Induced Regeneration of Skin and Peripheral Nerves in the Adult

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Abstract

Injury to the mammalian fetus is reversible during early stages of gestation and the spontaneous wound response is capable of restoring the structure and function of the original organ, a process called *regeneration*. By contrast, the unimpaired response to severe injury in adult mammals is an irreversible *repair* process leading to closure of the injured site by contraction and formation of scar, a nonphysiological tissue. The consequences of irreversible healing at the organ scale are far-reaching: they typically result in an essentially nonfunctional organ.

Numerous approaches have been investigated to restore the loss of organ function in adults following irreversible injury. These strategies include transplantation, autografting, implantation of permanent prostheses, the use of stem cells, in vitro synthesis of the organ, and regenerative medicine (Yannas, Tissue and organ regeneration in adults. Springer; 2001). The last of these strategies is also referred to as *induced organ regeneration*, or the recovery of physiological structure and function of nonregenerative tissues in an organ (also known as de novo synthesis) by use of elementary reactants, such as biologically active scaffolds, either unseeded or seeded with cells.

There is accumulating evidence that the spontaneous healing process of an injured organ in the adult mammal can be modified to yield a partially or completely regenerated organ. Regenerative medicine is an emerging field of study involving the implantation of biomaterials to facilitate formation (regeneration) of tissue in vivo. This field is undergoing rapid growth at this time, as evidenced by observation of regeneration or reported progress in on-going research efforts in a wide range of organs including skin (Butler and Orgill, Adv Biochem Eng Biotechnol 94:23–41, 2005),

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conjunctiva (Hatton and Rubin, Adv Biochem Eng Biotechnol 94:125–140, 2005), peripheral nerves (Zhang and Yannas, Adv Biochem Eng Biotechnol 94:67–89, 2005), bone (Mistry and Mikos, Adv Biochem Eng Biotechnol 94: 1–22, 2005), heart valves (Rabkin-Aikawa et al., Adv Biochem Eng Biotechnol 94:141–178, 2005), liver (Takimoto et al., Cell Transplant 12(4):413–421, 2003) articular cartilage (Kinner et al., Adv Biochem Eng Biotechnol 94:91–123, 2005), urological organs (Atala, Adv Biochem Eng Biotechnol 94:179–208, 2005), and the spinal cord (Verma and Fawcett, Adv Biochem Eng Biotechnol. 94:43–66, 2005).

Keywords

Irreversible injury in skin and nerves • Repair • Skin regeneration • Regenerative similarity • The tissue triad • Anatomically well-defined defect • Synthetic protocol • In Vitro • In Vivo • Induced organ regeneration • Collagen-based scaffolds • Dermal regeneration template • Peripheral nerve regeneration • NeuraGen • Collagen-based scaffolds • Defect closure rule • Spontaneous healing • Contraction • Repair • Contractile fibroblast • Scaffold regeneration

Introduction

Injury to the mammalian fetus is reversible during early stages of gestation and the spontaneous wound response is capable of restoring the structure and function of the original organ, a process called *regeneration*. By contrast, the unimpaired response to severe injury in adult mammals is an irreversible *repair* process leading to closure of the injured site by contraction and formation of scar, a nonphysiological tissue. The consequences of irreversible healing at the organ scale are far-reaching: they typically result in an essentially nonfunctional organ.

Numerous approaches have been investigated to restore the loss of organ function in adults following irreversible injury. These strategies include transplantation, autografting, implantation of permanent prostheses, the use of stem cells, in vitro synthesis of the organ, and regenerative medicine [1]. The last of these strategies is also referred to as *induced organ regeneration*, or the recovery of physiological structure and function of nonregenerative tissues in an organ (also known as de novo synthesis) by use of elementary reactants, such as biologically active scaffolds, either unseeded or seeded with cells.

There is accumulating evidence that the spontaneous healing process of an injured organ in the adult mammal can be modified to yield a partially or completely regenerated organ. Regenerative medicine is an emerging field of study involving the implantation of biomaterials to facilitate formation (regeneration) of tissue in vivo. This field is undergoing rapid growth at this time, as evidenced by observation of regeneration or reported progress in on-going research efforts in a wide range of organs including skin [2], conjunctiva [3], peripheral nerves [4], bone [5], heart valves [6], liver [7], articular cartilage [8], urological organs [9], and the spinal cord [10].

The basic outline of a hypothetical mechanism for induced organ regeneration has become clear. It relies on regenerative studies in three organs (skin, conjunctiva, and peripheral nerves), which started much earlier and have progressed much further than research in other organs. From these studies a pattern has emerged, based on two observations: (a) regeneration was successfully induced, at least partially, when contraction was blocked, following grafting with a class of scaffolds that

were characterized by a very highly specific structure (collectively referred to as "regeneration templates") and (b) when a class of "inactive scaffolds" with slightly different properties than their biologically active counterparts was used, regeneration was thwarted and vigorous contraction ensued. The available data support the hypothesis of contraction blocking as a plausible mechanism for induced organ regeneration in the adult mammal. In almost all such processes, the critical reactant supplied by the investigators was a scaffold, a highly porous, degradable macromolecular solid that has a specific contraction-blocking activity as well as the ability to mimic the in vivo environment, and particularly the stroma, of the organ.

In this chapter, we present elements of a theory of induced regeneration that is organ nonspecific. We proceed by discussing, in order, the macroscopic outcome of irreversible healing in adults, the evidence for induced regeneration, the association between contraction blocking and regeneration, and a proposed mechanism for the regenerative activity of certain scaffolds.

Most regeneration data available to date comes from acute wound models that prove to be far more amenable to control by the investigator than chronic wound models. When healing is unimpaired, the adult mammalian spontaneous healing response to severe acute injury is contraction and scar synthesis. Nevertheless, the results of recent clinical studies indicate that the discussion in this chapter is relevant to severe chronic wounds. Increasingly, FDA-approved versions of collagen-based devices are demonstrating efficacy in the clinical management of these injuries [11–15]. Detailed indications for their use in the treatment of chronic wounds (classified by both anatomy and pathology/diagnosis) have been presented [16].

The majority of available induced regeneration data described in this chapter comes from skin [17–24] and peripheral nerve models [25–34]; these are the two organs that have been studied most extensively in this respect to date. Skin wounds can be studied with relative ease and for this reason studies of skin wound healing comprise the bulk of quantitative wound healing data in the literature. In organs other than skin, wound healing has been studied mostly qualitatively. Nevertheless, the observations made so far form a body of evidence that suggests certain strong similarities, as well as identifying differences, between wound healing in skin and in less studied organs, such as peripheral nerves. Taken together, the wealth of regenerative data for these two very different organs has aided the development of a general theory of induced regeneration that may have value in studies of induced regeneration in other organs as well [1].

Irreversible Injury in Skin and Nerves

The complex inflammatory response of the adult mammal to injury is elucidated by on-going research at the cellular and molecular level. While the formation of an accurate mechanistic perspective of wound healing is essential both in understanding the effect of current clinical treatment and in the development of emergent therapies, an examination of the macroscopic outcome of healing also provides a uniquely valuable viewpoint. An introductory phenomenological discussion of spontaneous wound healing at the tissue level provides a framework that forms a focus for future discussion of detailed cellular/molecular mechanisms and facilitates the derivation of concepts and rules of induced regeneration that may conceivably apply to almost any organ in the body.

