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Review

Proteinases in cutaneous wound healing

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Abstract. Cutaneous wound healing is a complex and highly coordinated process where a number of different cell types participate to renew the damaged tissue under the strict regulation of soluble and insoluble factors. One of the most versatile processes involved in wound repair is proteolysis. During cell migration, proteins of extracellular matrix are cleaved, often creating biologically active cleavage products, and proteolysis of cellular contacts leads to increased cell motility and division. Moreover, proteases activate various growth factors and other proteases in wound and regulate growth factor signaling by shedding growth factor receptors on cell surface. Normally, proteolysis is strictly controlled, and changes in protease activity are associated with alterations in wound closure and scar formation. Here, we present the current view on the role of metalloproteinases and the plasmin-plasminogen system in normal and aberrant cutaneous wound repair and discuss their role as potential therapeutic targets for chronic ulcers or fibrotic scars.

Keywords. ADAM, ADAMTS, fibrosis, MMP, plasminogen, skin, wound.

Overview of cutaneous wound repair

Wound healing in mammalians is a rapid and effective process, the main purpose of which is to stop bleeding and reconstitute the structural and functional barrier, preventing drying and invasion of microbes to the body. Timely and effective repair of the outermost layer of the organism has been one of the most important processes in the evolution enabling life on dry land. In general, cutaneous wound healing is divided into three major phases: 1) hemostasis and inflammation, 2) re-epithelialization and granulation tissue formation, and 3) tissue remodeling [1]. These phases are histologically and functionally distinct but they overlap temporally, and the complete healing requires interactive, carefully orchestrated communication of numerous cell types in distinct tissue compartments. Fig. 1 demonstrates the phases of cutaneous wound healing and the important events in chronological order. It should be noted, that the time scale is suggestive and depends on the size of a wound. Immediately following the cutaneous injury extending to the dermal layer, blood extravasates to the open wound from disrupted blood vessels. The inflammatory phase is initiated by hemostasis as a result of vasoconstriction, and adhesion, aggregation, and degranulation of platelets. Activation of the cascade of coagulation factors on the damaged cells and platelets leads to the cleavage of fibrinogen by thrombin and formation of insoluble fibrin fibers. These fibers then bind to platelets and together they

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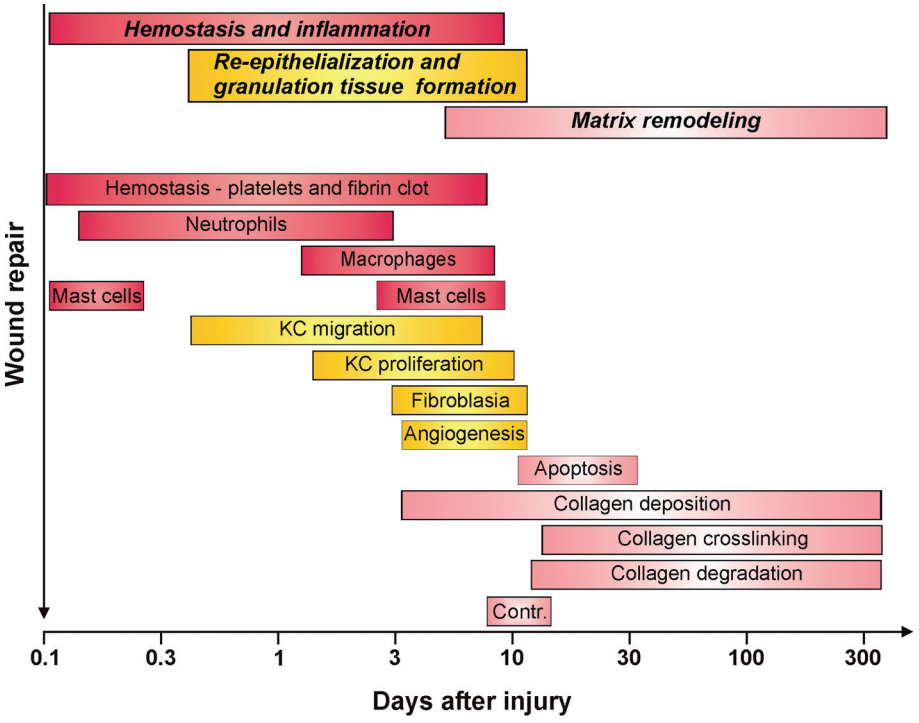

Figure 1. The phases and functional events of cutaneous wound healing. KC, keratinocyte; Contr., contraction. Modified from [1].

form a clot, which serves as a physical plug to stop bleeding. The fibrin clot also includes plasma fibronectin and vitronectin and provides a provisional matrix for cell migration. Fragments of coagulation factors, activated complement components, and growth factors derived from damaged cells and activated platelets within blood clot proffer chemotactic stimuli for inflammatory cells, keratinocytes, fibroblasts and endothelial cells. As wound healing progresses, the provisional fibrin matrix is invaded by migrating keratinocytes, degraded by proteinases, mainly by plasmin, and finally disentangled from the wound site as an eschar [2].

The first inflammatory cells arriving at the site of injury are neutrophils, which appear in skin wound a few hours after injury, at least in part attracted by the resident mast cells in the wounded tissue [3]. The main function of the neutrophils is phagocytosis of infectious agents and devitalized tissue in the wound. They also secrete various factors that amplify clot formation and inflammation, and stimulate the repair process [4]. Within two days of injury blood monocytes immigrate to the wound site and become activated macrophages which function as antigen-presenting cells and phagocytes, and contribute to the regulation of wound healing by secreting numerous growth factors, such as transforming growth factors (TGF-β, TGF-α), basic fibroblast growth factor (bFGF) and platelet-derived growth factors (PDGF) [4].

Parallel to the inflammatory phase of wound healing, re-epithelialization and granulation tissue formation are initiated. In a few hours after injury, epidermal keratinocytes at the wound edge and in the remnants of the skin appendages, e.g. hair follicles, detach from the underlying basement membrane and from adjacent cells and start to migrate into the wound, typically underneath the scab [2]. In two days, wound keratinocytes distant from the wound edge obtain hyperproliferative phenotype providing cells to fill the gap in the epithelium [2]. There is evidence that stem cells of the skin epithelium located in the hair follicle bulge regions adjacent to the wound site supply proliferative potential needed during re-epithelialization and maintenance of the intact epidermis [5]. Once the wound gap is closed, the basement membrane is re-established and the cellular contacts are re-formed, keratinocytes differentiate to constitute the multilayered epidermis of skin.

Wound granulation tissue contains numerous new blood vessels, fibroblasts, tissue macrophages and extracellular matrix (ECM) molecules. In skin, formation of granulation tissue begins in a few days after injury by activation of fibroblasts in undamaged dermis by growth factors, such as PDGF and TGF-β, which stimulate cell proliferation and migration [2]. Intriguingly, recent evidence suggests that a portion of fibroblasts is derived from the bone marrow-derived mesenchymal progenitor cells [6]. Fibroblasts deposit and remodel wound ECM, which initially consists

mainly of fibronectin and hyaluronan, both of which stimulate cell migration. At later stage, proteoglycans and type III and I collagens are deposited and become the major components of wound ECM [1]. During the second week of healing, largely as a result of TGF-β and the mechanical tension generated by the open wound and remodeling of granulation tissue ECM, fibroblasts obtain myofibroblast phenotype characterized by the expression of α-smooth muscle actin [7, 8]. These cells benefit wound closure by contracting the tissue and pulling wound edges closer to each other. Myofibroblasts are eliminated via apoptosis when mechanical stress decreases [9].

The early granulation tissue, rich in blood clot fibrin and fibronectin, stimulates the sprouting of new blood vessels into the tissue. Angiogenesis, as a result of the migration and proliferation of endothelial cells, is initiated by tissue destruction and hypoxia, and subsequently stimulated by various soluble factors, such as bFGF, vascular endothelial growth factor (VEGF) and TGF-β, secreted by wound macrophages, keratinocytes and endothelial cells. Angiogenesis is dependent on proteolytic activity of plasmin and matrix metalloproteinases (MMPs) [2].

