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Regulation of Wound Healing by Growth Factors and Cytokines

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Werner, Sabine, and Richard Grose. Regulation of Wound Healing by Growth Factors and Cytokines. *Physiol Rev* 83: 835–870, 2003; 10.1152/physrev.00032.2002.—Cutaneous wound healing is a complex process involving blood clotting, inflammation, new tissue formation, and finally tissue remodeling. It is well described at the histological level, but the genes that regulate skin repair have only partially been identified. Many experimental and clinical studies have demonstrated varied, but in most cases beneficial, effects of exogenous growth factors on the healing process. However, the roles played by endogenous growth factors have remained largely unclear. Initial approaches at addressing this question focused on the expression analysis of various growth factors, cytokines, and their receptors in different wound models, with first functional data being obtained by applying neutralizing antibodies to wounds. During the past few years, the availability of genetically modified mice has allowed elucidation of the function of various genes in the healing process, and these studies have shed light onto the role of growth factors, cytokines, and their downstream effectors in wound repair. This review summarizes the results of expression studies that have been performed in rodents, pigs, and humans to localize growth factors and their receptors in skin wounds. Most importantly, we also report on genetic studies addressing the functions of endogenous growth factors in the wound repair process.

I. INTRODUCTION

Injury to the skin initiates a cascade of events including inflammation, new tissue formation, and tissue remodeling, which finally lead to at least partial reconstruction of the wounded area (57, 176; Fig. 1). The repair process is initiated immediately after injury by the release of various growth factors, cytokines, and low-molecular-weight compounds from the serum of injured blood vessels and from degranulating platelets. Disruption of blood vessels also leads to the formation of the blood clot, which is composed of cross-linked fibrin, and of extracellular matrix proteins such as fibronectin, vitronectin, and thrombospondin (56, 57, 176). Apart from providing a barrier against invading microorganisms, the blood clot also serves as a matrix for invading cells and as a reser-

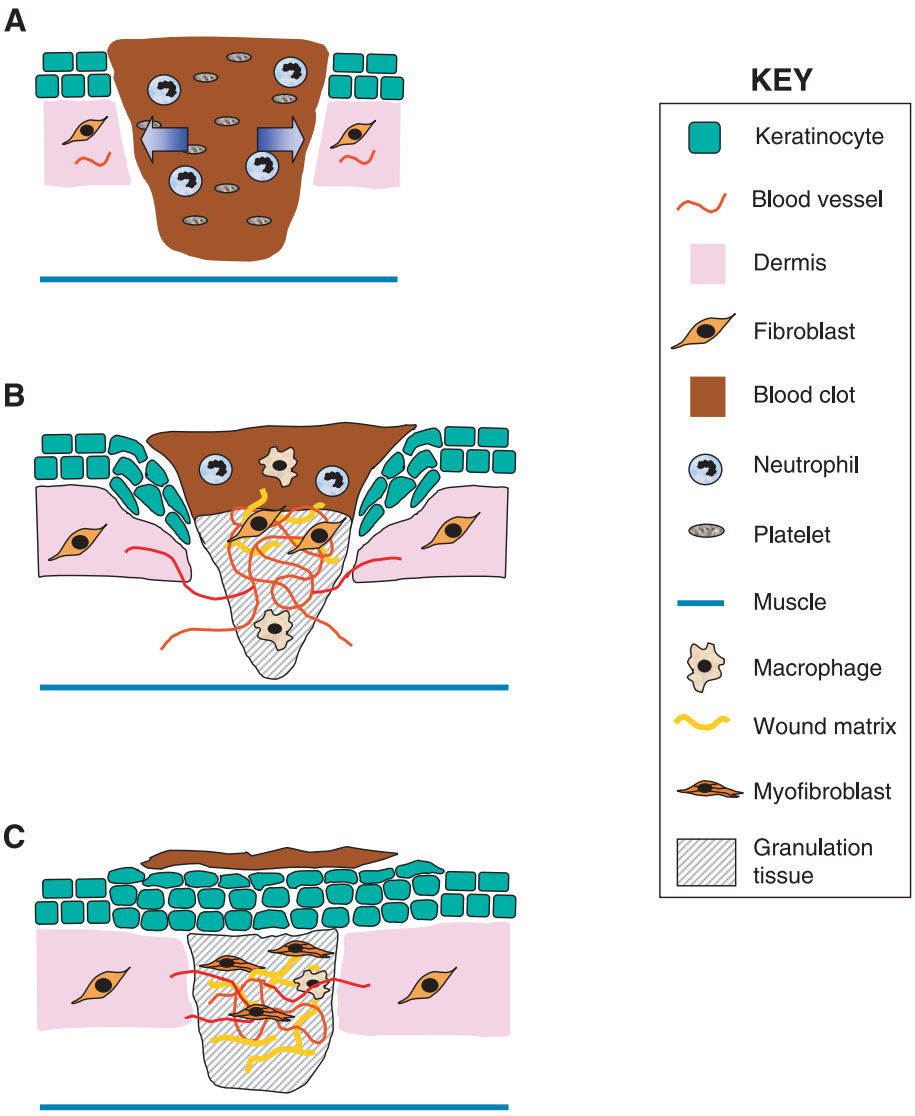

FIG. 1. Schematic representation of different stages of wound repair. *A*: 12–24 h after injury the wounded area is filled with a blood clot. Neutrophils have invaded into the clot. *B*: at *days 3–7* after injury, the majority of neutrophils have undergone apoptosis. Instead, macrophages are abundant in the wound tissue at this stage of repair. Endothelial cells migrate into the clot; they proliferate and form new blood vessels. Fibroblasts migrate into the wound tissue, where they proliferate and deposit extracellular matrix. The new tissue is called granulation tissue. Keratinocytes proliferate at the wound edge and migrate down the injured dermis and above the provisional matrix. *C*: 1–2 wk after injury the wound is completely filled with granulation tissue. Fibroblasts have transformed into myofibroblasts, leading to wound contraction and collagen deposition. The wound is completely covered with a neoepidermis.

voir of growth factors required during the later stages of the healing process. Within a few hours after injury, inflammatory cells invade the wound tissue. Neutrophils arrive first within a few minutes, followed by monocytes and lymphocytes. They produce a wide variety of proteinases and reactive oxygen species as a defense against contaminating microorganisms, and they are involved in the phagocytosis of cell debris. In addition to these defense functions, inflammatory cells are also an important source of growth factors and cytokines, which initiate the proliferative phase of wound repair. The latter starts with the migration and proliferation of keratinocytes at the wound edge and is followed by proliferation of dermal fibroblasts in the neighborhood of the wound. These cells subsequently migrate into the provisional matrix and deposit large amounts of extracellular matrix. Furthermore, wound fibroblasts acquire a contractile phenotype and transform into myofibroblasts, a cell type which plays a major role in wound contraction. Massive angiogenesis leads to the formation of new blood vessels, and nerve sprouting occurs at the wound edge. The resulting wound connective tissue is known as granulation tissue because of the granular appearance of the numerous capillaries. Finally, a transition from granulation tissue to mature scar occurs, characterized by continued collagen synthesis and collagen catabolism. The scar tissue is mechanically insufficient and lacks appendages, including hair follicles, sebaceous glands, and sweat glands. Scarring can also be excessive, leading to hypertrophic scars and keloids. In contrast, wound healing in mammalian embryos until the beginning of the third trimester results in essentially perfect repair, suggesting fundamental differences in the healing process between embryonic and adult mammals (57, 168, 176).

In addition to the importance of cell-cell and cell-matrix interactions, all stages of the repair process are

controlled by a wide variety of different growth factors and cytokines. Multiple studies have demonstrated a beneficial effect of many of these growth factors, e.g., platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), and granulocyte-macrophage colony stimulating factor (GM-CSF) on the healing process, both in animal models and also in patients suffering from different types of wound healing disorders (1, 79, 107, 115, 196). However, the roles of endogenous growth factors in the healing response have been only partially elucidated, and in most cases, the suggested function of these molecules is based on descriptive expression studies and/or functional cell culture data. However, in vivo functions of many growth factors remain largely unconfirmed.

The development of transgenic and knock-out mouse technologies has provided new insights into the function of many different genes during embryonic development. These technologies allow gain of function experiments (overexpression of genes) as well as loss of function experiments (gene knock-outs by homologous recombination in embryonic stem cells or overexpression of dominant-negative mutants). Most importantly, spatial and temporal control of gene ablation or overexpression, using both inducible and cre-lox technologies, makes it possible to determine the functions of proteins formerly precluded due to embryonic lethality. A large number of viable genetically modified mice are now available that can be used to elucidate the role of the deleted, mutated, or overexpressed genes in different types of repair processes. Indeed, the past years have seen an exponential growth in the number of genetically modified mice that were used for wound healing experiments, and these studies have provided interesting, and often unexpected, results concerning the in vivo function of growth factors in wound repair (see http://icbxs.biol.ethz.ch/members/grose/woundtransgenic/home.html). In this review, we summarize the reported expression and function of endogenous growth factors and cytokines in cutaneous wound repair. Results of experiments with exogenous growth factors for the treatment of wound repair are only mentioned briefly, and reviews are cited wherever possible. In addition, we focus on those growth factors and cytokines for which results from functional in vivo studies are available.

II. PLATELET-DERIVED GROWTH FACTOR FAMILY

PDGFs comprise a family of homo- or heterodimeric growth factors, including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD (reviewed in Ref. 120). They exert their functions by binding to three different transmembrane tyrosine kinase receptors, which are homo- or heterodimers of an α- and a β-chain (120, 121).

PDGF was the first growth factor shown to be chemotactic for cells migrating into the healing skin wound, such as neutrophils, monocytes, and fibroblasts. In addition, it enhances proliferation of fibroblasts and production of extracellular matrix by these cells. Finally, it stimulates fibroblasts to contract collagen matrices and induces the myofibroblast phenotype in these cells (56, 121). Thus it has long been suggested to be a major player in wound healing. Indeed, a series of experimental and clinical studies have demonstrated a beneficial effect of PDGF for the treatment of wound healing disorders (121). Furthermore, PDGF was the first growth factor to be approved for the treatment of human ulcers (80, 169).

A. Expression of PDGF at the Wound Site

In addition to its therapeutical potency, a series of studies suggest an important role of endogenous PDGF in the repair process. Upon injury, PDGF is released in large amounts from degranulating platelets (233), and it is present in wound fluid, particularly early after injury (35, 116, 173, 183, 204, 255, 275, 281). Furthermore, expression of PDGFs and their receptors has been demonstrated in various cells of murine, pig, and human wounds using in situ hybridization and immunohistochemistry (5, 6, 21, 223, 294). The patterns of PDGF and PDGF receptor expression suggest a paracrine mechanism of action, since the ligands are predominantly expressed in the epidermis, whereas the receptors are found in the dermis and the granulation tissue. Interestingly, expression of PDGFs and their receptors was reduced in wounds of healing-impaired genetically diabetic *db/db* mice and glucocorticoid-treated mice (19, 21), indicating that a certain expression level of PDGFs and their receptors is essential for normal repair. This hypothesis was supported by the finding that impaired wound healing in aged mice is associated with a delay in appearance of PDGF A and B isoforms, and α- and β-receptors (10). Finally, the levels of PDGF in nonhealing human dermal ulcers were strongly reduced compared with surgically created acute wounds (216), further supporting an important role of PDGF for normal healing.

On the other hand, augmented PDGF production might be involved in the pathogenesis of hypertrophic scars and keloids as suggested by the potent effect of PDGF on fibroblast proliferation and extracellular matrix production by these cells (see above), the presence of enhanced levels of this growth factor in hypertrophic scar tissue (198), and the increased responsiveness of keloid fibroblasts to PDGF (114).

B. Inhibition of PDGF Action in Healing Skin Wounds

Based on its expression pattern in the healing wound and its known in vitro activities, PDGF has been suggested to have two major but distinct roles in wound repair: an early function to stimulate fibroblast proliferation and a later function to induce the myofibroblast phenotype (56). This hypothesis was supported by the finding that addition of neutralizing PDGF antibodies to human wound fluid caused a 45% reduction in the mitogenic effect of the wound fluid for cultured fibroblasts (143). However, a recent study demonstrated that the PDGF-B chain of hematopoietic origin is not necessary for granulation tissue formation and that its absence even enhances vascularization (42). In this study, the authors prepared hematopoietic chimeras, in which the hematopoietic system of a normal adult mouse was replaced by that of a PDGF B-chain −/− donor. In these chimeras the extent of local granulation tissue was not affected, and vascularization was increased. These findings suggest that the production of PDGF by other cell types in the wound is sufficient for normal healing. The use of neutralizing antibodies for wound healing studies or analysis of tissue-specific PDGF or PDGF receptor knock-out mice will help to further clarify the role of endogenous PDGF in wound repair.

III. FIBROBLAST GROWTH FACTOR FAMILY

FGFs comprise a growing family of structurally related polypeptide growth factors, currently consisting of 22 members (206). They transduce their signals through four high-affinity transmembrane protein tyrosine kinases, FGF receptors 1–4 (FGFR1–4) (138), which bind the different FGFs with different affinities. Additional complexity is achieved by alternative splicing in the extracellular domains of FGFR1–3, which dramatically affects their ligand binding specificities. Most FGFs bind to a specific subset of FGF receptors. FGF1, however, binds to all known receptors, and FGF7 specifically interacts with a splice variant of FGFR2, designated FGFR2IIIb (207). A chararacteristic feature of FGFs is their interaction with heparin or heparan sulfate proteoglycans, which stabilizes FGFs to thermal denaturation and proteolysis, and which strongly limits their diffusibility. Most importantly, the interaction with heparin or heparan sulfate proteoglycans is essential for the activation of the signaling receptors (205).

Most members of the FGF family have a broad mitogenic spectrum. They stimulate proliferation of various cells of mesodermal, ectodermal, and also endodermal origin. The only exception is FGF7 (keratinocyte growth factor, KGF), which seems to be specific for epithelial cells, at least in the adult organism (289). In addition to their mitogenic effects, FGFs also regulate migration and differentiation of their target cells, and some FGFs have been shown to be cytoprotective and to support cell survival under stress conditions (17, 206, 289).

Numerous in vivo effects of FGFs have been demonstrated, which suggest a role of these growth factors in wound repair. In particular, FGF1 and FGF2 were shown to stimulate angiogenesis in various assay systems (226). Furthermore, FGFs are mitogenic for several cell types present at the wound site, including fibroblasts and keratinocytes (1). Thus FGFs are clear candidates for contributing to the wound healing response, and this hypothesis has been corroborated by a number of studies where local application of FGF1, FGF2, FGF4, FGF7, or FGF10 stimulated tissue repair (1, 289).