Macroscopic Outcomes of Healing: Repair Versus Regeneration

When exposed to injury, in the form of either acute trauma or chronic insult, the organism mounts a spontaneous wound healing process that typically closes the discontinuity in organ mass caused by the injury in a matter of days. Two macroscopic outcomes to injury have been observed experimentally: regeneration and repair. These fundamentally different processes are clearly distinguished by the identity of tissue present in the final state, that is, the newly synthesized tissue that closes the injured site. In the

early mammalian fetus and in many species of amphibians, wound healing is largely reversible and proceeds via *spontaneous regeneration*, a process that restores the structure and physiological function through synthesis of the missing organ structures [1]. Certain adult urodeles exhibit an impressive capacity for spontaneous regeneration: replacement of an amputated appendage occurs by direct outgrowth of the severed cross section (epimorphic regeneration), a reversible process [35].

In clear contrast, severe injury to normal adult mammalian tissue typically results in an irreversible healing response. Spontaneous healing of severe skin wounds proceeds via *repair*, in which the wound closes with a combination of tissue deformation and translation (collectively referred to as contraction) and synthesis of a nonphysiological tissue (scar) in place of the normally functioning tissue that has been injured [1]. By replacing the lost organ mass with scar, the injured organ is condemned while the organism is spared as a result of the healing process. The immediate consequence of irreversible injury is a loss of normal organ function. On a broader scale, skin injury may have additional detrimental effects, such as loss of mobility and lack of social acceptance, e.g., following formation of disfiguring scars from burns. It appears that nearly every adult mammalian organ can be injured irreversibly and the extent of irreversibility seems to depend both on the identity of the tissue injured and the severity of the injury [1].

Regenerative Similarity: The Tissue Triad

Standard pathology texts describe three generic tissue types that comprise the majority of organs in the body: epithelia, basement membrane, and stroma [1, 36–38] (Fig. 9.1). Collectively, we will refer to these three tissue types as the tissue triad for a specific organ. This classification provides a useful framework for comparing the regenerative capacity of specific tissue types from one organ to another. The composition of each member of the triad is markedly different. Epithelial tissue forms a completely cellular covering on every surface, tube, and cavity in the body, performing a wide array of vital functions including protection, secretion, absorption, and filtration. As epithelial tissue is devoid of extracellular matrix (ECM) and blood vessels, it is sustained by the diffusion of nutrients from the underlying vascular connective tissue, or stroma. Epithelia are separated from underlying stroma by the basement membrane (basal lamina), a very thin, noncellular tissue layer, comprising exclusively ECM. The stroma is a connective tissue layer that is vascularized, containing both cells and ECM.

The skin, as one example, consists of the epidermis (epithelia) attached to the basement membrane and the underlying dermis (stroma). Considerable evidence from peripheral nerve studies indicates that Schwann cells function as epithelial cells following synthesis of a completely cellular layer (myelin sheath) around axons [39]. Nerve fibers (Schwann cell-axon units) are attached to a basement membrane that separates them from the outlying endoneurial stroma, a tissue consisting of a vascularized ECM. Further evidence for the epithelial nature of the myelin sheath comes from the observed polarity of Schwann cells which is very similar to that of keratinocytes, the epithelial cells that form the epidermis in skin. In each case, one epithelial cell surface is firmly attached to a basement membrane and another is part of the epithelial tissue, endowed in each case with function unique to the respective organ, that characterizes the epidermis (in the case of skin) or the nerve fiber insulation of peripheral nerves [39].

Tissues that are "regeneratively similar" appear in different organs yet share a common spontaneous healing response, be it regeneration or repair. The spontaneous healing behavior of each layer of the tissue triad in skin and peripheral nerves is well documented and will be briefly reviewed.

Provided the stroma is still intact to facilitate epithelial cell spreading, injury to the epithelial layer of either of the two organs (the epidermis in skin and myelin sheath in peripheral nerves, respectively) results in spontaneous regeneration of the injured tissue by remaining epithelial cells in the defect [1, 40–43]. Following nerve crushing

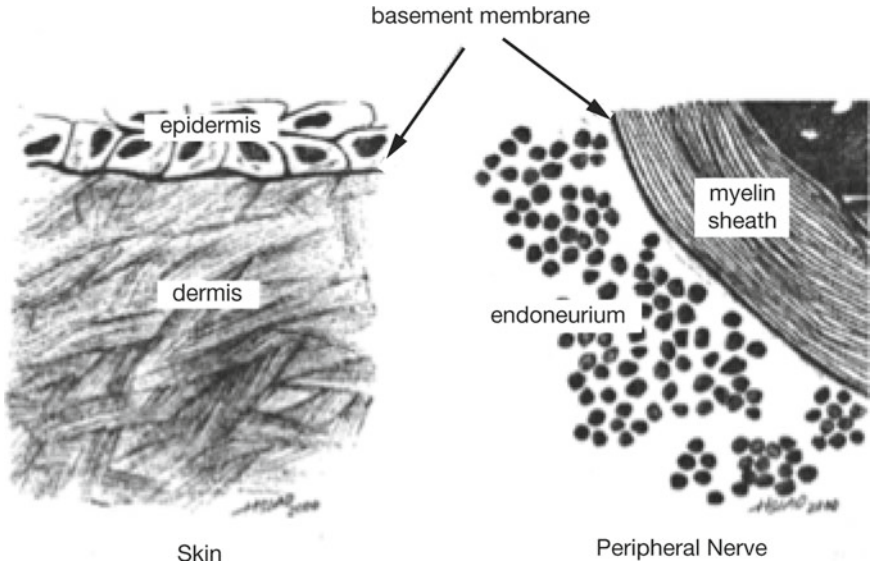

Fig. 9.1 The tissue triad structure in skin and peripheral nerves. The basement membrane (basal lamina), a thin noncellular layer consisting of extracellular matrix, separates the cellular, nonvascular epithelia (epidermis, myelin sheath) from the stroma (dermis, endoneurium) which contains cells, ECM, and blood vessels. Epithelia and basement membrane regenerate spontaneously; stroma does not (Adapted from Yannas, I.V. (2001) Tissue and Organ Regeneration in Adults. Springer, New York)

with myelin disruption but with no injury to the endoneurium, the myelin sheath regenerates spontaneously and no contraction is observed. Similarly, epidermal excision is a reversible injury that closes exclusively by spontaneous regeneration rather than contraction. The epidermis in skin and the myelin sheath in peripheral nerves exhibit spontaneous regeneration, a reversible healing response leading to a full recovery of structure and function, and are therefore regeneratively similar [1]. Injuries that interrupt the continuity of the basement membrane in both organs without injuring the stroma also exhibit spontaneous regeneration by epithelial cells; basement membranes are regeneratively similar in the two organs. However, when a wound is severe enough to cause injury to the stroma of either organ (the dermis in skin or the endoneurial stroma in peripheral nerves), the organism achieves wound closure by a combination of contraction and scar synthesis (irreversible healing response) [44]. The dermis and non-neuronal peripheral nervous tissue, such as the endoneurium, heal by repair; since they are both nonregenerative, they are considered to be regeneratively similar.

In summary, when the spontaneous regenerative capacity of corresponding tissue types in skin and peripheral nerves is directly compared, a useful similarity emerges [1]: Epithelia and basement membrane are regeneratively similar tissue layers, exhibiting a reversible healing response even in the case of severe injury. Likewise, the stroma in both organs is distinctly nonregenerative. Hence, the central objective of induced organ regeneration is synthesis of the nonregenerative stroma.

