue and organ and is “by definition nature’s ideal biologic scaffold material” (Booth et al. 2002; Badylak 2007). In mammalian tissues, the ECM is characterized by a complex 3D composition of structural and nonstructural molecules, including collagen as a main structure, glycosaminoglycans (GAGs) such as hyaluronic acid, and glycoproteins like fibronectin. The ECM is in a state of dynamic equilibrium as it is manufactured by the resident organ-specific cells and similarly guides morphogenesis of the organ (Kleinman et al. 2003). The molecular structure of the ECM activates adjacent cells to migrate and differentiate, allows for the diffusion of nutrients, and supports ingrowth of blood vessels, nerves, and lymphatics (Rosso et al. 2004).

### 14.2.3 Manufacturing Process of pECM Scaffolds

The pECM scaffolds for tissue restoration can be harvested from allogeneic and xenogeneic tissues such as heart valves (Booth et al. 2002; Grauss et al. 2005), small intestinal submucosa (SIS) (Abraham et al. 2000), or skin (Chen et al. 2004).

The specific structure and composition of the pECM molecules destine host immune response and downstream degradation of the device (Record et al. 2001; Gilbert et al. 2007; Badylak and Gilbert 2008).

Altering the tissue-specific properties of the harvested ECM graft during the manufacturing process thus decisively affects clinical outcome as well as quality of regenerated tissue (Badylak et al. 2009).

Decellularization of the harvested xenogeneic or allogeneic material is an essential step in the manufacturing process, since antigenic epitopes, in particular the GAL epitope, associated with cell membranes or intracellular components may cause adverse immunologic or inflammatory host responses (Gilbert et al. 2006).

Cross-linking the structural molecules of the pECM is a convenient method to increase its mechanical strength and to slow down degradation. However, cross-linking might change the host tissue response from an anti-inflammatory constructive remodeling response to a proinflammatory, foreign body response after implantation (Badylak and Gilbert 2008; Badylak et al. 2008).

Lyophilization or freeze-drying is a further step during the manufacturing process to facilitate handling and storage of the devices (Hafeez et al. 2005; Freytes et al. 2008a).

At the end of the manufacturing process, the scaffold is sterilized, e.g., via gamma irradiation (Gouk et al. 2008).

All of these manufacturing steps might considerably alter the bioactive properties of the ECM-derived material.

Nevertheless, the molecular structure and composition of manufactured ECM-derived devices and the structure/function relationship after scaffold implantation have not yet been comprehensively investigated. However, preserving selective mechanical and structural properties of the natural ECM during the manufacturing process seems to be of advantage. The native structure of molecules and fibrils in the pECM, such as integrins and collagens provides binding sites and guidance for host cells and facilitates constructive host tissue response (Brown et al. 2010).

#### **14.2.4 The Process of Tissue Remodeling After Scaffold Implantation**

Scaffold implantation starts a dynamic cell-matrix interaction between invading host cells and adhesion points of matrix molecules that serve as inductive structures. A highly porous structure of the pECM scaffold facilitates nutrient transport and rapid tissue ingrowth, while the scaffold material is degraded and replaced by regenerated tissue (Lawrence and Madhally 2008).

The downstream tissue remodeling process is orchestrated by the individual host immune response, which is, in turn, influenced/bestimmt by the specific tissue properties the pECM graft is composed of (Valentin et al. 2006; Badylak et al. 2009). The long-term remodeling response varies from low-grade chronic inflammation and fibrosis in pECM products that resist or retard degradation, to the formation of organized, site-appropriate tissue in rapidly degrading devices.

Degradation of the pECM scaffold apparently is a precondition for successful tissue remodeling. During degradation, antimicrobial peptides as well as growth factors are released, which facilitate angiogenesis, mitogenesis, and cell differentiation (Hodde et al. 2002; Badylak et al. 2003; Mcdevitt et al. 2003; Malmsten et al. 2006). Low-molecular-weight peptides, formed during degradation, have chemoattractant potential to multipotent progenitor cells and bone marrow-derived cells that participate in the long-term remodeling process of the ECM (Li et al. 2004).

At the end of a successful remodeling process, the implanted pECM scaffold material is replaced by site-specific host ECM.

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### **14.3 Clinical Applications of pECM Scaffolds**

Although the process of tissue remodeling after scaffold implantation is not yet completely understood, the numerous successful clinical applications of ECM-derived devices are a testament for their reconstructive potential.

Currently, pECM matrices of different tissue origin and produced under various manufacturing procedures have been commercialized and are utilized in multiple surgical procedures for neurosurgical, intestinal, soft tissue, and dermal reconstruction. SIS-ECM alone has been applied in more than one million patients (Booth et al. 2002).

A partial list of commercially available ECM-derived scaffolds is shown in Table 14.1.

#### **14.3.1 Soft Tissue and Dermal Restoration with Dermis-Derived pECM**

Acellular dermis-derived matrices (ADM) of allogeneic and xenogeneic origin have been successfully used for soft tissue and dermal remodeling in different anatomic sites.

**Table 14.1** Partial list of commercially available ECM-derived scaffolds

Product	Company	Material	Chemical modification	Form	Use
Oasis®	Healthpoint	Porcine small intestinal submucosa (SIS)	Natural	Dry sheet	Partial- and full-thickness wounds, superficial and second-degree burns
Xelma™	Molnlycke	ECM protein, PGA, water		Gel	Venous leg ulcers
AlloDerm	LifeCell	Human skin	Cross-linked	Dry sheet	Abdominal wall, breast, ENT/head and neck reconstruction, grafting
CuffPatch™	Arthrotek	Porcine small intestinal submucosa (SIS)	Cross-linked	Hydrated sheet	Reinforcement of soft tissues
TissueMend®	TEI Biosciences	Fetal bovine skin	Natural	Dry sheet	Surgical repair and reinforcement of soft tissue in rotator cuff
Durepair®	TEI Biosciences	Fetal bovine skin	Natural	Dry sheet	Repair of cranial or spinal dura
XenformT™	TEI Biosciences	Fetal bovine skin	Natural	Dry sheet	Repair of colon, rectal, urethral, and vaginal prolapse, pelvic reconstruction, urethral sling
SurgiMend™	TEI Biosciences	Fetal bovine skin	Natural	Dry sheet	Surgical repair of damaged or ruptured soft tissue membranes
PriMatrix™	TEI Biosciences	Fetal bovine skin	Natural	Dry sheet	Wound management
Permacol™	Tissue Science Labs.	Porcine skin	Cross-linked	Hydrated sheet	Soft connective tissue repair
Graft Jacket®	Wright Medical Tech	Human skin	Cross-linked	Dry sheet	Foot ulcers
Surgisis®	Cook SIS	Porcine small intestinal submucosa (SIS)	Natural	Dry sheet	Soft tissue repair and reinforcement
Durasis®	Cook SIS	Porcine small intestinal submucosa (SIS)	Natural	Dry sheet	Repair dura matter

(continued)

**Table 14.1** (continued)

Product	Company	Material	Chemical modification	Form	Use
Stratasis®	Cook SIS	Porcine small intestinal submucosa (SIS)	Natural	Dry sheet	Treatment of urinary incontinence
OrthADAPT™	Pegasus Biologicals	Horse pericardium	Cross-linked		Reinforcement, repair, and reconstruction of soft tissue in orthopedics
DurADAPT™	Pegasus Biologicals	Horse pericardium	Cross-linked		Repair dura matter after craniotomy
Axis™ dermis	Mentor	Human dermis	Natural	Dry sheet	Pelvic organ prolapse
Suspend™	Mentor	Human fascia lata	Natural	Dry sheet	Urethral sling
Restore™	DePuy	Porcine small intestinal submucosa (SIS)	Natural	Sheet	Reinforcement of soft tissues
Veritas®	Synovis Surgical	Bovine pericardium		Hydrated sheet	Soft tissue repair
Dura-Guard®	Synovis Surgical	Bovine pericardium		Hydrated sheet	Spinal and cranial repair
Vascu-Guard®	Synovis Surgical	Bovine pericardium			Reconstruction of blood vessels in neck, legs, and arms
Peri-Guard®	Synovis Surgical	Bovine pericardium			Pericardial and soft tissue repair
MatriDerm®	Dr. Suwelack SHC AG	Bovine skin (collagen, elastin)	Natural	Dry sheet	Dermal regeneration, grafting in partial- and full-thickness wounds and burns
Integra®	LifeCell	Bovine skin (collagen), shark chondroitin	Cross-linked	Dry sheet	Dermal regeneration, grafting in partial- and full-thickness wounds and burns
Strattice®	LifeCell	Porcine skin	Cross-linked	Dry sheet	Hernia repair, breast reconstruction

The table exemplifies the numerous clinical applications of pECM scaffolds derived from allogeneic and xenogeneic tissues (From Badylak 2007, adapted)

Allogeneic ADM increased soft tissue padding in implant breast reconstruction (Gamboa-Bobadilla 2006) and improved soft tissue drapery around breast implants (Namnoum 2009). In further clinical applications, the dermal scaffold was utilized

**Fig. 14.1** Schematic outline of subdermal application of an ADM scaffold for soft tissue restoration. The ADM device is inserted under the widely mobilized skin in the infraorbital region to restore degenerated soft tissue and improve infraorbital contour



to provide an interface for musculofascial component separation in abdominal wall defects (Kolker et al. 2005).

In animal models, implantation of ADM induced neovascular and collagenous fiber proliferation in eyelid reconstruction (Li et al. 2007) and produced a vascularized interface in chest wall reconstruction (Holton et al. 2007). Moreover, xenogeneic ADM is commonly used as dermal substitute in reconstructive and burn surgery to improve dermal regeneration (Lamme et al. 2000; Dantzer and Braye 2001; Van Zuijlen et al. 2002; Haslik et al. 2009; Boyce et al. 2010).

### 14.3.2 New Application of a Xenogeneic ADM for Facial Contour Correction

The subdermal implantation of dermis-derived biomatrices represents a potential new application of such scaffolds. A recent clinical trial investigated the tissue remodeling effect of a dermis-derived scaffold on subdermal and dermal tissue (Meyer et al. 2009, 2010).

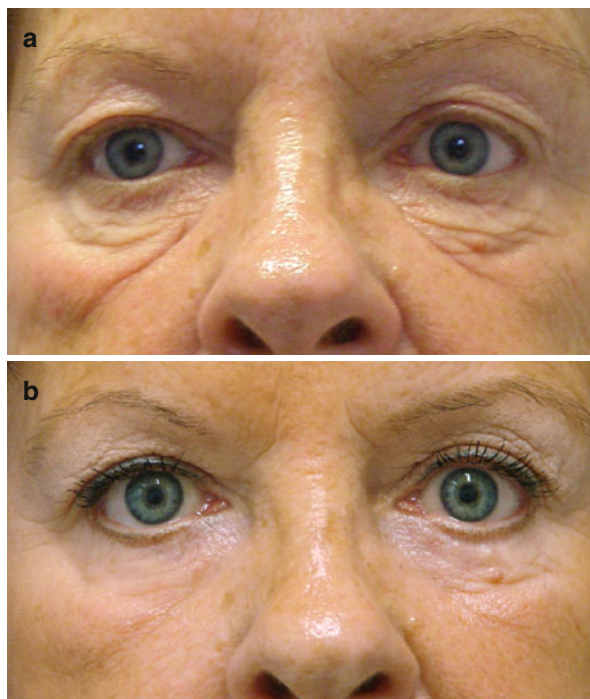
Based on previously described successful applications of dermal matrices in deeper anatomic layers and under split-skin grafts, the ECM scaffold was now implanted in the subdermal layer under a mobilized full-thickness flap of intact facial skin (Fig. 14.1).

The aim of these investigations was to characterize the potential effect of the matrix-induced soft tissue remodeling process on infraorbital facial contour and skin. Study cohort included  $n=40$  patients with degenerative defects of facial profile.

In a pertaining safety phase, clinical outcome and reactogenicity was analyzed in a test area at the upper arm, which was subjected to the same implantation procedure. In this context, the readout was extended to specific immunohistochemical and histological diagnostics and to the physical changes of dermal and subdermal tissue after scaffold implantation.

The porous scaffold used in these explorative clinical studies was a bovine dermis-derived collagen-elastin matrix. The acellular matrix mainly preserves the natural, non-cross-linked fibrillar structure of the genuine ECM during manufacturing process.

**Fig. 14.2** (a) Preoperative photograph of a 56-year-old patient with typical age-related defects of infraorbital facial contour. These changes are caused by quantitative and qualitative loss of dermal and subdermal tissue. (b) Long-term clinical result after subdermal ADM scaffold application shows increased soft tissue padding in the infraorbital region and substantially improved facial contour 12 months postoperatively



Clinical evaluation during the postoperative observation period of 13 months did not reveal allergic or adverse reactions correlating with scaffold implantation. The implanted dermis-derived matrix was well tolerated by all patients and led to complete movability of the overlaying skin flap both in the test area and in the infraorbital facial region. Subdermal matrix implantation in the infraorbital and upper mid-facial region substantially improved facial contour (Fig. 14.2a, b).

Histologic and immunohistologic analysis of biopsies taken 7 and 13 months after scaffold implantation proved the absence of chronic inflammatory or foreign body reaction. Compared to the preoperative dermal architecture, enhanced number of capillaries in the upper dermis and increased cell proliferation in the basal cell layer of the epidermis was observed in the skin area overlaying the implanted matrix.

Overall, the clinical and histological results indicate that the laminar application of a dermal ECM-derived matrix significantly influences the tissue remodeling process in the subdermal layer. Thus, subdermal matrix implantation in the infraorbital region may decrease the degenerative loss of dermal and subdermal tissue and improve tissue quality. Long-term postoperative evaluation of facial profile suggests that laminar matrix implantation bridges contour irregularities and improves soft tissue padding of bony structures.

Probably the most remarkable result of these investigations was the complete lack of postoperative adhesions or secondary contracture in the mobilized skin. Laminar



ADM implantation leads to complete mobility and natural pliability of the overlying dermal flap, even in the extremely delicate infraorbital skin. Moreover, components of the ADM support capillarisation during degradation and potentially enhance viability of the mobilized skin flap (Daamen et al. 2008).

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## 14.4 The Future of Subdermal Tissue Regeneration

### 14.4.1 Biologic Scaffolds and Stem Cells

The ongoing experimental and clinical research substantiates the obvious potential of biomatrices to specifically influence cell proliferation and differentiation (Guillot et al. 2007; Lawrence and Madihally 2008).

Experiences from the above-mentioned clinical trial suggest the subdermal application of ADM as an interface to treat or prevent postoperative adhesion and contracture in reconstructive surgery of the integument. Moreover, ADM-assisted soft tissue padding of musculoskeletal structures could improve the aesthetic 3D silhouette of posttraumatic or degenerative defects of the body surface.

The processing of ECM-derived scaffolds to optimize mechanical and bioactive properties for site-specific tissue restoration leads to clinical applications as scaffolding material for chondrogenesis (Shainer et al. 2010; He and Pei 2011) and the creation of functional mucosal and dermal substitutes (Xiao et al. 2008; Golinski et al. 2009; Zhang et al. 2009).

Further interests are targeted on the refinement of bioactive signalling (Vandevondele et al. 2003; Vogel and Baneyx 2003) or on adding mechanical strength to the pECM devices by combining the biologic materials with appropriate synthetic polymers (Ho et al. 2004; Lee et al. 2004; Stankus et al. 2008).

Recently, the growing clinical experience and easy accessibility of adipose tissue-derived stem cells (ASCs) opens up a new field of combining pECM scaffolds and ASCs for soft tissue regeneration (Flynn et al. 2008, 2009).

Micronized ADM and pECM scaffolds in liquid format have been shown to retain bioactive properties of the genuine ECM (Ho et al. 2004; Freytes et al. 2008b; Badylak, et al. 2009; Zhang et al. 2009) (Turner and Flynn 2012). Injectable devices processed in this way might have the potential to combine biocompatibility and long-term host tissue restoration with the physical properties of conventional fillers. Thus, micronized ADM devices could be used for soft tissue augmentation and ASC proliferation in patients with posttraumatic or degenerative contour defects of the integument.