A. Expression of FGFs in Healing Skin Wounds

Some FGFs have been detected at the wound site, indicating that the endogenous proteins are also regulators of wound healing. FGF2 was found in human and porcine wound fluid, particularly at early stages after injury (35, 50, 61, 106, 199, 281). Using immunohistochemistry, this FGF has been localized in injured skin. In a mouse incisional wound model, FGF2 was found extracellularly at the surface of the wound and within the dermis adjacent to the wound. Interestingly, this staining pattern was only seen in wounds of adult mice but not in fetal wounds where FGF2 immunoreactivity was undetectable. It was suggested that this difference could explain at least in part the reduced amount of capillary formation seen in the fetal versus adult wounds (294). In a full-thickness excisional wound model in mice, FGF2 was associated with hair bulbs at the wound edge and with basal keratinocytes of the normal and hyperproliferative wound epidermis (152). In rat burn wounds, FGF2 immunoreactivity was detected in the regenerated epidermis, in a bandlike zone near the regenerated epidermis, in renewed capillaries, and in cells infiltrating in the granulation tissue (145). Finally, a diffuse extracellular staining was seen at the edge of human burn wounds (101). The observed differences are probably due to species-specific differences or to different cross-reactivities of the antibodies with other members of the FGF family. To overcome this problem, two groups determined the expression of FGFs during wound healing at the mRNA level. Using in situ hybridization, Antoniades et al. (7) found upregulation of FGF1 and FGF2 expression in keratinocytes of porcine wound epidermis. Werner et al. (291) determined the mRNA levels of different FGFs in full-thickness excisional mouse wounds by RNase protection assay. Expression of FGF1, FGF2, FGF5, and FGF7 was found in normal and wounded skin, and expression of all

these FGFs increased after skin injury. The most dramatic effect was seen with FGF7, which was more than 100-fold upregulated within 24 h after wounding. The strong upregulation of FGF7 expression was subsequently also confirmed for acute human wounds (172). In both mouse and human wounds, FGF7 mRNA was predominantly detected in dermal fibroblasts adjacent to the wound and in fibroblasts of the granulation tissue (172, 291). In addition, γδT cell receptor-bearing dendritic epidermal T cells (DETCs) were recently identified as a major source of FGF7 in murine skin wounds (134). Finally, FGF10 (KGF-2) was also shown to be expressed in mouse wounds (20, 134, 267), although upregulation of this type of FGF was only found in one study using RT-PCR (267), but not in another study where expression was determined by RNase protection assay (20). Similar to FGF7, FGF10 was predominantly expressed by DETCs (134) and fibroblasts (unpublished data).

In addition to the ligands, all FGF receptors are expressed in normal and wounded mouse skin (291; Werner, unpublished data). FGFR2IIIb, the only high-affinity receptor for FGF7, is expressed in keratinocytes of the normal and wounded epidermis as well as in hair follicles of murine, porcine, and human wounds (69, 172; Werner, unpublished data), and FGFR1 was found in the regenerating epidermis as well as in blood vessels of rat burn wounds (269).

Three different studies demonstrated a correlation between reduced FGF expression/responsiveness and wound healing disorders. Thus the mRNA levels of FGF1, FGF2, and FGF7 were reduced during wound healing in healing-impaired genetically diabetic mice compared with control mice (290). Furthermore, impaired would healing was seen in aged mice, and this impairment was associated with reduced levels of FGF2 and with a reduced angiogenic response in the skin of these mice upon addition of FGF2 (265). Finally, a member of the FGF family, most likely FGF2, was identified in a search for wound-regulated proteins (250). Expression of this FGF was found to be upregulated after injury in normal but not in diabetic rats.

B. A Role for FGF2 in Wound Repair

To provide functional evidence for a role of FGF2 in wound repair, Broadley et al. (37) used a neutralizing polyclonal antibody that was raised against human FGF2. They incorporated the purified IgG into pellets, which were placed in the center of a polyvinyl alcohol sponge disk, and the disks were then implanted subcutaneously under ventral panniculus carnosus of rats. The continuous release of the antibody caused a striking reduction in cellularity and vascularization compared with the granulation tissue formed in the control IgG sponges. In addition, DNA, protein, and collagen levels in the anti-FGF2 sponges were reduced by ~25–35% relative to control at *day 7* after implantation. This study strongly suggested an important role of endogenous FGF2 in wound repair, although cross-reactivity of this antibody with other members of the FGF family could not be excluded. The role of FGF2 in wound repair was finally clarified when FGF2 null mice were used for wound healing studies. Interestingly, these mice appeared superficially indistinguishable from wild-type littermates. However, when they were challenged by full-thickness excisional wounding, they showed delayed healing (208). In addition to a retardation in the rate of reepithelialization, mice null for FGF2 showed reduced collagen deposition at the wound site, and they had thicker scabs. In contrast, no wound healing abnormalities were observed in FGF1 knock-out mice, and in FGF1/FGF2 double knock-out mice, the defects were similar in extent to those seen in the FGF2 null animals (188). These results demonstrate that FGF1 is dispensable for wound healing in mice.

C. FGF Receptor Signaling Is Important for Reepithelialization

In addition to FGF2, several studies have provided evidence for an important role of FGF7 and its receptor (FGFR2IIIb) in cutaneous wound repair. The strong upregulation of this FGF in fibroblasts and DETCs after skin injury and the expression of its receptor in keratinocytes (see above) suggested that FGF7 stimulates wound reepithelialization in a paracrine manner. To test this hypothesis, transgenic mice were generated that express a dominant-negative FGFR2IIIb mutant in the epidermis (292). The mutant receptor lacks a functional tyrosine kinase domain and, upon ligand binding, forms nonfunctional heterodimers with full-length wild-type receptors, thereby blocking signal transduction (278, 279). The truncated FGFR2IIIb is known to bind FGF7, FGF10, FGF1, FGF3, and, although with lower affinity, also FGF2 (130, 207). Therefore, it should inhibit the action of all these ligands. The skin of the animals expressing the dominant-negative receptor mutant was characterized by epidermal atrophy, disorganization of the epidermis, hair follicle abnormalities, and dermal hyperthickening (292). Histological analysis of full-thickness excisional wounds revealed a severe delay in wound reepithelialization in the transgenic mice compared with control littermates. At *day 5* after injury, the number of proliferating keratinocytes in the hyperproliferative epithelium was 80–90% reduced compared with control mice. These results demonstrated an important role for FGF receptor signaling in wound repair, although the type of FGF that is responsible for this defect was not defined by this study.

D. FGF7-Deficient Mice Show No Defect in Wound Healing

To further determine the role of FGF7 in development and repair, Guo et al. (113) used embryonic stem cell technology to generate mice lacking FGF7. Their knock-out mice revealed no obvious defects, with the exception of the fur, which appeared matted and greasy, especially in male animals. Most surprisingly, the healing process of full-thickness incisional wounds was not obviously affected by the lack of FGF7, and the proliferation rate of the keratinocytes at the wound edge was not altered. These data demonstrate that incisional wounds can heal in the absence of FGF7. It would, however, be interesting to study the healing process of excisional wounds in these animals, since the extent of reepithelialization is much higher in excisional than in incisional wounds.

The lack of obvious phenotypic abnormalities in the FGF7 null mice is contradictory to the results obtained with the dominant-negative FGFR2IIIb mutant (see above). Although it might be possible that FGF7 is indeed not involved in reepithelialization of skin wounds, this seems unlikely, since the pattern of FGF7 expression correlates well with its postulated functions in normal and wounded skin. The most likely explanation for the discrepancies between the knock-out and the dominant-negative receptor results is a redundancy in ligand signaling. Although FGF7 might normally be the most important ligand of FGFR2IIIb in the skin, the lack of this gene could be compensated for by other known ligands of this receptor. More recent data suggest that FGF10 is the principal candidate for effecting this compensation, since it is also expressed in normal and wounded skin (20, 134, 267). Furthermore, mice lacking DETCs have a significant delay in wound reepithelialization, most likely due to the lack of DETC-derived FGF7 and FGF10 in the healing wound (134). Studies using neutralizing FGF7 and/or FGF10 antibodies during wound repair should help to further clarify the roles of FGF7 and FGF10 in the healing process. The tissue-specific knockout of FGFR2IIIb, as well as double knock-outs of different ligands of this receptor, will shed more light on the role of FGFR2IIIb and the various types of FGF in normal and wounded skin.

IV. EPIDERMAL GROWTH FACTOR FAMILY

The epidermal growth factor (EGF) family of mitogens comprises several members, including EGF, transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin, epiregulin, betacellulin, neuregulins, the recently discovered epigen, as well as proteins encoded by *Vaccinia* virus and other poxviruses (263, 276, 303). In addition, more distantly related proteins known as neuregulins (heregulins, neu differentiation factors, NDF 1–4) can also bind to some EGF receptor family members (303). All these growth factors exert their functions by binding to four different high-affinity receptors, EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 (Fig. 2*A*). Upon ligand binding, these receptors form homo- or heterodimers (303). Overexpression of these receptors, in particular of HER2, is often found in human cancers and is likely to have a causative role in tumorigenesis. In addition, a series of experimental and clinical studies have demonstrated a positive effect of EGF, TGF-α, and HB-EGF on wound repair, suggesting that the endogenous growth factors are also involved in the healing process (107, 240, 259).

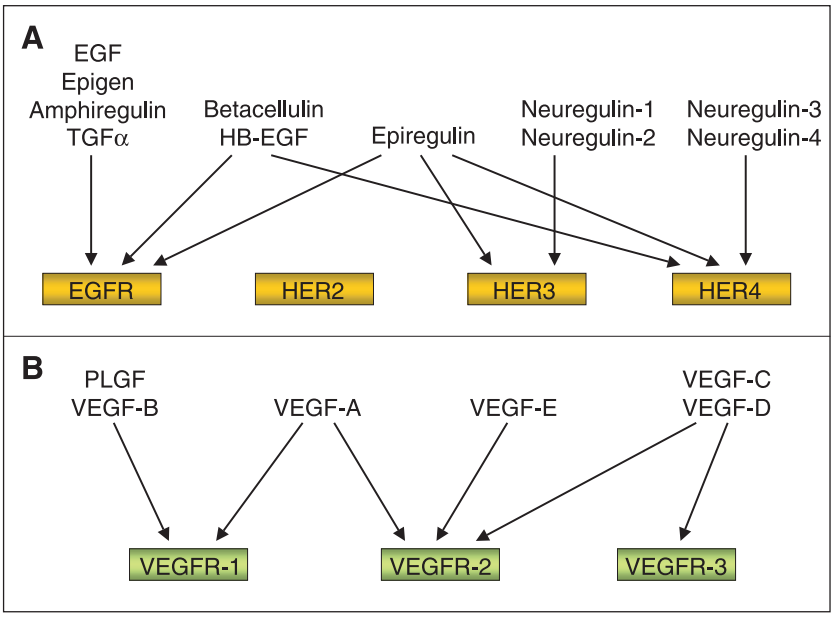

FIG. 2. Epidermal growth factor (EGF; *A*) and vascular endothelial growth factor (VEGF; *B*) family members and their receptors. Upon ligand binding, receptors form homo- or heterodimers. Note the lack of a ligand for HER2 homodimers. However, this receptor binds the ligand of a partner upon heterodimerization.

A. Expression of EGF, TGF-α, and HB-EGF at the Wound Site

First evidence for a role of EGF receptor ligands in wound healing came from the analysis of wound fluid. Grotendorst et al. (111) detected EGF-like factors in wound fluid collected from rats. Acid extracts from this type of wound fluid contained a chemotactic activity for endothelial cells that was neutralized with anti-EGF antisera (111). In addition, substantial levels of EGF and TGF-α were found in wound fluid from skin graft donor site wounds in patients with small to moderate-sized burn injuries (106). The result was confirmed for TGF-α in another study (204). However, this group detected only very low levels of EGF in the same type of wound fluid. Several publications report on the presence of HB-EGF in wound fluid. Thus this growth factor was shown to be present at high levels in human burn wound fluid (184). In addition, HB-EGF was identified as the major heparin-binding growth factor in wound fluid of porcine partial-thickness excisional wounds (173). Because HB-EGF is mitogenic for fibroblasts and keratinocytes, it was suggested to play an important role in reepithelialization and granulation tissue formation. Interestingly, it was shown to act synergistically with insulin-like growth factor (IGF) I, another growth factor present in wound fluid, in stimulating keratinocyte proliferation in vitro (174).

In a search for the cellular source of these EGFR ligands in wounds, Rappolee et al. (219) detected TGF-α mRNA in isolated wound macrophages. With the use of in situ hybridization and immunohistochemistry, this growth factor was also detected in eosinophils in a rabbit cutaneous open wound model and also in hamster wounds (272, 297). In addition, epidermal keratinocytes at the wound edge as well as hair follicle epithelial cells were identified as a source of TGF-α in partial-thickness murine burn wounds, in particular during the phase of keratinocyte proliferation (65). EGF immunoreactivity was found to be associated with the presence of wound inflammatory cells and wound fibroblasts in early rat CO_2 laser wounds (304). Finally, HB-EGF was localized in the advancing epithelial margin, islands of regenerating epithelium within human burn wounds, and in eccrine sweat glands (184). In another study, the same growth factor was detected in marginal surface keratinocytes and hair follicle epithelial cells of murine partial-thickness burn wounds, with maximal levels being found during the period of keratinocyte proliferation (65).

B. Expression of EGF Receptors at the Wound Site

EGF, TGF-α, and HB-EGF exert their function via binding to the EGFR, a transmembrane protein tyrosine kinase that is expressed on many different cell types. Consistent with the expression of the ligands at the wound site, EGFR mRNA and protein were also detected in healing wounds. With the use of enzyme-linked immunosorbent assay and histological methods, an increase in the number of immunoreactive receptors was found in a tape stripping wound model before an increase in epidermal thickness. This early increase was followed by a decline in EGFR levels, which was followed by a decline in epidermal thickness (262). This expression pattern suggested a role of the EGFR in reepithelialization of skin wounds. In early human full- and partial-thickness burn wounds, EGFR was detected in undifferentiated, marginal keratinocytes, in keratinocytes of the hyperproliferative wound epidermis and hair follicles, as well as in sweat ducts and sebaceous glands (288). At later stages after injury, immunoreactive EGFR was still detected in the hyperthickened wound epidermis and in all appendages, but was absent from leading epithelial margins (288). This expression pattern of the EGFR in human burn wounds provided further evidence for a role of EGFR signaling in reepithelialization. In addition, the observed delayed appearance of EGF and EGF receptors in incisional wounds of aged mice compared with young mice (10) further suggests a functional role of these proteins in the healing process.

C. Ectodomain Shedding of EGF Receptor Ligands Is Required for Keratinocyte Migration During Wound Healing

In addition to these correlative data, recent functional studies revealed an important role of EGFR ligands in wound repair. All EGFR ligands are synthesized as membrane-anchored forms, which are proteolytically processed to the bioactive soluble forms (180). Interestingly, the transmembrane forms are also able to stimulate the growth of adjacent cells in a juxtacrine manner, indicating that both transmembrane and soluble forms might play a role in wound healing. However, processed HB-EGF was detected in wound fluid (173), suggesting that ligand shedding could play an important role in wound healing. Indeed, in vitro scratch wounding of a keratinocyte monolayer induced shedding of EGFR ligands, particularly of HB-EGF. Shedding was inhibited by the compound OSU8–1, and this in turn suppressed keratinocyte migration. Most interestingly, the application of this compound to full-thickness mouse wounds caused a strong retardation of reepithelialization as a result of impaired keratinocyte migration. This inhibition was reversed by addition of recombinant soluble HB-EGF along with OSU8–1 (273). These results indicate an important role of EGFR

ligand shedding for keratinocyte migration in vitro and in vivo.