Experimental Considerations

Importance of an Anatomically Well-Defined Defect

The appropriate experimental volume for studies of induced organ regeneration is the anatomically well-defined defect [1]. The above discussion of

the differential regenerative capacity of the various layers of the tissue triad calls for an experimental injury that is free of nonregenerative tissue. In this manner, the effects of an exogenous regenerative agent on the potential synthesis of nonregenerative tissue can be evaluated without ambiguity. In addition, the experimental volume should also have well-defined anatomical boundaries to reduce contributions from extraneous healing processes occurring elsewhere in the organ (e.g., caused by collateral damage during the surgical procedure) and to improve the reproducibility of the surgical protocol from one animal to the next as well as between independent laboratories. The treatment of the defect should include prevention of loss of extravascular tissue fluid (exudate), which contains important growth factors and regulators that are crucial both to regeneration and to repair. Inability to prevent exudate loss from the injured site radically affects the outcome of both spontaneous and induced healing processes in both skin and peripheral nerves [45–47]. Physical containment is also necessary to prevent detrimental extraneous processes, such as bacterial infection in skin, from interfering with the outcome of the healing response.

For studies of induced regeneration in skin, the most widely used well-defined defect is the dermis-free full-thickness wound in the rodent or swine. In the case of peripheral nerves, the fully transected peripheral nerve in the rat or mouse has been studied extensively [1]. Both the introduction of various grafts or sheet-like covers to skin defects and tubulation to transected nerves using a variety of materials typically imparts significant activity that either assists or hinders regeneration; their use must be controlled carefully.

Synthetic Protocol: In Vitro or In Vivo?

A detailed comparison of the synthetic regeneration processes carried out in vitro and in vivo shows that in studies of skin and peripheral nerves, various protocols for in vitro synthesis have so far resulted largely in the formation of epithelia and the associated basement membrane but not the physiological stroma. By contrast, several protocols conducted in vivo have yielded not only the physiological epithelia and basement membrane, but a near-physiological stroma as well. The following section highlights these observed cases of induced regeneration.

Overview of Induced Organ Regeneration

Evidence of Induced Organ Regeneration in Adults

Studies that started in the early 1970s in the Fibers and Polymers Laboratory at Massachusetts Institute of Technology have shown that the adult mammal can be induced to regenerate selected organs that have been accidentally lost or excised. In every case, it had been established previously that the excised adult organ in question does not regenerate spontaneously; that is, in the absence of experimental intervention, the adult excised site generally closed spontaneously by contraction and scar formation rather than by regeneration. The organs in question were induced to regenerate partially with the aid of certain insoluble substrates (scaffolds) that were optionally seeded with cells (Fig. 9.2).

The most extensive data on induced organ regeneration are available with skin and peripheral nerves (see ref. [1] for a detailed review). Data with other organs from the work of several investigators were presented in a recent volume [3–10]. We review below the induced organ regeneration data obtained in our laboratory.

The three anatomical sites which were induced to regenerate partially were: (1) full-thickness skin wounds, with epidermis and dermis completely excised, in the adult guinea pig, adult swine, and adult human; (2) full-thickness excision of the conjunctiva, with complete excision of the stroma, in the adult rabbit; (3) the fully transected rat sciatic nerve, with stumps initially separated by a gap of 15 mm (later 22 mm and recently 30 mm). A summary of induced regeneration data for the constitutive tissues of each organ is presented in Table 9.1.

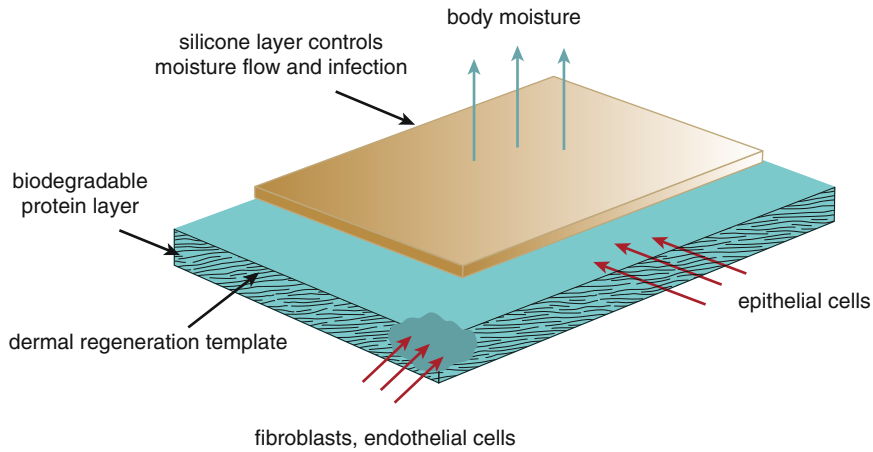

Fig. 9.2 Schematic diagram of a bilayer device that induced regeneration of dermis in full-thickness skin wounds in the guinea pig [7]. The two-layer device consists of a top layer which is a thin film of poly(dimethyl siloxane) that limits bacterial invasion and controls moisture flux to physiologic levels. Underneath is a highly porous scaffold, a graft copolymer of type I collagen and chondroitin-6-sulfate, that induces regeneration of the dermis in the full-thickness skin wound. Regenerative devices based on collagen-glycosaminoglycan scaffolds have been described in a number of patents, either as a cell-free device that induces dermis regeneration or as a keratinocyte-seeded device that induces simultaneous regeneration of dermis and epidermis. Several FDA-approved versions of this device are now used for regeneration of acute and chronic skin wounds in humans (Adapted from Yannas IV, Burke JF, Orgill DP, Skrabut EM. Wound tissue can utilize a polymeric template to synthesize a functional extension of skin. Science 1982;215:174–176)

Table 9.1 Constitutive tissues of skin, peripheral nerves, and conjunctive that were induced to regenerate in adults

Organ	Regeneration observed	Regeneration not observed	Regeneration not studied
Skin (guinea pig, swine, human)	Keratinized epidermis, basement membrane, dermis, nerve endings, blood vessels	Appendages (e.g., hair follicles, sweat glands)	
Peripheral nerve (mouse, rat, cat, monkey, human)	Myelin sheath, nerve fibers (large and small diameter), blood vessels, endoneurial stroma?		Endoneurial stroma? Perineurium
Conjunctiva (rabbit)	Epithelia, conjunctival stroma		Basement membrane

Adapted from Yannas, I.V. (2001) Tissue and Organ Regeneration in Adults. Springer, New York

Observations of induced regeneration in adults made over the years have been tested repeatedly by morphological and functional tests as follows: (a) confirmation of partial regeneration of skin (including both a dermis and an epidermis but lacking skin organelles) was made by histological, immunohistochemical, ultrastructural, and functional studies [7–14]; (b) confirmation of regeneration of the conjunctiva (including the conjunctival stroma) was made using histological data [34]; (c) confirmation of regeneration of peripheral nerves was made using both morphological and functional (electrophysiological and neurological) data [25, 26, 28–33, 48].

The available evidence in the above studies strongly supports the conclusion that these severely injured anatomical sites did not close by contraction and scar formation.