The previously outlined applications of ECM-derived scaffolds exemplify the matrix-immanent potentials on morphogenesis and the various possibilities of its implementation into tissue restoration procedures. The available data are promising and will inspire the rapidly growing new field of regenerative surgery.

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P. Golinski, S. Groeger, and J. Meyle

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## 15.1 The Oral Mucosa

### 15.1.1 Structure and Function of the Oral Mucosa

The epithelium is a unique barrier that separates the body from its environment. The basic structure of the oral mucosa and the human skin is similar.

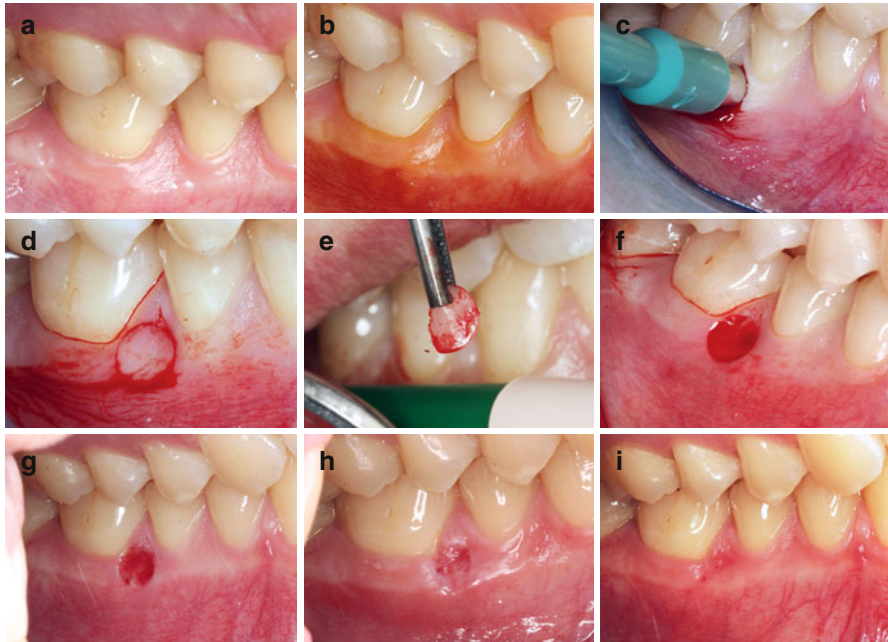
They consist of two layers, an epithelium with a basal membrane and an underlying connective tissue (Squier and Kremer 2001). The main tissue components of oral mucosa are the oral epithelium and the underlying connective tissue (lamina propria) that includes the supra-alveolar fibre apparatus, blood and lymphatic vessels, and nerves. Essential functions of the oral mucosal barriers are resistance against pathogens, exogenous substances and mechanical stress (Presland and Jurevic 2002). Gingival tissues are designed for peripheral body defence (Schroeder and Listgarten 1997). The tissue surrounding the teeth provides a seal to resist the frictional forces of mastication and to defend the potential space between the teeth and the soft tissues against foreign invaders, such as microorganisms. The gingiva is a combination of epithelial and connective tissues that forms a collar of masticatory mucosa around the teeth of the complete deciduous or permanent dentition and is attached to the teeth and the alveolar process. It covers the alveolar crest, the interdental bone septum and the coronal portion of the alveolar process to the mucogingival junction.

In contrast to the epidermis of skin, which is orthokeratinised, all three major differentiation patterns of keratinocytes occur in normal oral epithelia.

The gingiva is a keratinised anatomical and functional unit with variations in shape, contour and clinical topography that result in part from tissue adaptation to

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P. Golinski (✉) • S. Groeger • J. Meyle  
Department of Periodontology,  
Zentrum fuer Zahn-, Mund- und Kieferheilkunde,  
Justus-Liebig-University Giessen,  
Giessen, Germany  
e-mail: Sabine.E.Groeger@dentist.med.uni-giessen.de



**Fig. 15.1** Vestibular view of the lower right mandibula (a) prior to biopsies. (b) Characterisation of the gingival mucosal border (with Schiller's iodine, yellowish brown), (c) punch biopsy after local anaesthesia, (d) the biopsy is located in the gingiva (4 mm Ø), (e) excision was performed within the underlining connective tissue, (f) situation after excision, (g) first follow-up control 1 day after surgery, (h) second follow-up after 7 days, (i) wound closure with complete keratinisation after 15 days

the specific location around fully erupted teeth. In regions that are exposed to mechanical forces like mastication, such as the gingiva and the hard palate, a keratinised epithelium similar to the epidermis occurs. The keratinised gingiva can be differentiated histochemically by Schiller's iodine (Fig. 15.1) solution revealing stored glycogen in the lining mucosa (Fasske and Morgenroth 1958).

The mucogingival junction demarcates the boundary of the gingiva from the movable alveolar mucosa and the floor of the mouth mucosa. The floor of the mouth and the buccal part need flexibility for chewing, speech or swallowing and are covered with a lining mucosa that shows a non-keratinising epithelium. The specialised mucosa on the dorsum of the tongue contains numerous papillae and is covered by an epithelium, which may be either keratinised or non-keratinised. A characteristic feature of the epithelium is its exceptionally high rate of cellular turnover that is much faster than in skin (Narayanan and Page 1983; Sodek 1977). This high turnover rate provides an effective barrier to bacterial penetration. In primates the turnover time was found to be 4.6–10.9 days and in mice 3–5 days (Demetriou and Ramfjord 1972; Kirschner and Ruhl 1969; Skougaard 1970). The protective function

of the gingival epithelium is also enhanced by the salivary secretion that physically lubricates the epithelial surfaces and provides antimicrobial protection. For laboratory analyses, *in vitro* generated mucosa is cultured based on collagen matrices, on decellularised dermis or in inserts (Golinski et al. 2011; Groger et al. 2008; Izumi et al. 1999).

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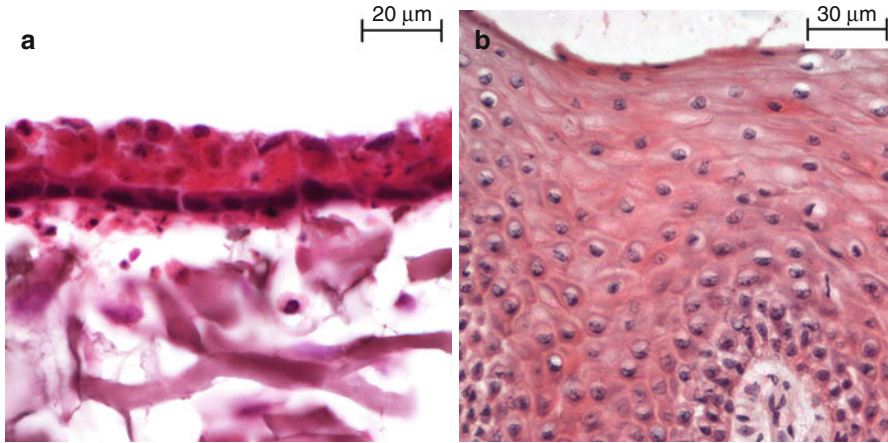
## 15.2 In Vitro Model of Oral Mucosa

### 15.2.1 Establishment of a Mucosa Model Based on Collagen–Elastin Matrix

Regeneration of the structure and function of the gingiva is one of the main objectives in periodontal health care. Tissue engineering represents an excellent approach to overcome difficulties encountered in periodontal health care such as diseased sections of soft tissues in the oral cavity and a limited amount of gingival tissue. Based on the experiments of Rheinwald and Green (Rheinwald and Green 1975) which successfully performed serial culture and colony formation of epidermal keratinocytes, the development of soft tissue models has been of growing interest. Tissue-engineered skin replacements, including biological substances such as fibrin sealant, various types of collagen and hyaluronic acid, were cultured on acellular or cellular matrices and cell culture inserts. These components were brought together as scaffolds and were found to perform essential structural and physiological functions during the construction of *in vitro* three-dimensional tissue substitutes (Horch et al. 2005; Liu et al. 2010).

AlloDerm<sup>®</sup> was employed as an underlying (dermal) structure for the development of fully differentiated oral tissue (Izumi et al. 1999, 2000). In further studies, this *ex vivo* oral mucosa equivalent also was shown to be usable for intra-oral grafting in oral surgery (Izumi et al. 2003). In addition, the function of this scaffold was used *ex vivo* to develop a test system to determine radiation-induced effects on oral keratinocytes (Tobita et al. 2010).

The primary indication of the collagen–elastin matrix (Matriderm<sup>®</sup>) as dermal substitute is for the treatment of deep partial- and full-thickness burn wounds (Haslik et al. 2007; Kolokythas et al. 2008; Ryssel et al. 2008). Matriderm<sup>®</sup> has been proved to be an effective dermal substitute and was recently described as a suitable scaffold for tissue engineering in the generation of three-dimensional skin scaffolds (Golinski et al. 2009). Based on this technique, the biocompatibility of Matriderm<sup>®</sup> with gingival fibroblasts as well as with keratinocytes was tested. In further experiments, a three-dimensional oral mucosa model using tissue-engineering techniques and the architecture of this model was investigated by histological analysis and electron microscopy. Important characteristics and measures of tissue quality are a multilayered epithelium, interactions between keratinocytes by desmosomes as well as the development of a basement membrane during coculture (Golinski et al. 2011).



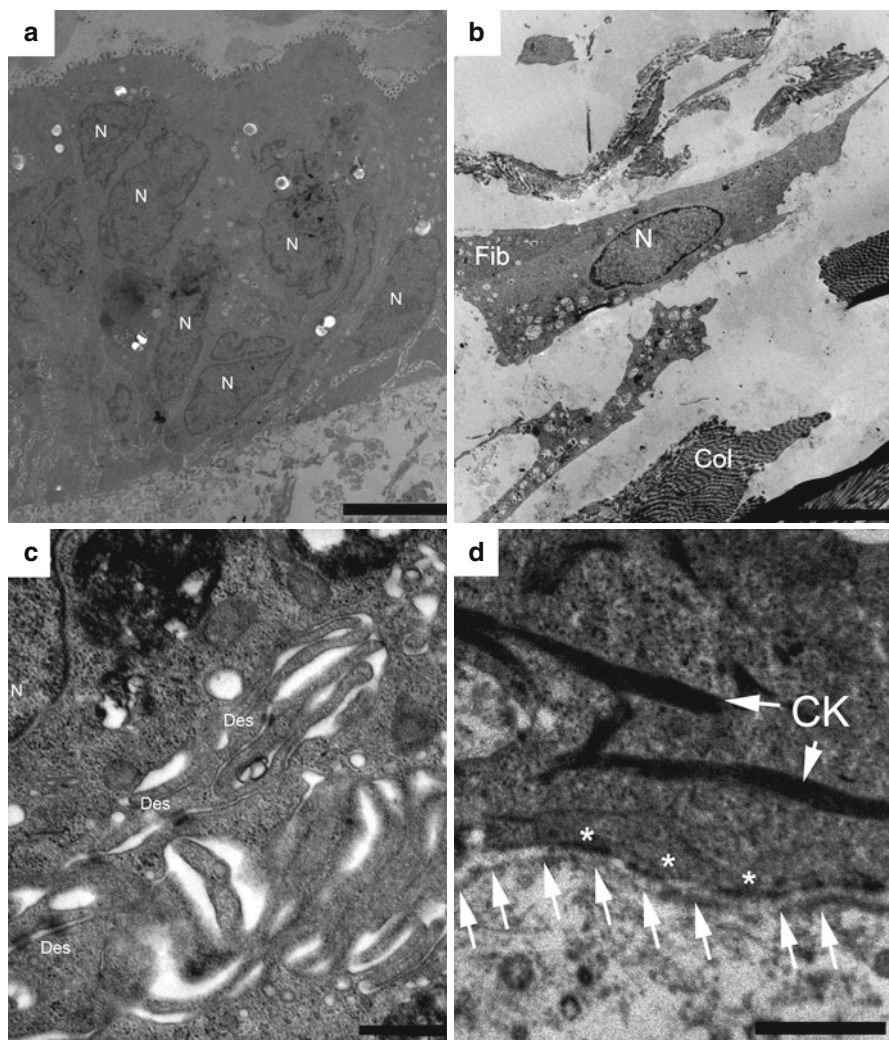
**Fig. 15.2** Haematoxylin and eosin staining of an oral mucosa model. **(a)** Oral mucosa after 10 days of culture at the air-liquid interface (ALI) in comparison with naïve gingival tissue. **(b)** The stratum basale was identified as the bottom layer of keratinocytes on the matrix. Cells that moved towards the apical region (into upper layers) changed from a columnar shape to a polygonal shape

## 15.2.2 Analysis of an Oral Mucosal Model

### 15.2.2.1 Structural and Immunologic Analysis

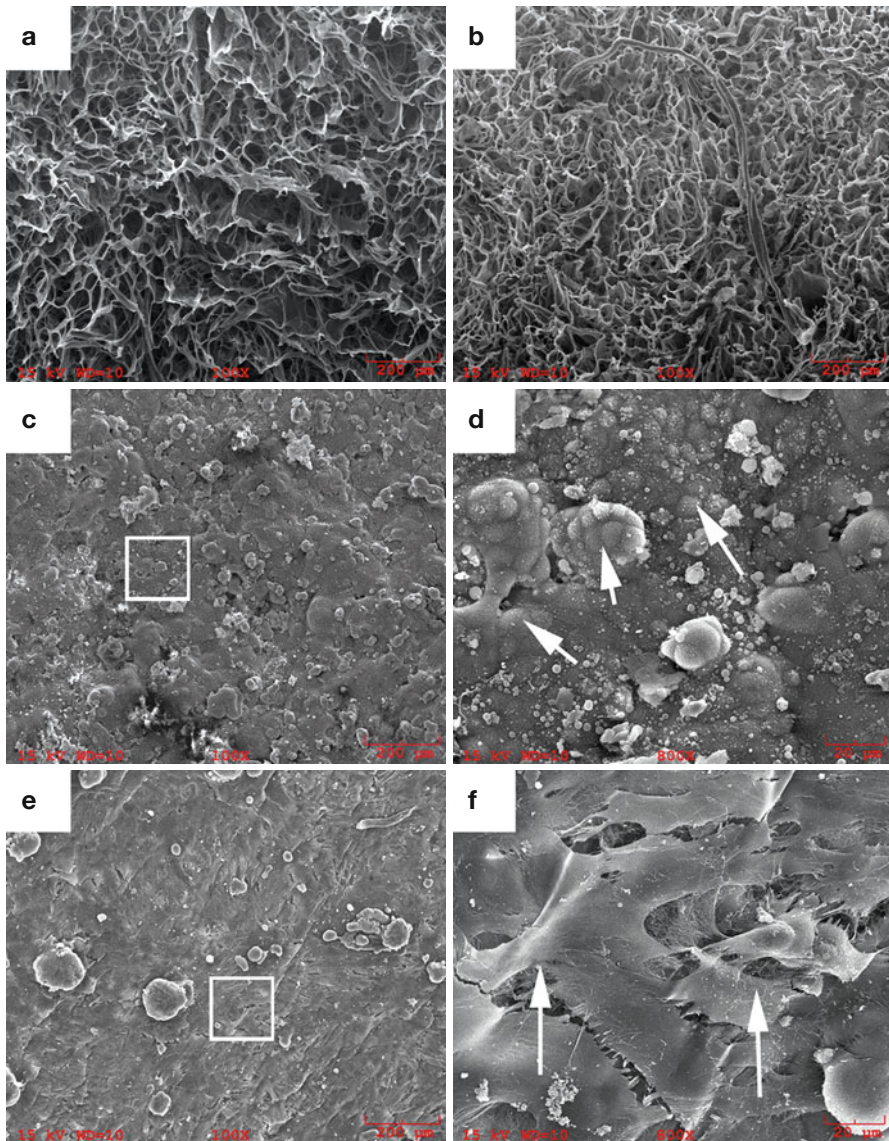
After 30 days of culture, an ex vivo differentiated oral mucosa model was generated on Matriderm®. In this model, fibroblasts were seeded onto Matriderm® (Fig. 15.3b). Then, after 14 days of incubation, keratinocytes were added to the fibroblast scaffold, and histological analysis using standard haematoxylin and eosin staining was performed (Fig. 15.2). Apically, a stratum basale was visible and characterised as a single layer of cuboidal shape. Cells in the superficial layer appeared extended and compact (Fig. 15.2). To characterise the epithelial junction zone, an immunohistochemical staining against collagen IV was performed and a basement membrane was demonstrated (Fig. 15.5b). Transmission electron microscopy analysis showed a multilayered formation of gingival keratinocytes (Fig. 15.3a) and a basement membrane (arrows) with hemidesmosomal structures (Fig. 15.3d). Fibroblasts incorporated into a dermal substrate may play a role in the formation of a basal membrane and provided a suitable environment in supporting mucosa formation (El Ghalbzouri et al. 2002). Correspondingly microenvironmental ex vivo studies performed with skin substitutes revealed the need for dermal components (fibroblast) for the generation of a stratified epithelium (Andriani et al. 2003). Desmosomal cell-cell interactions were detected by electron microscopy (Fig. 15.3c). These structures are cadherin based and highly specialised anchoring junctions that link intermediate filaments (Green and Simpson 2007 28). The naïve matrix presented irregularly shaped pores of approximately 50 µm in diameter (Fig. 15.4a) and was not suitable as an underlying structure for freshly seeded keratinocytes alone. Incubation of Matriderm® in fibroblast growth medium resulted in macroscopic shrinkage (≈30 %) and reduced the pore diameter (Fig. 15.4b). Fibroblasts were then seeded onto the scaffold, almost to confluency, and the remaining gaps closed after 14 days of incubation