The regenerative phase of cutaneous wound healing is followed by the maturation of the wound, leading to formation of collagenous scar. This remodeling phase of wound repair is characterized by reduction of the fibroblast number and disintegration of the majority of the blood vessels via apoptosis, which is probably regulated by the cease of physical tension of the open wound, by special cell-cell contacts and by different growth factors. The resident fibroblasts in the maturing wound continue remodeling of collagenous dermis for several months by depositing and degrading collagen molecules and arranging collagen fibers into orientation that supports the tensile strength of skin, which, however, never quite reaches the original strength of intact skin [2, 9].

The renewal of tissue after injury necessitates highly coordinated function of different cell types. Many important signals are mediated by cell-ECM contacts, intercellular contacts and by changes in cellular mechanical stress. However, in wound, there are a number of different growth factors and cytokines that regulate cell behavior, which are secreted or released from cell surface or ECM by proteinases. As shown by wound healing studies with genetically modified animals and neutralizing antibodies against specific growth factors, it is obvious that many of them, e.g. hepatocyte growth factor/scatter factor (HGF/SF), are crucial for normal wound closure and maturation [10]. The effects of the most characterized growth factors and cytokines involved in cutaneous wound repair, and the producer cell types are listed in Table 1 ([4, 11–13]). PDGF and TGF-β can be considered as the two main growth factors in wound that regulate the formation of granulation tissue while HGF, TGF-α and HB-EGF seem to be the most important growth factors for re-epithelialization. As growth factors and cytokines regulate the expression of proteases by various cell types involved in wound healing, active proteases regulate the bioavailability of a variety of soluble factors by shedding, releasing them from ECM, and by activating them.

PA-plasmin system

Plasmin is the main fibrinolytic enzyme in the body responsible for fibrin homeostasis. The classical fibrinolytic system involves active serine protease plasmin and its liver-derived inactive precursor plasminogen (Plg) abundantly present in blood plasma, urokinase and tissue plasminogen activators (uPA and tPA), inhibitors of serine proteases (serpins), such as α2-antiplasmin, α2-macroglobulin and PA inhibitors -1 and -2 (PAI), and the cellular receptors for plasmin and PAs [14]. In MEROPS peptidase database, serine endopeptidases plasmin, uPA and tPA are included in large subfamily S1A [15]. In general, uPA converts Plg to plasmin within tissues while tPA generates plasmin for thrombolysis in the vascular system. Activation of Plg usually takes place in association with fibrin by tPA or at the cell membrane by uPA or tPA bound to a cellular receptor such as the uPA receptor, αMβ2 integrin or annexin 2. Plg is proteolytically activated by peptide bond cleavage generating an active dimeric plasmin [14]. In addition to PAs, serine protease plasma kallikrein and coagulation factors XIa and XIIa are able to directly activate Plg [16–18]. Plasmin cleaves fibrin, dissolves the fibrin-containing clot which may cause occlusion of the vessel or block cell migration during tissue healing, and generates fibrin fragments that have other biological functions, such as chemotaxis and inhibition of platelet function [14]. Plasmin also digests ECM proteins, including laminin and fibronectin [19, 20]. In addition, it activates and inactivates coagulation factors V and IX and may play a role in activation of TGF-β [14, 21]. Moreover, at least *in vitro,* plasmin activates several latent MMPs, including MMP-1, -8, -13, -9, -3, -7 and MT1-MMP [22]. Furthermore, it appears that plasmin and PAs can contribute to ECM turnover by regulating the production of MMPs [23–25]. Finally, Plg also has a well-established role in regulating angiogenesis. Angiostatin, an angiogenesis inhibitor, which is generated from Plg by internal proteolytic cleavage, regulates migration and proliferation of endothelial cells and induces their apoptosis [26].

Table 1. Growth factors and cytokines affecting wound healing.

Growth Factor/ Cytokine	Major source	Regulated wound healing events	Reference
EGF	platelets	epithelialization, fibroplasia, ECM production and degradation	[2, 224–226]
TGF-α	macrophages, neutrophils, KC	epithelialization	[11]
HB-EGF	KC	KC migration	[227]
TGF-β	platelets, macrophages, KC, fibroblasts	fibroplasia, ECM production, contraction, leukocyte recruitment	[11, 12]
HGF/SF	fibroblasts, KC	epithelialization, leukocyte recruitment, angiogenesis	[10, 228]
FGF-1	KC	pleiotropic mitogen, angiogenesis	[11]
FGF-2	KC, fibroblasts, macrophages, endothelial cells	pleiotropic mitogen, angiogenesis, ECM production	[2, 11]
KGF	fibroblasts	KC proliferation and differentiation	[229, 230]
PDGF	platelets, macrophages, KC	fibroplasia, leukocyte recruitment, ECM production, contraction	[11]
VEGF	KC, macrophages	angiogenesis, vascular permeability	[231]
CTGF	platelets, fibroblasts	fibroplasia, ECM production, angiogenesis	[11]
IGF-I	fibroblasts, KC, plasma	epithelialization, ECM production	[232]
TNF-α	leukocytes, KC	expression of growth factors, leukocyte recruitment, ECM degradation	[11]
IL-1	leukocytes, KC	expression of growth factors, leukocyte recruitment, ECM degradation	[11]

KC, keratinocyte; fibroplasia defined as proliferation and migration of fibroblasts to wound site.

Matrix metalloproteinases (MMPs)

MMPs comprise a group of structurally related enzymes that are collectively able to cleave practically all types of ECM molecules and various other substrates including other proteases, growth factors and cytokines, of which MMPs often function as activators [27]. Comprehensive lists including physiologic and *in vitro* substrates for MMPs can be found in protease databases (http://cutdb.burnham.org/, http://merops.sanger.ac.uk) and in recent reviews such as [28–30]. All 23 MMPs identified in humans are classified into metalloendopeptidase subfamily M10A [15]. Based on their substrate specificity and structural similarity MMPs can be divided into distinct subgroups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and to the group of other MMPs [28–30]. All MMPs have a pro-domain and a catalytic domain. They have a signal peptide, which is removed during translation, and generally they are secreted from cells as inactive zymogens or proenzymes. The pro-domain maintains the MMP in latent form, because it contains a conserved cysteine residue that interacts with the catalytic Zn^{2+} ion bound to the highly conserved HExxHxxGxxH sequence in the catalytic domain. During proteolytic activation of proMMPs, the "bait" region of the pro-domain is first cleaved generating an MMP intermediate which subsequently is processed by cleavage of the remnants of the pro-domain by MMP intermediate itself or by other active proteinases, generating a fully active MMP. Most of the MMPs are activated pericellularly but membrane type MMPs (MT-MMPs), MMP-11, -21, -23 and -28 contain a cleavage site for furin-like proteases between the pro- and catalytic domains, and are likely to be activated within the secretion pathway. In addition to proteolytic activation of MMPs, they can be activated *in vitro* by chemicals that alter the molecular structure, such as mercurial compounds and SH reagents or by oxidants reacting with cystein of the cystein switch [29]. Increasing evidence is emerging that MMP-1, MMP-11 and MMP-2 have also intracellular functions and that MMP-11 can be directly translated as an active protein as a resultant of alternative splicing and promoter activity [31–33]. MMPs, except matrilysins (MMP-7 and MMP-26) and MMP-23, possess a C-terminal hemopexin domain, which mediates molecular interactions and is involved in substrate recognition. The catalytic domains of gelatinases MMP-2 and -9 also have three repeats of fibronectin type II sequence, which mediate the recognition of large ECM proteins such as type IV collagen, gelatin and elastin. MMPs are mainly secreted proteins but MT-MMPs are linked to the plasma membrane via the transmembrane domain or by GPI anchor. MMP-23 associates to cell membrane via N-terminal signal

anchor and is secreted after the activating cleavage [29].

The physiologic and pathologic roles of MMPs depend on their capacity to specifically digest a variety of proteins, including ECM macromolecules, latent growth factors and cytokines, their receptors and inactivating binding proteins, cell-ECM receptors, proteins mediating cell-cell contacts, as well as other pro-proteinases and proteinase inhibitors [34]. The action of MMPs is often associated with pathologic events with focal tissue destruction such as osteoarthritis and rheumatoid arthritis [35], heart and vascular diseases [36], different kinds of lung diseases [37], cancer progression [38, 39], periodontitis [40] and chronic cutaneous and intestinal ulcerations [41, 42]. Although the expression levels of MMPs are usually very low in intact tissues, their expression and function is elevated in various dynamic physiologic situations such as in different aspects of embryonic tissue development, during reproductive cycle, and in every phase of wound healing [41, 43].