Nevertheless, induced regeneration observed to date is described as "partial" since perfectly

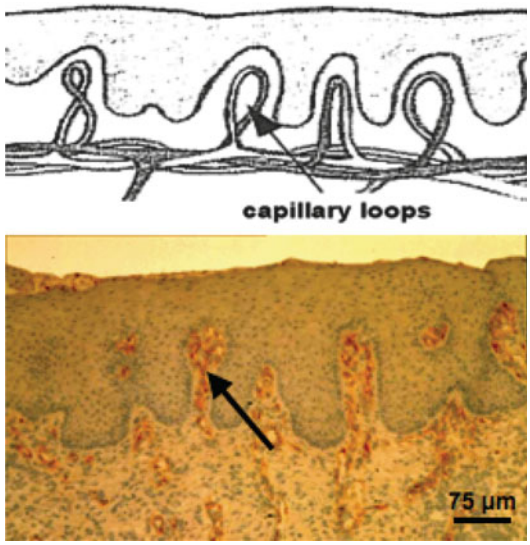

Fig. 9.3 Evidence for induced regeneration of skin using collagen-glycosaminoglycan scaffold. (*Top*) A schematic diagram of physiologically normal skin shows characteristic rete ridges at the dermal–epidermal junction and is contrasted with that of partially regenerated skin in the swine, following grafting with the keratinocyte-seeded dermal regeneration template scaffold (*bottom*). The new skin is not scar, as evidenced by the presence of rete ridges and capillary loops inside the ridges. Immunostaining for Factor VIII 35 days after grafting revealed that capillary loops had formed in the rete ridges of the regenerated dermis (*arrow*) similar to those observed in physiological skin. Bar: 75 µm (*Top*, from Burkitt HG, Young B, Heath JW. Wheater's Functional Histology. Edinburgh, Scotland: Churchill Livingstone; 1993. *Bottom*, from Compton CC, Butler CE, Yannas IV, Warland G, Orgill DP. Organized skin structure is regenerated in vivo from collagen-GAG matrices seeded with autologous keratinocytes. J Invest Dermatol. 1998;110:908–916)

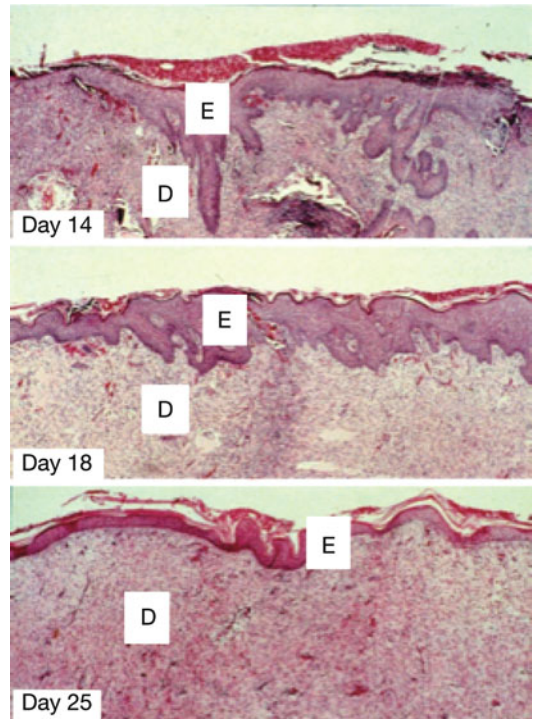

Fig. 9.4 Kinetics of early skin synthesis in the swine with collagen-glycosaminoglycan scaffold between days 14 and 25. In this case, the collagen-glycosaminoglycan scaffold was seeded with autologous keratinocytes before grafting onto full-thickness skin wounds in the swine. Newly formed epidermis is denoted as *E* and the neodermis is denoted as *D*. The scaffolds degrade with a half-life of 15 days (Reproduced from Butler CE, Orgill DP, Yannas IV, Compton CC. Effect of keratinocyte seeding of collagen glycosaminoglycan membranes on the regeneration of skin in a porcine model. Plast Reconstr Surg. 1998;101:1572–1579)

physiological organs have not yet been regenerated. Regenerated skin was histologically and functionally different from scar and identical to physiological skin in almost all respects, including a physiological epidermis, well-formed basement membrane, well-formed capillary loops at the rete ridges of the dermal–epidermal junction, nerve endings with confirmed tactile and heat-cold feeling, and a physiological dermis; however, the regenerate lacked certain organelles (hair follicles, sweat glands, etc.). Evidence for the induced regeneration of partial skin is presented in Fig. 9.3 and the kinetics of this process are presented in Fig. 9.4. The supportive data for induced regeneration of peripheral nerves is presented in Fig. 9.5.

Clinical Experiences with Collagen-Based Scaffolds

The clinical significance of induced regeneration studies is readily apparent. Two collagen-based regenerative devices have been approved thus far

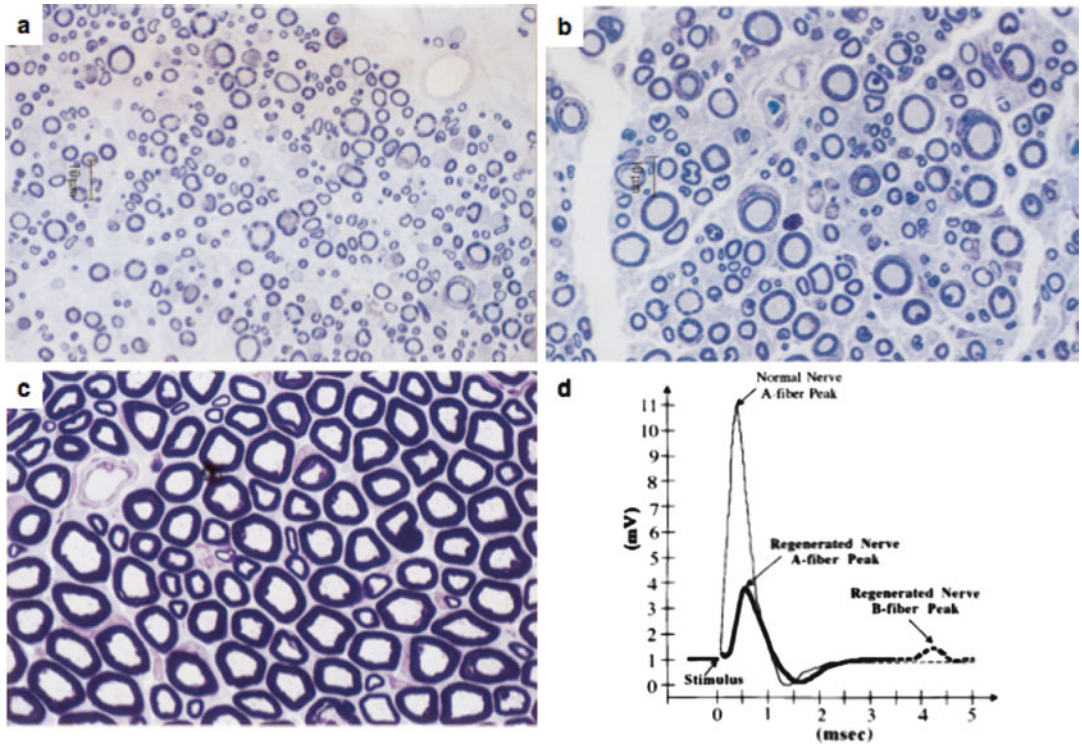

Fig. 9.5 Evidence of induced regeneration of peripheral nerve using collagen-based nerve regeneration template. Histological micrographs of nerve tissue postfixed with osmium tetroxide and stained with toluidine blue. The magnification for each micrograph is the same; scale bars, 10 μm. (**a**) Tissue regenerated through the midportion of a matrix-filled large-pore collagen (LC/M) implant at 30 weeks. Note the large number of axons in this cross-section with the majority of axons being small in diameter. The largest axons have diameters of approximately 7 μm. Many Schwann cells are visible with some actively participating in myelination. The blood vessel that is visible in this micrograph was characteristic of the caliber of most vessels present in the regenerated tissue. (**b**) Tissue regenerated through the midportion of a LC/M implant at 60 weeks. Compared to 30 weeks, the axons are much larger (diameters up to 12 μm) and have thicker myelin sheaths. Also, fewer small diameter axons are visible. Few nonmyelinating Schwann cells are visible at 60 weeks. (**c**) Normal nerve tissue from the level of the lesion is shown as a control. Note the number of large diameter fibers and the thickness of the myelin sheaths compared to the regenerated nerves. (**d**) Typical oscilloscope tracings of A-fiber and B-fiber compound nerve action potentials for normal sciatic nerve and nerve regenerated through a LC/M implant at 60 weeks postimplantation. The A-fiber peak for the regenerated nerve has a significantly smaller amplitude than the normal nerve control. This was typical of all regenerated groups. By contrast, the conduction velocity of the regenerated nerve, although significantly slower than normal, was approaching normal values. The latency is measured along the *x*-axis from the stimulus to the peak and then combined with the constant distance between electrodes to determine conduction velocity. The *dashed line* indicating that the B-fiber peak has been added on to the tracing for reference. Note that the normal nerve tracing has no visible B-fiber peak. In the regenerated nerves, the B-fiber peak was similar and visible in all groups (Reproduced from Chamberlain LJ, Yannas IV, Hsu HP, and Spector M. Collagen-GAG Substrate Enhances the Quality of Nerve Regeneration through Collagen Tubes up to Level of Autograft. Exp Neurol 1998: 154: 315–329)

by the Food and Drug Administration (FDA), one each for the regeneration of skin and peripheral nerves. Increasingly, these devices are establishing themselves as a viable alternative to autografting.