D. Wound Healing in Mice Deficient in TGF-α

Based on the presence of TGF-α in wound fluid (see above), its strong upregulation early after injury (113), and the beneficial effect of exogenous TGF-α for wound healing, TGF-α was expected to play an important role in the repair process. To test this possibility, two groups generated mice lacking this growth factor (164, 171). Surprisingly, these mice appeared normal with the exception of eye abnormalities and waviness of whiskers and fur. The epidermis of these animals was indistinguishable from that of control mice. Most interestingly, no significant wound healing abnormalities were observed in these mice, whereby two different wound models (full-thickness back skin excisions and tail amputation) were used. However, one group observed more variability in the rate of wound closure in TGF-α-deficient mice (164), suggesting that the lack of this mitogen can be compensated for to a variable extent by other growth factors. Such compensation could be achieved by other EGFR ligands, in particular HB-EGF. This hypothesis is supported by the severe phenotypic abnormalities of mice lacking the EGF receptor (187, 252) and of transgenic mice expressing a dominant-negative EGF receptor in the epidermis (193), although the wound-healing process in these animals has not been analyzed yet. In contrast, the lack of TGF-α is unlikely to be compensated for exclusively by FGF7, since incisional wound healing also appeared normal in mice lacking both TGF-α and FGF7 (113).

Although these initial studies suggested that TGF-α is dispensable for wound healing, a more detailed analysis revealed a role of this factor in the early phase of reepithelialization (146). These investigators generated full-thickness head wounds and partial-thickness ear wounds in the TGF-α knock-out mice. In the ear model, where healing is mainly achieved by reepithelialization, the knock-out mice had significantly larger epithelial gaps compared with control animals at *days 3* and *5* after injury, and the epithelial thickness was reduced at these time points. However, wounds of both genotypes were completely reepithelialized at *day 8* postwounding. In contrast, head wounds that heal by reepithelialization and granulation tissue formation were indistinguishable in TGF-α null mice and control animals. These data suggest a role of TGF-α in the early phase of reepithelialization, but the lack of this factor is compensated if healing is accompanied by granulation tissue formation. These results demonstrate the importance of the chosen wound model for the analysis of growth factor function in wound repair.

E. A Role of Neu Differentiation Factor in Wound Repair?

In addition to the EGF receptor ligands, Neu differentiation factor (NDF) might also play a role in the regulation of wound repair. Thus recombinant NDF-α2 stimulated epidermal migration, epidermal thickness, and keratinocyte differentiation in a rabbit ear model of excisional wound repair (68). Endogenous NDF was found to be upregulated during the healing process of full-thickness excisional wounds, possibly as a response to increased levels of FGF7 and HGF which were found to be potent inducers of NDF expression in cultured keratinocytes (46). With the use of in situ hybridization, NDF α-isoforms were found to be expressed in dermal fibroblasts of wounded and unwounded rabbit ear skin. HER2 and HER3 receptors, which mediate the function of NDF, were expressed in unwounded epidermis and dermal adnexa. After injury, expression of HER2 decreased in the wound neoepidermis, while neoepidermal HER3 expression was strongly upregulated (68). These results suggest that NDF stimulates keratinocyte migration during cutaneous wound repair in a paracrine manner.

V. VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY

The VEGF family currently includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PLGF). They exert their biological functions by binding to three different transmembrane tyrosine kinase receptors, designated VEGFR-1, VEGFR-2, and VEGFR-3 (95; Fig. 2*B*). The biological functions of VEGF-A and its receptors VEGFR-1 and VEGFR-2 have been characterized in most detail. Based on a series of in vitro and in vivo studies, VEGF-A has been identified as a major regulator of vasculogenesis and angiogenesis during development (95), indicating that it might also be involved in the regulation of angiogenesis during wound healing.

A. Expression of VEGF-A and Its Receptors in Skin Wounds

In support for a role of VEGF-A in wound repair, expression of this gene was shown to be strongly induced after cutaneous injury, with keratinocytes and macrophages being the major producers (40, 90). In addition, its receptors were detected on blood vessels of the granulation tissue (153, 213). This expression pattern suggested that VEGF-A stimulates wound angiogenesis in a paracrine manner. The important role of VEGF-A for the healing process was supported in several studies where reduced expression of VEGF-A or its accelerated degradation were found to be associated with wound healing

defects (90, 140, 153, 265). Furthermore, treatment of ischemic wounds with VEGF-A or VEGF-A-overexpressing fibroblasts accelerated the healing process (33, 55), and adenovirus-mediated VEGF-165 gene transfer enhanced wound healing in diabetic mice by promoting angiogenesis (232).

B. A Role for VEGF-A in Wound Angiogenesis

The important role of VEGF-A in wound healing was recently revealed in a study where application of neutralizing VEGF-A antibodies caused a striking reduction in wound angiogenesis, fluid accumulation, and granulation tissue formation in a pig wound model (124). Furthermore, the angiogenic activity present in human wound fluid from later time points after injury was strongly inhibited by VEGF neutralization (200). Finally, retroviral delivery of a dominant-negative VEGFR-2 to murine skin wounds caused a strong reduction in angiogenesis and granulation tissue formation (277). However, wound closure was not affected in these animals due to increased wound contraction. These findings support the important role of endogenous VEGF in wound angiogenesis, although functional VEGFR2 signaling is obviously not critical for normal closure of acute excisional wounds.

C. Lack of PLGF Results in Impaired Wound Angiogenesis

In addition to VEGF-A, PLGF was recently identified as a regulator of wound angiogenesis. Expression of PLGF mRNA and protein was strongly upregulated in migrating keratinocytes of acute human skin wounds. Furthermore, endothelial cells of capillaries adjacent to the wound expressed PLGF (84). This upregulation appears to be of functional importance, since PLGF knockout mice were characterized by impaired wound healing as a result of a defect in angiogenesis (45). Interestingly, a synergy between VEGF-A and PLGF was detected in these studies, indicating that the presence of both growth factors is important for normal wound angiogenesis.

D. Expression of VEGF-C and Its Receptor in Healing Skin Wounds

Besides the formation of new blood vessels, lymphangiogenesis occurs during the healing of skin wounds. Several groups have shown the formation of lymphatic vessels to be regulated via VEGFR-3 and its ligands VEGF-C and VEGF-D (142). In a recent study using a pig wound model, VEGFR-3-positive lymphatic vessels were found in the wound granulation tissue (209). These vessels appeared in the wound concurrently with blood vessels but regressed earlier. The responsible ligand is probably VEGF-C, which is expressed in normal and wounded mouse skin (unpublished data). Interestingly, a relative absence of lymphatic vessels was found in chronic human wounds (209), which might be one of the reasons for their impaired healing. Taken together, members of the VEGF family are likely to be major regulators of angiogenesis and lymphangiogenesis not only during development but also during cutaneous wound repair.

VI. ANGIOPOIETINS

In addition to the VEGFs, angiopoietins comprise a second family of growth factors acting on the vascular endothelium. Up to now, four different angiopoietins have been discovered that bind to a transmembrane tyrosine kinase receptor, Tie2, that is exclusively present on endothelial cells. Interestingly, angiopoietins-1 and -4 were identified as activators of this receptor, whereas angiopoietins-2 and -3 are likely to block the activity of this receptor under most circumstances. Unlike VEGFs, angiopoietins do not regulate endothelial cell proliferation; rather, angiopoietin-1 is responsible for the stabilization of blood vessels, whereas angiopoietin-2 causes vessel destabilization and remodeling (95).

A. Expression of Angiopoietins and Their Receptor in Healing Skin Wounds

First evidence for a role of angiopoietins in wound healing came from studies by Wong et al. (296), who demonstrated upregulation of Tie2 protein and mRNA in rat and mouse skin wounds, respectively. Moreover, Tie2 was found to be tyrosine-phosphorylated in the healing wound, indicating active downstream signaling. In addition to the receptor, two groups demonstrated expression of angiopoietins-1 and -2 in normal and wounded mouse skin. Whereas angiopoietin-1 expression was not affected by skin injury, angiopoietin-2 expression was transiently upregulated during the period of granulation tissue formation in normal mice (30, 140). In healing-impaired genetically diabetic mice, the period of angiopoietin-2 upregulation was extended (140). Thus wounds in diabetic mice are characterized by high levels of angiopoietin-2 but low levels of VEGF-A (90), a situation that has been suggested to lead to blood vessel regression during tumorigenesis (123). These findings suggest that the strongly impaired angiogenic response in diabetic animals could result from an imbalance in the levels of VEGF-A and angiopoietins.

VII. INSULIN-LIKE GROWTH FACTORS

IGF-I and IGF-II are potent stimulators of mitogenesis and survival of many different cells types, and they

exert their functions in an autocrine, paracrine, or endocrine manner. Their actions are mediated through the type I IGF receptor, a tyrosine kinase that resembles the insulin receptor. In addition, IGF-II also binds to the IGF type II/mannose-6-phosphate receptor, which results in internalization and degradation of IGF-II (201). The availability of free IGF for interaction with the IGF-I receptor is modulated by six IGF-binding proteins (IGFBPs). In addition, IGFBPs have also been shown to have IGF-independent effects on cell growth (54). Several studies have revealed a beneficial effect of exogenous IGF-I on wound healing, in particular in combination with other growth factors (167). In addition, liposome-mediated IGF-I gene transfer improved the pathophysiology of a thermal injury (136). These findings suggested important activities of IGFs in the healing wound.

A. Expression of IGFs and Their Receptors in Skin Wounds

Several groups demonstrated expression of IGF-I and IGF-II in wounds of different species. Thus IGF-I was found in rat and porcine wound fluid (174, 230, 260), and minimal degradation of this protein was observed (231). In an attempt to localize IGFs and their receptors at the wound site, one group used a rat ear freeze-thaw injury model to study IGF-I expression by immunohistochemistry (135). In normal skin, only a few cells in the dermis and epidermis expressed this protein. However, all epidermal cells as well as macrophages and some other inflammatory cells were positive within 1–3 days after wounding. Others used an incisional wound model as well as a subcutaneous sponge implant model to determine expression of IGF-I and IGF-II in the wound (97). Interestingly, the mRNA levels of both IGFs increased significantly after injury in both models. Increased IGF-I mRNA levels but unaltered IGF-I receptor expression were observed in a rat wound model where steel wire mesh cylinders were implanted in the subcutaneous tissue of the back (260). Finally, in situ hybridization studies on porcine wounds revealed expression of IGF-I, IGF-I receptor, and IGF-II receptor mRNAs in epithelial cells of normal and wounded skin. In this study, however, no major differences between nonwounded and wounded skin were observed (7).

B. Impaired Wound Healing Is Associated With Abnormal Expression of IGFs and Their Receptors

Several studies suggest a role of the IGF system in the wound healing abnormalities associated with diabetes and glucocorticoid treatment. Thus one group found that streptozotocin-induced diabetes in rats caused a 42% reduction in wound fluid IGF-I levels (27). Others analyzed the expression of IGF-I and IGF-II during wound healing in normal and genetically diabetic mice (39). The normal induction of IGF-I mRNA expression was severely delayed and reduced in diabetic mice. Delayed induction was also seen for IGF-II, although peak concentrations of IGF-II mRNA were higher in diabetic compared with control mice. Consistent with the RNA data, a delayed appearance of the proteins was noted in diabetic animals. In another study, subcutaneously implanted polyvinyl sponges and stainless steel mesh chamber models were used to analyze the levels of IGF-I, IGF-I receptor, and IGFBP3 mRNAs in wound tissue of healing-impaired diabetic and glucocorticoid-treated rats (26). Interestingly, expression of all these genes was strongly reduced in the healing-impaired animals, further supporting the importance of the IGF system for normal healing. These findings are likely to be important for the pathogenesis of chronic human wounds, since IGF-I protein was absent in the basal layer of the epidermis and in fibroblasts of diabetic patients but not of healthy control patients. Furthermore, it was absent in the basal keratinocyte layer at the edge of human diabetic foot ulcers (28). Taken together, these studies suggest that reduced expression of IGFs and/or their receptors leads to impaired wound healing, although this hypothesis has yet to be confirmed by functional studies.

On the other hand, enhanced expression of IGF-I might lead to excessive scarring as suggested by the observed overexpression of IGF-I in postburn hypertrophic scar tissue compared with control skin. Because IGF-I was shown to increase the expression of the pro alpha 1(I) chain of type I procollagen and the pro alpha 1 (III) chain of type III procollagen in cultured dermal fibroblasts, these findings indicate a causative role of elevated IGF-I levels in the pathogenesis of hypertrophic scars (98).

VIII. SCATTER FACTORS

The family of scatter factors (SF), also known as plasminogen-related growth factors (PRGF), encompasses two members to date: hepatocyte growth factor (HGF)/SF, also called PRGF-1, and macrophage-stimulating protein (MSP), also called hepatocyte growth factor-like protein (HGFL) or SF2 or PRGF-2. They are both secreted as large inactive precursors, which are proteolytically cleaved to produce active, disulfide-linked heterodimers (59).

HGF was independently discovered as a powerful mitogen for hepatocytes and as a stimulator of dissociation of epithelial cells. Due to these features it was designated HGF or SF. It is predominantly produced by cells of mesenchymal origin and acts via a high-affinity trans-

membrane tyrosine kinase receptor (MET) on various cell types. In addition, heparan sulfate proteoglycans act as low-affinity receptors for HGF and allow accumulation of the ligand in the proximity of its target cells (59). Because HGF stimulates migration, proliferation, and matrix metalloproteinase production of keratinocytes (78, 182), as well as new blood vessel formation (43), it has been suggested to play a role in cutaneous wound repair.

MSP is a liver-derived serum protein that regulates proliferation and differentiation of various cell types. In the serum MSP is predominantly present in the inactive precursor form, whereas active MSP is only generated at the surface of its target cells. The latter express RON, the only known high-affinity receptor for this protein. It is present on many different cell types, including macrophages and keratinocytes, suggesting a function of MSP in wound repair (155, 253).

A. Overexpression of HGF Enhances Granulation Tissue Formation and Wound Angiogenesis

Expression of HGF and its receptor MET was found to be strongly upregulated in keratinocytes of the wound epidermis as well as in several cell types in the granulation tissue during the healing of excisional wounds in rats (63). This upregulation is likely to be of functional importance, since transgenic mice overexpressing HGF under the control of the metallothionein promoter were characterized by enhanced granulation tissue formation after full-thickness excisional wounding, and the number of blood vessels in the granulation tissue was strongly increased. This effect on wound angiogenesis seems to be at least partially mediated via VEGF-A, since the latter was overexpressed in these transgenic mice (274). In contrast, reepithelialization was obviously not affected by overexpression of HGF. These results revealed important activities of HGF during wound healing, although the role of the endogenous protein in the healing process remains to be determined.