In vivo, the activity of MMPs is regulated in principle at three levels: 1) by proteolytic activation of the zymogen as described above, 2) by small biological tissue inhibitors of metalloproteinases (TIMPs) and 3) by transcriptional control of MMP genes. In mammals, there are four known endogenous TIMPs, namely TIMP-1, -2, -3 and -4. All TIMPs bind all MMPs and inhibit them with 1:1 stoichiometry, although TIMP-1 only poorly inhibits MMP-19 and most MT-MMPs. TIMPs are secreted proteins, which are primarily soluble in the extracellular space. However, they can associate with certain membrane-bound proteins, and TIMP-3 adheres to ECM proteoglycans. In addition to TIMPs, most MMPs are also inhibited by binding to plasma proteinase inhibitors, such as α1-proteinase inhibitor or α2-macroglobulin, and to thrombospondin-1 and -2 [44]. Furthermore, at least certain MMPs are inhibited by reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and endocytosis among some other mechanisms [45–47].

The regulation of MMP gene expression was recently comprehensively reviewed [48]. Shortly, at the transcriptional level, the expression of MMPs is regulated initially by extracellular signals that are generated via ligand binding to a growth factor or cytokine receptor or integrin receptors, or by alterations in cadherin-mediated cell-cell junctions. This triggers cellular signals e.g. activation of mitogen-activated protein kinase (MAPK) pathway, Smad-pathway or NF-κB-pathway by a growth factor or a cytokine, activation of focal adhesion kinase (FAK) by integrin activation, or activation of β-catenin by cadherins, leading eventually to activation/inactivation of an appropriate gene promoter region and shift of MMP expression [48, 49]. Many MMP promoters share much similarity and are simultaneously expressed. Factors that are abundantly present also in cutaneous wounds and regulate MMP expression include at least TGF-β, PDGF, TNFα, IL-1β, bFGF, EGF and KGF. For example, the expression of MMP-1, which is induced by collagen contact in migrating wound keratinocytes *in vivo*, is down-regulated *in vitro* by bFGF and KGF in keratinocytes [50]. Moreover, the expression of MMP-1 by human fibroblasts is enhanced, e.g. by PDGF, while TGF-β down-regulates MMP-1 expression [51, 52]. Furthermore, the expression of certain MMP genes is regulated by epigenetic modification of chromatin, such as methylation or packing of DNA around the histones, as well as post-transcriptionally by stabilizing or destabilizing mRNA [48].

ADAMs and ADAMTS proteases

ADAMs, a disintegrin and metalloproteinases, are transmembrane proteins. Most of them possess adhesive and proteinase activities in their ectodomains and putative signaling activities in the cytosolic compartment. In the MEROPS peptidase database, ADAMs are classified into the metalloendopeptidase subfamily M12B [15], as metalloproteinases. However, only 13 of the 27 known human ADAMs contain the complete sequence of Zn-dependent catalytic site [53, 54]. Hence, not all possess proteolytic properties. Similar to MMPs, ADAMs consist of several domains. As extracellular proteins, ADAMs have a signal peptide, the latency-associated pro-domain, a metalloprotease domain, a disintegrin-like and a cysteine-rich domains that have intercellular and cell-ECM adhesive roles, an epidermal growth factor (EGF)-like domain, a transmembrane domain, and a cytoplasmic tail which contains potential residues for tyrosine and serine/threonine phosphorylation and interaction motifs for several cytosolic signalling molecules [53, 54].

Some ADAMs have very restricted expression pattern e.g. in testis. However, ADAMs 8, 9, 10, 11, 12, 15, 17, 19, 22, 23, 28 and 33 are expressed more widely in somatic tissues [53]. The biologic functions of ADAMs include cytokine and growth factor shedding, cell migration, myotube formation and fertilization. Gene knock-out experiments with transgenic mice indicate that at least ADAMs 10, 17 and 19 are crucial for the normal embryonal development of various tissues [55–58]. Much of the activity of an individual ADAM can most probably be compensated by the enzymatic redundancy, which could explain the mild phenotype of the most transgenic animals with ADAM gene deficiency.

The majority of the ADAMs containing an active metalloprotease domain function as sheddases, cleaving the ectodomain of a membrane bound protein releasing it into the extracellular space. Often, the shed protein is activated by the cleavage adding bioavailability of the protein, or in the case of cytokine or growth factor receptors, the number of active receptors can be regulated by shedding, and a soluble receptor may act as a decoy, competitor for a functional receptor. The proteins shed by ADAMs collectively include cytokines, growth factors, cytokine and growth factor receptors, adhesion proteins, proteases and various other cell surface-linked proteins. ADAM-17, the TNFα-converting enzyme (TACE) was first shown to release and activate TNFα but now more than 30 substrates for TACE have been identified [54]. One important set of sheddase-substrates are EGF receptor (EGFR, ErbB) ligands, namely TGFα, heparin binding-EGF (HB-EGF), amphiregulin, epiregulin, EGF, betacellulin and epigen, which all are produced as membrane associated inactive molecules and are substrates for ADAM-10 or ADAM-17 [59, 60]. In addition to "shed" substrates, ADAMs 9, 10, and 15, found in humans, have been reported to cleave ECM substrates, including fibronectin, basement membrane type IV collagen and gelatin, which may facilitate cell migration or release growth factors that adhere to ECM proteins [61–63]. Disintegrin domains of several ADAMs, ADAM-7, -9, -12, -15, -28, -33 and additional murine ADAMs 1, 2 and 3, have been shown to interact with α4/5/6/9β1, α4β7 and αVβ3/5 integrins of the neighboring cells. The suggested roles for these interactions include counter-receptor action of an ADAM and an integrin in cell-cell contacts, possible inhibition of an ADAM by an integrin and the direction of a sheddase to a convenient substrate by an integrin, although not much direct evidence is yet available for these potential functions [64].

In addition to the ability of certain integrins to inhibit active ADAMs, the proteolytic activity of ADAMs is restrained by endopeptidase inhibitor α2-macroglobulin and to some extent by TIMPs. TIMP-3 has been shown to inhibit the activity of ADAMs 10, 12, 17, 28 and 33, TIMP-1 has been shown to inhibit ADAM-10, and TIMP-4 ADAMs 28 and 33 [45, 65, 66].

ADAMTS proteases, a disintegrin and metalloproteinases with thrombospondin type 1 motifs, are structurally closely related to ADAMs. However, they are secreted proteins and in contrast to ADAMs, which have cell membrane associated proteins as their main substrates, ADAMTS proteases prefer ECM proteins. There are 19 human members in the ADAMTS family with nomenclature from ADAMTS-1 to ADAMTS-20, ADAMTS-11 being identical to ADAMTS-5. ADAMTS proteases are widely expressed in tissues. Their involvement in several biological events, such as organization of connective tissue, blood clotting, inflammation, angiogenesis and cell migration, suggests a role for this protease family in wound healing [67, 68]. ADAMTS-2, highly expressed in skin, cleaves aminoterminal peptide of fibril forming procollagens I, II, and III essential for collagen fibril formation. Recently, it was shown to digest also aminopeptide of fibrillar type V procollagen which, in turn, is also needed in type I collagen fibrillogenesis [69]. ADAMTS-3 and ADAMTS-14 process type II and type I procollagens, respectively [70]. ADAMTS-1 and -8 are demonstrated to inhibit angiogenesis by mechanism that most probably involves binding to $VEGF_{165}$ and thus blocking of signaling via VEGF receptor-2 in endothelial cells [71, 72].

ADAMTS proteases are more susceptible to inhibition by TIMPs than ADAMs. Matrix associated TIMP-3 potently inhibits the activity of aggrecanases ADAMTS-4 and -5, as well as ADAMTS-2 and ADAMTS-1, the latter of which is also inhibited by TIMP-2 [73]. Thus, TIMP-3 appears to be an important regulator of ADAMTS activity in tissues.