Skin Regeneration with Dermal Regeneration Template®

In 1996, the FDA approved the Integra Dermal Regeneration Template® (DRT, described briefly earlier), as an urgent treatment modality for

patients suffering from severe burns. Since that time, DRT has been approved by regulatory agencies in several other countries. In 2002, the FDA-approved DRT for a second application: restorative or reconstructive surgery of skin scars. The efficacy of DRT for the induced regeneration and treatment of chronic and pathological deep skin ulcers (chronic skin wounds) has been established, and modified versions of this device have been designed specifically for the treatment of these wounds. DRT has been studied recently by several clinical investigators [49–55].

Recent reports demonstrate the efficacy of the DRT in healing foot wounds in diabetic patients [11–16]. A recent study of 30 diabetic patients who underwent surgical debridement of diabetic foot wounds followed by grafting with DRT reported an 86.7% healing rate and a significantly more distal level of amputation ($p<0.003$). A recent retrospective review of 105 patients with diabetic foot ulcers receiving DRT for lower extremity salvage indicates DRT as a viable option for stable closure of these wounds in patients with low risk of amputation. DRT efficacy for patients with an already high risk of amputation (based on available blood supply and presence of infection) seems to be limited. In another study, 111 patients received DRT grafting as a method of closure for selective refractory pathological wounds. Patients were treated predominantly in an outpatient setting with an average healing time of 7 months. Detailed indications for the use of Integra DRT in the treatment of chronic wounds (classified by both anatomy and pathology/diagnosis) have been presented [11–16].

Using a DRT has the potential to replace the need for a full-thickness autograft in a patient population already suffering from large and deep wounds, in those patients where a simplified reconstruction can be designed, and in those in whom less scarring may be desired [56].

Peripheral Nerve Regeneration with NeuraGen™

In 2001, Neuragen®, an early version of the collagen-based tubular devices that have been described above, was approved for the regeneration of peripheral nerves to treat individuals suffering from paralysis of the extremities. Further studies have shown that, as with studies of skin regeneration [4], the structure of collagen requires extensive optimization in order to increase the regenerative activity of this natural protein. One such study identified an optimized version of the device: a cell-permeable collagen tube with controlled degradation rate, higher cell-permeability, and an overall superior quality of regeneration [30].

Recently a multicenter human trial (using randomized, blind, parallel groups), compared the NeuraGen™ nerve guide to direct suturing repair (control group), which is the current clinical gold standard for treatment of short gap injuries [58]. The study followed 32 patients who had complete traumatic nerve injuries to the median and/or ulnar nerves in the distal third of the forearm over 2 years. Patients treated with the collagen devices had significantly lower postoperative pain scores than controls at early time points and at completion of the study demonstrated sensory and motor function performance equal to the direct repair group. Taken together, the results indicate that the collagen nerve guide tube is a realistic alternative to conventional end-to-end nerve repair, but requires extensive redesign in order to optimize the regenerative activity of collagen, thereby making it useful at longer gap lengths.

Limitations of Collagen-Based Scaffolds as Regenerative Devices

As stated earlier, these clinically approved devices do not induce regeneration of entirely normal organs. For example, skin regenerated by the use of collagen-based scaffolds lack several appendages (sweat glands, hair follicles). Future work may focus on speeding the rate of cell migration and angiogenesis within the device (angiogenesis peaks in 7–14 days in a murine model) with the goal of improving regeneration, increasing the safety profile of the device, and decreasing the risk of infection [81]. Peripheral nerves that regenerated with tubular collagen-based scaffolds exhibit conduction velocity profiles that are somewhat slower and weaker than with normal nerves (although scaffolds that have been recently synthesized, but are not commercially available, improve significantly on the clinically available device).

The Defect Closure Rule

Careful review of the literature suggests that no more than three distinct processes are used to close an anatomically well-defined defect (dermis-free defect) in skin wounds: contraction originating from the edges of the defect, scar formation by stromal fibroblasts (followed by epithelialization of scar), and regeneration.

Kinetic data extending continuously over lengthy periods are rarely available from regeneration experiments and often difficult to compare from one study to another. One approach to studying the regenerative activity of exogenous agents on the healing process is to establish two standardized configuration states (e.g., an initial and a final state) and to evaluate the total change that is caused during this fixed period in the healing process. In the absence of kinetic data, the *defect closure rule* bridges the gap by presenting a quantitative description of the healing process through comparison of snapshots of the initial and final stages of wound healing. The initial state of configuration is the anatomical description of the recently generated defect, characterized by the loss of structural continuity in one or more tissues, the beginning of exudate flow, and the loss of physiological homeostatic control of the organ. As defect healing progresses, the original area, A_0, eventually diminishes spontaneously due to one or more of the three processes mentioned above. The area of the closed defect (the closed wound) comprises tissues that result either from contraction (fractional amount, $\%C$), scar formation ($\%S$), or regeneration ($\%R$) and the configuration of the final state can be described by the following simple relation, called the defect closure rule:

$$C + S + R = 100. \qquad (9.1)$$

Equation 9.1 states that the defect closure in any organ can be described by only three outcomes: contraction, scar formation (neuroma or fibrosis), and regeneration (partial or total).

For the idealized case of early fetal wound healing (spontaneous regeneration), contraction and scarring is absent (C, $S = 0$) and

$$R = 100 \quad \text{(regeneration)}.$$

For normal defect closure in adult mammals following irreversible injury (repair), regeneration is absent ($R = 0$) and

$$C + S = 100 \quad \text{(repair)}.$$

The literature describes several assays to determine the configuration of the final state (recently closed defect) [1]. Functional assays can be used to qualitatively identify the physiological nature of the tissue and assist in providing a quantitative measure of its incidence in the final state in terms of the numerical values of these three quantities (C, S, or R). The defect closure rule may be interpreted as a conservation principle: provided that the magnitude of two individual terms (e.g., C and S) has been determined, the magnitude of the remaining process may be calculated. Defect closure data is expressed using the following convention: ($\%C$, $\%S$, $\%R$).

The defect closure rule is useful in evaluating the activity of unknown reactants as inductive agents of regeneration. This quantitative description of the structure and function of the injured organ at its final state has shed interesting light on the relationship between the characteristic elements of the adult healing response (contraction or scar synthesis, or both) and regeneration.

Prevalence of Contraction During Spontaneous Healing

In the skin, the defect closure rule has been used to present data on the configuration of the final state following spontaneous healing of the anatomically well-defined defect (dermis-free defect) in several species. In all cases of spontaneous healing of full-thickness skin wounds, it was ensured that the contribution of regeneration to defect closure was negligible ($R = 0$). Skin contraction was measured directly as the reduction in initial wound surface area by inward (centripetal) movement of skin from the margins of the wound. Scar formation was studied qualitatively by histology. In a few cases, scar formation was confirmed quantitatively by the use of laser light scattering, used to measure the average degree of

collagen fiber orientation and thereby deduce on the identity of the tissue present in the healed injury site [60]. Values for the percentage of initial defect area closed by epithelialized scar (S) were determined using the simplified defect closure rule for repair ($S = 100 - C$).