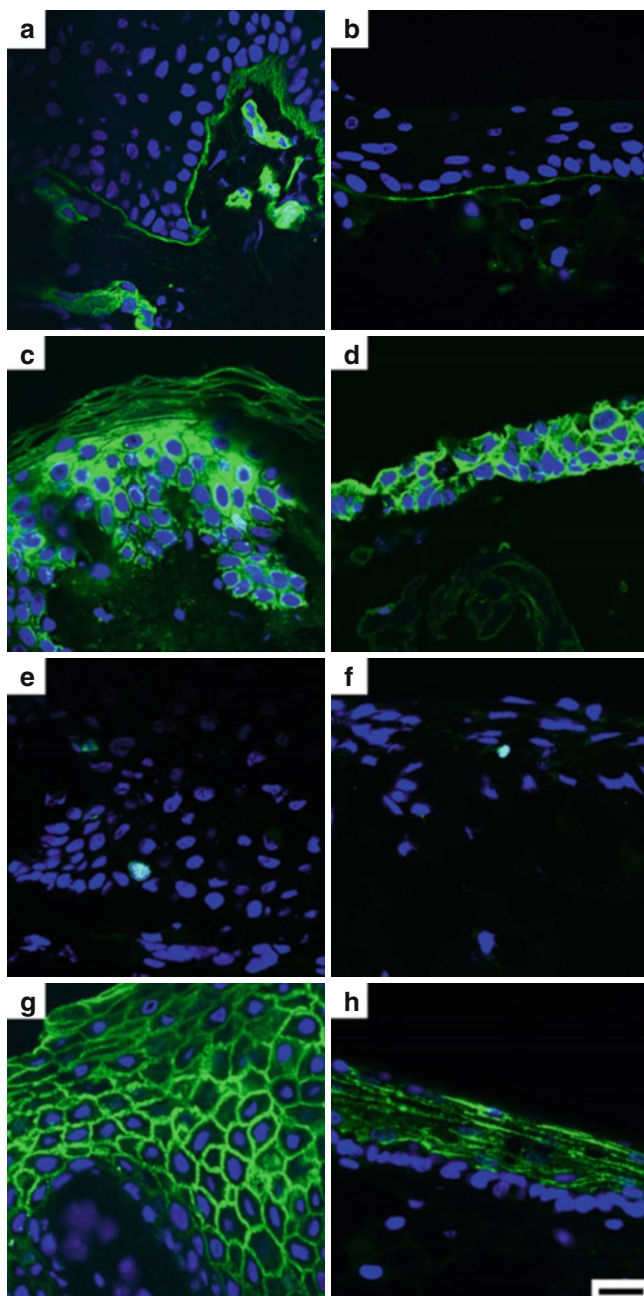


**Fig. 15.3** Analysis of the oral mucosa model by transmission electron microscopy. (a) Transmission electron microscopy of a multilayered oral mucosa model: gingival keratinocytes with nuclei (*N*) are shown. (b) Gingival fibroblasts (*Fib*) were grown inside the matrix, and nuclei (*N*) and collagen fibres (*Col*) are visible. (c) Gingival keratinocytes were connected via desmosomes (*Des*). (d) A basement membrane (*arrows*), hemidesmosomal structures (*\**) and cytokeratin filaments (*CK*) were detectable between the dermal and the epidermal parts of the mucosa

(Fig. 15.4c, d). Thereafter, the gingival keratinocytes were seeded on top of the fibroblast layer and cultured submersed for 6 days. After an additional incubation period of 10 days in close proximity to the air–liquid interface (ALI), the cells formed a continuous epithelial layer (Fig. 15.4e, f). Immunohistological analysis with an antibody against pancytokeratin demonstrated cytokeratin expression in all layers (Fig. 15.5d). The Ki-67 protein, a nuclear protein, is a cellular marker for proliferating cells and was detectable



**Fig. 15.4** Analysis of an oral mucosa model by scanning electron microscopy. (a) Naïve collagen–elastin matrix (Matriderm®) without media or cells. (b) Matrix without cells, after 1 day of incubation in fibroblast growth medium. (c) Gingival fibroblasts cultured for 14 days; the *white square* marks the area of higher magnification shown in panel d. (d) Magnification (800) of the *white square* shown in panel c; fibroblasts are indicated with *arrows*. (e) Oral mucosa model after 30 days of culture with differentiated gingival keratinocytes; the *white square* marks the area of higher magnification shown in panel f. (f) Magnification (800·) of the *white square* shown in panel e; keratinocytes are marked with *arrows*



**Fig. 15.5** Immunohistochemical analysis of an oral mucosa model after 10 days of culture at the air–liquid interface (ALI) compared with naïve gingival tissue. Collagen IV staining (*green*) of a basement component in (a) naïve oral mucosa and (b) the oral mucosa model. Pancytokeratin staining (*green*) of (c) naïve oral mucosa and (d) the oral mucosa model. Staining against Ki-67 (*green*) as a proliferation marker (e) naïve oral mucosa and (f) oral mucosa model. Staining against involucrin (*green*) as a differentiation marker in (g) naïve oral mucosa and (h) oral mucosa model. Nuclei were stained *blue* using TOPRO-3 iodide. Scale bar=30  $\mu$ m

in the stratum basale of the mucosal model (Fig. 15.5f). Because of the presence of Ki-67 protein during all active phases of the cell cycle (G(1), S, G(2) and mitosis), and its absence in resting cells (G(0)), Ki-67 is a useful marker for determining the proliferating fraction of a cell population (Gibbs and Ponc 2000; Scholzen and Gerdes 2000). Involucrin was also detected in the mucosa model. It can be demonstrated in stratified layers of keratinocytes and is cross-linked to membrane proteins by transglutaminase (Kalinin et al. 2001) (Fig. 15.5h) and is a suitable marker for differentiation.

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## 15.3 In Vitro Model of the Epithelial Barrier

### 15.3.1 The Gingival Epithelial Barrier

The bacterial biofilm on the tooth surface and in the gingival crevice is the primary etiologic causative agent of periodontal inflammation. Epithelial tissues provide a barrier between the body and the environment, and keratinocytes form the first line of defence against the bacterial challenge (Presland and Jurevic 2002). Gingival keratinocytes are connected to each other by a variety of specialised transmembrane proteins.

#### 15.3.1.1 Structure and Function of Tight Junctions (TJ)

Tight junctions demarcate the border between apical and basolateral membrane domains (Niessen 2007) and act as a semipermeable barrier to the paracellular transport of ions, solutes and water (Anderson 2001; Tsukita et al. 2001; Dejana 2004). They coordinate a variety of signalling and trafficking molecules, regulating cell differentiation, proliferation and polarity (Matter et al. 2005; Kohler and Zahraoui 2005). Structural components of tight junctions consist of a large number of different proteins, like occludin (Furuse et al. 1993), occludin 1B (Muresan et al. 2000), zonula occludens proteins ZO-1 (Stevenson et al. 1986), ZO-2 (Gumbiner et al. 1991) and ZO-3 (Haskins et al. 1998), as well as members of the protein family of claudins (Furuse et al. 1998a, b; Tsukita and Furuse 1998). Occludin seems to function as a signal transmitter and can associate with signalling molecules such as the non-receptor tyrosine kinase c-Yes, atypical protein kinase C (aPKC) and PI3-kinase, as well as protein phosphatases 2A and 1 (Chen et al. 2002; Seth et al. 2007).

Claudins have barrier properties (Furuse et al. 1998a, b, 2002; Tsukita and Furuse 2002) and directly regulate the gate function as paracellular tight junction channels (PTJC). These structures have biophysical properties similar to those of traditional ion channels (Tang and Goodenough 2003). Another property is that they also recruit occludin to tight junctions (Furuse et al. 1998a, b). In adjacent mouse liver cells, different members of the claudin family were shown to build homophilic or heterophilic polymers. They also formed paired strands to the membrane of adjacent cells (Furuse et al. 1999). Claudin 1 and occludin were detected in gingival epithelium (Hatakeyama et al. 2006).

### 15.3.1.2 Transepithelial Electrical Resistance (TER)

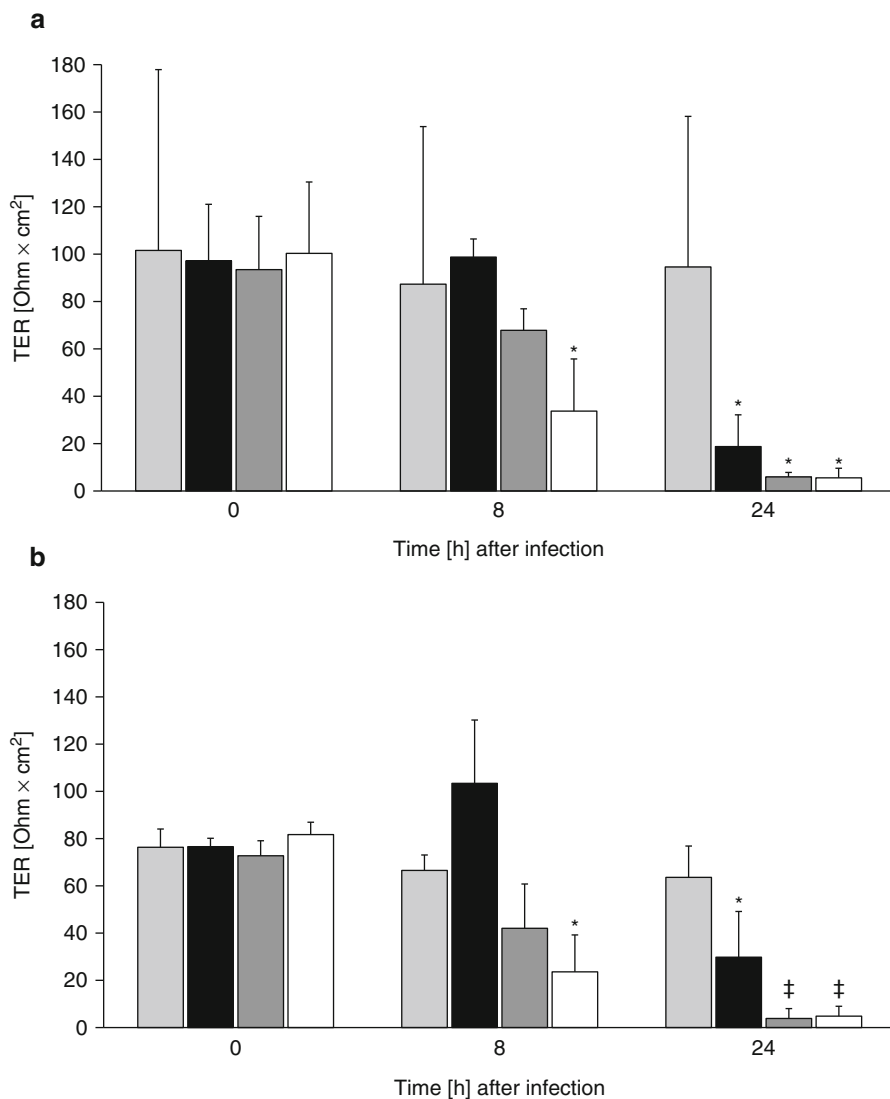
To study the permeability of mucosal barriers in vitro, the transepithelial electrical resistance (TER) measurement has been widely used, and changes of TER values have been directly related to the function of the paracellular occluding barrier (Gumbiner and Simons 1986; Staddon et al. 1995). TER measurements are commonly used to assess the integrity of tight junctions. A close correlation between the number of junctional strands and junctional tightness, as judged by transmural resistance values, was demonstrated (Claude 1978). The correlation between tight junctions and TER was also shown in primary human gingival keratinocytes (Meyle et al. 1999). TER depends on the intracellular  $\text{Ca}^{2+}$  concentration, i.e. increasing  $\text{Ca}^{2+}$  levels are followed by a decreasing NaCl diffusion potential (Palant et al. 1983).

### 15.3.2 Bacterial Influence on a Gingival Epithelial Barrier Model

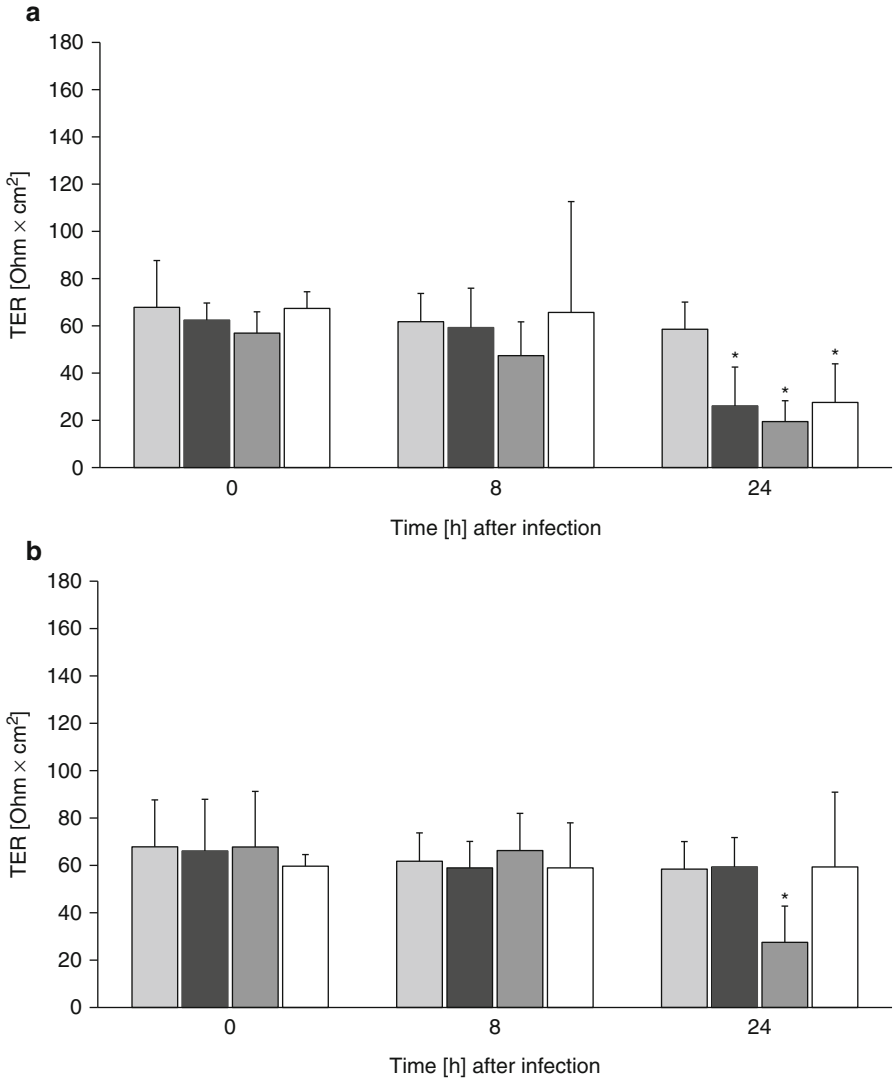
Among all microorganisms in the oral cavity (Paster et al. 2001), some periodontal pathogens (*Aggregatibacter actinomycetemcomitans*=*A. actinomycetemcomitans*, *Porphyromonas gingivalis*=*P. gingivalis*, *Tannerella forsythensis*=*T. forsythensis*, *Treponema denticola*=*T. denticola*) are able to induce inflammatory responses that lead to attachment loss and periodontal destruction (van Winkelhoff et al. 2002; Tatakis and Kumar 2005).