Proteolysis in cutaneous wound

The four major groups of proteases that mediate various proteolytic processes in a healing wound were discussed above. They generate proteolytic cleavage products with different bioactive properties and may regulate cell behavior by altering cell-cell and cell-ECM contacts. The changes in strictly regulated proteolysis are associated with scarring and alterations in wound closure. Next, we discuss in detail the current view of the roles of PA-plasmin system, MMPs, ADAMs and ADAMTS proteases in cutaneous wound repair.

PA-plasmin system

Plasma derived Plg is activated to plasmin mainly by uPA in skin wounds. The expression of the key components of the Plg activation system, uPA and its receptor (uPAR), as well as its inhibitor PAI-1, is induced early during re-epithelialization in the migrating epithelial sheet in murine cutaneous wound [74–76]. In acute human wounds, uPA-expression coincides with the expression of MMP-1 (see below) [77]. uPA and uPAR are up-regulated also by migrating keratinocytes *in vitro* although migration on plastic is not dependent on the function of uPA [78]. In cultured keratinocytes, TGF-β, HGF and KGF up-regulate uPA, and recently IL-1α was implicated in tPA induction in murine keratinocytes [79–81]. Dur-

Table 2. Protease gene targeting in mice – cutaneous wound phenotypes.

Gene	Modification	Wound phenotype	Reference
hMMP-1	overexpression in KCs	Delayed re-epithelialization	[104]
MMP-8	knockout	Delayed re-epithelialization, delayed onset and persistent inflammation	[115]
MMP-13	knockout	Unaltered	[112]
MMP-9	knockout	Enhanced re-epithelialization	[126]
MMP-3	knockout	Impaired wound contraction	[117]
MMP-10	overexpression in KCs	Unaltered closure, scattered epithelial sheet	[120]
MT1-MMP	knockout	Unaltered, impaired epithelialization *ex vivo*	[147]
PLG	knockout	Severely impaired re-epithelialization	[76]
ADAMTS-1	knockout	Delayed closure, increased angiogenesis	[184]

ing keratinocyte migration *in vitro*, uPA and uPAR are enhanced by activation of protein kinase C, suggesting regulation by cytoskeletal alterations [78]. In addition, uPA is detected in macrophages and fibroblasts, and uPAR in macrophages *in vivo* in human wound granulation tissue [82].

The major role of plasmin in wound healing has been demonstrated using Plg-deficient mice, which show severely impaired wound repair [76] (Table 2). Wound re-epithelialization in these animals is dramatically affected due to disability of migrating keratinocytes to dissect the fibrin-containing wall from their way. Indeed, additional deletion of fibrinogen gene rescues the wound phenotype in Plg-null mice [83]. Although delayed, skin wounds of Plg-deficient mice eventually close in 60 days, but if these mice are simultaneously treated with wide spectrum metalloproteinase inhibitor galardin, the healing is virtually blocked demonstrating the parallel roles of plasmin and metalloproteinases in re-epithelialization [84]. Interestingly, wound closure in uPA/tPA double-deficient mice is not as severely impaired as in Plg-deficient mice, and the treatment with galardin further delays but does not block wound healing. Serine protease plasma kallikrein was demonstrated to regulate the closure of wounds of galardin-treated uPA/tPA –null mice, most likely by directly activating Plg [16].

Wound granulation tissue formation is unaltered in Plg-deficient mice probably due to the adequate enzymatic redundancy [76]. However, in a normal situation the induction of uPA, uPAR and PAI-1 expression in endothelial cells, and of PAI-1 in accompanied stromal and epithelial cells, during angiogenesis is established. *In vitro*, their expression is up-regulated by bFGF and VEGF, both of which are potent angiogenic factors [85]. Subsequently, plasmin most likely functions to degrade fibrin clots which may disturb angiogenesis and also releases bFGF and VEGF bound to ECM associated with blood vessels, generating an autostimulatory loop for the PA-plasmin system during angiogenesis [86, 87]. In addition, PAI-1 appears to be important in tumor angiogenesis as well as in physiologic angiogenesis. PAI-1 may protect ECM from excessive proteolysis and in this way maintain the scaffold for endothelial cell migration. Alternatively, it may regulate adhesion of endothelial cells via interaction network of PAI-1, uPAR, integrins and vitronectin [85]. Finally, angiostatin, which consists of the first four kringles of Plg is generated by proteolytic cleavage by, e.g. MMPs, inhibits angiogenesis by regulating endothelial cell migration, proliferation and inducing apoptosis [26].

MMPs and TIMPs

In general, the expression level of MMPs in intact skin is very low. Only MMP-7 and MMP-19 are constitutively produced in sweat and sebaceous glands [88, 89]. In addition, MMP-28 expression is detected in basal and suprabasal keratinocytes of intact skin *ex vivo* [90] and MMP-19 is detected in basal keratinocytes in intact epidermis and in hair follicles as well as in endothelial and smooth muscle cells of the veins and arteries [89]. Also, a consistent low level expression of MMP-2 and MT1-MMP, and MMP-2 has been detected in dermis of intact mouse and porcine skin, respectively [91, 92]. However, due to the chemical and physical changes in the cellular environment after skin injury, the expression of multiple MMPs is induced. MMPs that have been identified in normally healing skin wounds include collagenases MMP-1 and MMP-8 [93, 94], gelatinases MMP-2 and MMP-9 [95], stromelysins MMP-3 and MMP-10 [77], metalloelastase MMP-12 [91], MT1-MMP [95], MMP-19 [96], MMP-26 [97] and MMP-28 [90]. The expression of TIMP-1, -2 and -3, but not TIMP-4, in acute skin wounds is also reported [98]. The expression and cellular source of MMPs in acute cutaneous wound is illustrated in Fig. 2.

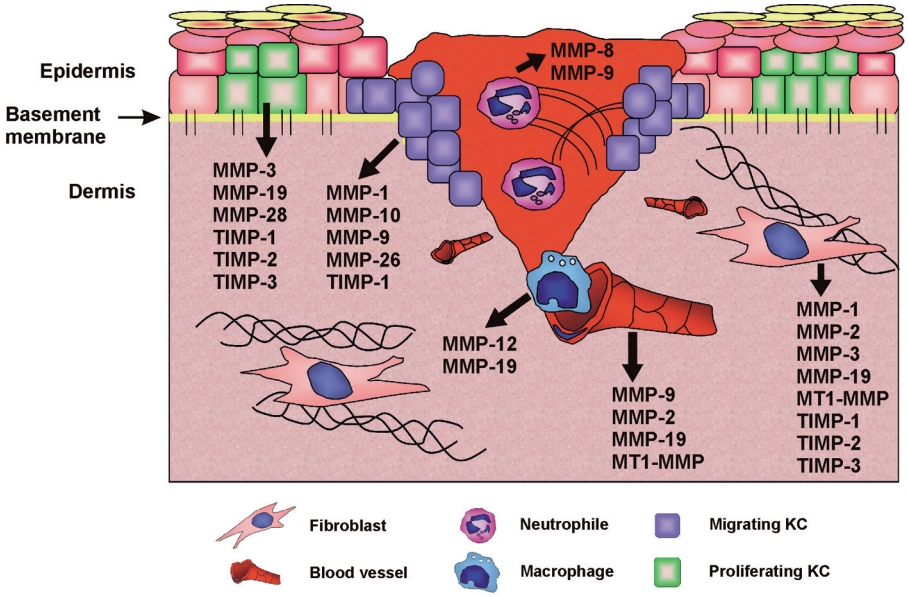

Figure 2. The expression and cellular source of matrix metalloproteinases (MMP) and tissue inhibitors of MMPs (TIMP) in acute cutaneous wound.