The contribution of the various methods of defect closure in anatomically well-defined defects is species-dependent. In rodents, where the integument is mobile, contraction is by far the main engine of closure of skin wounds, while scar formation has been shown to be quantitatively much less important.

The spontaneous healing of a full-thickness skin wound in the guinea pig is characteristic of several rodents and lagomorphs (rabbits) and results in the following final state configuration: [91, 9, 0] [7, 8]. In general, $C \gg S$ and defect closure for adult rodents and rabbits reduces to $C \approx 100$.

In humans, where the integument is tethered more securely onto subcutaneous tissues, contraction and scar formation contribute approximately equally to wound closure. Experimentally, the spontaneous healing of full-thickness skin defects in the human ($R = 0$) results in a final state represented by [37, 63, 0] [59].

In the absence of direct quantitative observations, histological analysis was used to describe the closure of the fully transected peripheral nerve in the adult rat. Spontaneous healing results in reduction of the initial area of cross sections of nerve trunks by 95% with neuroma formation (neural scar) accounting for the remaining 5%. The resulting estimation of the final state configuration was [95, 5, 0] [1].

The contraction of a wide array of organs in response to trauma is well documented in both animals and humans, yet these reports are almost exclusively of a qualitative nature [57, 61–80]. The organ in which contraction has been studied systematically to date is the skin. Despite the dearth of widespread quantitative data, the prevalence of contraction must not be overlooked; it appears to be a critical outcome of the spontaneous healing response throughout the adult organism.

The Antagonistic Relation Between Contraction and Regeneration

The characteristic elements of the adult healing response (contraction or scar synthesis, or both) must be controlled in order for induced regeneration to occur. Extensive data, including empirical data on the final state of the defect in response to various reactants, suggest that during healing of a severe injury, contraction antagonizes regeneration [1].

Induced regeneration of skin, a peripheral nerve trunk, and the conjunctival stroma was accompanied in each case by direct observation of a significant reduction in contraction as a mode of defect closure. Conjunctival and peripheral nerve regeneration studies were guided by earlier studies of skin regeneration. Partial skin was first induced to regenerate in the adult guinea pig. The spontaneous healing behavior of the untreated dermis-free defect in this organism resulted in a final configuration of [91, 9, 0]. Grafting an identical well-defined skin defect with a highly porous copolymer of type I collagen and chondroitin 6-sulfate (referred to as a dermis regeneration template, DRT) (Fig. 9.6a) abolished scar synthesis and led to the regeneration of a small mass of dermis and subsequent synthesis of an overlying epidermis within the defect. In the context of the defect closure rule, the regenerative activity of the cell-free DRT on the configuration of the final state [1] was as follows:

$$[92,8,0] \rightarrow [89,0,11] \quad (\text{DRT}).$$

In addition, the DRT led to a significant delay in wound contraction over 25 days.

When a DRT seeded with keratinocytes (KC) was grafted into an identical defect, the result was much more pronounced:

$$[91,9,0] \rightarrow [28,0,72] \quad (\text{DRT} + \text{KC}).$$

KC-seeded DRTs accomplished rapid wound closure through partial regeneration of skin (simultaneous synthesis of a physiological dermis and epidermis, described earlier) and completely arrested contraction at 35–40 days [1].

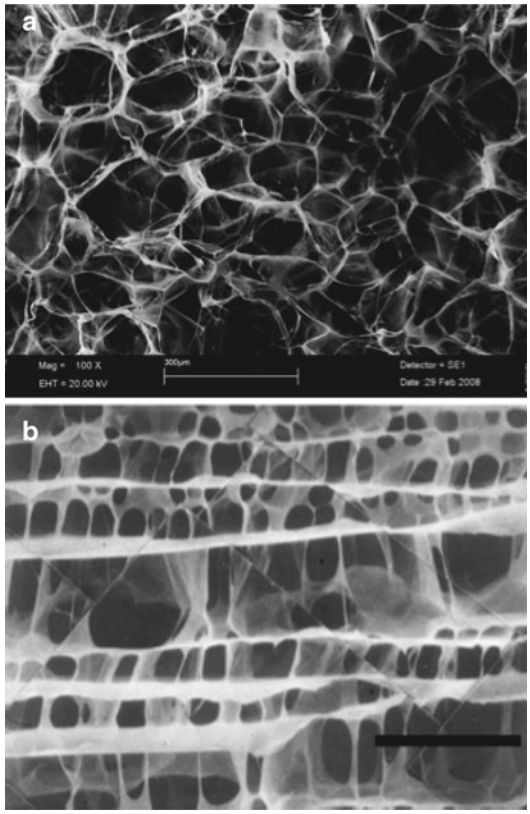

Fig. 9.6 (**a**, *Top*) A scaffold that has induced regeneration of the dermis in animals and humans. Composition: graft copolymer of type I collagen and chondroitin 6-sulfate. Scanning electron micrograph. Pore-channel orientation is almost completely random. Average pore diameter, 80 µm (Courtesy of E. Soller, MIT). (**b**, *bottom*) Peripheral nerve was induced to regenerate across a 15-mm gap (and eventually longer gaps) in the rat sciatic nerve using this scaffold as a bridge between the two stumps inside a silicone tube. In later studies, the chemical composition of this scaffold was changed to GAG-free type I collagen. Pore-channel orientation along the major nerve axis. Scanning electron micrograph. Average pore diameter, 20 µm

The cell-free DRT that induces partial skin regeneration comprises the regenerative component of the two-layer device (Integra DRT®, Fig. 9.6a) approved by the FDA for restoration of a physiological epidermis and dermis in patients suffering from severe burns as well as those undergoing plastic and reconstructive surgery of the skin, as described in an early study [21] and reviewed recently [81].

Growth factors [82, 83], epidermal cell suspensions, and cell sheets [84] exhibited negligible regenerative activity when added to full-thickness skin wounds in other rodent models. These reactants did not significantly alter the configuration of the final state or the extent of contraction delay. Similarly, a number of synthetic polymer scaffolds [85, 86] failed to induce physiological dermis (or skin) regeneration. These observations focus attention on the mechanism of scaffold regenerative activity, to be discussed later.

Quantitative studies of induced regeneration of peripheral nerves were conducted in the adult rat. The spontaneous healing behavior of the untreated transected peripheral nerve in this organism resulted in a final configuration of [95, 5, 0] that was estimated using histological analysis. Insertion of the fully transected nerve stumps into a silicone tube filled with a collagen-based tubular regeneration template (referred to as a nerve regeneration template or NRT, Fig. 9.6b) resulted in reduced contraction (as determined by histological analysis of cross-sectional areas of regenerates) and partial regeneration over a 10-mm gap length [30, 31]. Contraction was abolished and the quality of regeneration improved significantly when the NRT was used in conjunction with a degradable collagen tube. In the context of the defect closure rule, the regenerative activity of the NRT in each experimental configuration can be evaluated by inspecting the estimated characteristics of the final state, as follows (the arrow indicates the change observed following use of the scaffold):

$$[95,5,0] \rightarrow [53,0,47] \quad \text{(NRT inside silicone tube)},$$
$$[95,5,0] \rightarrow [0,0,100] \quad \text{(NRT inside collagen tube)}.$$

The relative importance of each method of defect closure (*C*, *S*, and *R*) changes during animal development. A sharp change occurs during the fetal-adult transition in mammals (roughly during the third trimester of gestation), in which contraction replaces regeneration as the dominant method of closure [87–89]. Similarly, as amphibian (frog) development progresses, contraction becomes a more prominent method of wound closure, as regeneration recedes and scar formation becomes more evident [90, 91].