*P. gingivalis* is an anaerobic gram-negative coccoid rod exhibiting a variety of virulence factors. This microorganism is considered as one of the primary microbiological factors involved in the pathogenesis of periodontitis (Moore and Moore 1994; Kumagai et al. 2005; Holt et al. 1999). Some strains produce a number of proteolytic enzymes, like gingipains, amino peptidases, invasins, cytotoxic substances, collagenases (Birkedal-Hansen et al. 1988), LPS (Soolari et al. 1999; Kesavalu et al. 1992), outer membrane proteins (Kesavalu et al. 1992), fimbriae (Amano 2003; Amano et al. 2004; Jotwani and Cutler 2004) and capsule proteins (Preshaw et al. 1999). *P. gingivalis* ATCC 33277, one of the best characterised strains, expresses fimbriae (Hamada et al. 1994), is able to produce gingipains (Grenier 1992) and to invade oral cells (Njoroge et al. 1997), which can be inhibited by protease inhibitors (Lamont et al. 1995). Fimbriated *P. gingivalis* strains are more effective in entering human dendritic cells than fimbriae-deficient strains (Jotwani and Cutler 2004). The physical association between fimbrillin and  $\beta 1$  integrin seems to be a prerequisite for the invasion of *P. gingivalis* ATCC 33277 into primary gingival keratinocytes (Yilmaz et al. 2002). *P. gingivalis* W83 is a virulent strain that barely expresses fimbriae, shows low adherence to human fibroblasts (Watanabe et al. 1992) and produces gingipains (Shah et al. 1990; Wolburg and Lippoldt 2002).

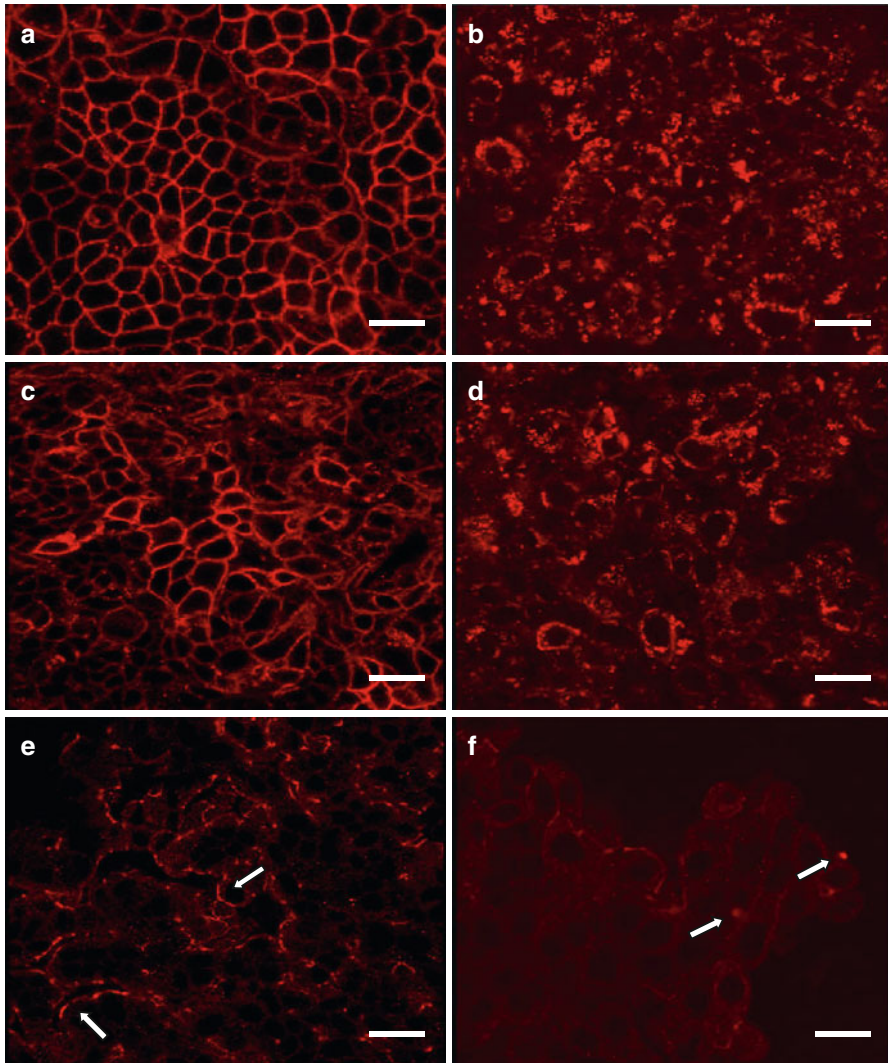
The use of human gingival keratinocytes with functional characteristics of the gingival epithelial barrier provides a valuable in vitro model to study the early steps of gingival/periodontal infections that are demonstrated in Figs. 15.6, 15.7, 15.8 and 15.9 (Groeger et al. 2010). The results of this study give some insights into the initial stages of infection processes leading to gingivitis and periodontitis.



**Fig. 15.6** Influence of *P. gingivalis* W83 (MOI  $10^4$ ) on the TER of human gingival keratinocytes. Mean values from three different experiments ( $n=6$ ) are shown. Significant differences to the negative control are depicted (bars = standard deviation,  $*=p<0.05$  and  $\ddagger=p<0.01$ ). **(a)** Primary human gingival keratinocytes (PHGK), **(b)** immortalised human gingival keratinocytes (IHGK). □ = control, ■ = apical infection, ▒ = basolateral infection, ◻ = apical plus basolateral infection



**Fig. 15.7** Influence of *P. gingivalis* ATCC 33277 (**a**) and KDP 136 (**b**) (MOI 10<sup>4</sup>) on the TER of immortalised human gingival keratinocytes. Mean values from three different experiments (n=6) are shown. Significant differences to the negative control are depicted (bars = standard deviation, \* = p < 0.05). (a) *P. gingivalis* ATCC 33277, (b) *P. gingivalis* KDP 136. □ = control, ■ = apical infection, ▒ = basolateral infection, ◻ = apical plus basolateral infection

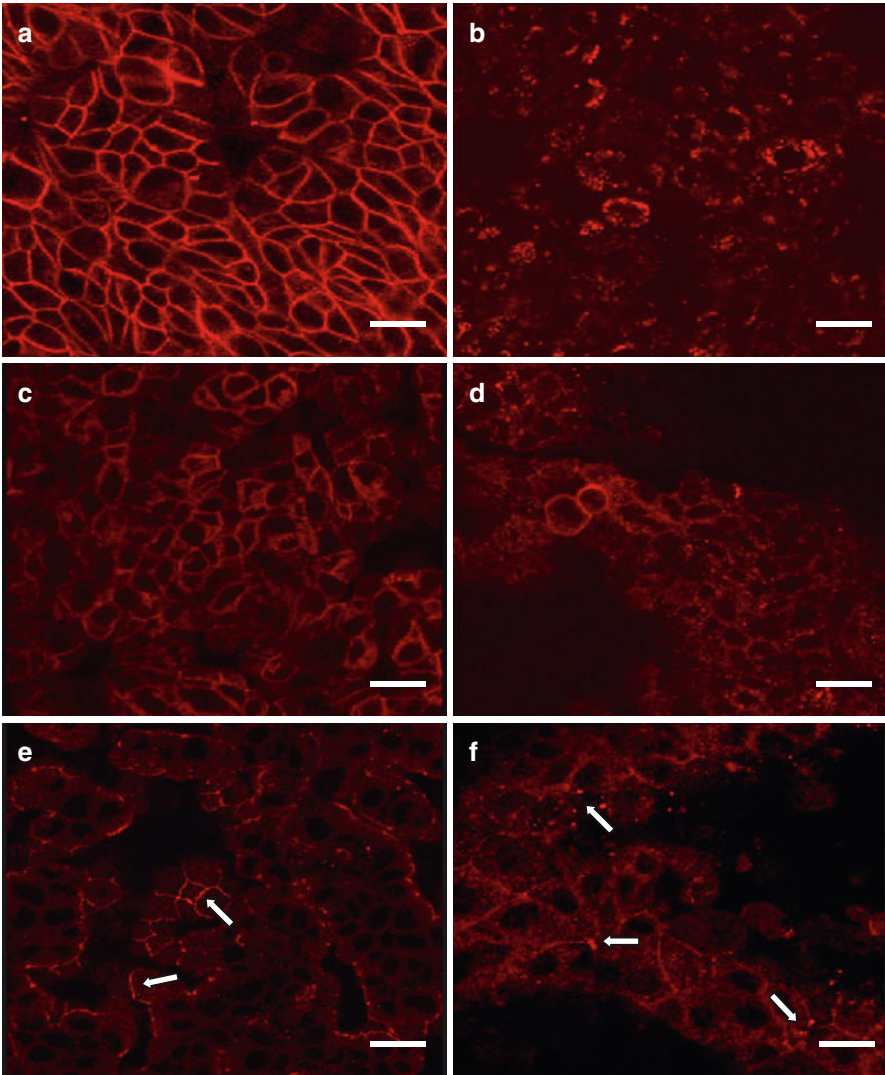


**Fig. 15.8** Immunostaining of the tight junction proteins in primary human gingival keratinocytes, claudin 1 (**a, b**) claudin 2 (**c, d**) and occludin (**e, f**); **a, c, e** = cells in culture medium; **b, d, f** = cells infected apically plus basolaterally with *P. gingivalis* W83 (MOI  $10^4$ ) for 4 h. *Arrows* (**e, f**) show curved occludin strains in the walls of noninfected cells; in infected cells the *arrows* point to occludin aggregations, marker = 20  $\mu$ m

### 15.3.2.1 Infection of the Gingival Barrier Model with Periopathogenic Bacteria: Development of the TER

Immortalised human gingival keratinocytes (IHGK) (Groger et al. 2008; Huibregtse and Beaudenon 1996) and in comparison primary cells seeded on collagen-coated filter inserts attach to the collagen-coated surface of the insert and are polarised so that the basal parts of the cells are located on the collagen-coated surface of the





**Fig. 15.9** Immunostaining of the tight junction proteins in immortalised human gingival keratinocytes, claudin 1 (**a, b**) claudin 2 (**c, d**) and occludin (**e, f**); **a, c, e**=cells in culture medium; **b, d, f**=cells infected apically plus basolaterally with *P. gingivalis* W83 (MOI  $10^4$ ) for 4 h. *Arrows (e, f)* show curved occludin strains in the walls of noninfected cells; in infected cells the arrows point to occludin aggregations, marker=20  $\mu$ m

insert and the apical part is oriented upwards. The 3D culture model shows two different parts where the culture medium and the bacteria-containing solutions can be added: the apical part above the cells and the basolateral part at the bottom of the wells surrounding the inserts.

Differentiation includes the formation of tight junctions, one prerequisite for the development of the TER. The cells have to be confluent, which is controlled by light

microscopy. Gingipain-producing *P. gingivalis* strains ATCC 33277, W83 and the RGP/KGP defect mutant strain KDP 136 (Shi et al. 1999) were cultured and prepared as described before (Groeger et al. 2010), can be applied either apically (api) or basolaterally (baso) or, in a third approach, can be added from api, as well as from baso (a+b). The transepithelial electrical resistance (TER) as marker for the epithelial barrier function can be detected with a volt ohm meter.

Primary human gingival keratinocytes (PHGK) infected with *P. gingivalis* W83 in MOI  $10^4$  (Fig. 15.6a) show no significant changes in the TER upon apical or basolateral infection after 8 h, while the decrease upon apical plus basolateral infection is statistically significant ( $p < 0.05$ ). After 24 h, decrease is significant in all three modes of infection ( $p < 0.05$ ). The IHGK cells show a significant decrease in TER after 8 h of apical plus basolateral infection (Fig. 15.6b) ( $p < 0.05$ ). After 24 h, there is a significant decrease upon apical ( $p < 0.05$ ), basolateral and apical plus basolateral infection ( $p < 0.01$ ). PHGK and IHGK cells behaved very similarly.

Using *P. gingivalis* strain ATCC 33277 for infection of IHGK, no significant changes of the TER after 8 h are shown, independent of the mode of infection, while decrease after 24 h is statistically significant in all modes of infection (Fig. 15.7a) ( $p < 0.05$ ).

When IHGK are infected with *P. gingivalis* KDP 136, a gingipain defect mutant strain, after 8 h, there are no changes of the TER. After 24 h, only the basolaterally infected cells show a statistically significant decrease (Fig. 15.7b) ( $p < 0.05$ ).

Infection of IHGK cells with a fimbriated *P. gingivalis* strain (ATCC 33277) results in comparable effects on TER, as the effects caused by the barely fimbriated strain W83. The TER of the cells infected with the gingipain-producing strains is reduced or decreased to zero within 24 h. In contrast, the RGP/KGP gingipain mutant strain KDP 136 shows very low effects on the epithelial barrier after 24 h.

### 15.3.2.2 Infection of the Gingival Barrier Model with Periopathogenic Bacteria: Immunolocalisation of the TJ Proteins Claudin 1, Claudin 2 and Occludin

For immunostaining, cells are seeded on inserts and infected with *P. gingivalis* W83 like described before. Alterations in claudin 1 (Figs. 15.8 and 15.9a, b), claudin 2 (Figs. 15.8 and 15.9c, d) and occludin (Figs. 15.8 and 15.9e, f) expression caused by *P. gingivalis* W83 are demonstrated. In primary cells (Fig. 15.8), the controls show a typical chicken wire pattern of claudin 1 (a) and claudin 2 (b) that disappears after infection. Claudin 1 and claudin 2 appeared as protein aggregations (Fig. 15.8b, d). Occludin is localised in curved strands on the cellular walls of the control (Fig. 15.8e) (arrows). The strands disappear in infected cells and some aggregations are stained (Fig. 15.8f) (arrows). Immortalised cells show a staining pattern that is very similar to primary cells, except for a less intensive labelling of claudin 2 in the controls (Fig. 15.9a, c). The penetration and damage of the epithelial layer by infecting organisms is an important step in the pathogenesis of periodontitis. Among the putative periodontal pathogens, *Porphyromonas gingivalis* possesses a variety of virulence factors which enable this organism to colonise oral soft tissues and evade immune responses.

Infection with *P. gingivalis* of human gingival keratinocyte barriers shows an initial TER increase 8 h after apical application in primary and immortalised cells. This might reflect a defence reaction, while the underlying mechanisms are still unknown. Infection from the apical side of the barriers corresponds to the initial epithelial infection in vivo. Basolateral infection can be considered as a state where tissue destruction proceeds and bacteria might invade the underlying connective tissue. Basolateral infection caused an accelerated destruction of the barrier. This could be the result of an early detachment of the cellular layer from the insert surface. Proteolysis of a variety of focal contacts and adherence junction proteins (shown in HOK-16 cells) led to reduced adhesion of the cells to the extracellular matrix proteins (Hintermann et al. 2002). The demonstrated results suggest rapid tissue destruction in vivo if the bacteria are able to overcome the epithelial barrier.