B. Expression of MSP at the Wound Site

First evidence for a role of MSP in wound healing came from studies by Nanney et al. (195), who demonstrated the presence of MSP in wound exudates of burn patients. Interestingly, a large percentage of the wound exudate-derived MSP was found to be in the active form, and MSP was shown to be responsible for the stimulatory effect of wound exudate on macrophages. In the same study, a marked upregulation of RON expression was demonstrated in burn wound epidermis and accessory structure as well as on macrophages and capillaries of the granulation tissue (195). Because MSP stimulates macrophage pinocytosis and phagocytosis in vitro (253), this study suggested that MSP may enhance macrophage-dependent wound debridement. In another study, the localization of MSP and RON was determined in full-thickness excisional wounds in rats (63). MSP-positive cells were identified by immunofluorescence at the wound edge as well as in cells within the wounds, and some of them were shown to be monocytes. In addition, RON was detected in the granulation tissue, but not in the wound epidermis.

C. MSP Is Dispensable for Wound Repair

To determine the role of MSP in cutaneous wound repair, mice lacking the *msp* gene were generated (24). Although these animals were characterized by delayed macrophage activation, no macroscopic differences in the healing of incisional wounds were observed. However, it is still possible that these mice have subtle wound healing abnormalities that are only detectable upon histological and/or molecular analysis.

IX. NERVE GROWTH FACTOR

Nerve growth factor (NGF) is the prototype for the neurotrophin family of polypeptides, which are essential for the development and survival of certain sympathetic and sensory neurons in both the central and peripheral nervous systems (158). In addition, it plays a key role in the initiation and maintenance of inflammation in various organs. Thus it has been suggested that NGF is also involved in cutaneous wound repair. This hypothesis was supported by the observation that removal of the submandibular glands of mice retards the rate of contraction of skin wounds and that licking of wounds enhances contraction (128). Because NGF is present at high levels in saliva, this growth factor was thought to be responsible for this effect. Indeed, exogenous NGF was shown to accelerate wound healing in normal and healing-impaired diabetic mice (159, 181) and to promote the healing of pressure ulcers in humans (23).

A. Expression of NGF in Skin Wounds

A role of endogenous NGF in wound healing was further supported by studies of Constantinou et al. (60), who found a marked increase in NGF levels after wounding of neonatal but not of adult rats. Subsequently, a rise in serum NGF levels after generation of full-thickness wounds in mice was demonstrated, which was shown to be due to release of NGF from the salivary gland (181). In addition, NGF levels also increased at the wound site in the same wound model, and NGF mRNA was detected in newly formed epithelial cells at the wound edge and in granulation tissue fibroblasts (181). A particular high ex-

pression of NGF was found in myofibroblasts within the granulation tissue of rat wounds, with much higher levels being found in myofibroblasts of neonatal compared with adult animals (118).

B. Multiple Roles for NGF in Wound Healing?

Due to its potent effects on sensory nerves, the major function of NGF in the wound tissue appears to be the stimulation of nerve ingrowth. This hypothesis is supported by results obtained in an in vitro coculture model, which demonstrated a potent effect of adult rat dorsal foot skin on dorsal root ganglia neurite outgrowth. This function was blocked by neutralizing antibodies to NGF (224). However, the activity in wounded neonatal skin was not blocked by these antibodies, suggesting the presence of other factors in neonatal wounds that induce neurite outgrowth (224).

Because innervation has been shown to be essential for normal wound healing (117 and references therein), the stimulatory effect of NGF on the wound repair process is likely to be at least partially due to its effect on nerves. This might be of particular importance in diabetic patients who suffer from peripheral neuropathy, which often results in impaired wound healing. Indeed, NGF administration was shown to protect against experimental diabetic sensory neuropathy (8), and NGF depletion was found in keratinocytes in diabetic human skin (4), suggesting that NGF might be helpful for the treatment of diabetic foot ulcers.

In addition to its effect on nerves, NGF affects other cell types present in the healing skin wound. Thus NGF stimulates proliferation and inhibits apoptosis of keratinocytes in vitro (217), and enhances proliferation and adherence molecule expression on human dermal microvascular endothelial cells (220). Finally, a recent report demonstrated that NGF has a potent effect on fibroblast migration and increases α-smooth muscle actin expression and collagen gel contraction by these cells (186), indicating that NGF regulates various processes during cutaneous wound repair. Independent from its mechanisms of action, the presence of higher levels of NGF in neonatal compared with adult wounds suggests that this growth factor is at least partially responsible for the faster healing observed in neonatal animals.

X. TRANSFORMING GROWTH FACTOR-β

The TGF-β superfamily encompasses a diverse range of proteins, many of which play important roles during development, homeostasis, disease, and repair. The structurally related but functionally distinct mammalian members include TGF-β1–3, bone morphogenetic proteins (BMPs), Mullerian inhibiting substance, nodals, inhibins, and activins (178). Their biological effects are mediated by heteromeric receptor complexes, which activate intracellular signaling cascades (282).

The three mammalian TGF-β isoforms (TGF-β1, -β2, and -β3) are synthesized as latent precursors, usually being secreted as a complex with latent TGF-β-binding protein, which is then removed extracellularly via proteolytic cleavage (227; Fig. 3). Active TGF-βs then exert their biological functions via binding to a heteromeric receptor complex, consisting of one type I and one type II receptor, both of which are serine-threonine kinases. In addition, they bind with high affinity to a nonsignaling type III receptor, which functions mainly to present TGF-β to the type II receptor (227; Fig. 3). The three TGF-β isoforms have both distinct and overlapping functions. In vitro, they have been shown to be mitogenic for fibroblasts, but

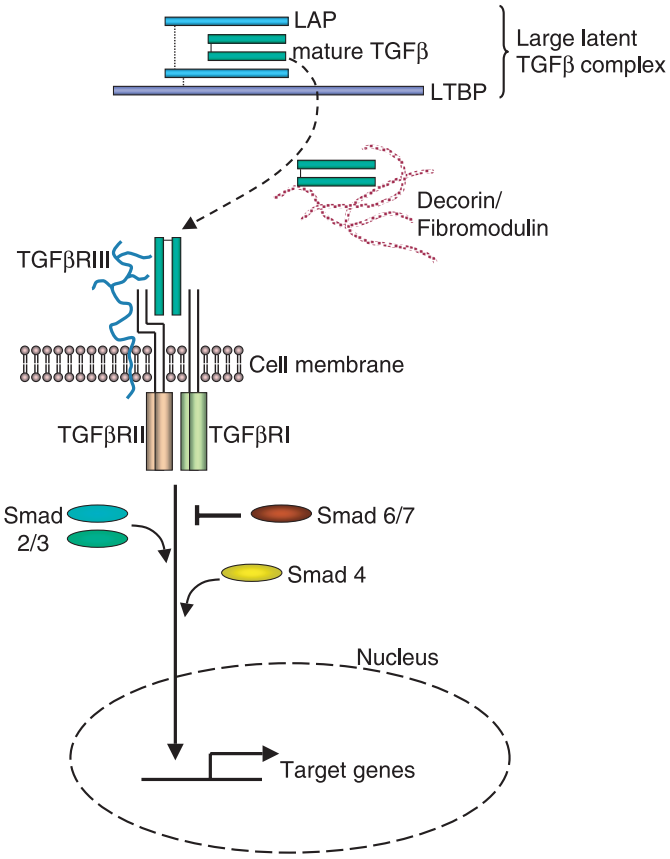

FIG. 3. Activation of Smad proteins by transforming growth factor (TGF)-β receptors. TGF-β is first produced as an inactive precursor that binds to latency-associated protein (LAP). The latter is covalently bound to latent TGF-β binding protein (LTBP). Upon activation, TGF-β is either sequestered by extracellular binding proteins (decorin, fibromodulin) or it binds to a type III receptor that presents it to the signal-transducing receptors (type II and type I). Upon ligand binding, TGF-β type II receptor recruits and phosphorylates the type I receptor. The latter subsequently binds and phosphorylates Smad2 and Smad3. Phosphorylated Smad2 and Smad3 bind to Smad4 and translocate to the nucleus where they bind to other transcription factors that confer specificity, leading to activation of target genes. Other signaling pathways that are also used by the TGF-β receptor (282) are not included in the figure.

they inhibit proliferation of most other cells, including keratinocytes. Furthermore, TGF-βs are very potent stimulators of the expression of extracellular matrix proteins and integrins (178, 179, 228). Thus they possess the properties expected of wound cytokines and indeed are among the most studied molecules in the wound healing scenario.

A. Expression of TGF-β at the Wound Site

Immediately after wounding, TGF-β1 is released in large amounts from platelets (13) (Fig. 4). This initial kick-start of active TGF-β1 from platelets serves as a chemoattractant for neutrophils, macrophages, and fibroblasts, and these cell types further enhance TGF-β1 levels in various cell types (Fig. 4). As well as active forms, latent TGF-βs are also produced and sequestered within the wound matrix, allowing sustained release by proteolytic enzymes. This combination of different cellular sources and temporary storage ensures a continuous supply of TGF-β throughout the repair process (228). Several publications report on the presence of TGF-βs in wound fluid of different species (27, 35, 204, 281). Furthermore, expression of all three isoforms was detected in many different cell types during repair, with each isoform having a characteristic distribution in the wound tissue (91, 141, 157, 238, 270, 271). In most studies, a rapid induction of TGF-β1 and -β2 was observed, whereas an increase in TGF-β3 expression was seen at later stages of repair. These results have been reviewed in detail (203). Interestingly, at least some of the TGF-β present at the wound site was shown to be active as determined by a new in situ activity assay (301). In addition to the ligands, the type I and the type II TGF-β receptors are present in various cell types within the healing wound (104, 238, 239).

On the basis of the expression pattern of TGF-βs and their receptors in the healing skin wound and on the observed effects of exogenous TGF-β, it has been suggested that TGF-βs stimulate reepithelialization and granulation tissue formation. The effect of TGF-β on reepithelialization appears paradoxical; its expression by keratinocytes after wounding together with the inhibitory effect of TGF-β on keratinocyte proliferation in vitro and in vivo (58, 243) suggests TGF-β as a negative regulator of reepithelialization. On the other hand, it also induces the expression of integrins necessary for keratinocyte migration across the fibronectin-rich provisional wound matrix (94, 305), and exogenous TGF-β was shown to stimulate keratinocyte migration and wound reepithelialization (119, 305). However, treatment of hairless mouse ear wounds with neutralizing antibodies to TGF-β1 and -β2 suggested that the endogenous growth factors are not essential for reepithelialization and neovascularization in this healing model (82). Furthermore, treatment of porcine burn wounds with a synthetic TGF-β antagonist accelerated wound reepithelialization (125). Most importantly, several results obtained with transgenic and knock-out mouse models revealed an inhibitory role of endogenous TGF-β1 in wound reepithelialization (see below).

The presence of TGF-β in the granulation tissue was expected to be important for efficient healing, since TGF-β was shown to stimulate angiogenesis, fibroblast proliferation, myofibroblast differentiation, and matrix deposition (71, 228, 229). This hypothesis is supported by a series of studies in several animal models that demonstrated a beneficial effect of exogenous TGF-β for wound repair, in terms of both the rate of healing and the strength of the healed wound (228). Complementary to these data are findings suggesting that aberrant expression of TGF-βs is associated with the wound healing defect seen in glucocorticoid-treated (91) and aged mice (10) as well as in diabetic rats (27).

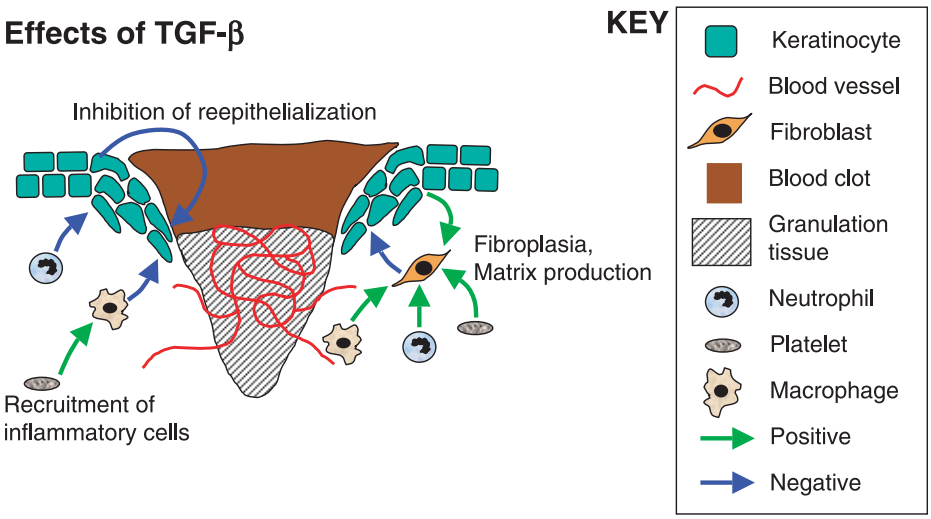

FIG. 4. Multiple functions of TGF-β during wound healing. Upon local hemorrhage, TGF-β is released in large amounts from platelets. In the healing wound, it is produced by leukocytes, macrophages, fibroblasts, and keratinocytes and acts on these cells to stimulate infiltration of inflammatory cells, fibroplasia, matrix deposition, and angiogenesis. In contrast, endogenous TGF-β has been shown to inhibit reepithelialization.

B. Neutralizing Antibodies to TGF-β1 and -β2 Reduce Scarring

Several studies support an important role of TGF-βs in cutaneous scarring. First of all, a reduced and/or more transient expression of TGF-βs and their receptors was observed in nonscarring fetal wounds compared with adult wounds (62, 177, 197, 264, 294). In addition, a strong and persistent expression of TGF-βs and their receptors was detected in fibroblasts of human postburn hypertrophic scars (99, 239, 241, 283, 306), and overexpression of TGF-β1 and -β2 was found in keloid tissues and keloid-derived fibroblasts (154, 211). Finally, the activity of TGF-β appears to be increased in scar tissue. Thus the expression of decorin, an extracellular matrix proteoglycan that inhibits TGF-β bioactivity (Fig. 3), was downregulated in postburn hypertrophic scars (241). Fibromodulin, another TGF-β binding protein (Fig. 3), was expressed at significantly higher levels in nonscarring fetal wounds compared with scarring wounds at later stages of gestation (256). On the other hand, decorin was shown to be downregulated in scar-free healing embryonic rat wounds compared with later wounds that develop a scar (18). Thus the role of decorin in the scarring response remains to be determined.

Interestingly, treatment of fetal wounds with different concentrations of TGF-β1 caused marked scarring of these wounds, demonstrating a direct involvement of TGF-β1 in cutaneous scarring (264). This finding was further supported by studies from Shah et al. (244, 245). In these experiments, incisional rat wounds were treated with neutralizing antibodies to TGF-β1 or to a combination of TGF-β1 and -β2. This treatment caused a significant reduction in extracellular matrix deposition and subsequent scarring, suggesting that endogenous TGF-β1 and -β2 induce cutaneous scarring in adult animals. A reduced scarring response was also observed in mouse wounds that were topically treated with antisense TGF-β1 oligodeoxynucleotides (51). Finally, topical application of a synthetic TGF-β antagonist reduced scarring in porcine burn and excisional wounds as well as in rabbit skin excisions (125).