In various types of cutaneous wounds, in which the basement membrane is disrupted, skin injury evokes within hours temporarily and spatially restricted expression of MMP-1 in migrating keratinocytes that face the dermal compartment of skin and lack the contact with intact basement membrane. This expression peaks 24 hours after wounding and subsides by completion of re-epithelialization [93, 99]. The early induction of MMP-1 is important especially in the re-epithelialization kick-off when keratinocytes come into contact with type I collagen and the provisional matrix has not yet been formed. It has been shown that native type I collagen induces MMP-1 expression in these cells *in vitro* while basement membrane proteins, fibronectin or type III collagen do not [100]. Moreover, the activity of MMP-1 seems to be essential for keratinocyte migration on native type I collagen and its collagen-induced expression is mediated via α2β1 integrin [101]. It has been demonstrated that proMMP-1 and activated MMP-1 can bind to cell surface integrin α2β1 in keratinocyte cultures on collagen-coated dishes, suggesting a specifically directed collagenolysis by MMP-1 at the sites where it is needed [102]. MMP-1 cleaves type I collagen, generating fragments that at body temperature denature to gelatin, which is a less adhesive ligand for α2β1 compared to native collagen and therefore a more suitable substrate for migration. Thus, in humans, the α2β1-MMP-1 complex is suggested to function as a motor stimulating migration of keratinocytes on type I collagen during re-epithelialization. Surprisingly, α2-deficient mice show normal re-epithelialization, which may reflect the different composition of human and mouse dermis or perhaps compensation by another collagen receptor integrin [103].

Following re-epithelialization and establishment of new basement membrane the expression of epidermal MMP-1 is shut down, apparently mediated by cellular contacts with basement membrane proteins. Indeed, in the presence of type I collagen, contact with laminin-111 (previously called laminin-1) represses production of MMP-1 by keratinocytes [100]. The importance of the strict regulation of MMP-1 activity during wound healing has been demonstrated by overexpression of human MMP-1 in mouse epidermis. These mice display markedly delayed closure of full-thickness wound and hyperproliferative epidermis [104] (Table 2). However, in mice with collagenase-resistant mutation in type I collagen, the closure of incisional skin wound was severely impaired due to impaired wound contraction and delayed re-epithelialization [105]. Thus, controlled epidermal as well as dermal collagenolysis is required for proper wound healing.

MMP-1 is also expressed by fibroblasts in granulation tissue [93, 106], where it is believed to participate in remodeling of collagenous ECM [107]. Collagenase-3 (MMP-13) displays a similar expression pattern in mouse skin as MMP-1 in human skin [91] and is expressed by fibroblasts in human fetal cutaneous wounds [108]. MMP-1 and MMP-13 may also regulate survival of fibroblasts during dermal wound healing by affecting fibroblast-mediated matrix contraction and matrix rigidity, and by revealing cryptic binding sites from native collagen for integrins, such as αV-integrin, which promotes cell survival in collagen lattice [109–111]. Mice deficient in MMP-13 displayed, however,

no alteration in healing of excisional wounds, but possess normal wound closure, inflammatory response, granulation tissue formation and matrix remodeling [112]. The lack of wound healing phenotype in MMP-13 knock-out mice could be explained by enzymatic redundancy since these mice have elevated expression level of collagenase-2 (MMP-8) in wounds [112]. In addition, MMP-2 and MT1-MMP, also capable of cleaving fibrillar collagens, are present in murine and human skin wounds [91, 112]. Finally, the need for collagenolytic activity in the re-epithelialization of excisional wound in mice has been questioned due to differences in the amount of collagenous ECM in mouse full thickness wound compared to humans [113]. Thus, in murine skin wounds, MMP-13 may play more important role e.g. in removal of provisional matrix proteins from the way of migrating keratinocytes, which however, can also be performed by other proteases.

MMP-8 is mainly expressed by neutrophils, in which it is stored in cellular granules and secreted on neutrophil activation [114]. In human cutaneous excisional wounds, MMP-8 was discovered to be the most abundant collagenase [94]. Its relevance in wound healing has still been somewhat obscure until knock-out studies with MMP-13-deficient mice first alluded the enzymatic compensation of MMP-13 by MMP-8 [112], and recently with mice deficient in MMP-8, revealed a significant delay in wound healing due to the impaired re-epithelialization, a lag in neutrophil infiltration, and persistent inflammation [115]. These studies suggest a role for neutrophil-derived MMP-8 in keratinocyte migration together with epidermal collagenase, and in migration and resolution of neutrophils at the wound site.

Stromelysins, i.e. MMP-3 and MMP-10, are also expressed by epidermal cells during wound repair in human and mouse wounds. MMP-3 is expressed by the basal proliferating keratinocytes behind the migrating cells, while MMP-10 is produced exclusively at the tip of the migrating keratinocyte sheet [77, 91, 116]. In addition, MMP-3 is produced by wound fibroblasts [77, 91]. MMP-3-deficient mice display delayed wound closure due to impaired wound contraction, although the re-epithelialization is not altered [117]. Thus, the role of MMP-3 in epithelium remains unclear. However, MMP-3 can activate several proMMPs, digest several ECM substrates including basement membrane proteins, and increase activity and bioavailability of many cytokines and growth factors e.g. HB-EGF and bFGF [86, 118]. These functions propose a role for MMP-3 in the organization of newly formed basement membrane or in the regulation of cell migration and proliferation. MMP-3 also cleaves E-cadherin ectodomain on the surface of human breast cancer cells resulting in inhibition of intercellular contacts and enhanced invasion [119]. Thus, MMP-3 may act to loosen the epithelial structure of epidermis as a prerequisite for keratinocyte proliferation and migration.

Epidermal MMP-10 is induced about three days post-wounding in humans, and at least *in vitro* does not seem to be regulated by matrix contacts but rather by cytokines such as EGF, TGF-β1 and TNF-α [116]. MMP-10 is suggested to regulate keratinocyte organization and migration during re-epithelialization, since the overexpression of constitutively active MMP-10 in basal keratinocytes of transgenic mice severely scatters the migrating epithelial sheet. This is probably due to the increased processing of laminin-332 (previously called laminin-5) by MMP-10 and alterations in cell-ECM adhesion, which disturbs the controlled keratinocyte migration. However, at the end, wound healing in these mice is virtually unaffected and characterized by normal closure rate and normally established basement membrane [120].

Gelatinases, i.e. MMP-2 and MMP-9, exhibit distinct expression pattern during cutaneous wound repair. MMP-9 is detected at the migrating epithelial front [84, 91, 95], whereas MMP-2 is exclusively expressed in the dermal compartment of skin by fibroblasts, especially adjacent to regenerating epidermis, and by endothelial cells in mouse excisional wounds and human partial-thickness acute wounds [91, 95]. MMP-9 is also present in inflammatory cells including T cells and neutrophils [121, 122]. The relatively stable and long-lasting enhancement of MMP-2 expression in porcine skin wound suggests a role in prolonged ECM remodeling [92]. Indeed, in addition to direct processing of dermal ECM proteins, several studies have implicated MMP-2 in activation of TGF-β, a potent growth factor regulating matrix deposition and remodeling, by cleaving latency-associated peptide (LAP) of proTGF-β and latent TGF-β binding protein (LTBP), and releasing TGF-β bound to ECM [123–125]. MMP-9 is also able to activate latent TGF-β and disentangle it from LTBP [123, 124]. Based on the study with MMP-9-deficient mice, in contrast to most MMPs, MMP-9 seems to have an inhibitory or regulatory role in epidermal wound healing and it was not needed for wound closure. These mice show enhanced re-epithelialization of cornea and skin probably due to the enhanced epithelial cell proliferation. Interestingly, the remodeling of basement membrane after re-epithelialization, and degradation of fibrin-containing provisional matrix are defective in MMP-9-deficient mice [126]. In another study, MMP-9 was shown to potently digest fibrin [127]. Thus, MMP-9 may be involved in the final adjustment of the epidermal tissue after wound healing by remodeling of

the basement membrane zone and by complete resorption of provisional matrix from the wound bed. Both MMP-2 and MMP-9 are expressed in endothelial cells as well as are MT1-MMP and MMP-19 [95, 96, 128, 129]. MMP-2 and MMP-9 have been shown to play pivotal roles in both physiologic and tumorigenic angiogenesis [130–133]. These two gelatinases digest various constituents of the vascular basement membrane, which is obligatory for generation of new blood vessels. Importantly, gelatinases contribute to angiogenesis also by activating angiogenic cytokines and growth factors, such as TNF-α, which is upregulated after injury or during infection [11, 134]. Cancer stroma-derived MMP-9 has been shown to release VEGF from ECM [133] and recently MMP-2 was reported to release VEGF from inactivating complexes with CTGF and pleiotrophin/heparin affin regulatory peptide (HARP) [135]. Interestingly, gelatinases, as well as several other MMPs, not only promote angiogenesis but they also may inhibit blood vessel formation by generating anti-angiogenic peptides from other proteins. MMP-3, -7, -9, -13, and -20 have been shown to generate endostatin from type XVIII collagen [136] and MMP-2, -3, -7, -9 and -12 angiostatin from plasminogen *in vitro* [137, 138]. In addition, MMP-2 can shed cellular FGF receptor -1 still preserving the receptor capable of binding FGF [139]. Thus, MMP-2 may regulate FGF-mediated mitogenic and angiogenic signals by affecting the number of functional cell surface receptors and availability of active FGF. It is conceivable that potent angiogenic stimulation in physiologic situation, such as wound repair, is strictly controlled and in this respect MMPs play an important role in generating the peptides carrying anti-angiogenic stimuli.