Gingipains are extracellular cysteine proteinases and constitute one of the major virulence factors of *P. gingivalis* (Bedi and Williams 1994). Endothelial cell adhesion was disrupted by the influence of gingipains from *P. gingivalis* W83 (Chen et al. 2001). Gingipains also increase collagenolytic activity by induction of host matrix metalloproteinases. The cleavage of cellular receptors was demonstrated by Sheets et al. (2006). Moreover, gingipains are able to lyse the cellular adhesion molecules N-cadherin, VE-cadherin and  $\beta$ -integrin and cause detachment of endothelial cells. Furthermore, *P. gingivalis* is able to produce several other proteolytic enzymes. Among these are eight endopeptidases and a number of exopeptidases (Potempa et al. 2000). It is possible that these proteases are involved in the decrease of the TER after 24 h in cells infected basolaterally with a gingipain defect mutant strain. In noninfected cell layers, claudin 1 and claudin 2 showed a grid pattern marking the cellular walls. These proteins are supposed to be present at the apical surface of the cells (Will et al. 2008). Infection changed the pattern of adhesion molecules dramatically and led to a denaturation of claudin 1, claudin 2 and occludin. Besides proteolytic damage, active internalisation of epithelial adhesion complexes could be responsible for the observed changes. In intestinal epithelial cells (T84 cells), interferon- $\gamma$  induces the internalisation of tight junction proteins (claudin 1, occludin, jamA) via micropinocytosis, resulting in a leakage of the epithelial layer (Bruewer et al. 2005). The impact of infection on tight junctions and epithelial barrier has been analysed with other cell systems and species. In an epidermal keratinocyte cell line (HaCaT), it was shown that infection with exfoliative *Staphylococcus* strains (*S. aureus*, *S. epidermidis*) resulted in downregulation of tight junctions. Claudin 1 and occludin changed in a similar pattern (Ohnemus et al. 2008). Infection of gastric epithelial cells with *Helicobacter pylori* induced a time-dependent redistribution of occludin from the cellular walls to the cytosol which was preceded by a decrease of the TER (Wroblewski et al. 2008). *Campylobacter jejuni*, a major pathogen for human enterocolitis, induced a decrease of the TER of colonic epithelial cells and a redistribution of occludin, resulting in a similar staining pattern (Chen et al. 2006). The results demonstrate the reaction of a human gingival epithelial in vitro model to the major oral pathogen *P. gingivalis*. The results suggest the involvement of further soluble virulence factors in addition to gingipains and also indicate that the destruction of the epithelial barrier is associated with the degradation of cell–cell junctional complexes, thus allowing the spread of the bacterium.

## 15.4 Mucosa Models for Pharmaceutical Testing

Currently, based on the fields of dermatology and wound surgery, Matriderm® was found to be applicable in tissue regeneration as well as in the aesthetic and elective treatment of patients with deep and partial burn wounds (Haslik et al. 2007; Kolokythas et al. 2008; Ryssel et al. 2008). The characteristics of Matriderm® include potential use in the generation of engraftable skin equivalents for the purpose of transplantation. Based on this scaffold, a multilayered oral mucosa-like structure was generated. Histological, immunohistochemical and electron microscopic analysis of the dermal–epidermal junction showed a typical basement membrane and hemidesmosomal structures. Neighbouring keratinocytes formed desmosomes in the epidermal sections. Cytokeratin was detectable in all epidermal layers. Involucrin was found in stratified layers Ki-67 protein as proliferation marker was demonstrated in the basal cell layer. These results revealed that the collagen–elastin matrix was highly biocompatible with gingival cells under ex vivo conditions. The biocompatibility with gingival cells indicates that Matriderm®, as a supportive structure for the formation of tissue scaffolds, might be suitable for the treatment of oral cavity defects, surgery grafting (e.g. periodontal) or pharmaceutical testing. Considering these recent findings, our oral mucosa scaffold incorporates the advantages of the dermal (fibroblasts) and epidermal (keratinocytes) structures in combination with the collagen–elastin matrix (Matriderm®). This strategy is adequate for laboratory use and holds perspective for clinical indications (e.g. for oral grafting procedures). Complex mucosal models as the reconstructed mucosa described in Sect. 15.2 appear to be useful for the investigation of interactions between the different cell types (fibroblasts and keratinocytes), particularly regarding the epidermal–dermal junction. Simple model systems of cellular functions like the TER are more suitable for studies that aim to investigate specific markers like epithelial barrier. TER measurements are commonly used to assess the integrity of tight junctions. The (TER) was shown to be a useful marker for the epithelial barrier function to investigate pathologic events during bacterial infection (Groeger et al. 2010). Furthermore, the investigation of soluble products that are secreted or released from gingival keratinocytes such as growth factors or cytokines is easier in a less complex structure. A biological gel or a dermis may trap molecules that are released from the cells, while inert interfaces with pores like cell culture inserts allow the passage of macromolecules towards the medium bathing the tissue from the other side of the insert (Poumay and Coquette 2007).

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Anna I. Arno and Marc G. Jeschke

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## 16.1 Definition of Dermal Substitutes

Dermal substitutes are biomatrices that fulfil the functions of the cutaneous dermal layer: control of pain and scarring. They act as matrices or scaffolds and promote new tissue growth and enhance wound healing (Lee 2000; Pham et al. 2007), with enhanced pliability and a more favourable scar. Dermal substitutes play a major role in repairing full-thickness skin defects, both in acute and chronic wounds (van der Veen et al. 2010), and there is evidence that they improve scar quality (Box 16.1).

### Box 16.1 Dermal Substitutes' Basics

They have been considered to be a life-saving tool and currently that shifted to improve scar quality following acute thermal trauma (Hodgkinson and Bayat 2011).

They are biomaterials composed of a matrix  $\pm$  cells.

They serve as a scaffold into which cells can migrate and repair the injury (Truong et al. 2005).

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A.I. Arno, MD  
Plastic Surgery Department and Burn Unit,  
Ross Tilley Burn Centre, Sunnybrook Health Sciences Centre,  
University of Toronto, 2075 Bayview Avenue,  
Toronto, ON M4N 3M5, Canada

Plastic Surgery Department and Burn Unit, Vall d'Hebron University Hospital,  
Autonomous University of Barcelona, Passeig de la Vall d'Hebron 119-129,  
Barcelona 08035, Spain

M.G. Jeschke, MD, PhD (✉)  
Ross Tilley Burn Centre, Sunnybrook Health Sciences Centre,  
University of Toronto, 2075 Bayview Avenue,  
Toronto, ON M4N 3M5, Canada  
e-mail: marc.jeschke@sunnybrook.ca

## 16.2 History of Dermal Substitutes

Advances in acute treatment, acute care and intensive care have resulted in decreased mortality in major burns (Saffle 1998). The current focus in burn care has now shifted towards improving the long-term function and appearance of the healed skin in conjunction with quality of life (Richters et al. 2008). The loss of the dermis in extensive full-thickness wounds like burns poses a serious problem which is not completely solved by the application of split-thickness autografts (Machens et al. 2000). It is important to have an effective *dermal* replacement because dermal tissue does not regenerate into normal dermis *in vivo* after full-thickness dermal injuries. Application of a dermal substitute underneath the autologous skin graft may, possibly, improve the wound healing process (Zuijlen van et al. 2002) and the treatment of burns, skin ulcers, various deep wounds and unstable scar replacement (Machens et al. 2000) (Box 16.2).

### Box 16.2 Why Were Dermal Substitutes Developed?

The lack of dermal tissue in full-thickness wounds and the poor quality of the scars after treatment with split-thickness autografts or cultured epithelial grafts, which contain little or no dermal component, respectively, initiated the development of dermal substitutes during the 1990s (van der Veen et al. 2010; Hodgkinson and Bayat 2011).

All in all, dermal substitutes play a role in control of scarring (Pham et al. 2007). Pathologically excessive skin scarring (hypertrophic scars and keloids) represents the main morbidity cause in surviving burn patients and has a varying prevalence of up to 67 %, and this increases with the time a wound takes to heal (Bombaro et al. 2003). Scars impose several problems clinically, both aesthetically and functionally, due to contractures and may lead to the formation of scar carcinoma, e.g., Marjolin's ulcer (Akguner et al. 1998). Patients with keloids or hypertrophic scars suffer a severe impairment of quality of life, causing physical, psychological and social sequelae (Leventhal et al. 2006).

## 16.3 Design Considerations

### 16.3.1 Functional Requirements of Dermal Substitutes

Dermal substitutes are designed to mimic the basic properties of the extracellular matrix (ECM) (Hodgkinson and Bayat 2011) and should share the same functions as normal dermis:

1. *Restoration of skin anatomy and physiologic function*: Due to their scaffolding properties, dermal substitutes help to control pain, contracture and scarring, with reduced healing times (van der Veen et al. 2010). If the dermal substitute is provided with an impermeable wound cover, like a silicone layer, it can also function as a protection of the wound from infection and fluid loss. The vascularisation period of the dermal component is usually 21 days, and it correlates with wound

infection rates. After that, the silicone layer is removed and replaced by an autologous split-skin graft. This procedure is known as the two-step procedure (e.g., the classical Integra<sup>®</sup>, see below for further description). To avoid infection and two operations, an advantageous method has been developed: immediately after debridement, the dermal substitute is placed in the wound and covered by an autologous split-skin graft (for instance, Matriderm<sup>®</sup> or Integra single layer<sup>®</sup>). This method provides earlier wound closure but may hamper graft survival, and not all dermal substitutes are compatible with this one-step procedure (depending on pore size and influx of cells, mainly) (van der Veen et al. 2010).

2. *Biocompatibility*: Refers to tissue integration, host tolerance or immunocompatibility and biodegradation. Biocompatibility is demonstrated by the ingrowth of fibroblasts and blood vessels (Richters et al. 2008). Vascularisation of the substitutes is mandatory to ease the take rate of split-skin grafts on top of them (der Veen van et al. 2011). Apart from rapid adherence and vascularisation, other related factors to take into account are mechanical stability and durability. Biopolymers may be tissue derived or synthetic (Boyce 1996). Biopolymers used are collagen (the most popular one), hyaluronic acid, fibrin, laminin, elastin, polylactic acid (PLA) and polyglycolide (PGA), among others (Hodgkinson and Bayat 2011). When using natural materials (allogeneic or xenogeneic), immunogenicity and disease transmission (for instance, prion disease and porcine retroviruses in xenograft products (Alisky 2004)) remain a concern (Hodgkinson and Bayat 2011). The use of synthetic materials has in some cases been found to lead to a foreign body response and fibrous capsule formation. Therefore, controlled rate of biodegradation, nontoxic metabolites, low or absent antigenicity, and inflammatory or foreign body reactions (Burke et al. 1981) are mandatory. On the other hand, synthetic materials are more cost-effective than the natural ones.
3. *Hosting or enabling the influx of cells that will function as dermal cells*: Facilitation of invasion of normal fibroblasts and capillaries to synthesise new dermis is influenced by the composition, pore size and degradability of the dermal substitute (Burke et al. 1981; Wang et al. 2005). Although a scaffold material can be designed as “permanent”, generally it is considered desirable that the transplanted scaffold be safely assimilated into the body as new matrix is generated by the populating cells (Hodgkinson and Bayat 2011).
4. *Resistance to shear forces*: The dermal substitute should be strong enough to be held in place in difficult anatomic areas such as joints (der Veen van et al. 2011).

### 16.3.2 Future Directions

Regarding current bioengineered skin substitutes, to date there is still none that replaces skin in its entirety, nor functionally, nor morphologically. Furthermore, although dermal substitutes have achieved some clinical success in restoring damaged skin, some milestones may still remain to be achieved, such as decreasing or abrogating prolonged healing times and scarring without skin appendages, objectively proving that they improve cutaneous scars comparing to the use of split-thickness graft alone, the need for a second surgery, limited tissue functionality and high cost (Boyce 2001; Shakespeare 2001; Krueger et al. 1994).

**Box 16.3 New Dermal Substitutes: Tissue-Engineered Scaffolds with Stem Cells (Hodgkinson and Bayat 2011)**

The most successful scaffolds are acellular polymer matrices, prepared through lyophilisation and phase separation techniques, designed to mimic the dermal extracellular matrix.

Cell-containing scaffolds have short shelf life, high cost and low viability of transplanted cells.

However, the use of stem cells within substitutes containing 3D scaffolds or microenvironments that control stem cell behaviour may decrease cell death and lead to new-generation dermal substitutes.

Preceded by the introduction of composite epithelial autografts (CEA) by Green in 1975 (Atiyeh and Costagliola 2007), dermal substitutes lack the epidermal component necessary for restoring fully functional skin, so further research is devoted to developing combined dermal-epidermal bioengineered cultured skin substitutes and, most recently, to stem cell therapy and tissue engineering (Arno et al. 2011; Leclerc et al. 2011) (Box 16.3). Dermal substitutes may serve as a three-dimensional scaffold to host stem cells and be programmed to accelerate wound healing with minimal fibrosis, eventually improving function and cosmetics (Zhang and Fu 2008).

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## 16.4 Classification of Dermal Substitutes

Although classical classification of dermal substitutes has distinguished between natural and synthetic materials, the current literature categorises dermal substitutes based on the source of the dermal substitute as synthetic versus biological materials. The latter are further divided between natural and constructed or artificial materials (van der Veen et al. 2010; der Veen van et al. 2011). In the classic literature, “natural” materials would only refer to “natural biological” materials.

### 16.4.1 Biological Materials

#### 16.4.1.1 *Natural Biological Materials*

Natural biological materials consist of human (the gold standard) or porcine tissue which is treated to obtain an acellular scaffold (van der Veen et al. 2010). Cells are eliminated to decrease the risk of immune responses (Richters et al. 2008). At the same time, natural dermal substitutes have the best dermal qualities in terms of porosity, microtopology and presence of basement membrane (Prunieras et al. 1983; Andreadis et al. 2001). However, it is very difficult to entirely remove all cell remnants. This drawback, in addition to the risk of disease transmission, forces the use of this kind of materials as temporary dressings, rather than permanent dermal substitutes (van der Veen et al. 2010). However, their main advantages are (1) high similarity to native dermis (especially when glycerol sterilisation is used

**Table 16.1** Natural biological materials' characteristics

Risks	Currently used solutions		
	Types		Similarity to native dermis?
Disease transmission	Sterilisation	Ethylene-oxide	No
		$\gamma$ -irradiation	No
		Glycerol	Yes
Immune rejection	Decellularisation	NaCl-SDS	Yes
		NaOH	Yes <sup>a</sup>

<sup>a</sup>But it decreases basement membrane

(Ghosh et al. 1997)) because they provide a 3D-ECM of collagen and elastin without cells (der Veen van et al. 2011), therefore with less theoretical risk of excessive scarring, and (2) partial conservation of basement membrane (van der Veen et al. 2010), which favours keratinocyte adherence (Ralston et al. 1999) (Table 16.1).

### Human-Derived Natural Biological Materials

#### Alloderm<sup>®</sup>

Alloderm<sup>®</sup> (LifeCell Corporation, Woodlands, TX) is human cadaver skin that has been chemically treated to decellularise dermis and is one of the oldest and most utilised matrices (Jones et al. 2002). It is processed from fresh cadaver skin treated with high salt to remove the epidermis and extracted to remove the cellular material. After a freeze-dry step, the immunologically inert acellular dermal matrix with intact basement membrane complex is obtained. Following its application to a wound bed, it is repopulated by host cells, revascularised and incorporated into the tissue (Jones et al. 2002). Therefore, it functions as a template for dermal regeneration. Approved and considered as banked human tissue by the FDA, it has been used to treat burns since 1992, and it has also been used to treat severe soft tissue defects. Alloderm<sup>®</sup> has been shown to have good graft take rates and to reduce subsequent scarring of full-thickness wounds, with results similar to Dermagraft<sup>®</sup> (Jones et al. 2002), although the graft take of split-skin grafts on top of Alloderm<sup>®</sup> in a one-step procedure was lower than split-skin graft applied alone (Wainwright et al. 1996). In summary, there are many advantages of Alloderm<sup>®</sup>: it is acellular and immunologically inert; it provides a template with natural dermal porosity for regeneration with the presence of an intact basement membrane and it allows the use of thinner autografts. The disadvantages include risk of transmitting infectious diseases, the need for two surgical procedures, high cost and donor site (Bello et al. 2001; Wainwright 1995).

#### Glyaderm<sup>®</sup>

Glyaderm<sup>®</sup> (Glycerol-preserved acellular dermis) is a novel dermal substitute, produced and distributed by Euro Skin Bank in the Netherlands. It is an acellular dermal collagen-elastin matrix obtained from human donorskin, preserved in 85 % glycerol (specifically, a NaOH treated glycerol preserved cadaver skin (Richters et al. 2008)). Its thickness is 0.3 mm, and it is best used in a two-step procedure. Glyaderm<sup>®</sup> is currently under a European multicentre burn unit clinical trial.