On the other hand, treatment of the same type of wounds with recombinant TGF-β3 also inhibited scarring, indicating that this type of TGF-β antagonizes the effect of the other TGF-β isoforms (245). However, studies from other authors have yielded contradictory results concerning the effect of TGF-β3 on connective tissue deposition. They demonstrated an increase in new dermal matrix by exogenous application of TGF-β3 to wounds in age-impaired animal models (64). Independent of the effect of exogenous TGF-β3 on the scarring response, the role of the endogenous protein in wound healing and scar formation remains to be determined.

Reflecting the importance of TGF-β in wound repair, several groups have conducted wound healing studies on mice genetically modified such that they have either deficiency or gain of function at various levels of the TGF-β signaling pathway.

C. TGF-β1-Deficient Mice Show Severely Impaired Late-Stage Wound Repair

In the first study to use a knock-out approach to further clarify the role of the TGF-β1 isoform in wound repair, Brown et al. (41) wounded transgenic mice deficient in TGF-β1 due to a targeted disruption of the *tgf-β1* gene (151). These mice exhibit no obvious developmental abnormalities and appear phenotypically normal until, at ~3 wk of age, they develop a severe wasting syndrome accompanied by a pronounced multifocal inflammatory response and tissue necrosis, resulting in multisystem organ failure and death. To overcome this problem, the animals were wounded at *day 10* after birth.

Full-thickness excisional wounds healed almost normally for the first few days in the TGF-β1-deficient mice. However, histological analysis of the wounds at *day 10* after injury revealed a thinner, less vascular granulation tissue in the knock-out mice, which was dominated by a marked inflammatory cell infiltrate. Furthermore, decreased reepithelialization and collagen deposition were observed in mutant animals when compared with control mice (41). Superficially, this suggests that other TGF-β isoforms or even different growth factors can compensate for the lack of TGF-β1 in early wounds, but implies that TGF-β1 plays a crucial role later in the repair process. Alternatively, maternal rescue of TGF-β1 by transmission in the milk (156) might explain the lack of abnormalities in early wounds, with differences only becoming apparent as the mice are fully weaned and lack any maternal TGF-β1. The lack of TGF-β1 ultimately caused a severe inflammatory response in the wound, but since this was also seen in many other tissues it may not be of great significance to understanding the function of TGF-β1 at the wound site. The defects in wound repair are likely to be a secondary effect, perhaps due also to the severe wasting syndrome observed in these mice. Malnutrition and weight loss have been associated with impaired wound healing (108), and the weight loss which accompanies the inflammatory response is also likely to exert an adverse effect on repair.

D. Immunosuppressive Approaches Allow the Study of TGF-β1 Function in Adult Wounds

Two independent groups have used different approaches to dissect the TGF-β1-dependent wound healing defects from the effects of severe inflammation. Although the studies used independently generated knock-out

mice, the unwounded mice were reported to have essentially identical phenotypes (151, 251).

In a pharmacological approach, Koch et al. (149) used the immunosuppressive drug rapamycin to subdue the multifocal inflammatory disease phenotype seen in their TGF-β1 null mice (251), extending their lifespan from <30 days to up to 60 days. This drug has no effect on repair in wild-type mice. Based on the observation that maternal TGF-β1 protein is still present in wounds made to 10-day-old knock-out mice, they studied incisional wound repair in 30-day-old mice. At this age, immunohistochemistry revealed TGF-β1 protein to be markedly reduced in wounds of knock-out mice, but the mice did not show any of the inflammatory foci characteristic of untreated TGF-β1-null littermates. Wounds to TGF-β1-null mice showed enhanced healing, with narrower, scabless wounds, less granulation tissue, and reduced collagen deposition. The rate of reepithelialization increased such that, 3 days postwounding, wounds in the knock-out mice were 90% covered with the neoepidermis. In contrast, only 22% of the wound surface was reepithelialized in control wounds, suggesting that endogenous TGF-β1 is inhibitory to reepithelialization. However, these differences in repair come with the caveat that the unwounded skin of these TGF-β1-null mice already shows clear differences compared with skin of wild-type littermates. Histological analysis revealed the epidermis, dermis, and panniculus carnosus of control mice to be 52, 58, and 48% thicker, respectively, compared with TGF-β1 knock-out mice. Such differences could be at least partially responsible for the observed wound healing phenotype.

In a genetic approach, Crowe et al. (66) crossed TGF-β1 null mice onto the immunodeficient Scid −/− background (66). Scid −/− mice lack T and B cells and, therefore, do not have the machinery to mount the large inflammatory response seen in nonimmunocompromised TGF-β1-null mice (41). This enabled excisional wound healing experiments to be performed on mice of 8–10 wk of age. In contrast to what was predicted, the absence of inflammation in TGF-β1 −/− Scid −/− mice resulted in a remarkable delay in repair, delaying all of the major phases by at least a week compared with TGF-β1 +/+ Scid −/− controls. The wounds of TGF-β1 −/− Scid −/− mice had still not fully repaired by 21 days postwounding, in contrast to the controls, 100% of which were fully healed by 16 days. This delay was not singly due to either the lack of TGF-β1 or the lack of lymphocytes, but to the combination of the two. This suggests that TGF-β1 and lymphocytes may affect compensatory pathways during repair. Alternatively, the delay may be a side effect of absence of TGF-β1 in wounds leading to delayed expression of the other two TGF-β isoforms, TGF-β2 and -β3. Although unable to distinguish between which of these hypotheses may be true, this study presents a valuable method for bypassing a knock-out phenotype that would otherwise mask a defect in wound repair.

E. TGF-β1 Overexpression Studies Yield Contrasting Results, Dependent on the Transgenic Strategy

In contrast to the knock-out approaches described above, two groups have investigated the effect of excess levels of TGF-β1 on wound repair. Shah et al. (246) began with the hypothesis that elevated levels of circulating TGF-β1 would accelerate healing but also enhance scarring. Mice with elevated plasma levels of active TGF-β1 were generated by overexpressing a constitutively active porcine TGF-β1 mutant in the liver under the control of the mouse albumin promoter. Using a dorsal incisional wound model, complemented by ventral subcutaneous implantation of PVA sponges, they were able to study both normal cutaneous wound repair and cellular infiltration as a model of granulation tissue formation.

Surprisingly, they found that, while the PVA sponges yielded the expected results, with increased cellularity, granulation tissue formation and collagen deposition in transgenic animals, local TGF-β1 levels were lower in the incisional wounds of transgenic mice than in their control littermates. As such, the data show that increased circulating levels of TGF-β1 do not necessarily lead to increased levels of TGF-β1 at the wound site. Concomitant with the decreased TGF-β1 level in the wounds of transgenic mice, they observed an increase in levels of TGF-β3 and type II TGF-β receptor at the wound site, and this might be the reason for the improved neodermal architecture in the healed wounds of the transgenic mice.

In a different approach, Yang et al. (300) generated mice constitutively overexpressing latent human TGF-β1 in the epidermis under the control of the human keratin 14 promoter (300). They showed increased levels of latent TGF-β1 protein in unwounded keratinocytes, as well as a dramatic increase in both latent and active TGF-β1 following wounding. A CO_2 laser wounding model was used to generate partial thickness dorsal burns, ablating cells down to the adipose tissue but not damaging the underlying musculature. In wild-type mice, such wounds normally (92%) complete reepithelialization within 12 days, but in hemizygous and homozygous TGF-β1 overexpressing mice only 42 and 25% of wounds, respectively, had healed at this time point. Quantitative studies revealed transgenic mice to have significantly higher levels of active TGF-β1 at the wound site, in contrast to the systemically overexpressing mice discussed above (246). The major effect of the excess TGF-β1 was to inhibit keratinocyte proliferation, hence the delayed reepithelialization, although it also acted in a paracrine fashion to increase the expression of type I collagen mRNA and hydroxypro-

line in transgenic wounds. Similar findings were obtained when these mice were subjected to full-thickness excisional wounding (47). The different results obtained in the Yang/Chan studies on the one hand and the Shah study on the other hand may be due in part to the stability of latent TGF-β1 (half-life 100 min) relative to active TGF-β1 (half-life 2–3 min), as well as to the more specific targeting of transgene expression to the site of injury.

F. Mice Expressing a Dominant-Negative Type II TGF-β Receptor in the Epidermis Show Accelerated Reepithelialization and Reduced Keratinocyte Apoptosis

Rather than adopting a ligand-based approach to understanding the role of TGF-β at the wound site, Amendt and colleagues (2, 3) chose to target the type II TGF-β receptor by overexpressing a dominant-negative human type II TGF-β receptor in the basal layer of the epidermis of transgenic mice using a keratin 5 promoter. The dominant-negative receptor lacks most of the intracellular domain, including the kinase domain, and upon dimerization blocks signaling by wild-type receptors (2). This approach blocks the action of all TGF-β isoforms in basal keratinocytes. Excisional wounds to hemizygous transgenic mice showed an enhanced rate of reepithelialization, characterized by increased proliferation (between 50–100% higher dependent on the transgenic line) and decreased apoptosis (~50% lower) in keratinocytes at the wound edge. These data fit well with the study discussed below (12), where abrogation of the TGF-β downstream signaling pathway led to enhanced cutaneous repair.

G. Impaired Wound Healing in Mice Lacking the TGF-β Type II Receptor in Fibroblasts

To determine the role of TGF-β receptor signaling in fibroblasts for cutaneous wound repair, Bhownick et al. (25) developed a mouse model that exhibits a conditional knock-out of the TGF-β type II receptor in fibroblastic cells. Mice carrying two floxed TGF-β receptor type II alleles were crossed with animals expressing Cre recombinase under the control of the promoter of the fibroblast specific protein 1 (FSP-1). The latter is expressed in the mesenchymal cells of fibroblastic origin beginning embryonic *day 9*. When these mice were challenged by excisional or incisional wounding, wound closure and keratinocyte organization were unaffected. However, the number of suprabasal keratinocytes was increased in the remodeled excisional wounds of the mutant mice compared with control littermates. Most importantly, the tensile strength of the wounds was severely reduced 7 days after wounding compared with control littermates, demonstrating the importance of TGF-β for granulation tissue formation during wound healing (25).

H. Accelerated Cutaneous Wound Healing With an Increased Rate of Reepithelialization and Reduced Inflammation in Smad3-Null Mice

Downstream of receptor activation, TGF-βs and activin, both of which regulate key cellular functions during cutaneous wound repair, are known to activate different signaling pathways (282). One of the major pathways uses the transcriptional regulators Smad2 and Smad3 (11, 70, 179; Fig. 3). These signaling proteins are recruited to ligand-bound TGF-β and activin receptor complexes, where they are phosphorylated by the type I receptor. The phosphorylated Smads 2 and 3 undergo a conformational change, which allows them to bind to cytoplasmic Smad4, shuttle to the nucleus, and activate their downstream targets (52; Fig. 3).

In contrast to Smad2 null mice, which die during embryogenesis (287), mice lacking functional Smad3 survive into adulthood (302). Following full-thickness incisional wounding, Smad3-null mice show accelerated healing, characterized by an increased rate of reepithelialization and reduced inflammation (12). In addition to neutrophils and monocytes being almost absent in the Smad3 knock-out wounds, granulation tissue formation was dramatically reduced, and there was an overall decrease in the wound area. Wounds of Smad3 knock-out mice also showed significantly lower levels of TGF-β1 expression, likely due to the decreased monocyte concentration, since these cells are a key source of TGF-β1 in the early wound.

To determine whether the lack of TGF-β was a cause of rather than effect of the lack of inflammatory response, exogenous TGF-β1 was applied to the wounds of control and Smad3-null mice. While this treatment resulted in an augmented neutrophil infiltration into the wounds of control mice, it failed to rescue the inflammatory response in Smad3-null animals, indicating that Smad3 signaling may underpin TGF-β1-mediated inflammatory cell chemotaxis. Contrastingly, exogenous TGF-β1 did rescue the granulation tissue phenotype, resulting in a stimulation of matrix production in the wounds of Smad3-null mice, although the fibroblast numbers were not increased. Thus TGF-β1-dependent matrix deposition seems to function in a Smad3-independent fashion in these mice, in agreement with previous studies that revealed an involvement of c-Jun in the TGF-β-mediated fibronectin expression (122).

In summary, these data suggest that Smad3 signaling plays an inhibitory role during wound repair, since its abrogation leads to enhanced reepithelialization and contraction of wounds, at least in an incisional wound healing scenario.

Using the same mice in a study to determine the role of TGF-β signaling in the response to ionizing radiation, the same laboratory found that Smad3 signaling was responsible for the skin injury resulting from a single dose of 30–50 GΩ of γ-irradiation (89). Radiation-induced fibrosis shows several similarities to the fibrosis that results after repair of cutaneous wounds in the adult; there is an extensive infiltration of inflammatory cells, dermal fibroblasts misexpress α-smooth muscle actin, fibrous extracellular matrix is aberrantly deposited, and TGF-β is implicated in its pathogenesis (175). Additionally, the epidermis becomes hyperthickened. Analysis of skin biopsies taken 6 wk postwounding revealed Smad3-null mice to have reduced inflammation (~50% cell number), to express lower levels of TGF-β, and to have 40% less blood vessels and myofibroblasts compared with wild-type mice. Thus Smad3-null animals seem to be largely protected from the cutaneous fibrosis caused by radiation injury.

XI. ACTIVINS

Activins are members of the TGF-β superfamily, which regulate various aspects of cell growth and differentiation in many tissues and organs. They are dimeric proteins, consisting of two β_A subunits (activin A), two β_B subunits (activin B), or a β_A and a β_B subunit (activin AB). In addition, β_C, β_D, and β_E subunits have been identified, although little is as yet known about the corresponding proteins. The biological functions of activins are mediated by two type I and two type II receptors that bind activin with high affinity. In addition to these transmembrane serine/threonine kinase signaling receptors, the biological activities of activin are also regulated by follistatin and follistatin-related protein, soluble activin-binding glycoproteins, which inhibit activin function in vitro and in vivo (178, 215).

A. Increased Expression of Activin After Skin Injury

First evidence for a role of activin in wound healing came from studies of Hübner et al. (127). In these experiments, full-thickness excisional wounds on mouse back skin were analyzed for the expression of activins at different time points after injury. Most remarkably, expression of the activin β_A and to a lesser extent of the β_B subunit was strongly induced within 24 h after injury and remained high until the repair process was completed. Follistatin, follistatin-related protein, as well as the activin receptors were also expressed in normal and wounded skin, but their levels were not affected by skin injury (127, 285). In situ hybridization studies revealed that activin β_A mRNA was predominantly expressed in the granulation tissue adjacent to the hyperproliferative epithelium and below the eschar, whereas highest levels of activin β_B mRNA were detected in suprabasal keratinocytes of the hyperproliferative epithelium at the wound edge and in the migrating epithelial tongue (127). The upregulation of activin expression is likely to be important for normal wound repair, since the severe delay in wound healing observed after cyclosporin A treatment of rats was associated with a strong downregulation of activin βA expression in granulation tissue fibroblasts (214).