The important role of MT1-MMP in wound angiogenesis may relate to its fibrinolytic and collagenolytic activity needed for vessel invasion through fibrin barriers and collagenous obstruction in the tissue stroma [140, 141]. Also its role in TIMP-2-mediated activation of proMMP-2 is well characterized [142]. *In vitro*, MT1-MMP co-localizes with β1 and αVβ3 integrins at the intercellular contacts and in migration-associated cellular processes, respectively, in endothelial cells, suggesting a regulatory role for endothelial cell adhesion and migration [129]. Furthermore, MT1-MMP is detected in wound stromal cells [143, 144]. It has been shown to control the migration and invasion of fibroblasts through collagenous matrix [145]. Surprisingly, although MT1-MMP null mice display severe abnormalities in bone development and defective angiogenesis in cartilage and cornea [146], cutaneous wound healing is unaffected in 3-day-old animals [147].

During cutaneous wound repair in humans, MMP-19 has been detected in proliferating epithelium, microvascular endothelial cells, fibroblasts and in macrophages [96, 148]. In keratinocytes, MMP-19 has been implicated in regulation of migration, proliferation and adhesion via releasing insulin-like growth factor (IGF) by IGF binding protein -3 cleavage and via laminin-332 cleavage [149, 150], and regulation by transcription factors Tst-1 and Skn-1 suggests that its expression is linked to cellular differentiation [151]. Increasing evidence is suggesting that MMP-19 would play an inhibitory role in angiogenesis involving destabilization of ECM necessary for capillary morphogenesis [152, 153].

MMP-12, or metalloelastase, is produced by wound macrophages in acute murine excisional wounds especially around the blood vessels reaching the maximum expression at completing the re-epithelialization [91]. Abundant expression of MMP-12 by macrophages was detected in various human cutaneous granulomas although the authors could not find MMP-12 in majority of the examined acute or chronic skin wounds despite the presence of macrophages [154]. MMP-12 is a potential regulator of angiogenesis through its ability to generate angiostatin and degrade fibrinogen interfering blood clotting [138, 155].

Matrilysin-2 (endometase, MMP-26), the smallest MMP, and epilysin (MMP-28), are also expressed during cutaneous wound healing. MMP-26 was detected in epithelial tip bordering the wound gap during re-epithelialization in normally healing acute skin wounds as well as in chronic ulcers, while the granulation tissue was MMP-26-negative. The immunostaining for MMP-26 was detected 1 day post-injury and sustained at least 9 days in the open wound [97]. Another study with different antibody reported MMP-26 immunostaining in extracellular stroma of dermal compartment near the basement membrane zone in acute wounds at day 1 disappearing thereafter [156]. Regulation by Wnt-signaling [157] and altered migration of mucosal keratinocytes treated with MMP-26 antibody [156] suggest that MMP-26 may be involved in keratinocyte proliferation and migration. Finally, MMP-28 was detected in human skin excisional wounds and in suction blisters in the suprabasal keratinocytes distal to the epithelial tip in the region where the cell phenotype changed from migratory to stationary and showed virtually intact basement membrane [158]. *Ex vivo*, in cultured skin biopsy, the immunostaining for MMP-28 was observed also in the migratory tip of the biopsy epithelium [90]. Recently, MMP-28 was shown to cleave LTBP-1 leading to increase in active TGF-β in the cell culture medium of A549 lung adenocarcinoma cells [159]. Thus, the spatial expression pattern of MMP-28 and its

ability to activate TGF-β suggest a role in the regulation of basement membrane establishment and/or keratinocyte proliferation.

The activity of secreted MMPs can be regulated by TIMPs also in wounds. The expression of TIMP-1 has been detected in the epithelium of healing excisional wounds and burn wounds in humans [77, 98, 160] but not in murine wounds [91]. In addition, TIMP-1 was detected in wound fibroblasts, especially around blood vessels [98, 160]. Both TIMP-2 and TIMP-3 have been shown to be expressed by skin epithelial cells and fibroblasts. Occasional blood vessels of acute wounds were also found to be positive for TIMP-1, TIMP-2 and TIMP-3. No TIMP-4 expression was detected in acute human wounds [98]. TIMPs are acknowledged to play other roles in human physiology and pathology in addition to MMP-inhibition, e.g. regulating proliferation and survival of various normal and tumour cells and this should be recognized also when clarifying regulatory mechanisms of wound healing [161].

Finally, various studies have demonstrated the importance of collective action of MMPs in wound healing *in vitro* and *in vivo* with wide spectrum chemical inhibitors of MMPs. For instance, treatment of fibroblasts cultured in 3-dimensional collagen matrix with marimastat (BB-2516) impairs fibroblast-mediated collagen contraction [162] and batimastat (BB-94) totally blocked TGF-β-promoted migration of keratinocytes *in vitro* [163]. Moreover, systemic administration of galardin (GM6001, ilomastat) to murine skin wounds attenuates wound contraction and interferes with migration of keratinocytes [84, 164]. However, the specificity of these small-molecule inhibitors is not restricted to MMPs but e.g. batimastat and galardin can also inhibit meprin metalloproteinases, capable of cleaving basement membrane proteins, and ADAMs, especially sheddases, among of some other proteases [165, 166].

ADAMs and ADAMTS proteases

The specific roles of ADAMs and ADAMTS proteases in wound healing are not yet well characterized. Their extensive expression analysis in a healing cutaneous wound remains to be done and the data from skin wound healing studies using ADAM or ADAMTS transgenic animals is currently limited.

ADAM-9, -10 and -17 are expressed by keratinocytes in intact human epidermis, and ADAM-9 also by fibroblasts *in vitro* [167, 168]. The sheddase activity of these epidermal ADAMs may play an important role in keratinocyte biology. HB-EGF and other EGF receptor ligands are key regulators of keratinocyte proliferation and migration, as well as of granulation tissue formation [11, 169–171]. The ectodomain shedding of HB-EGF is particularly needed for keratinocyte migration in skin wound [172]. ADAMs 9, 10, and 17 are potent sheddases/activators of EGF receptor ligands [59, 60, 173]. In addition, ADAM-10 plays a significant role in regulating keratinocyte adhesion, migration and proliferation *in vitro* by shedding intercellular E-cadherin. The cleavage of E-cadherin leads to receding of cell-cell contacts, β-catenin translocation and subsequent signaling, and enhanced proliferation [174]. The ability of ADAM-10 and ADAM-17 to shed, e.g. CD44, may also regulate keratinocyte migration and proliferation during wound healing [175]. Additionally, shedding of hemidesmosome-associated collagen XVII from keratinocytes by ADAMs 9, 10, and 17 is implicated in reduced motility of the cells due to the inhibitory effect of the shed collagen XVII ectodomain on migration [167]. Moreover, ADAM-9 has been shown to bind α3β1-integrin on HaCaT cells and induce the expression of MMP-9 and subsequent cell migration in a manner that involves activation of ERK-mitogen activated protein kinase [168]. These observations provide evidence that ADAMs modulate cell migration during wound repair.