Glyaderm<sup>®</sup> is recommended to be used 5 or 7 days after wound debridement and allograft coverage, to assure an appropriate wound bed. In the two-step procedure,

after 5–7 days, a meshed split-thickness skin graft is applied on top of the Glyaderm® (Pirayesh et al. 2007).

Application of Glyaderm® in a two-step procedure, allowing the dermal substitute to become well vascularised before split-skin application, resulted in better take rates and reduced wound contraction compared to control wounds treated with only split-skin grafts, similarly to Alloderm® (van der Veen et al. 2010).

Experiments performed by Pirayesh et al. indicated that the take rate of split-skin grafts was reduced when applied as a one-step procedure on top of Glyaderm® or Alloderm® in a porcine full-thickness wound model (van der Veen et al. 2010).

Compared to Alloderm®, Glyaderm® offers a more cost-effective method to eliminate the dermal antigenic structures (Richters et al. 2008).

### Porcine-Derived Natural Biological Materials

Porcine dermal matrices are very similar to human dermal matrices, and although they have the disadvantages of xenografts, they represent the first choice of non-human-derived natural biological dermal substitutes (Rennekampff 2009), and many researchers consider them as a substitute for acellular human dermal matrices in the future (Ge et al. 2009). Currently, there are three acellular porcine dermal matrices in the market: Permacol®, which is used in full-thickness defects such as burns and for soft tissue reconstruction, such as hernia repair; Stratattice®; and Xenoderm®.

#### 16.4.1.2 Constructed or Artificial Biological Materials

These materials contain designed, controlled and purified biological molecules – usually and mainly collagen – by means of lyophilisation, which can be supplemented with glycosaminoglycans (GAGs) and also cross-linked in order to modulate its properties (Yannas et al. 1980), reducing immune rejection and improving the composition of the matrix with specific growth factors. Disadvantages include the still not complete knowledge of what should be incorporated and what should be avoided to design the best material and that they not mimic native dermis in its entirety (for instance, there is usually absence of basement membrane and different architecture) (van der Veen et al. 2010) (Table 16.2).

**Table 16.2** Constructed biological materials

Design characteristics	Advantages	Disadvantages
Cross-linking	<ol style="list-style-type: none"> <li>1. Stability<sup>a</sup></li> <li>2. Increases matrix longevity in the wound area</li> </ol>	<ol style="list-style-type: none"> <li>1. Foreign body response Less split-skin graft take rate More contraction and HS risk</li> <li>2. Increased matrix rigidity</li> <li>3. Reduced cell viability</li> </ol>
GAGs	<ol style="list-style-type: none"> <li>1. Increase collagen resistance to collagenases</li> </ol> <p>Avoid excessive cross-linking</p>	<ol style="list-style-type: none"> <li>1. Many GAGs (as chondroitin-6-sulphate) are anti-angiogenic</li> </ol>

*Abbreviations:* HS hypertrophic scarring, GAGs glycosaminoglycans

<sup>a</sup>Stability of collagen dermal substitutes can also be increased adding fibronectin, hyaluronic acid or elastin

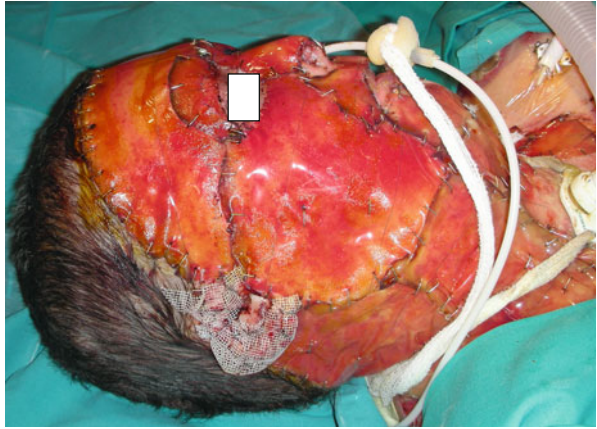
## Integra®

Integra® (Integra Life Science Corporation, Plainsboro, NJ, USA) artificial skin is currently the most widely accepted artificial skin substitute (Jones et al. 2002), for acute deep partial-thickness and full-thickness burns and for burn reconstruction (Burke et al. 1981; Dantzer et al. 2003). Furthermore, Integra® has also been used for chronic non-healing wounds and for reconstruction of cutaneous lesions, even with bone exposure –as a second treatment strategy after a flap – and to fill soft tissue defects in reconstructive and aesthetic surgery (Gottlieb and Furman 2004; Muangman et al. 2006; Chalmers et al. 2010; Murray et al. 2011). It was introduced in 1981 by Yannas and Burke (Yannas and Burke 1980; Yannas et al. 1980; Shores et al. 2007; Burke et al. 1981; Clark et al. 2007), who were the pioneers in developing an acellular, artificial skin substitute (Krejci and McGuire 1992). Integra is currently approved by the FDA for clinical use in burns and for elective unstable scar replacement (Akguner et al. 1998; Clark et al. 2007).

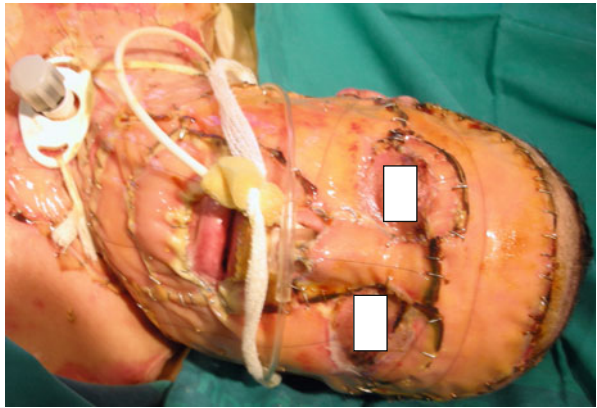
Integra® has a bilayer (original and classical form) and a single layer (IntegraSL®, the newer and more expensive one) form. Integra® is composed of a cross-linked bovine tendon collagen-based dermal analogue layer or matrix with glycosaminoglycans (GAG) – specifically, chondroitin-6-sulphate from shark cartilage – and no cells (Shores et al. 2007; Bello et al. 2001; Jones et al. 2002; Nguyen et al. 2006; Ruszczak 2003). The bilayer form also has an outer layer or membrane, composed of silicone (synthetic polysiloxane polymer or silastic) (Ruszczak 2003), 0.1 mm thick (Jaksic and Burke 1987), which works as a temporary epidermis and serves to control moisture loss from the wound (Akguner et al. 1998; Nguyen et al. 2006; Sheridan et al. 1994; Lee 2000). In fact, water flux across this silicone membrane is the same as that across normal epidermis, 0.5 ml/cm<sup>2</sup>/h (Yannas and Burke 1980). This superficial silicone layer is imbedded with monofilament nylon sutures to easily distinguish it from the collagen dermal layer (Shores et al. 2007). The dermal layer contains many pores, and it is manufactured with a defined degradation rate and controlled porosity (Machens et al. 2000). An appropriate pore size is critical to allow the optimal ingrowth of patients' own fibroblasts and endothelial cells and is carefully controlled at 50 +20 µm (Sheridan et al. 1994) or 70–200 µm (Jones et al. 2002). Larger pores prevent the cell attachment process and smaller pores delay bio-integration (Jones et al. 2002). Freeze-drying procedures are used to control pore size (van der Veen et al. 2010).

The collagen dermal replacement layer serves as a matrix for the infiltration of fibroblasts, macrophages, lymphocytes and capillaries derived from the wound bed. As the healing progresses, an endogenous collagen matrix is deposited by fibroblasts; simultaneously, the dermal layer of the artificial dermis is degraded, usually in approximately 30 days (Sheridan et al. 1994; Nanchatal et al. 2002). The degradation rate of the collagen-GAG sponge is controlled by glutaraldehyde-induced cross-links (Ruszczak 2003; Sheridan et al. 1994). Upon adequate vascularisation of the dermal layer and availability of donor autograft tissue, usually in 21 days (although it has also been described in only 10–14 (Sheridan et al. 1994) or even up to 8 weeks (Shores et al. 2007; Jones et al. 2002)), the temporary silicone layer is removed and an ultrathin, possibly meshed split-skin autograft is placed over the vascular “neodermis.” (Figs. 16.1–16.4).

**Fig. 16.1** Integra®: Full-thickness facial burn, already debrided and primarily covered with Integra®, 4 days after OR in a patient in the burn intensive care unit, with a full-thickness and deep-dermal 55 % TBSA burn. (Vall d'Hebron University Hospital, Barcelona, Spain; chair: Dr JP Barret)



**Fig. 16.2** Integra®: The same patient as in Fig. 16.1, but at 3 weeks after Integra® placement. It is ready to proceed to the second step, or surgical removal of the silicone Integra® layer and autografting. (Vall d'Hebron University Hospital, Barcelona, Spain; chair: Dr JP Barret)



**Fig. 16.3** Integra®: Integra® over left knee, distal thigh and proximal lower leg, after acute debridement of a 35 % TBSA full-thickness burn. (Vall d'Hebron University Hospital, Barcelona, Spain; chair: Dr JP Barret)





**Fig. 16.4** Integra®: The same patient as in Fig. 16.3, showing now bilateral lower limbs with Integra®, at 4 weeks after the first surgical step. Integra® adopts a yellowish-salmon “peach” colour, indicating it is time to perform the second step. (Vall d’Hebron University Hospital, Barcelona, Spain; chair: Dr JP Barret)



The main advantage of using Integra® is that it allows a neodermis to develop (Shores et al. 2007), and this is supposed to lead to improved scar cosmetic appearance and elasticity when compared to SSG alone (Nguyen et al. 2010), with good aesthetic and functional outcomes (Chalmers et al. 2010; Branski et al. 2007). Furthermore, it allows for the use of thinner grafts, resulting in more rapid healing of donor sites and decreased hospital stay (Fette 2005), and it allows vascularisation in poor recipient sites, such as bone or tendon (Muangman et al. 2006). Despite there having been described more benefits than harms, and Integra® is currently the most commonly used dermal skin substitute worldwide, a review of the literature demonstrates that there is still not enough scientific evidence (Nguyen et al. 2010; Fette 2005). Regarding Integra®’s design, shelf storage and immediate availability are also advantageous aspects (Fette 2005).

The main complication is collection of fluid under Integra® (hematoma, seroma), which increases susceptibility to infection (Bello et al. 2001; Nguyen et al. 2010). This can be prevented by careful surgical technique (Sheridan et al. 1994). As Integra® has no intrinsic antibacterial qualities, careful wound preparation with Betadine® wash and meticulous haemostasis is essential.

The scientific literature describes a proflavin wool tie-over dressing and prophylactic courses of oral flucloxacillin or erythromycin (if allergic to penicillins); Betadine® irrigation catheters above the Integra® sheet; Integra® washed in Diflucan, meropenem and vancomycin with an overlying Acticoat® dressing (Smith & Nephew, UK); and the use of V.A.C. NPD (Chalmers et al. 2010). Infection may be subtle and amenable to needle aspiration but again relies on regular dressing reviews and experience. Indeed, regular follow-up and wound assessment, as well as continuity of care by a core specialist team, is vital in ensuring optimum outcomes (Chalmers et al. 2010).

A minor disadvantage is separation of the silastic membrane at the periphery of the material, which is considered a not so serious problem (Sheridan et al. 1994). Other disadvantages include the relatively high cost, relative difficulty of use (with the need for sequential operative procedures) and the risk of (animal) virally transmitted diseases.

**Table 16.3** Advantages and disadvantages of Integra® (Muangman et al. 2006; Fette 2005)

## Advantages

1. No immune reaction
2. No histological harm
3. Thinner epidermal grafts and smaller mesh possible
4. Better aesthetic and functional outcomes (less itching, less hypertrophic scarring rates, better movements)
5. Immediate availability
6. Prolonged shelf time; off-the-shelf product
7. Capable of vascularising over exposed bone and tendon

## Disadvantages

1. Collection fluid risk (hematoma, infection, seroma)
2. Virus and prion-transmission risk
3. 2-step operational procedures (in the bilayer or classical form)
4. High cost
5. Steep learning curve
6. Inability to replace both dermal and epidermal components.
7. Still weak scientific evidence\*

\*Integra functional and cosmetic results generally reported as positive, but there is still need of multicentric and large clinical trials to strengthen scientific evidence

Social, cultural and ethical problems may arise as well, because of animal tissue components (Fette 2005). Furthermore, Integra® must be avoided in patients that have developed allergic reactions to bovine products (Clark et al. 2007).

In fact, currently Integra® – as the rest of the dermal substitutes – has the common disadvantage of being unable to replace the dermal and epidermal layer simultaneously, due to the lack of a real and permanent epidermal component (Bello et al. 2001). Experimental trials with keratinocytes are ongoing (Fette 2005) (Table 16.3).

**Matriderm®**

Matriderm® (Skin and Health Care AG, Billerbeck, Germany) is a multiporous membrane from bovine origin, composed of collagen (types I, III and V) and a hydrolysate of elastin-alpha, treated with gamma rays (Shores et al. 2007). Its function is to enhance skin elasticity and improve the resulting scar quality in wounds, especially in the case of burns (Haslik et al. 2010; Ryssel et al. 2008; Schneider et al. 2009; Bloemen et al. 2010; Rnjak et al. 2011).

As with Integra®, Matriderm® is utilised for dermal regeneration, and its indications are similar. It also has two presentation forms, 2 and 1 mm thick, requiring two-step and a single-stage procedure, respectively. In contrast to current most used Integra®, Matriderm® is usually used in single-stage procedures (Bloemen et al. 2003). Classical or bilayer Integra® contains chondroitin-6-sulphate, which has anti-angiogenic properties, in contrast to elastin. This explains why Integra® requires up to 3 weeks to become fully vascularised, in contrast to Matriderm®, which can sustain a split-skin graft when applied in a one-step procedure.

Matriderm® serves to treat soft tissue defects, full-thickness or deep dermal burns, and chronic wounds, but especially those located in cosmetic and functional

anatomically relevant areas, such as hands or joints (Haslik et al. 2010), and it is especially useful in the paediatric population in general, to enhance scar quality. Matriderm® has been shown to accommodate immediate split-thickness skin grafting with no diminished take (Kolokythas et al. 2008) and turned out to be feasible for use in critical-care patients in a one-stage procedure (Haslik et al. 2010).

The matrix serves as a support structure for the ingrowth of cells and vessels; its elastin component improves the stability and elasticity of the regenerating tissue. In fact, elastin-based dermal substitutes have the potential to decrease wound contraction and improve scar appearance and functionality (Rnjak et al. 2011).

As the healing process advances, fibroblasts deposit native extracellular matrix and the Matriderm® resorbs (Ryssel et al. 2008). Schneider et al. (2009) compared the engraftment rate and vascularisation of Matriderm® and Integra® in a rat model, finding no major differences between the two different types of dermal substitutes (Schneider et al. 2009).

In experimental models, the matrix composition of Matriderm® reduces wound contracture. In a porcine full-thickness wound model, elastin-coated collagen matrices reduced granulation tissue formation, fibrosis, and contraction and stimulated collagen deposition by fibroblasts (van der Veen et al. 2010). Clinical trials with a long-term clinical evaluation showed no difference in scar elasticity between the described dermal substitute and split-thickness grafts alone (Kolokythas et al. 2008). However, there is still lack of clinical data on the development of wound contracture and weak scientific evidence.

### **Hyalomatrix®/Hyalograft 3D®**

Hyalomatrix® (*Anika Therapeutics, former Fidia Advanced Biopolymers, Padua, Italy*) is a bilayer, sterified hyaluronic acid (Hyaff) matrix or scaffold with an outer silicone membrane (Halim et al. 2010). The scaffold delivers hyaluronan to the wound bed, allowing cellular invasion and capillary growth, being promptly colonised by fibroblasts and ECM components (Gravante et al. 2007), and is biodegradable. The silicone membrane acts as a temporary epidermal barrier (Myers et al. 2007). Hyalomatrix® allows wound closure via spontaneous re-epithelialisation and can also act as a suitable dermal layer for skin grafting (<http://www.anikatherapeutics.com/products/dermal/hyalomatrix.html>). It is indicated in traumatic, burn and chronic wounds (all types of ulcers, draining wounds, donor sites/grfts, etc.) ([http://www.accessdata.fda.gov/cdrh\\_docs/pdf7/K073251.pdf](http://www.accessdata.fda.gov/cdrh_docs/pdf7/K073251.pdf)).