While scar has been widely considered, the key barrier to regeneration in adults, quantitative study reveals that contraction is the dominant mode of spontaneous closure in skin and peripheral nerve defects. Studies of induced regeneration in skin, peripheral nerves using analogs of the ECM indicate that scar formation is a process that is secondary to contraction: in studies of induced regeneration in these organs, when contraction was even slightly inhibited, scar formation was totally abolished [1].

$$[92,8,0] \rightarrow [89,0,11] \quad (\text{skin}, \text{DRT}),$$
$$[91,9,0] \rightarrow [28,0,72] \quad (\text{skin}, \text{DRT} + \text{KC}),$$
$$[95,5,0] \rightarrow [53,0,47] \quad (\text{peripheral nerve}, \text{NRT in silicone tube}),$$
$$[95,5,0] \rightarrow [0,0,100] \quad (\text{peripheral nerve}, \text{NRT in collagen tube}).$$

Suppression of contraction in certain cases of impaired healing, e.g., following use of pharmacological agents, such as steroids was not accompanied by regeneration, indicating that suppression of contraction alone did not suffice to induce regeneration [1].

The available evidence supports the theory that selectively suppressed contraction in adult defects is required, but not sufficient to induce regeneration of skin and peripheral nerves. This can be expressed in the context of the defect closure (9.1) rule as follows:

$$\Delta R > 0 \text{ and } S \rightarrow 0 \quad \text{if } \Delta C < 0. \qquad (9.2)$$

This condition describes an antagonistic relationship between contraction and regeneration in the closure of a defect. It suggests that successful induced regeneration strategies consist of reactants that block contraction without blocking other aspects of the healing process.

Repair: Mechanism of Contraction

Similarities in the mechanistic hypotheses for inducing regeneration of skin and peripheral nerves originate in their common response to irreversible injury. Both organs spontaneously respond to injury by recruiting contractile cells that, if not properly suppressed, drive closure of the defect by contraction and scar synthesis rather than by regeneration. Contraction of skin defects starts from a cell cluster at the edge of the defect and later extends across the entire defect area. In peripheral nerves, contraction primarily results from the activity of a circumferential sheath of contractile cells.

The Contractile Fibroblast Is the Main Cell Type Associated with Contraction

The well-documented, macroscopic contraction that drives the closure of skin defects finds its origin at the cellular scale, arising from the individual contribution of contractile forces generated by differentiated myofibroblasts (MFB) [92–98]. The current consensus is that MFB that are present in granulation tissue following skin wounding derive directly from fibroblasts and comprise an intermediate, contractile, cellular phenotype between the fibroblast and the smooth muscle cell [99]. There is also evidence that undifferentiated fibroblasts may contribute to macroscopic contraction by applying traction to the ECM very soon after coming into contact with it [100–103].

In response to external tension, fibroblasts exert sustained isometric force on their surrounding environment via a Rho/Rho-kinase (ROCK)-mediated, actomyosin contractile apparatus [104–106]. This 3D, transcellular structure consists of bundles of actin and nonmuscle myosin microfilaments called “stress fibers.”

Of the many ultrastructural and biochemical factors that distinguish MFB from their fibroblast precursors, the most useful operational distinction of MFB differentiation is expression of the α-smooth muscle actin (α-SMA) phenotype [107–109]. Stress fibers of immature MFB (called proto-MFB) contain only beta- and gamma-cytoplasmic actins [89]. Additionally, differentiated MFB exhibit stress fibers typically arranged parallel to the long axis of the cell, nuclei which consistently show multiple indentations or deep folds, and two cell-matrix adhesion macromolecules (vinculin and fibronectin) [107–109].

Simplistically, the myofibroblast differentiation process can be described as a positive feedback loop that requires the concurrent action of at least three factors: the cytokine transforming growth factor-beta1 (TGF-β1), the presence of mechanical tension, and the ED-A splice variant of cellular fibronectin (an ECM component) [92]. Fibroblasts respond to the development of mechanical tension by upregulating TGF-β1 production and expressing the α-SMA isoform; in turn, α-SMA expression strengthens the contractile apparatus and increases tension development [92]. Recent work suggests that mature MFB in skin granulation tissue link their cytoskeletons together using cadherin proteins, which allow them to generate even higher levels of force to drive wound closure [109].

Mechanism of Scaffold Regenerative Activity

Structural Determinants of Scaffold Regenerative Activity

Scaffolds that induce regeneration of partial skin (Fig. 9.6a) possess a highly specific structure that is distinctly different in pore structure and degradation rate from scaffolds that regenerate peripheral nerves (Fig. 9.6b). The nature and duration of the contractile response as well as the structure of the two organs differ greatly as do the values for several of the structural parameters of the early scaffolds that were used to control contraction and induce regeneration in each organ (Table 9.2). The scaffolds are type I collagen-based yet they differ in average pore diameter (higher in the case of the DRT), the pore-channel orientation (axial for the nerve guide, random for the DRT), and degradation rate (a higher average molecular weight between cross-links, *M*c (kDa) in the nerve guide leads to faster degradation).

In skin wounds, the mechanism of induced regeneration has been elucidated through careful modulation of the DRTs structural properties that impart contraction-blocking activity. DRTs that actively block contraction in skin wounds (and induce regeneration) have structural properties that accomplish three main processes: (1) reduction in MFB number present in the wound, possibly due to inhibition of TGF-β synthesis, leading to downregulation of myofibroblast recruitment; (2) blocking orientation of MFB axes in the plane of the defect where macroscopic contraction is observed; and (3) ensuring that DRT degradation time is sufficiently long to ensure that contraction blocking persists for the duration of the interim MFB contractile response but not so long as to interfere with key regenerative processes.

1. *Apparent downregulation of TGF-β synthesis.* The quaternary structure of collagen fibers is a requirement for the aggregation of platelets, an early component of the wound response. Platelet aggregation initiates a cascade of events that include the release of the cytokine TGF-β1, one of the main inductors of the myofibroblast phenotype. Collagen fibers in the DRT maintain their tertiary (triple helical) structure but are practically free of banding (due to treatment with acetic acid during scaffold preparation). DRT apparently disrupts platelet aggregation within the defect, reducing production of TGF-β1, and the recruitment of contractile MFB to the wound site [110].
2. *Blocking orientation of MFB axes in the plane of the wound as well as MFB–MFB binding*. Contraction of wound edges appears to require orientation of MFB axes in the plane of the wound as well as MFB–MFB binding and MFB–ECM binding. MFB binding on the extensive surface of the highly porous 3D scaffold inhibits such orientation as well as inhibiting MFB–MFB and MFB–ECM binding. It is suggested that these mechanisms are additionally responsible for contraction blocking by the scaffold. According to this suggested mechanism, contraction blocking requires extensive MFB binding onto a sufficiently large scaffold surface, which must take place via specific integrin–ligand interactions. Fibroblasts bind onto a specific GFOGER ligand on a collagen surface via the α1β1 and α2β1 integrins [111]. When other structural properties are held constant, the ligand density

Table 9.2 Structural determinants of regenerative activity of CGSs

Structural parameter required for regenerative activity	Skin regeneration[a]	Nerve regeneration[b]	Structural feature hypothetically responsible for contraction blocking
Type I collagen/GAG (w/w) residual collagen fiber banding	98/2 approximately 5% of native collagen	98/2 approximately 5% of native collagen	Ligand identity Reduction in recruitment of contractile cells
Average molecular weight between cross-links (kDa)	5–15	40–60	Controls duration of undegraded scaffold during contraction
Average pore diameter (μm)	20–120 random	5–10 axial	Ligand density
Pore-channel orientation			Ligand orientation

[a]Approximate levels of structural determinants observed in skin regenerative studies conducted by grafting the scaffold on a full-thickness skin wound (Yannas et al. [20])
[b]Approximate levels of structural determinants observed in peripheral nerve regeneration studies performed by inserting the scaffold inside a nerve conduit (the conduit connected the two stumps of the transected nerve across an experimental gap of defined length) (Chang et al. [27]; Chang and Yannas [28])
Adapted from Yannas et al. [81]

of a scaffold increases with decreasing average pore size (since the specific surface area of the scaffold available for attachment is thereby increased). An appropriate ligand density appears to be necessary to disrupt extensive MFB–ECM binding responsible for the onset of macroscopic contraction in skin wounds (Fig. 9.7).