ADAM 10 and 17 may also play a role in wound repair via regulation of inflammation. They have been shown to shed endothelial cell transmembrane chemokines, which, in addition to generating chemoattractive soluble chemokines, promotes detachment of mononuclear leukocytes bound via chemokine receptor to endothelium, this way regulating leukocyte trafficking [176, 177]. Finally, ADAM-15 is implicated in angiogenesis by several findings. For instance, its expression is up-regulated in endothelial cells during angiogenesis, and challenging of ADAM-15-deficient mice show reduced angiogenic response to changed concentration of air oxygen [178, 179]. However, disintegrin domain of ADAM-15 is shown to inhibit tumor angiogenesis [180]. Thus, although the mechanisms of ADAM-15 function in the regulation of angiogenesis remain largely unclear, it may be that different activities of ADAM-15 play balancing roles in the process. Finally, it has been reported that endothelial ADAM-15 can interact with platelet αIIbβ3-integrin and mediate platelet activation and aggregation, suggesting a role for ADAM-15 also in hemostasis during wound healing [181].

Among ADAMTS proteases, only a role for ADAMTS-1 has been reported specifically in cutaneous wound healing. ADAMTS-1 is constitutively expressed in the epidermal layer of murine skin, and mice deficient in *adamts1* gene have abnormal epidermal differentiation. During wound healing in mouse skin, the expression of ADAMTS-1 is enhanced in the basal keratinocytes after closure of the

wound, and the expression pattern appears to follow the differentiation level of keratinocytes. In HaCaT cells, the expression of ADAMTS-1 is up-regulated by TGF-β1 and TGF-β3. In the dermal compartment of cutaneous wound, ADAMTS-1 is up-regulated first in the macrophages, and by day 5, the expression is shifted to fibroblasts in the granulation tissue. *In vitro* migration studies have demonstrated that proteolytically active ADAMTS-1 significantly increases migration of fibroblasts at certain concentration but high doses of ADAMTS-1 inhibit migration via binding to bFGF [182]. ADAMTS-1 has been implicated in the inhibition of angiogenesis [183], and ADAMTS-1 knockout mice display delayed wound healing with elevated angiogenic response [184]. Inhibition of blood vessel formation attributed to ADAMTS-1 involves proteolytic release of angiostatic polypeptides from thrombospondin-1 and -2, and sequestration of angiogenic VEGF and bFGF [72, 182, 184]. Finally, plasma-derived ADAMTS-13 regulates platelet aggregation by cleaving of von Willebrand factor [185], thus it is likely to participate in hemostasis as well as in wound healing.

Proteolysis in aberrant cutaneous wound repair

Chronic skin wounds

Healing of a skin wound is an extremely dynamic process where the protective barrier function of skin is reconstituted in a timely and efficient manner. However, for example, increasing age, diabetic vasculopathy, venous insufficiency and arteriosclerosis increase the risk of getting chronic ulcers. Chronic wounds with delayed, insufficient or missing wound closure are characterized by pathologic inflammation, fibroblast senescence and uncontrolled proteolysis [186].

Although MMPs are important players in the wound healing process, the regulation of MMP expression and activity and the equilibrium between proteases and their inhibitors are pivotal during wound repair. Excess proteolysis may lead to destruction of wound ECM, disturbance of cell migration, or degradation of momentous growth factors and their receptors. A number of studies show, that in the non-healing chronic ulcer, the activity of MMPs is upregulated while the expression of TIMPs is decreased compared to acute wounds. MMP-2, -9, -1 and -8 levels are elevated in wound fluid of chronic ulcers [94, 187–189]. Moreover, increased activity of MMP-2 and -9 has been reported [187, 190], and similar expression pattern of gelatinases has been described in chronic wounds of diabetic patients [191]. One factor explaining the increased MMP activity and the altered distribution of MMPs in chronic wounds is, for instance, a dramatic infiltration of inflammatory cells to the chronic wound [94, 95]. Non-healing ulcers also possess less TIMP-1 and TIMP-2 as compared to normally healing wounds suppressing the inhibition of MMP activity, but also, in the case of TIMP-2, may alter activation of MMP-2 [94, 98, 190]. In contrast to fibroblasts in acute wounds, fibroblasts from chronic ulcers express TIMP-4 [98]. Despite general up-regulation of MMP activity in chronic wounds, stromal fibroblasts from chronic leg ulcers have been reported to express lower levels of active MMP-2 and proMMP-1, and more TIMP-1 and -2 compared to fibroblasts from an acute wound, when cultured inside three-dimensional collagen gel [192]. Thus, it must be noted that the total MMP levels detected in wound fluids reflect the sum of MMPs expressed by variety of different cell types. A distinct feature of chronic ulcers is the expression of MMP-13 by wound fibroblasts embedded in collagenous stroma [106]. This may provide a survival mechanism for the fibroblasts and contribute to the remodeling of the ECM of a chronic ulcer [110]. Interestingly, compounds derived from a common infectious microbe in chronic leg ulcers, *Staphylococcus aureus*, appear to up-regulate the expression of multiple MMPs, namely MMP-1, -2, -3, -7, -10, -11, and -13, as well as TIMP-1 and TIMP-2 by normal dermal fibroblasts [193]. In contrast to human chronic wounds, genetically diabetic mice, a well established model for impaired wound repair, show lower levels of MMP-2 and MMP-9 in wound tissue extracts and also increased expression of ADAMTS-1 mRNA compared to control animals during early wound healing (up to day 7 and day 1, respectively) [182, 194]. While immunoreactivity of MMP-26 starts to decline in acute skin wounds one day after injury, in chronic wounds with different etiologies, MMP-26 is consistently detected in stroma beneath the basement membrane at the ulcer margin where it may participate in the activation of MMP-9 and release of IGF from IGFBP-1 [156].

Keratinocytes at the edge of a chronic ulcer express uPA comparable with keratinocytes at the leading edge of acute wound epidermis [77]. In contrast, abundant tPA mRNA and protein was found in the basal and suprabasal keratinocytes at the margin of chronic venous leg ulcers while tPA in acute wound epidermis was weakly expressed [195]. In addition, higher expression level of tPA in the fibroblast- and macrophage-like cells of acute wound granulation tissue compared to chronic ulcer stroma was also reported. That is, both stromal and epidermal compartments differ in acute and chronic wounds in their fibrinolytic capacity with respect to plasminogen

activation, which in turn may affect activation of MMPs.

Fibrotic cutaneous wounds

As chronic wounds display delayed or missing closure, fibrotic wounds, characterized by massive formation of collagenous scar tissue, can be considered to heal too effectively. Excessive scarring may occur e.g. in healing of burn wounds or skin grafts and as a result, skin loses elasticity and in extremities, this may lead to contractures and functional disability. Hypertrophic scars and keloids are two types of local fibrosis that may exist in skin after defective wound maturation. Keloids are fibrous, reddish and firm nodules that grow beyond the borders of the original wound. Susceptibility of developing keloids appears to often have genetic background [196]. Hypertrophic scars are more common, raised scars, smaller than keloids, and they typically restrict to the boundaries of the initial injury. They may, however, develop severe contractures affecting tissue functionality.

Large area, depth, delayed closure and tension attributable to motion or loss of tissue, are common risks for scar formation in a skin wound. However, the molecular events behind the shift from normal healing to healing that leads to excessive scarring are still largely unknown. Up-regulation of TGF-β1 detected in hypertrophic scars and keloid fibroblasts, and TGF-β2 in keloid fibroblasts, is implicated in fibrosis [197, 198]. This probably contributes to elevated collagen synthesis detected in keloid and hypertrophic scar fibroblasts [198–200]. In hypertrophic scars, TGF-β may also regulate the existence of myofibroblasts that participate in the formation of scar contracture [201]. In addition to increased ECM synthesis in fibrotic scars, hypertrophic scar fibroblasts show reduced levels of MMP-1 mRNA [202]. In contrast, up-regulation of MMP-1, as well as MMP-2 and TIMP-1 proteins has been reported for keloid fibroblasts [199]. Tissue extracts of both of these abnormal scars show markedly increased levels of MMP-2 activity compared to normal skin samples, and very low level of MMP-9 [203]. Moreover, the expression level of MMP-13 seems also to be elevated in keloid tissues, and certain treatments inducing keloid regression are reported to further up-regulate its expression [204]. Thus, the expression of MMP-13 by keloid fibroblasts may reflect their attempt to remove the excess collagen in tissue. In hypertrophic scar fibroblasts, the down-regulation of MMP-1 is suggested to be due to IGF-1 [202], although it is also possible that fibrotic scar collagen receptor integrins dysfunction during collagen ligation, resulting in abnormal regulation of collagenases and collagen synthesis [205]. Furthermore, a marked increase of PAI-1 with a concomitant decrease of uPA expression levels is observed in keloid fibroblasts. This abolishes the ability of keloid fibroblasts to degrade fibrin but may also reduce activation of other proteinases such as MMPs [206].