B. Overexpression of Activin in the Epidermis of Trangenic Mice Enhances Wound Repair and Scarring

To gain insight into the function of activin in wound repair, transgenic mice that overexpress the activin βA subunit specifically in the epidermis were generated (192). The skin of these animals was characterized by epidermal hyperthickening and dermal fibrosis. The latter effect is most likely due to diffusion of activin from the epidermis to the mesenchyme and suggests a role of the protein in fibrotic processes. The epidermal hyperthickening in transgenic mice was reminiscent of the phenotype seen in hyperproliferative human skin disease. Indeed, a two- to threefold increased proliferation rate of the epidermal keratinocytes of the transgenic mice was observed. This effect of activin on keratinocyte proliferation in vitro is probably indirect, since activin was shown to inhibit proliferation of human keratinocytes (242, 247). Thus activin might induce the expression of growth factors in dermal fibroblasts, which stimulate keratinocyte proliferation in a paracrine manner. In addition, the differentiation pattern of the epidermal keratinocytes was affected.

Analysis of full-thickness excisional wounds in these mice revealed a remarkable increase in granulation tissue, with a higher cell density and an enhanced deposition of extracellular matrix, compared with wild-type mice. The latter effect appears to be at least partially due to an earlier induction of fibronectin and tenascin-C expression in the wounds of activin overexpressing mice. In contrast, collagen type I expression was similar in normal and transgenic mice, indicating that the effects of activin on the synthesis of extracellular matrix proteins are selective, whereas TGF-β seems to stimulate the synthesis of extracellular matrix in a more general manner (229).

C. Impaired Wound Healing in Transgenic Mice Overexpressing the Activin Antagonist Follistatin in the Epidermis

The results obtained with the activin-overexpressing mice demonstrated novel activities of activin in the regu-

lation of the healing process. However, they do not allow conclusions regarding the roles of endogenous activin in wound healing. To address this question, Wankell et al. (286) overexpressed the soluble activin antagonist follistatin in the epidermis of transgenic mice (286). The skin of these animals was characterized by a mild dermal and epidermal atrophy. After injury, a severe delay in wound healing was observed. In particular, granulation tissue formation was strongly reduced, leading to a major reduction in wound breaking strength. The wounds, however, finally healed, and the resulting scar area was smaller compared with controls (286; Werner, unpublished data). These results are complementary to the results obtained with activin overexpressing mice and thus provide first evidence for an important function of endogenous activin in the control of wound repair and scar formation.

XII. BONE MORPHOGENETIC PROTEINS

In addition to TGF-βs and activins, BMPs have also been suggested to play a role in wound repair. Fifteen BMPs have as yet been identified which exert their functions by binding to heteromeric receptor complexes of a type II receptor and two different type I receptors (189).

A. Expression of BMPs at the Wound Site

BMP-2, BMP-4, and BMP-7 are expressed in normal and wounded adult mouse skin, although their expression is not regulated by skin injury (286). The sites of expression of these proteins in wounded skin and their roles in wound repair have as yet not been determined, but exogenous BMP-2 induced massive dermal and epidermal growth in fetal wounds of lambs and an adultlike pattern of scar formation (261).

In contrast to other BMPs, the expression of BMP-6 in healing skin wounds has been well documented. It is highly expressed in the regenerating epidermis at the wound edge as well as in fibroblasts of the granulation tissue. After completion of wound closure, BMP-6 accumulated throughout the suprabasal layers of the newly formed epidermis (139). This localization suggested a role of BMP-6 in the inhibition of keratinocyte proliferation and/or induction of differentiation, a hypothesis which is supported by the finding that BMP-6 induces keratinocyte differentiation in vitro (77, 185).

B. Delayed Reepithelialization in Transgenic Mice Overexpressing BMP-6 in the Epidermis

To determine the activities of BMP-6 in the skin, Blessing et al. (29) generated transgenic mouse lines overexpressing this protein in the suprabasal layers of the epidermis. Interestingly, strong and uniform expression of the BMP-6 transgene inhibited cell proliferation but had little effect on differentiation, whereas weak and patchy expression resulted in keratinocyte hyperproliferation and in a psoriasis-like phenotype. Most importantly, reepithelialization was significantly delayed in the transgenic mice that overexpress low levels of BMP-6 in the epidermis (139), suggesting that this protein inhibits keratinocyte proliferation in wounded skin and is necessary for the reestablishment of a fully differentiated epidermis. Wound healing studies with BMP-6-deficient mice (254) will help to determine whether the endogenous protein indeed fulfills this function.

XIII. CONNECTIVE TISSUE GROWTH FACTOR/ CYSTEINE-RICH 61/NEPHROBLASTOMA OVEREXPRESSED (CNN) FAMILY

The CNN family comprises as yet six different members, including connective tissue growth factor (CTGF), cysteine-rich 61 (cyr61), nephroblastoma overexpressed (nov), WISP-1, WISP-2, and WISP-3. They are secreted proteins that contain 38 conserved cysteine residues that are organized into 4 distinct structural modules. Members of this family appear to be involved in embryonic development, differentiation, as well as pathological processes (36). In addition, CTGF and cyr61 have been suggested to play a role in wound repair. CTGF is expressed in many different tissues and organs and stimulates proliferation and chemotaxis of fibroblasts directly (31). Most interestingly, it is a potent inducer of extracellular matrix proteins, such as collagen type I and fibronectin and their integrin receptors (93), and it acts as a mediator of TGF-β1 in these processes (150). Due to this function and to the fact that it is overexpressed in various types of fibrotic disease, CTGF has been suggested to be a major player in the pathogenesis of fibrotic processes (36).

A. Expression of CTGF in Skin Wounds

First evidence for a role of CTGF in cutaneous wound repair came from studies by Igarashi et al. (129). Using a rat wound model consisting of a subcutaneously implanted stainless steel mesh chamber, they demonstrated the presence of CTGF mRNA in wounded but not in normal skin. Highest levels of CTGF transcripts were observed at *day 9* after injury that coincides with the initial ingrowth of granulation tissue (129). In another study CTGF mRNA levels were analyzed in full-thickness excisional mouse wounds in mice. In this model, CTGF mRNA was most abundant at *day 1* after injury and declined to basal levels within the next 5 days (67). Due to the potent effect of CTGF on fibroblast proliferation and matrix deposition by these cells, the upregulation of

CTGF expression after injury is likely to be important for granulation tissue formation and subsequent scar formation. In addition, it was recently demonstrated that CTGF promotes endothelial proliferation, migration, survival, and adhesion in vitro and angiogenesis in vivo (14, 248), suggesting that this protein might also be involved in wound angiogenesis.

B. Expression of Cyr61 in Skin Wounds

In addition to CTGF, Cyr61 is likely to play a role in cutaneous wound healing. Cyr61 was shown to promote chemotaxis of fibroblasts and to enhance the mitogenic effect of other growth factors for these cells (147). Furthermore, it was identified as an angiogenic inducer in vivo (15). To determine the expression pattern of the *cyr61* gene in healing skin wounds, the expression of this gene was examined in full-thickness incisional wounds of transgenic mice that express the bacterial *lacZ* gene encoding β-galactosidase under the control of the endogenous *cyr61* gene promoter (49). These studies revealed a strong expression of Cyr61 in dermal fibroblasts of the granulation tissue. In vitro, Cyr61 activated a genetic program for wound repair in cultured skin fibroblasts, indicating that Cyr61 regulates inflammation, angiogenesis, cell-matrix interactions, and matrix remodeling after skin injury (49).

XIV. CHEMOKINES

In addition to the "classical" growth factors, several cytokines have been shown to play important roles in wound repair. Cytokines are small, secreted proteins that affect the behavior of immune cells but also of other cells. They include the interleukins, lymphokines, and several related signaling molecules such as tumor necrosis factor-α (TNF-α) and interferons. Chemokines (chemotactic cytokines) are a subset of small cytokines that stimulate chemotaxis and extravasation of leukocytes. This large protein family with nearly 50 members in the human system is subdivided into four subfamilies, α- (CXC-) and β- (CC-) chemokines, which include most of the chemokines, and two additional subfamilies, the CX3C chemokines and C-chemokines with only one or two members each. Chemokines exert their functions via binding to G protein-coupled receptors on the surface of target cells, the CXC-receptors and the CC-receptors that only recognize chemokines of the corresponding subfamily (16, 53, 234). Recent studies have provided evidence for an important role of chemokines in the recruitment of inflammatory cells to the wound site. In addition, the presence of chemokine receptors on resident cells suggests that chemokines also contribute to the regulation of reepithelialization, tissue remodeling, and angiogenesis. Expression of a wide variety of different chemokines has been detected at the wound site, and these results have been reviewed in detail (103). Here we only report on those chemokines for which functional wound healing data are available.

A. A Role for Macrophage Chemoattractant Protein in the Regulation of Inflammation, Granulation Tissue Formation, and Reepithelialization

The CC chemokine macrophage chemoattractant protein (MCP-1/CCL2) is one of the major chemoattractants for monocytes/macrophages, and it also acts on a subset of T cells and on mast cells carrying the CCR3 receptor (16, 53). In a murine excisional wound model, mRNA encoding the murine MCP-1/CCL2 homolog JE was found at high levels between 6 and 24 h after wounding and the levels subsequently declined (75, 133, 293). By in situ hybridization, JE transcripts were predominantly found in monocytic and macrophage-like cells, as well as in a few fibroblasts and other interstitial cells (75). In two other studies, keratinocytes of the hyperproliferative wound epidermis were identified as the major source of MCP-1/CCL2 mRNA or protein in the wound (133, 293). Consistent with the data obtained in the mouse, MCP-1/CCL2 mRNA was found in keratinocytes of early human burn wounds as well as in human excisional wounds. In addition, some endothelial cells and inflammatory cells in the granulation tissue expressed this chemokine (81, 100).

In all studies, the time course of MCP-1/CCL2 expression correlated well with macrophage infiltration, suggesting a role of MCP-1/CCL2 in the recruitment of these cells during wound healing (75). In addition, it might attract T cells and mast cells to the wound site. Most interestingly, the prolonged persistence of neutrophils and macrophages in the wounds of healing-impaired diabetic *db/db* mice correlated with a large and sustained induction of MCP-1/CCL2/JE. Treatment of wounds from these mice with neutralizing antibodies to MCP-1/CCL2/JE and macrophage inflammatory protein (MIP-2) caused a reduction in the number of neutrophils and macrophages at the wound site, suggesting a direct involvement of these chemokines in the late infiltration of inflammatory cells into *db/db* wounds (293).

To determine the role of MCP-1/CCL2 for normal repair, mouse wounds were treated with either MCP-1/CCL2 or neutralizing antibodies to this chemokine (76). Treatment with MCP-1/CCL2 resulted in a substantial increase in the number of macrophages that was accompanied by a slight increase in wound-breaking strength. On the other hand, treatment with neutralizing antibodies reduced the number of macrophages at the wound site. In contrast to these results, the number of wound macro-

phages was not altered in wounds of MCP-1/CCL2/JE knock-out mice (163). These differences between the neutralizing antibody studies and the data obtained with the knock-out mice might either be due to cross-reactivity of the antibody with other chemokines or to compensatory upregulation of other chemokines in the knock-out mice. Although the number of macrophages was not altered, MCP-1/CCL2/JE knock-out animals were characterized by significantly delayed wound reepithelialization and angiogenesis, and collagen synthesis was also reduced in these mice (163). These data revealed an important role of MCP-1/CCL2 in wound repair, probably by influencing gene expression/protein synthesis in murine macrophages. However, in humans, MCP-1/CCL2 seems to be mainly involved in macrophage trafficking rather than in the regulation of growth factor production by these cells, since stimulation of human macrophages with MCP-1/CCL2 did not induce expression of major growth factors (81).

B. Macrophage Inflammatory Protein 1α: A Chemoattractant for Macrophages in the Healing Wound?

Another important monocyte chemoattractant is MIP-1α/CCL3. Both MIP-1α/CCL3 mRNA and protein were detectable in mouse wounds between 12 h and 5 days after injury, with levels peaking at *day 1* after wounding. This time point correlates with maximum macrophage infiltration. Treatment of mice with a neutralizing antiserum to this chemokine before injury resulted in a reduced number of macrophages at the wound site, followed by reduced collagen production (74). Whereas this study suggested an important role of MIP-1α/CCL3 in wound repair, analysis of wounds in MIP-1α/CCL3 knock-out mice revealed no obvious defect in either reepithelialization, angiogenesis, or collagen production (163). In contrast to the murine situation, MIP-1α/CCL3 was not detected at significant levels in acute human incisional wounds (81), indicating species-specific differences in the expression and function of this chemokine in wound repair.

C. Growth-Related Oncogene-α Regulates Macrophage Infiltration Into Healing Wounds

GRO-α/CXCL1 and its possible murine homolog macrophage inflammatory protein 2 (MIP-2) are potent regulators of neutrophil chemotaxis (53). The involvement of GRO-α/CXCL1 in wound healing was first suggested by its detection in inflammatory cells at *day 3* after injury in a model of wound healing using wound chambers (83). In acute human excisional wounds, GRO-α/CXCL1 mRNA was found at highest levels at *day 1* after wounding in the superficial wound bed and the underlying provisional matrix, and its expression was spatially and temporally associated with neutrophil infiltration. Furthermore, its expression profile correlated with keratinocyte migration and with neovascularization (81). In human burn wounds, GRO-α/CXCL1 immunoreactivity was detected in suprabasal layers of the wound epidermis as well as in the granulation tissue and the overlying exudate (194). The presence of GRO-α/CXCL1 in human burn wounds was confirmed by Rennekampf et al. (222), who detected high levels of this protein in donor site wound fluids from *days 1* to *5* after injury (222). In vitro experiments revealed a strong mitogenic activity of this chemokine for keratinocytes, suggesting its involvement in reepithelialization. This hypothesis was supported by the observed stimulatory effect of GRO-α/CXCL1 on reepithelialization of meshed split-thickness human skin grafts on athymic mice. In contrast, wound contraction was significantly reduced by GRO-α/CXCL1 (222).

In a mouse excisional wound model, expression of MIP-2 was found to be upregulated between *days 1* and *5* after injury in normal mice. Immunohistochemistry revealed the presence of MIP-2 in keratinocytes of the outer root sheath of hair follicles adjacent to the wound, but not in the granulation tissue (293). Similar to MCP-1/CCL2, the upregulation of MIP-2 expression was prolonged in healing-impaired *db/db* mice. Treatment of wounds from these animals with neutralizing antibodies to MCP-1/CCL2 and MIP-2 caused a reduction in the number of neutrophils and macrophages at the wound site, suggesting a direct involvement of these chemokines in the late infiltration of inflammatory cells into *db/db* wounds (293).