Hyalograft-3D® is a variant of the product, which incorporates autologous fibroblasts. Hyalograft-3D is not currently available for sale in the USA (<http://www.anikatherapeutics.com/products/dermal/hyalomatrix.html>).

## **16.4.2 Synthetic Materials**

### **16.4.2.1 Dermagraft®**

Dermagraft® (Advanced BioHealing, LaJolla, CA, USA) is a bioabsorbable polyglactin (vicryl) mesh seeded with cryopreserved neonatal allogeneic foreskin fibroblasts (Hansen et al. 2001; Clark et al. 2007), using the Cooper method (Cooper et al. 1991).

Dermagraft® does not contain macrophages, lymphocytes, blood vessels or hair follicles (Ruszczak 2003). This fibroblast collagen matrix can be used alone, or as a base for meshed split-thickness skin autografts or possibly epidermal cultures (Phillips 1993), and as a temporary or permanent covering on excised burn wounds (Hansen et al. 2001; Hansbrough et al. 1997). Indications for the usage of Dermagraft® are in burn wounds, chronic wounds and diabetic ulcers (van der Veen et al. 2010). It is advised that Dermagraft® should be used in patients that have adequate blood supply (Ruszczak 2003). In the case of diabetic ulcers, controversial results are encountered in the literature. In fact, Dermagraft® was primarily marketed for stimulating the healing of chronic lesions, rather than for closing burn wounds (Jones et al. 2002).

Dermagraft® appears to produce results as good as allograft with regard to wound infection, wound exudate, wound healing time, wound closure and graft take. It was also reported to be more easily removed than allograft, with significantly higher level of patient satisfaction (van der Veen et al. 2010; Purdue et al. 1997). There have been no adverse reactions to Dermagraft®, with no evidence of rejection, early deterioration or separation from wound (Hansbrough et al. 1997). There have so far been no remarkable safety issues regarding Dermagraft® (Purdue et al. 1997).

The advantages of this skin substitute include good resistance to tearing, ease of handling and lack of rejection (Hansbrough 1995).

ATS filed for bankruptcy in 2002 (Bouchle 2002) and was acquired by Smith and Nephew and was then closed. Although TransCyte® and Dermagraft® are currently off the market, these technologies have been licenced to Advanced BioHealing which was acquired by Shire in 2011, for further production and marketing to improve the product.

#### **16.4.2.2 TransCyte® (Dermagraft-TC®)**

TransCyte is a temporary, synthetic covering composed of a semipermeable silicone membrane and an extracellular matrix of newborn human dermal fibroblasts cultured on a porcine collagen-coated nylon mesh (Pham et al. 2007) (similar to a Biobrane® seeded with fibroblasts (Shores et al. 2007)).

It is a laboratory-grown temporary skin replacement (Bello et al. 2001), produced originally by Advanced Tissue Sciences Inc. (ATS, La Jolla, CA) (Clark et al. 2007), which was later acquired and transferred to Smith & Nephew, Largo, FL, USA (Ruszczak 2003). Human neonatal foreskin (allogeneic) fibroblasts are cultured and proliferate on an inner nylon mesh of fibres that are embedded in an outer silastic layer for 4–6 weeks (Clark et al. 2007; Bello et al. 2001), forming a dense cellular tissue which contains high levels of secreted human matrix proteins as well as multiple growth factors (Hansbrough 1995). The fibroblasts are rendered nonviable and disrupted by freezing, after a few weeks of being synthesising extracellular matrix products and growth factors, creating the final product. A multicenter, randomised, controlled, paired within patient study in 66 patients with 132 excised burn wounds showed TransCyte® to be as effective as human cadaver skin and was successful as a temporary wound coverage after excision of the eschar from burn wounds (Purdue 1997). TransCyte® is US FDA-approved for the treatment of burn wounds (it was the first human-based, bioengineered temporary skin substitute for the treatment of excised full-thickness and

**Table 16.4** Classification of dermal substitutes

Biological materials	Composition/number of layers	Thickness (mm)	Indications	Brand
<i>Natural biological materials</i>				
Alloderm	Acellular human cadaver dermis	0.79–3.3	Burn wounds, soft tissue replacement	LifeCell Corporation, Branchburg, NJ, USA
Glyaderm	Acellular human dermis	0.2–0.6	Full-thickness wounds	Euro Skin Bank, Beverwijk, Netherlands
Graftjacket	Acellular human dermis	1, 1.4 or 2	Chronic wounds, ligament repair, soft tissue replacement	Wright Medical Technology, Inc, Arlington, TN, USA
DermaMatrix	Acellular human dermis	0.2–1.7	Soft tissue replacement	Synthes, West Chester, PA, USA
AlloMax (formerly “NeoForm”)	Acellular human dermis (collagen + elastin)	0.8–1.8	Soft tissue repair	Bard Davol
Tiscover	Acellular human dermis with autologous fibroblasts	1–2	Chronic wounds	A-SKIN B.V., Amsterdam, Netherlands
SurgiMend	Acellular bovine dermis	0.4–1.54	Soft tissue reconstruction	TEI Biosciences, Boston, Mass
Strattice	Acellular porcine dermis	1.5–2	Soft tissue reconstruction	LifeCell, Branchburg, NJ
Permacol	Acellular porcine dermis	0.4 or 1.5	Full-thickness wounds	Covedien, Mansfield MSA
Xenoderm	Acellular porcine dermis	0.5–3	Full-thickness wounds	MBP (Asclepios Medizintechnik), Neustadt, Germany
Oasis (burn matrix)	Porcine small intestine submucosa acellular collagen matrix	0.15–0.30	Burn and chronic wounds	Healthpoint Ltd, Fort Worth, TX, USA
<i>Artificial biological materials</i>				
Integra	Bi-layer: 1. Human collagen I with GAG 2. Silicone (There is a single layer Integra with no silicone)	1.3	Burn wounds, chronic wounds, soft tissue defects	Integra life Sciences Corp, Plainsboro, NJ, USA

(continued)

Table 16.4 (continued)

Biological materials	Composition/number of layers	Thickness (mm)	Indications	Brand
Matriderm	Bovine collagen I+elastin	1 or 2 (2 forms)	Burn and chronic wounds	Skin and Health Care AG, Billerbeck, Germany
Terudermis	1. Calf collagen 2. Polyester mesh 3. Silicone (There is a monolayer type with only collagen)	3	Burn and other traumatic and mucosal defects	Olympus Terumo Biomaterials Corp.
Hyalomatrix	Hyaluronan-based scaffold with autologous fibroblasts	1.2	Burn and chronic wounds	Fidia
TransCyte	1. Collagen with neonatal fibroblasts 2. Nylon mesh 3. Silicone		Burns	Shire Regenerative Medicine, Inc., San Diego, California, USA
Apligraf <sup>®</sup>	1. Bovine collagen I gel with allogeneic neonatal fibroblasts +	0.4–0.75	Burn and chronic wounds, skin graft donor sites, epidermolysis bullosa	Organogenesis, Canton, MA, USA
Oreel (Formerly "Composite Cultured Skin")	2. neonatal keratinocytes Collagen I sponge + gel with allogeneic fibroblasts and keratinocytes	1	Chronic wounds, skin graft donor sites	Forticell Bioscience
Renoskin	Bovine collagen I with GAG	1.5–2.5	Burn wounds, tissue defects	Perouse Plastique
<i>Synthetic materials</i>				
Dermagraft	Polyglactin mesh + allogeneic fibroblasts	0.19	Burn and chronic wounds, diabetic ulcers	Shire Regenerative Medicine, Inc., San Diego, California, USA
Polyactive	Polyethylen oxide + polybutylterephthalate	0.25	Bone, cartilage repair	Octoplus Inc.

Note: None of the engineered skin substitutes containing living cells (even those approved in the USA, like Apligraf<sup>®</sup>, TransCyte<sup>®</sup>, Dermagraft<sup>®</sup> and OrCell<sup>®</sup>) have been approved in Europe (Ruszczak 2003)

<sup>a</sup>It is a bilayered cell therapy or composite skin substitute, not only a dermal substitute

partial-thickness burns approved by the US FDA in 1997 (Ruszczak 2003)), and advantages include immediate availability, ease of storage (Bello et al. 2001) (it must be stored frozen between  $-70$  and  $-20$  °C and defrosted directly before use) and direct visual monitoring of the wound bed, because it is transparent (Ruszczak 2003).

It has been indicated for use as a temporary covering for excised burns prior to autografting or burns that do not require autografting (partial-thickness burns). TransCyte® is applied to the burn and will peel away as the burn heals (Pham et al. 2007), or it must be removed or excised prior to grafting full-thickness wounds (Table 16.4).

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## 16.5 Final Remarks

Dermal skin substitutes have become an important part of acute and long-term burn care (Truong et al. 2005). However, at present there is still no ideal dermal substitute which completely mimics the morphology and functions of skin. Furthermore, although the literature describes better cosmetic and functional outcomes with their use in burns, scientific evidence is still scarce, and further research is warranted. Indeed, regenerative medicine and the application of tissue engineering to design appropriate scaffolds to feed viable stem cells appears to be the future of dermal and skin substitutes in general.

Finally, interdisciplinary cooperation between basic researchers, engineers, clinicians, and surgeons is mandatory to develop artificial skin and therefore contribute to reduced morbidity and mortality, as well as to improve quality of life, in burn patients.

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Lars-Peter Kamolz

The skin is the largest organ, consisting of different layers. Loss of skin integrity and skin functions due to injury or illness may acutely result in a substantial physiologic imbalance with long-term morbidity or even death (Williams et al. 2009; Kamolz 2010). The most common cause of severe skin loss is thermal injury. Over the past decades, extraordinary advances have been made in the understanding of cellular and molecular processes of wound healing. This knowledge has led to wound care innovations and new developments in burn care, having even improved survival of severe burn injuries. The trend in current treatment regimens is beyond the preservation of life; the ultimate goal is to turn burn victims back into society, as full participants into their families and communities.

One of the milestones resulting in improved outcome was a more aggressive surgical approach to burn injuries; early wound debridement and early wound coverage has led to higher survival rates but also to a higher number of patients requiring post-burn reconstruction.

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## 17.1 From the Reconstructive Ladder to the Reconstructive Clockwork

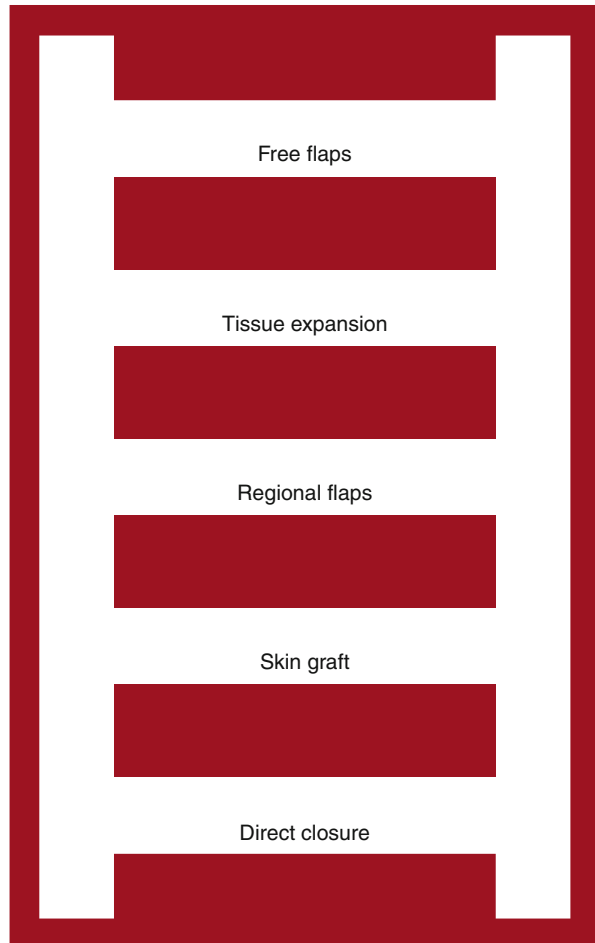
Mathes and Nahai have used the metaphor of the reconstructive ladder in 1982 in their book: *Clinical Application for Muscle and Musculocutaneous Flaps*. They used the term “reconstructive ladder” in order to integrate the use of free flap into the reconstructive repertoire (Mathes et al. 1982). The ladder reflected the idea of a stepwise approach to treat tissue defects starting with direct closure followed by

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L.-P. Kamolz, MD, MSc  
Division of Plastic, Aesthetic and Reconstructive Surgery,  
Research Unit for Tissue Regeneration, Repair and Reconstruction,  
Department of Surgery, Medical University of Graz, Graz, Austria  
e-mail: lars.kamolz@medunigraz.at

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**Fig. 17.1** The reconstructive ladder



skin grafting. Regional and local pedicled flaps, tissue expansion, and free tissue transfer were the next steps. This approach offered reconstructive surgeons a structured, thorough, and comprehensive algorithm for the treatment of wounds Fig. 17.1.

Nevertheless, a considerable medicine-technological progress has taken place. New areas like “composite tissue allotransplantation” of compound tissues like arms or parts of the face, robotics/bionics, and also regenerative medicine with “tissue engineering” have become part of a routine clinical application, not being mentioned in the conventional reconstructive ladder.

With the advancement in the understanding of the anatomy, operative techniques, instrumentation, and surgical skills, complex procedures are no longer considered as last resort procedures only. In the quest to reconstruct optimal form and function,

it is accepted to jump several rungs of the ladder, with the knowledge that some defects require more complex solutions. The reconstructive elevator (Gottlieb and Krieger 1994) allows one to ascend from the simplest to the more complex techniques, with the freedom to ascend directly to the chosen level of complexity. The decisions will be based upon the needs of the patient, in combination with the knowledge, experience, and technical ability of the surgeon and the multidisciplinary team. This elevator will ensure the use of the most appropriate surgical option necessary to reconstruct a defect, resulting in optimal restoration of form and function for the patient. The elevator acknowledges changes that have been made in plastic surgery. Many of these changes have occurred in recent decades; as with experience, they have become safe and reliable techniques, no longer considered a last resort. Or citing a headline from the journal “Plastic Reconstructive Surgery”: “Why climb a ladder when you can take the elevator?” (Bennet and Choudhary 2000).

Recalling these new developments, we suggest that the reconstructive sequence of the twenty-first century takes these developments into account. Neither the reconstructive ladders of Mathes and Nahai in 1982 nor the reconstructive elevator permits a real combination of the different reconstructive procedures. Often, combinations are applied in clinical daily routine, so the combination of new possibilities and methods will permit more and improved reconstructive possibilities for the patient.

The image of interlocking wheels in a clockwork (Knobloch and Vogt 2010) (Fig. 17.2) impressively illustrates the integration of different reconstructive methods, also with reference to their complexity.