Scarless wound healing

Numerous observations have revealed that unlike post-natal human skin wounds, fetal skin wounds (< 24 weeks gestation) as well as adult oral mucosal wounds heal rapidly and without or only with minimal scarring. There are number of external and internal factors that differ between adult and fetal skin or adult oral mucosal wounds. The latter two heal in moist environment rich in various soluble factors. The fetus is located in sterile environment and practically lacks the inflammatory response during epidermal wound healing while oral wounds face the oral microbe flora [207, 208]. Fetal skin fibroblasts also differ from adult skin fibroblasts by exhibiting faster migration rate, more dynamic production of ECM and in differentiation to myofibroblasts during wound healing. In addition, fetal and postnatal wounds differ with respect to composition of the ECM [207]. Both fetal skin and adult gingival fibroblasts can remodel three-dimensional collagen matrices more efficiently than adult skin fibroblasts [209]. A high expression ratio of TGF-β3/TGF-β1 in fetal skin wounds at early gestation time is associated with scarless repair and the relative presence of TGF-β isoforms, receptors and modulators of activity are suggested to contribute to scar formation [207]. Accordingly, exogenously added TGF-β3 and devitalizing TGF-β1 and-2 reduce scarring in a rat model [210].

The ability to process and remodel ECM during wound healing may also contribute to scarless healing. Several studies indicate up-regulation of MMPs and TIMPs in intact fetal skin as a function of gestation time from scarless healing to healing with scar formation. For instance, in human fetal skin, the levels of MMP-2, -9, -14, TIMP-1 and TIMP-2 increase during gestation time [211]. In rat fetal skin, the levels of interstitial collagenase, MMP-2, -3, -9, -14 and TIMP-2 were shown to increase in a similar manner. The level of TIMP-3 appeared to decrease during gestation time [212, 213]. However, in response to wounding, rat fetal skin at early gestation time shows stronger up-regulation of interstitial collagenase, MMP-9 and MMP-14, and MMP-levels relative to TIMP-levels are higher compared to wounds at later gestation time [213]. In wounded human fetal skin (gestational age 16–20 weeks) grafted onto SCID-mice, fibroblasts express wide spectrum collagenolytic ECM proteinase MMP-13 [108] that also can proteolytically activate

TGF-β3 [214]. MMP-13 is also expressed by gingival wound fibroblasts while it is absent in acute skin wounds in adults [106, 215]. Both gingival and fetal skin fibroblasts also express MMP-13 in response to TGF-β treatment *in vitro* [108, 215]. Finally, when compared to adult skin fibroblasts, oral fibroblasts exhibit markedly elevated activation of MMP-2 inside three-dimensional collagen while the levels of TIMP-1 and -2 were significantly lower [216]. Expanding proteolytic profile of fetal skin, the level of uPA has been shown to be higher in mouse skin at early gestation compared to later stage, while the expression of PAI-1 is contradictory regulated [217]. Therefore, it is likely, that different proteolytic expression profile may contribute to scarless wound repair in fetal skin and oral mucosa.

Prospects for cutaneous wound healing therapy by targeting proteolysis

Proteolysis is clearly required in all phases of physiological wound repair. However, uncontrolled proteolysis may attenuate wound closure. Even after extensive research, it is not quite clear why certain MMPs, e.g. gelatinases, show increased expression levels or activity in chronic wounds, whereas in some cases, as for MMP-13 expression by chronic wound fibroblasts, it remains unclear whether MMP production is contributing to poor healing or is actually induced because of the altered stimuli in the wound trying to promote tissue healing. However, the general view concerning the chronic wounds appears to be that controlled inhibition of enhanced MMP activity would stimulate healing.

One explanation for high MMP levels in chronic ulcers is the increased infiltration of leukocytes, which secrete high amounts of neutrophil elastase, MMP-8 and gelatinase [94]. Increased activity could be due to the higher number of zymogen activators present in a chronic wound. While compounds from bacteria colonizing the wound are also reported to enhance expression levels of several MMPs in fibroblasts, it is important to aim at reduction of inflammation and fulminant bacterial infection during the care of chronic wounds [193]. Studies with protease absorbing matrices with the aim of reducing excessive proteolytic activity in chronic venous leg ulcers and diabetic ulcers have shown somewhat promising results, especially when these have been used in combination with autologous growth factors [218–220]. Furthermore, an approach to neutralize the detrimental effect of high protease levels in chronic wounds has been taken in a pilot study by topical administration of doxycyline, an antibiotic with MMP and TACE inhibitor properties, on diabetic foot ulcers [221].

Some of the alterations in MMP expression of chronic wounds, such as the expression of MMP-13 by fibroblasts [106], the consistent expression of MMP-26 by stromal cells [156] and the expression of tPA in epidermis [195], are not explained by the changes in cell number but rather by changed levels of regulatory stimuli in the wound bed or altered regulation of the gene due to the altered response for growth factors and perhaps reduced signaling by one growth factor resulting in pronounced signaling by another. Understanding the growth factor regulation of protease expression and the various related signaling pathways gives us possibilities to modify protease production and provides a window for therapeutics to chronic ulcer treatment [41]. Finally, targeting specific protease inhibition would be of great importance since MMP and other protease activity in general is clearly beneficial for wound closure. In this respect, it is pivotal to understand the roles of individual proteases in normal and aberrant wound healing and further define the mechanisms of the diseases.

Excessive scarring may be a major problem not only for esthetic reasons but in severe cases it can also interfere with mobility of extremities and functionality of skin. There has been remarkable progression in understanding the mechanisms behind scarring. The role of TGF-β family in fibrosis of skin and other organs is well established [222]. In scars, such as keloids and hypertrophic scars, hindering redundant collagen deposition is of great interest as well as reduction of consistent contraction in the latter. In addition, enhancing collagen degradation and remodeling is a tempting hypothesis for reducing scar formation. Learning from scarless healing may give us hints to reduce scarring. Interestingly, athymic nude mice show scarless healing of skin wounds and high levels of MMP-9 21 days after injury suggesting potential anti-scarring effect for MMP-9 [223]. Moreover, MMP-13 is expressed by human fetal wound fibroblasts and may be involved in collagen remodeling and activation of TGF-β3 [108, 110, 214]. Using gene transfer tools in an appropriate model, it would be tempting to introduce a protease gene under specific regulation to the resident cells of the wound that is developing to e.g. fibrotic scar, and explore the potentials for gene therapy. Finally, modern molecular techniques such as siRNAs, cell type specific expression vectors or biomaterials as a vehicle of certain substances provide tools for more systematic studies concerning the roles of MMPs, plasmin and PAs, and the newcomers in the field, ADAMs and ADAMTS proteases, which are needed for generating novel therapeutics for aberrant wound healing.

Concluding remarks

While physiological wound healing in skin efficiently restores normal tissue function, defective wound repair, as exemplified by chronic ulcers and fibrotic scars, impair the quality of life of millions of people and burden the healthcare systems globally. Alterations in proteolytic profile of the wound tissue are associated with both of these conditions and controlling the proteolytic activity could offer new therapeutic options for treatment of abnormally healing wounds. In spite of the general interest for inhibiting excessive MMP activity in chronic wounds, there are surprisingly little data available on therapeutic feasibility of MMP inhibitors for these conditions. Moreover, data on controlled expression of certain proteases in fibrotic wounds would be of great interest. At present, there are a number of different types of vehicles available for delivery of small molecule inhibitors or proteins to the tissue. Accordingly, while our understanding of the specific roles of individual MMPs and other proteases in wound healing is increasing, the possibilities of achieving targeted inhibition or expression of specific genes have become available. Finally, although associated with many wound healing-related events, very little is currently known about specific roles of ADAMs and ADAMTS proteases in wound repair. It is expected, that elucidating their roles in normal and aberrant wounds will reveal novel pathogenic mechanisms and provide new possibilities for developing targeted therapy for chronic wounds or dermal fibrosis.

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