D. Interleukin-8 Stimulates Inflammation but Inhibits Wound Contraction

Interleukin (IL)-8/CXCL8, for which a murine homolog has not yet been identified, is also expressed in healing skin wounds. In acute human excisional wounds, it is coexpressed with GRO-α/CXCL1 at *day 1* after injury in the superficial wound bed, and its expression declines at *day 4* after wounding (81). In addition, IL-8/CXCL8 was found to be the major bioactive chemoattractant for neutrophils in human blister and skin graft donor site wound fluids (221). When human adult or fetal skin was placed subcutaneously in the SCID mouse and subsequently wounded, expression of IL-8/CXCL8 increased within 4 h after injury in fetal and adult wounds. However, by 12 h, no IL-8/CXCL8 mRNA was detected in fetal wounds, whereas expression of this chemokine persisted for up to 72 h in adult wounds. These results suggest that a reduced inflammatory cytokine response in fetal tissue may be responsible for the lack of inflammation in fetal wound healing that may contribute to scarless wound repair (161).

Studies from Iocono et al. (132) suggest that high levels of IL-8/CXCL8 are associated with impaired wound repair. Thus the levels of this chemokine were increased significantly in nonhealing human thermal wounds compared with healing wounds or normal skin. In vitro studies demonstrated an inhibitory effect of IL-8/CXCL8 on keratinocyte proliferation and collagen lattice contraction by fibroblasts, suggesting that elevated levels of this chemokine may directly contribute to retarded wound repair (132). In contrast to these studies, others found a stimulatory effect of IL-8/CXCL8 on keratinocyte proliferation in vitro (221). In addition, in vivo topical application of this chemokine on human skin grafts in a chimeric mouse model stimulated reepithelialization as a result of increased keratinocyte proliferation. Consistent with the observed effect of IL-8/CXCL8 on collagen lattice contraction (see above), wound contraction was diminished by topical application of IL-8/CXCL8 (221).

E. Impaired Wound Healing in CXCR2 Knock-out Mice

The effects of GRO-α/CXCL1/MIP-2 and several other chemokines are mediated, at least in part, by CXCR2 receptors that are expressed on keratinocytes, neovascularizing endothelial cells, and neutrophils. To determine the role of this receptor in wound repair, full-thickness excisional punch biopsy wounds were made to mice lacking CXCR2 (73). After wounding, these mice exhibited a defective neutrophil recruitment, delayed monocyte recruitment, and decreased secretion of the proinflammatory cytokine IL-1β. Histologically, they also showed a severe delay in reepithelialization. This effect is probably direct, since in vitro wounding studies with cultured keratinocytes from these animals revealed a retardation in wound closure compared with keratinocytes from wild-type mice (73). In addition, angiogenesis was severely impaired, most likely due to a diminished response to MIP-2 which is known to be angiogenic (22). These results demonstrate that CXCR2 and its ligands are not only involved in inflammatory cell recruitment, but also regulate the behavior of resident cells in the wound.

F. Overexpression of Interferon-γ-Inducible Protein 10 in the Epidermis of Transgenic Mice Stimulates Inflammation but Inhibits Reepithelialization

IP10/CXCL10 is a chemokine that is detected at high levels in several chronic inflammatory conditions, including psoriasis. It is a member of the CXC family of chemokines and acts primarily in the recruitment of lymphocytes carrying the receptor CXCR3 (53). Its expression was shown to be upregulated together with monokine induced by interferon-γ (MIG/CXCL9) following wounding, with an expression pattern that correlates well with recruitment of inflammatory cells to the wound site (81). To determine whether IP-10/CXCL10 could modulate an in vivo inflammatory response, Luster et al. (166) engineered mice that constitutively express IP-10/CXCL10 in keratinocytes. These mice showed no obvious abnormalities under normal laboratory conditions. After full-thickness injury, however, IP-10/CXCL10 overexpressing mice showed a more intense inflammatory phase, compared with control littermates, delayed reepithelialization, and a prolonged, disorganized granulation phase with impaired angiogenesis. The latter result was expected from the known inhibitory effect of IP-10 on angiogenesis (22). These data suggest that IP-10/CXCL10 is able to inhibit wound repair by disrupting the normal development of the granulation tissue. This adverse effect on wound healing could be at least partially achieved by the inhibitory effect of IP-10/CXCL10 on EGF-induced fibroblast motility (249).

G. Multiple Functions of Chemokines in Wound Repair

The results described above demonstrate the importance of chemokines for the recruitment of inflammatory cells into wounds. In addition, they have been shown to act directly or indirectly on resident cells, thereby regulating reepithelialization, angiogenesis (see above), and also myofibroblast differentiation as recently demonstrated for the chicken chemotactic and angiogenic factor (cCAF) (87). Finally, chemokines might also be involved in the regulation of skin homeostasis as suggested for stromal-derived factor 1 (SDF-1/CXCL12). This chemokine is constitutively expressed in dermal fibroblasts and blood vessels of human skin (210) but downregulated after injury in mice and humans (85; R. Gillitzer, personal communication). These results suggest that SDF-1/CXCL12 functions as a homeostatic regulator of tissue remodeling. The recent discovery of a plethora of additional chemokines (16) suggests that many of them might play a role in wound healing, and more exciting functions of these molecules in the repair process will undoubtedly be discovered.

XV. PROINFLAMMATORY CYTOKINES

It has long been thought that proinflammatory cytokines, including interleukins 1α and 1β (IL-1α and IL-1β), IL-6, and TNF-α, play an important role in wound repair. They likely influence various processes at the wound site, including stimulation of keratinocyte and fibroblast proliferation, synthesis and breakdown of extracellular ma-

trix proteins, fibroblast chemotaxis, and regulation of the immune response.

A. Expression of Proinflammatory Cytokines in Skin Wounds

In support of a role for proinflammatory cytokines in wound repair, expression of IL-1α, IL-1β, IL-6, and TNF-α was shown to be strongly upregulated during the inflammatory phase of healing (109, 110, 126). Polymorphonuclear leukocytes and macrophages were shown to be the major source of these cytokines, but expression was also observed in some resident cell types (86, 126). The coordinated expression of these cytokines is likely to be important for normal repair, since expression of these genes was strongly reduced after wounding of healing-impaired glucocorticoid-treated mice (126) and prolonged in genetically diabetic *db/db* mice (293).

B. IL-6 Knock-out Mice Show Severe Deficits in Cutaneous Wound Repair

With the use of a full-thickness punch biopsy wounding model on IL-6 knock-out mice, it was shown that wounds to IL-6 knock-out animals took up to three times longer to heal than those of wild-type controls (96). As expected by the mitogenic effect of IL-6 for keratinocytes (236), wounds in these animals were characterized by a dramatic delay in reepithelialization. In addition, granulation tissue formation was impaired. These abnormalities were completely rescued by administration of recombinant murine IL-6 protein 1 h before wounding. Thus it appears that IL-6 is crucial for kick-starting the healing response, both via its mitogenic effects on wound edge keratinocytes and via its chemoattractive effect on neutrophils.

Whereas a complete lack of IL-6 caused impaired wound healing, excessive levels of IL-6 have been associated with cutaneous scarring. Thus the increase in IL-6 expression after injury was only transient in fetal wounds but prolonged in adult wounds. Most importantly, IL-6 administration to fetal wounds resulted in scar formation (160). These results suggest that the more transient upregulation of proinflammatory cytokines such as IL-6 as well as of chemokines (see above) in fetal wounds compared with adult wounds might at least partially underlie the different scarring response in fetal versus adult mammals.

C. STAT-3-Mediated Transduction of Cytokine Signals Is Important for Wound Repair

STATs (signal transducers and activators of transcription) are cytoplasmic proteins that transduce signals from a variety of growth factors, cytokines, and hormones. Once activated by tyrosine phosphorylation, they dimerize and translocate to the nucleus, where they bind to specific DNA elements and thus regulate target gene expression (38). STAT-3 is activated upon binding of IL-6 to its receptor and is thus a likely candidate for a role in wound repair. Because STAT-3 null mice die during embryogenesis (268), Sano et al. (235) used a Cre-lox approach to specifically delete the *stat-3* gene in keratinocytes. They observed no effect on skin morphogenesis. However, following full-thickness excisional wounding, the healing process was severely compromised, with dramatically reduced reepithelialization. These abnormalities were remarkably similar to those seen in IL-6 knock-out mice (see above). However, the overall effect on repair was less dramatic than in the IL-6 null mice, since the cell types involved in both the inflammatory response and granulation tissue formation were unaffected by the keratinocyte-specific approach.

D. Accelerated Wound Healing in TNF Receptor p55-Deficient Mice

To determine the role of the TNF receptor p55 in cutaneous wound repair, Mori et al. (191) generated excisional wounds on the back of mice lacking this receptor. These animals showed an enhancement of angiogenesis, collagen content, and reepithelialization. These histological differences were accompanied by increased expression of TGF-β1, CTGF, VEGF, VEGFR-1, and VEGFR-2 at the wound site. In contrast, leukocyte infiltration and mRNA expression of adhesion molecules and cytokines were reduced. Overall, these changes resulted in accelerated healing of wounds in TNF receptor p55-deficient mice.

XVI. GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR

GM-CSF is a pleiotropic cytokine that was shown to be mitogenic for keratinocytes (144) and to stimulate migration and proliferation of endothelial cells (44). Together with its potent effect on hematopoietic cells, it has been suggested to play an important role in cutaneous wound repair. Indeed, a series of animal experiments and clinical studies have demonstrated a beneficial effect of GM-CSF for the treatment of normal wounds and chronic ulcers (112). Recently, Mann et al. (170) demonstrated a strong increase in the levels of GM-CSF in skin extracts after generation of full-thickness excisional wounds in mice, although the cellular source has not been determined (170).

A. Overexpression of GM-CSF in the Epidermis of Transgenic Mice Accelerates Wound Reepithelialization

To gain further insight into the possible role of GM-CSF in skin wound healing, the same group generated transgenic mice that overexpress this cytokine in the epidermis (34) and generated full-thickness excisional wounds in these animals (170). Interestingly, these animals displayed accelerated wound reepithelialization as a result of increased keratinocyte proliferation. Furthermore, neovascularization and granulation tissue formation were strongly enhanced. Interestingly, several cytokines that are known to be important for wound healing such as TGF-β1 were elevated in the wounds of these animals, indicating that GM-CSF stimulates wound repair directly but also indirectly via induction of secondary cytokines (170).

XVII. LEPTIN

Leptin is a 16-kDa protein that is produced by adipocytes and induces weight loss in both normal and genetically obese *ob/ob* mice via binding to its high-affinity receptor (295). The phenotype of *ob/ob* mice is due to a defective *ob* gene, leading to a lack of circulating leptin (307). These animals are obese and they suffer from multiple metabolic abnormalities, mimicking those seen in patients with type 2 diabetes mellitus. Most interestingly, the wound healing process in these animals is severely delayed (105). However, it has remained unclear whether this abnormality is secondary to the systemic defects or whether leptin is directly involved in wound healing.

A. Systemic and Topical Application of Leptin Accelerates Wound Repair

Two groups demonstrated that systemic and topical application of leptin accelerates wound healing in *ob/ob* mice (92, 225). Furthermore, topical leptin administration also stimulated the wound healing process in wild-type mice (92). Reepithelialization was particularly accelerated (92), whereas angiogenesis was not improved (225, 258). The stimulatory effect of leptin on wound healing was obviously mediated via the leptin receptor, since wound healing in leptin receptor-deficient, genetically diabetic *db/db* mice was not affected by leptin treatment. The finding that topical administration of leptin accelerates wound repair both in *ob/ob* and also in wild-type mice strongly suggests a direct stimulatory effect of leptin on wound repair.

B. Expression of Leptin Receptors at the Wound Site

The hypothesis of a direct effect of leptin on wound repair was supported by the detection of leptin receptor mRNA at the wound site. With the use of RNase protection assay, mRNAs encoding all forms of leptin receptor, including the signal-transducing long form, were found in normal and wounded skin (92, 225), although expression of this gene was downregulated after wounding (92). In situ hybridization revealed expression of leptin receptors in subcutaneous and dermal blood vessels (225) as well as in keratinocytes of the normal and wounded epidermis (92). Interestingly, the signal-transducing receptor was restricted to proliferation-competent keratinocytes (92). This finding together with the increase in keratinocyte proliferation seen in the wounds of leptin-treated animals suggested a mitogenic effect of leptin for these cells. Indeed, proliferation of human and murine keratinocytes was stimulated by leptin in vitro (92, 257), demonstrating that leptin is indeed a keratinocyte mitogen and a mediator of wound reepithelialization in vivo.

XVIII. INTERLEUKIN-10

In addition to proinflammatory cytokines, anti-inflammatory cytokines have also been shown to be important regulators of wound repair. In particular, the role of IL-10 in the healing response has been studied in some detail. This cytokine is thought to play a major role in the limitation and termination of inflammatory responses. In addition, it regulates growth and/or differentiation of various immune cells, but also of keratinocytes and endothelial cells (190). Based on these activities, a role of IL-10 in wound healing appeared likely.

A. Expression of IL-10 at the Wound Site

Two groups determined the expression pattern of this cytokine in the healing wound. Increased levels of IL-10 mRNA were observed after incisional wounding of mice, with a peak at 60 min after injury (202). Using the same wound model, Sato et al. (237) found two peaks of IL-10 expression after injury: an early peak with a maximum at 3 h after wounding and a second peak at *day 3* after wounding. Keratinocytes of the wound epidermis and infiltrating mononuclear cells were identified as the major producers of IL-10 mRNA and protein (237). Interestingly, increased expression of IL-10 was shown to be associated with impaired healing, since the levels of this cytokine were strongly increased in human chronic venous insufficiency ulcers compared with autologous donor wound tissue (165).

B. IL-10 Inhibits Inflammation and Scar Formation

To determine the function of IL-10 in wound repair, neutralizing antibodies to this cytokine were applied to incisional mouse wounds. This treatment demonstrated that endogenous IL-10 inhibits the infiltration of neutrophils and macrophages toward the site of injury as well as expression of several chemokines and proinflammatory cytokines (237). In another study, the role of IL-10 in fetal scarless healing was investigated (162). These investigators wounded embryonic skin from IL-10 null mice that had been grafted to strain-matched adult mice. Wounds to control embryonic skin grafts showed little inflammation and normal restoration of the dermal architecture. However, wounded IL-10 null grafts were characterized by a significantly higher inflammatory cell infiltration and collagen deposition and an adultlike scarring response. These results suggest an important role of IL-10 in regulating the expression of proinflammatory cytokines in fetal wounds, leading to reduced matrix deposition and scar-free healing.