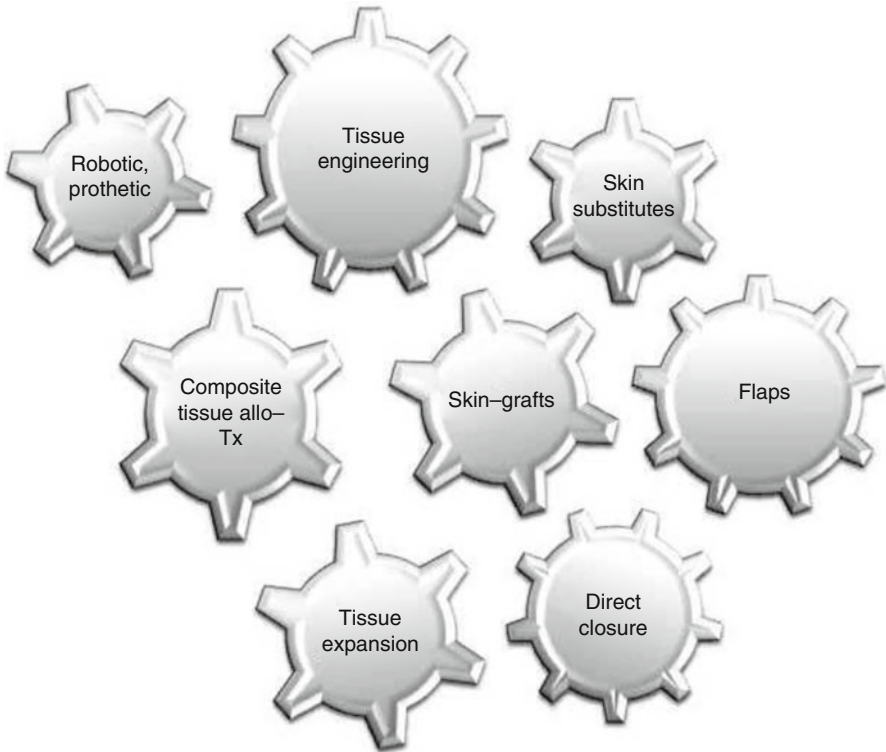
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## 17.2 General Principles

### 17.2.1 An Early Treatment of Deformity

Hypertrophic scars and scar contraction with concomitant functional impairment (i.e., of the eyelids, neck, axilla, elbow, hand, groin, knee, and foot) are two predominant problems following wound healing. The treatment regimens of applying pressure on scar tissues and the use of early mobilization of joints have been advocated to minimize their undesirable consequences.

Although the true efficacy of a nonsurgical regimen to control the deformities has not been established, the frequency of secondary joint release among individuals who had adequate therapy has been noted (Huang et al. 1975; Celis et al. 2003; Kamolz et al. 2009; Brou et al. 1989). The use of pressure garments, especially in the areas such as upper and lower limbs, with proper splinting of the hand and fingers (Kamolz et al. 2009), is strongly recommended early after trauma. The nonsurgical management of burn deformity must include daily physiotherapy and exercise to maintain joint mobility and to prevent muscle wasting.



**Fig. 17.2** The reconstructive clockwork

## 17.3 Reconstruction of Burn Deformities

Objective assessment of deformities and functional problems caused by scarring and scar contracture requires a detailed understanding of the extent of the original injury and a precise treatment approach to manage burn wounds. Formulating a realistic plan to restore physical problems and to alleviate pain and discomfort in the area of injury also needs a profound analysis of the physical deformities and psychological disturbance sustained by the patient. Psychiatric, psychosocial (Titscher et al. 2010), and physiotherapeutic care must be continued while a surgical treatment plan is instituted.

### 17.3.1 Indication and Timing of Surgical Intervention

For a surgeon, making a decision *how* to operate on a patient with burn deformities is quite simple. In contrast, deciding *when and where* to operate on a patient with burn deformities can be difficult. The dilemma, however, may be alleviated, if a basic principle is understood and followed:

- Restoring bodily deformities that impose functional difficulties must precede any surgical effort to restore the appearance.

In short, a surgeon's effort must concentrate on restoring the deformed bodily parts essential for physical functions, if not for patient survival alone. An exposed skull or calvarial defect, contracted eyelids, constricted nares, contracted major joints, and a urethral and/or anal stricture in individuals with severe perineal burns are the prime indications for an early surgical correction. In contrast, restoration of deformed regions in general can be performed in a later phase.

It is advocated that attempts to correct burn deformities should be delayed for at least 1–2 years (the time needed for scar maturation). During the interim, conservative treatment by using pressure garments and splinting is recommended to reduce scarring and to minimize joint contracture.

The 1- to 2-year moratorium on early burn reconstruction, in some instances, is appropriate and justifiable. Operating on an immature scar characterized by redness and induration is technically more cumbersome and will lead to a higher number of complications; e.g., a high rate of recontracture is expected, notably if partial-thickness skin grafts are used for releasing a wound still showing clinical evidence of active inflammatory processes.

### 17.3.2 The Techniques of Reconstruction

There are several techniques (Fig. 17.2) routinely used to reconstruct bodily deformities and to close defects in burn injuries, i.e., unsightly scars, scar and joint contractures:

- Direct closure techniques
- Skin grafting
- Skin grafting in combination with a dermal substitute
- Local and regional flaps
- Free flaps
- Tissue expansion techniques in combination with the above
- Composite tissue allotransplantation
- Tissue-engineered substitutes
- Bionics and prostheses

#### 17.3.2.1 Direct Closure

Scar excision with layered closure of the resultant wound is the simplest and most direct approach in burn reconstruction. The margins of the scar requiring excision are marked. It is important to determine the amount of scar tissue that can be removed so that the resultant defect can be closed directly.

#### 17.3.2.2 Skin Grafting

##### A Skin Graft Without the Combination of a Dermal Substitute

Covering an open wound with a skin graft harvested at a various thickness is the conventional approach of wound closure. Whole components of the skin removed



**Fig. 17.3** (a) Scar contracture and hypertrophic scars right arm. (b) Scar release and scar excision; wound bed already covered with the collagen/elastin matrix Matriderm(R); in the same operation coverage of the matrix with a split-thickness skin graft. (c) Fixation of the matrix and of the skin graft by use of V.A.C.<sup>TM</sup>. (d) Five days after the operation → removal of the V.A.C.<sup>TM</sup> dressing. 100 % take rate of Matriderm(R) and skin graft. (e) Early result after single-step reconstruction of dermis and epidermis

as an intact unit – i.e., epidermis and dermis – are defined as a full-thickness skin graft, and a piece of skin cut at a thickness varying between 8/1,000 of an inch (0.196 mm) and 18/1,000 of an inch (0.441 mm) is considered to be a partial- or a split-thickness skin graft. The thickness of a full-thickness skin graft is quite variable depending on the donor side region.

A paper template may be made to determine the size of the skin graft needed to close the defect. The skin graft is placed on the wound bed and anchored into place by suturing or stapling it to the wound edges. A continuous contact of the skin graft to the wound bed is essential to ensure an ingrowth of a vascular network in the graft within 3–5 days for its survival, well noting that any mechanical barriers – i.e., blood clot or pool of serous or purulent fluid – can restrain the vascularizing processes, leading to a graft loss. A gauze or cotton bolster tie-over dressing has been the traditional technique to anchor and to prevent fluid accumulating underneath a graft, while also other techniques exist (Mittermayr et al. 2006; Pallua et al. 2010; Roka et al. 2007). The use of negative pressure therapy or fibrin glue has been associated with a better take rate (Mittermayr et al. 2006) (Fig. 17.3).

The basis for using a skin graft of various thicknesses is not entirely clear. The use of a thin graft is appropriate for closing wounds with unstable vascular supplies, particularly if donor sites are scarce. The quality and the presence of dermis have influence on the extent of wound contraction with the more viable dermal components present the less graft contraction observed.

### Skin Graft in Combination with a Dermal Substitute

For the past several years, artificial dermal substitutes have been manufactured from alloplastic or xenographic materials, e.g., Alloderm<sup>TM</sup> and Integra<sup>TM</sup> (Nguyen et al. 2010), which have been found to form a layer of “neo-dermis,” thus providing improved dermal thickness and wound coverage in combination with an autologous





**Fig. 17.4** (a) Hypertrophic and unstable scar in the popliteal region. (b) Scar excision and grafting with Matriderm™. (c) In the same operation skin grafting (Mesh 1:1, 5). (d) Early result. (e) Longtime result (1 year after the operation) after combined reconstruction of dermis and epidermis in a single-step procedure

skin graft. While a staged approach with two required operations is more cumbersome to the patient, single-step matrices, like Matriderm™ (based on collagen and elastin), have become available (Haslik et al. 2007, 2010; Bloemen et al. 2010) (Figs. 17.3, 17.4, and 17.5).

The simultaneous use of Matriderm™ and split-thickness skin grafting is considered safe and feasible and leads to significantly improved results with respect to skin elasticity and range of motion (Haslik et al. 2007, 2010; Bloemen et al. 2010; Ryssel et al. 2010).

### 17.3.2.3 Skin Flap

The approach using a segment of skin with its intrinsic structural components attached to restore a destroyed and/or missed bodily part follows the fundamental principle of reconstructive surgery. The loss of the skin flap, more commonly encountered in burn patients because of altered vascular supply to the skin attributable to injuries and surgical treatment, could render this technique unsuitable. Despite

the drawbacks, the approach to restore a destructed bodily part with a piece of like tissue is technically sound and the procedure can provide restored bodily function and contour. The recent technical innovation of incorporating a muscle and/or facial layer in the skin flap design, especially in a burned area, further expanded the scope



**Fig. 17.5** (a) Hypertrophic scars and scar contracture at the dorsum of the right hand → hyperextension of the MCP joints → massive functional impairment. (b) Scar excision. (c) Longtime result (1 year after the operation) after combined reconstruction (Matriderm(R) and split-thickness skin graft) in a single-step procedure → no functional impairment → full range of motion achieved



**Fig. 17.5** (continued)

of burn reconstruction as more burned tissues could be used for flap fabrication. Despite its geometric advantage in flap design, fabricating a skin flap or skin flaps for z-plasty reconstruction burn deformities is not infrequently complicated by skin necrosis. Aberrant vascular supply to the skin attributable to the original injury and/or surgical treatment could be the factor responsible for these problems. In recent years, the use of a skin flap designed to include muscle or fascia underneath has expanded further the applicability of conventional z-plasty and the 3/4 z-plasty technique in burn reconstruction.

#### **17.3.2.4 Musculocutaneous (MC) or Fasciocutaneous (FC) Flap**

##### **Musculocutaneous z-Plasty**

While the skin pattern is identical to the conventional z-plasty technique, the muscle underneath must be included in the flap fabrication. Although physical characteristics of the normal skin – i.e., the skin pliability and expandability – are absent if scarred skin is included in the flap design, a “scarred-skin” MC or FC flap could be safely elevated and transferred to close an open wound. In practice, an MC z-plasty technique is useful in the neck release and in the eyelid because of the underlying muscle; i.e., platysma and orbicularis oculi muscles are thin, pliable, and easily movable.

##### **Fasciocutaneous z-Plasty**

This is a technical modification of the MC z-plasty technique by including the muscular fascia only. Separation of the skin and its subcutaneous tissues from the underlying fascia must be avoided in order not to impair the blood supply to the flap. In practice, the technique is useful in reconstructing contractures around the knee and ankle.

### 17.3.2.5 Tissue Expansion

Indications for the use of tissue expanders in burn reconstructions are instances, where there is not enough adjacent tissue to resurface or close a defect primarily or with a local flap. The same criteria used to select a suitable patient for a regional skin flap are applicable in the selection for tissue expanded skin or flap reconstruction. Ideally, the patient should have no serious medical problems (e.g., diabetes, hypertension) and should not be a heavy smoker.

Tissue expansion allows large areas of burn scar to be resurfaced by providing tissue of similar texture and color to the defect. It is combined with the advantage of reduced donor site morbidity. Issues and disadvantages that need to be addressed are that the technique of preexpansion requires additional office visits for serial expansion and at least one extra surgical procedure with potential for additional complications. A significant period between 9 and 12 weeks for progressive tissue expansion is required. Tissue expanders are very versatile tools in reconstructive burn surgery, but careful patient selection, correct indications and realistic treatment concepts, experience and well-chosen surgical technique, precise instruction of the medical staff, as well as detailed and continuous education of the patients are essential (Bozkurt et al. 2008).

### 17.3.2.6 Free Tissue Transfer

The use of free flaps was an important step to cover large and complex tissue defects due to trauma, tumor, or hereditary. After the description of the pedicled *M. latissimus dorsi* transfers by Tansini more than 100 years ago, the free transplantation of the *M. latissimus dorsi* also took place for defect coverage several decades ago. Nahai has described in 1979 his experiences with 60 transplanted *M. latissimus dorsi* flaps for functional muscle transfer to the shoulder and for defects of the upper extremity, for the use of mamma reconstruction and as a free flap (Bostwick et al. 1979). Other free flaps adjacent to the thoracodorsal bundle were also introduced in the 80s.

#### Free Flap: Perforator Flaps

Based on the septocutaneous perforator vessels, the perforator flap was developed. Song et al. described in 1984 that the lateral femoral region can serve not only as a skin harvest place but also as the donor side for the “anterolateral thigh flap” based on a long pedicle. Koshima and colleagues from Japan refined the ALT-transfer subsequently. In 1989, Koshima introduced an abdominal skin and fat flap based on the inferior epigastric vessels and muscle perforators. Recently, the theory of the perforasomes is under evaluation: every perforator supplies a unique vasculatory territory, the perforasome (Saint-Cyr et al. 2009).

The surgical progress concerning perforator flap surgery is directly related to increasing surgical experience, and new technologies improve the preoperative visualization of the vessels, also allowing a reduced duration for perforator dissection.

With the advent of microsurgical techniques, transplanting a composite tissue can be carried out with minimal morbidities. The regimen, in caring for burn victims, however, may be limited because of a paucity of donor sites. It is ironic that burn patients with suitable donor sites seldom require such an elaborate treatment,

but those, who are in need of microsurgical tissue transplantation, are inevitably without appropriate donor sites because of extensive tissue destruction.

### **17.3.2.7 Composite Tissue Allotransplantation**

“Composite tissue allotransplantation” (CTA) of parts of the face, or forearms and upper extremities (Brandacher et al. 2009; Siemionow et al. 2010; Gordon et al. 2009; Siemionow and Gordon 2010), is a young area of transplantation medicine. The first clinical results are promising in comparison to the first reports of the organ transplantation, although the medium-term and long-term problems, for example, tumor induction by the immunosuppression as well as the chronic rejection, have to be taken into account. This is not an unimportant fact, because CTA is normally not of vital importance. Nevertheless, for the affected individuals, who forced into social isolation with exhausted reconstructive measures or prostheses, such operations may lead to a dramatic improvement of quality of life. However, it is important to mention that, currently, only a highly selected small number of suitable patients are actual candidates for CTA.

### **17.3.2.8 Robotics/Bionics/Prostheses**

If all reconstructive measures fail, myoelectric prostheses are a promising option. This technique has improved tremendously by introducing targeted muscle transfers (TMR) to the armamentarium of reconstructive surgery (Aszmann et al. 2008; Hijjawi et al. 2006). Modern myoelectric prostheses have multiple degrees of freedom that mandate a complex control system to provide dependable use for the patient. Extremity reconstruction in the twenty-first century will see many new avenues to replace the loss of a limb and reconstruct the loss of function. Both biological and technical advances will provide possibilities that may well open up therapies that have been deemed unthinkable only a few years ago. Targeted muscle reinnervation in combination with a myoelectric prosthesis with several degrees of freedom is such an example and is a solid stepping stone leading to new strategies in extremity reconstruction and rehabilitation.

### **17.3.2.9 Regeneration: Tissue Engineering**

Tissue regeneration and engineering have gained more relevance in reconstructive surgery (Kamolz et al. 2008; Beier et al. 2010; Mansbridge 2009), with autologous fat transplantation being one of them. Czerny transplanted a lipoma for breast reconstruction in 1895. The fat injection was described among other inventions by Eugene Holländer in 1910 in a patient with “progressive decrease of the fatty tissue.” Erich Lexer dedicated the first part of his book to free fat transfers, comprising of nearly 300 pages. In 2001, it was demonstrated that beside fat cells also “adipose-derived stem cells” (ADSC) and other cell populations in the fatty tissue are usable for these purposes. The transplantation of ADSC was able to regenerate full-layered cartilage defects in the animal model (Dragoo et al. 2007). The stem cell-associated fat cell transplantation in patients with damaged skin following radiotherapy has led to healing. Moreover, fat cell transplantation is not only able to improve volume and contour defects but also skin quality (Klinger et al. 2008; Mojallal et al. 2009;

Rennekampff et al. 2010); thereby, it seems that fat transfer will play an important part in burn reconstruction.

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## 17.4 Summary

The regimen of burn treatment has changed dramatically over the past 50 years. The regimen of an early debridement and wound coverage has enhanced the survival rate. However, this improvement in survival rate has also caused an increase of patients who will require reconstructive surgery.

The difficulty concerning burn reconstruction is largely due to a lack of adequate donor sites, but due to the improvements in reconstructive surgical techniques, better results have become achievable. New areas like “composite tissue allotransplantation” of compound tissues and regenerative medicine approaches have become available in clinical routine and will lead to an improved final outcome in burn reconstruction.

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