When MFB bind to specific DRT integrins that are distributed evenly in a 3D, interconnecting porous network, the axes of their contractile apparatus becomes disoriented. At the cellular level, the randomized configuration of the preferential contractile axes that individual MFB adopt in the presence of DRT leads to approximate cancelation of the macroscopic mechanical forces that lead to 2D contraction and scar synthesis in ungrafted skin wounds. When the pore diameter of DRT is increased much beyond the level of 120 μm, the effective DRT ligand density drops to a value that does not provide sufficient binding of MFB and the contraction-blocking activity of the scaffold is lost [1, 109, 112]. Similarly, a minimal average pore size exists that is necessary to ensure MFB migration inside the scaffold. According to this interpretation if the pore size is too small, MFB does not infiltrate the scaffold, MFB–DRT ligand bonds do not form, and MFB contractile activity is not canceled. Experimentally, the highly planar orientation of myofibroblast axes that is characteristic of the spontaneous contractile response in ungrafted skin wounds is negligible in the presence of DRT [113].

3. *Duration of DRT in an undegraded state over the entire contraction process*. It is known that the regenerative activity of the scaffold depends sensitively on its degradation rate during skin regeneration [114] as well as during regeneration in the PNS [96]. To explain the data, it has been hypothesized that the DRT is required to undergo a process of *isomorphous tissue replacement*, in which the regenerate (dermis or nerve tissue) is synthesized at a rate which is of the same order as the rate of degradation of the DRT. The requirement for an optimal scaffold duration may reflect the need to have the scaffold persist in an undegraded (insoluble) state over a period that matches the length of the contraction process in skin wounds and nerve wounds, thereby ensuring that the contraction-blocking activity is operative when it matters. In skin wounds, the optimal half-life of degradation (t_b) for DRT in vivo is 14 days, roughly matching the irreversible contraction response in ungrafted wounds (t_h) [1, 112]. In peripheral nerve wounds, the optimal degradation half-life

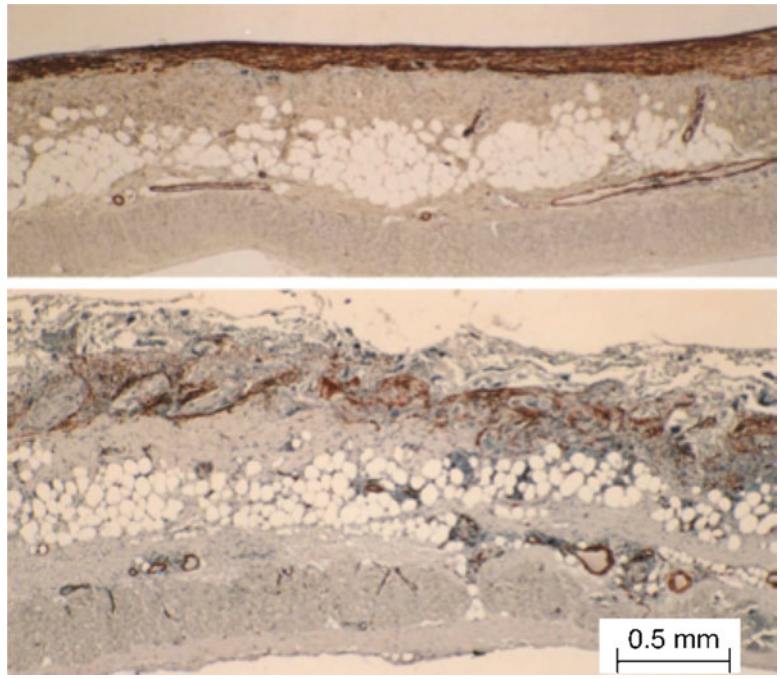

Fig. 9.7 Histological contrast between ungrafted and grafted full-thickness skin wound in the guinea pig. The ungrafted wound is contracting vigorously at day 10 after injury (*above*), whereas the wound grafted with the unseeded dermal regeneration template shows no contraction at the same time following injury (*below*). The tissue sections were stained with an antibody against α-smooth muscle actin, a protein that is synthesized when fibroblasts differentiate to the myofibroblast, the contractile phenotype. In the grafted wound, the myofibroblast density is reduced to approximately 20% of its level in the ungrafted wound; also, the long (contractile) axes of myofibroblasts become randomly oriented in space in the presence of the scaffold [22]. The result is blocking of wound contraction, a prerequisite for induced regeneration (From Yannas IV. Similarities and differences between induced organ regeneration in adults and early foetal regeneration. J R Soc Interface 2005;2:403–417)

is about 2 weeks, again matching roughly the half-life for the healing process in the transected nerve stump [29]. When the scaffold degraded at a slower rate ($t_b \gg 14$ days), the persisting DRT appeared to interfere with synthesis of the regenerate and scar formed around the scaffold. When the half-life of the DRT was significantly lower than the half-life of the contractile response ($t_b \ll 14$ days), the DRT had little effect on blocking contraction or scar synthesis and regeneration was not observed [20].

In summary, DRT dramatically blocks contraction while inducing skin regeneration. Scaffolds that are close in structure to DRT but do not block contraction, do not induce regeneration. There is evidence that DRT prevents recruitment of MFB and formation of oriented structures of MFB, two processes that characterize spontaneous healing in the adult mammal, over the duration of the normal contraction process.

Discussion and Conclusions

The experimental protocols that were used by several independent investigators to induce synthesis of elements of skin and peripheral nerves both in vitro and in vivo were analyzed in an effort to identify the minimal reactants required for organ regeneration. Despite the structural differences between the two organs, the simplest reactants required for induced regeneration of either skin or peripheral nerves were found to be similar. The empirical evidence supports the conclusion

that partial synthesis of either skin or peripheral nerves requires only the implantation of a scaffold with the requisite structure, appropriately seeded with epithelial cells dissociated from the organ of interest. The scaffold should possess a minimal density of specific ligands for contractile cells and an optimal persistence time in the insoluble state [1]. Exogenous reactants utilized widely in many regeneration protocols, notably cytokines and stromal cells (fibroblasts), were redundant. While the experimental evidence derives only from the two organs that have been studied extensively to date in this context, the conclusions reached above may be interpreted as a "trans-organ" approach for future regeneration efforts.

The evidence presented in this chapter shows that severe wounds in several organs in adults heal primarily by contraction, the same mechanism by which skin and peripheral nerves heal in adults. Contraction blocking in skin and in peripheral nerves is associated with induced regeneration. The available data suggest, therefore, the possibility that the mechanism of contraction blocking by scaffolds is similar in these two organs. It now becomes possible to seriously consider the possibility that the adult organism can be enabled to regenerate most of its organs.

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