XIX. TEMPORAL AND SPATIAL INTERACTION OF DIFFERENT GROWTH FACTORS AT THE WOUND SITE

The results described in this review demonstrate that a multitude of growth factors and cytokines are present at the wound site. Their expression dynamics show characteristic temporal and spatial regulation, and changes in the expression pattern of growth factors are associated with impaired wound healing. Most importantly, alterations in the levels of one factor are likely to affect the production of other growth factors and cytokines. Thus it has been shown that proinflammatory cytokines and serum growth factors that are released during the early phase of wound healing are potent stimulators of the expression of various growth factors. One example is the regulation of FGF7, a growth factor produced by fibroblasts at the site of injury (see sect. III). In vitro studies using cultured fibroblasts or organotypic cocultures of fibroblasts and keratinocytes revealed that the proinflammatory cytokines IL-1 and TNF-α as well as the growth factor PDGF are potent inducers of FGF7 expression (32, 48, 266). This suggests that PDGF released from platelets might be responsible for the early induction of FGF7. IL-1 and TNF-α, which are predominantly produced by invading neutrophils and fibroblasts, are likely to maintain the high levels of this growth factor within the first few days after injury (Fig. 5*A*). Another example is the regulation of VEGF, a major regulator of angiogenesis (see sect. V), which is produced by keratinocytes and macrophages at the wound site (40). It has been demonstrated that proinflammatory cytokines can induce expression of VEGF in both cell types (90, 212). In addition, several growth factors, including FGF7, EGF, TGF-α, and HGF, have been

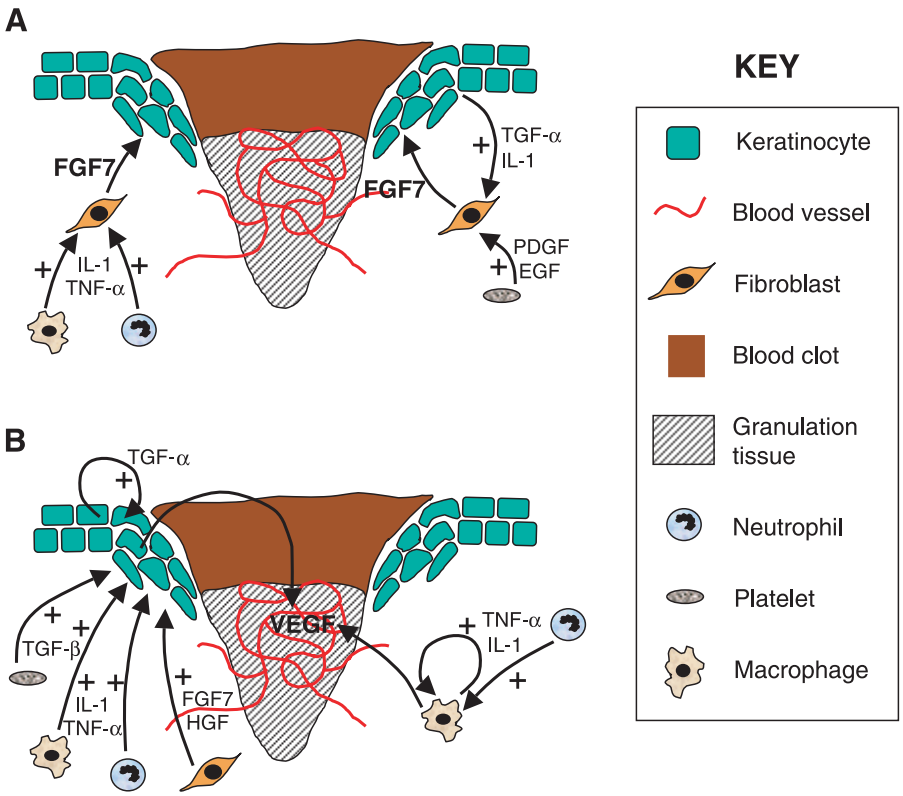

FIG. 5. Growth factor interactions at the wound site. *A*: regulation of fibroblast growth factor (FGF) 7 at the wound site—hypothetical model. Local hemorrhage causes extravasation of platelets and their release of PDGF and EGF. These mitogens stimulate FGF7 expression in fibroblasts. In addition, invading neutrophils and macrophages secrete the proinflammatory cytokines IL-1 and TNF-α which then cause a further induction of FGF7 expression in fibroblasts. Finally, IL-1 and TGF-α derived from keratinocytes also stimulate FGF7 expression in fibroblasts. *B*: regulation of VEGF expression at the wound site—hypothetical model. Local hemorrhage causes extravasation of platelets and their release of TGF-β. Invading macrophages also secrete this growth factor, together with the proinflammatory cytokines IL-1 and TNF-α. These factors stimulate VEGF expression in keratinocytes and macrophages. In addition, FGF7 and hepatocyte growth factor (HGF) derived from fibroblasts as well as keratinocyte-derived TGF-α stimulate VEGF expression in the epidermis.

TABLE 1. *Growth factors and cytokines and their effects on wound repair*

Process	Growth Factors/Cytokines Involved
Neutrophil infiltration	TGF-β, MCP-1, MIP2/GRO-α, IL-8, IL-6, IL-10(−)
Macrophage infiltration	TGF-β, MCP-1, MIP-1α, IL-10(−)
Angiogenesis	VEGF-A, PLGF, FGF2, Angiopoietins, HGF, Cyr61, MCP-1, IL-8, GRO-α, GM-CSF, IP-10(−)
Fibroplasia	PDGF, TGF-β, CTGF, GM-CSF, IGFs
Matrix deposition	FGF2, IGF-1, NGF, TGF-β, Activin, MCP-1, CTGF, Cyr61
Scarring	IGF-I, TGF-β, Activin, CTGF, IL-6, IL-10(−)
Reepithelialization	FGF2, FGF7, FGF10, EGF, TGF-α, HB-EGF, NDF, IGFs, NGF, Activin, MCP-1, IL-6, GM-CSF, Leptin, TGF-β(−), BMP-6(−), IP-10(−)

Note that a functional proof for these activities has only been obtained in very few cases. Furthermore, it is unclear whether the observed effects are direct or indirect. (−), Negative regulation. See text for definitions.

shown to stimulate the production of VEGF by cultured keratinocytes (72, 90, 102) (Fig. 5*B*). Recent studies revealed that these regulatory interactions are not only occurring in vitro but also in wounded skin. Thus nonviral liposomal FGF7 gene transfer increased both VEGF production at the wound site and neovascularization of wounded skin. The latter finding can explain how FGF7, an epithelial-specific mitogen, can indirectly affect angiogenesis. An even more complex interaction was shown for HGF and VEGF. Toyoda et al. (274) demonstrated that overexpression of HGF in transgenic mice caused a strong increase in VEGF expression in nonwounded and particularly in wounded skin. In this case, both growth factors are likely to act synergistically to stimulate wound angiogenesis, since HGF was recently shown to enhance VEGF-induced angiogenesis in vitro and in vivo (299).

Such examples highlight the complex growth factor interactions that occur during wound healing. These interactions have to be considered in the interpretation of results obtained by overexpression or elimination of a single growth factor at the wound site. It will be a major challenge in the future to study the interactions of different factors in vitro and particularly in wounded skin. Results from such studies are likely to be important for the development of novel strategies for the treatment of impaired wound healing, since they will facilitate the formulation of temporally and combinatorially optimized therapeutic approaches.

XX. CONCLUSIONS

The beneficial effect of exogenous growth factors in the treatment of wound repair as well as the identification of the in vitro activities of many growth factors and cytokines have implicated these proteins as key regulators of the wound healing process (Table 1). This hypothesis is strongly supported by the expression of multiple growth factors and their receptors in different cell types of healing skin wounds. In addition, upregulation of growth factor expression after injury is frequently observed, suggesting a need for high growth factor levels during the repair process. Finally, there are many examples where abnormal growth factor expression is associated with impaired wound healing or excessive scarring, indicating that a correct temporal and spatial expression of these genes is essential for normal repair.

To determine the in vivo function of endogenous growth factors and cytokines, several investigators have applied neutralizing antibodies to skin wounds or to wound fluid (Tables 2 and 3). In most cases, this treatment strongly affected the healing process and provided insight into the roles of growth factors in repair. However, these results have to be interpreted with care, since cross-reactivity with related growth factors cannot be excluded. Furthermore, it is unclear whether neutralizing antibodies get access to the complete wound area.

Given these limitations, a genetic approach to identify growth factor function in wound repair appears desirable. Indeed, the use of genetically modified mice for wound healing studies recently revealed crucial roles for several growth factors and cytokines in the repair process (Table 4). For example, these studies have demonstrated an inhibitory effect of TGF-β on wound reepithelialization

TABLE 2. *Use of neutralizing antibodies to block growth factor function in wound fluid*

Targeted Protein	Reference No.
EGF	111
PDGF	143
FGF2	199
VEGF-A	200

TABLE 3. *Use of neutralizing antibodies to block growth factor function in cutaneous wounds*

Targeted Protein	Reference No.
FGF2	37
TGF-β1 and -β2	82, 244,245
MIP-1α	74
VEGF-A	124
IL-10	237
MCP-1(CCL2)/MIP-2	293
MCP-1(CCL2)	76

TABLE 4. *Summary of genetically modified mouse studies of growth factor and cytokine function in wound repair*

Gene	Strategy	Reference No.
	PDGF family	
PDGF-B	Hematopoietic knock-out chimera	42
	FGF family	
FGF2	Knock-out	208
FGF1/FGF2	Double knock-out	188
FGFR2IIIb	Dominant negative: K14 promoter	292
FGF7	Knock-out	113
TCRγ	Knock-out	134
	EGF family	
TGF-α	Knock-out	164
TGF-α	Knock-out	171
TGF-α/FGF7	Double knock-out	113
TGF-α	Knock-out	146
	VEGF family	
PLGF	Knock-out	45
	Scattor factors	
HGF/SF	Overexpressor: metallothionein promoter	274
MSP	Knock-out	24
	TGF-β superfamily	
TGF-β1	Knock-out	41
TGF-β1	Knock-out (rapamycin treated)	149
TGF-β1	Knock-out (Scid −/− background)	66
TGF-β1	Overexpressor: albumin promoter	246
TGF-β1	Overexpressor: K14 promoter	300
TGF-βRII	Knock-out in fibroblasts	25
TGF-βRII	Dominant negative: K5 promoter	2
Smad3	Knock-out	12
Smad3	Knock-out	89
Activin	Overexpressor: K14 promoter	192
Follistatin	Overexpressor: K14 promoter	286
BMP6	Overexpressor: K10 promoter	139
	CTGF/CNN family	
Cyr61	Knock-out (heterozygous)	49
	Chemokines	
MCP-1	Knock-out	163
MIP-1α	Knock-out	163
CXCR2	Knock-out	73
IP-10	Overexpressor: K5 promoter	166
	Proinflammatory cytokines	
IL-6	Knock-out	96
Stat3	Knock-out in keratinocytes	235
TNFR p55	Knock-out	191
	GM-CSF	
GM-CSF	Overexpressor: K5 promoter	170
	Anti-inflammatory cytokines	
IL-10	Knock-out	162

(2, 12, 66, 149), an important role of FGF receptor signaling in this process (292), and a role of activin in granulation tissue formation and scarring (192, 286). In the latter case, the phenotype was more pronounced in a CD1 background compared with a B6D2F2 background (Werner, unpublished data), demonstrating the importance of the genetic background for the outcome of the repair process.

In spite of the potency of the genetic approach for the study of gene function in wound repair, some of the normal functions of targeted genes might not be revealed due to redundancy or compensation. Indeed, lack of wound healing abnormalities was often observed in mice deficient in a particular growth factor that belongs to a multiprotein family, e.g., in mice lacking FGF7 or TGF-α (113, 164, 171). In the latter case, however, a more detailed analysis of the healing process and the use of another wound model did finally reveal a function of TGF-α in epithelial repair (146). Nevertheless, the observed phenotype was more subtle than expected from its known in vitro activities. Although it cannot be excluded that FGF7 and TGF-α are indeed not important for wound repair, their strong induction in healing skin wounds supports their functional significance. In both cases other growth factors, which bind to the same receptor and which are also expressed in wounded skin, might compensate for the lack of these mitogens. Wound healing studies using animals, which are deficient in two or more homologous molecules, as well as studies with dominant-negative receptor mutants or with soluble inhibitors that block the function of several members of a protein family, will be very useful in answering these questions. For example, the overexpression of a dominant-negative FGFR2IIIb in the epidermis of transgenic mice demonstrated an important role of FGFR signaling in wound reepithelialization (292), whereas the knock-out of individual ligands did not reveal this function (113, 188, 208).

At the other extreme, systemic abnormalities caused by the transgene or by the general loss of a gene might obscure the normal function of a gene in wound repair. Thus it has long been known that systemic defects such as malnutrition and weight loss can strongly impair the healing process (108). This was observed for the TGF-β1 knock-out mice, which developed a strong inflammatory response in various tissues and organs, followed by severe weight loss at ~3 wk of age. These systemic defects appear to be responsible for the impaired wound healing seen in these animals (41), making it impossible to study the local effects of the lack of TGF-β1 on wound repair in this model. Suitable approaches to circumvent this problem, as mentioned above, were to cross the mice onto an immunodeficient background or to treat them with immunosuppressive drugs (66, 149). These studies serve to alert us to the difficulties of determining the functions of specific proteins in complex in vivo situations, but provide some elegant methods for circumventing these problems.

Another way to solve this problem is the generation of mice that have a tissue-specific knock-out or tissue-specific overexpression of a transgene (218). For example, overexpression of a dominant-negative TGF-β receptor in the epidermis of transgenic mice did not cause any

systemic abnormalities, but nevertheless allowed the identification of an inhibitory effect of TGF-β on wound reepithelialization (2). An even more elegant approach will be the use of inducible systems, which allow the induction of a transgene or the deletion of an endogenous gene in a time- and tissue-specific manner. The first successful results with inducible systems in the skin have recently been published. Two have adopted an estrogen receptor-based approach, where Cre recombinase was fused in frame with the tamoxifen-responsive hormone-binding domain of the estrogen receptor [CreER(tam)]. This fusion protein was expressed under the control of the keratin 5 (131) or keratin 14 promoters (280) that target transgenes to the basal layer of the epidermis and to the outer root sheath of hair follicles. Upon systemic or local application of tamoxifen, the latter binds to the hormone-binding domain of the fusion protein and causes activation of Cre recombinase. The activated enzyme then allows the deletion of the targeted gene in keratinocytes.

In an analogous approach, activation of other intracellular proteins can be achieved by fusing the cDNA encoding the protein of interest to the hormone-binding domain of the estrogen receptor. In this case, tamoxifen can be used to activate the protein as recently demonstrated for c-Myc (9).

Another group has used topical application of antiprogestin to induce expression of TGF-β1 in the epidermis (284). In this system, a fusion protein of a truncated progesterone receptor and the yeast GAL4 transcription factor was expressed under the control of the loricrin promoter. Thus by engineering a GAL4 binding domain, normally absent in mammalian cells, upstream of the target gene, transcription can be activated in a tissue-specific and temporally controlled manner. Finally, doxycycline-mediated gene expression (148) was recently used to inducibly express the erbB2 oncogene in the epidermis of transgenic mice (298).

Use of these types of systems will circumvent the problem of systemic defects and will also prevent abnormal skin development. The latter is important, since a wound healing phenotype might be secondary to a defect already present in nonwounded skin. Thus by induction of a transgene or deletion of an endogenous gene before wounding, the role of this particular gene in the healing response can be studied in the absence of secondary abnormalities. This type of study will undoubtedly unravel many exciting functions of growth factors and cytokines in normal wound repair as well as in impaired healing and scar formation.

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