Matrix metalloproteinases during wound healing – a double edged sword

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ABSTRACT

The extracellular matrix (ECM) provides a framework for cells and gives skin its tensile strength and elasticity. Loss of its integrity necessitates the clearing of damaged components and the deposition of firstly a provisional matrix and later remodelling of the ECM to support a functionally intact tissue. Matrix metalloproteinases (MMPs) are an important family of enzymes that function in the breakdown of the ECM and modulate the function of many biologically active molecules housed in the ECM. Through their enzymatic actions MMPs play a role in fundamental processes such as immune cell infiltration and ECM remodelling during wound repair. Their tight control is necessary for timely wound healing and excessive MMP activity participates in the development and persistence of chronic wounds, while reduced activity contributes to fibrosis. A number of inhibitors have been designed to target this activity and improve wound healing with limited success. Novel strategies are currently being investigated to improve wound healing by targeting MMP modulating molecules.

THE EXTRACELLULAR MATRIX

Within the skin the extracellular matrix (ECM) performs a number of strategic functions. These include the housing of growth factors and cytokines, the regulation of cell adhesion, chemotaxis and cell migration, and it acting as a scaffold, providing tensile strength for the cellular components and elasticity for the whole tissue[1,2]. It consists of both proteoglycans and fibrous proteins, such as collagens, laminins, elastins and fibronectin secreted by cells in the local environment and organised into a distinct meshwork. Early in the wound repair process a provisional matrix, consisting of fibrin, fibronectin, collagen and other ECM proteins, is laid down to provide a framework for cells to migrate into the site of injury[3]. This matrix also acts to stimulate cell proliferation and differentiation and to promote the synthesis of a new ECM[1,2]. During the repair process the degradation of the ECM and the release of its reservoir of housed growth factors and components mediating inflammation is tightly controlled to restore the structure and function of the injured tissue[2]. Degradation facilitates the breakdown of the provisional matrix and the remodelling of new granulation tissue, and promotes cell migration, re-epithelialisation, and angiogenesis. Its deposition within the wound is essential for timely healing and disturbances in its degradation, the laying down of new ECM and its remodelling are all thought to contribute to poor wound healing outcomes through uncontrolled proteolysis as seen in chronic wounds, or to fibrosis through reduced degradation of ECM components[3,4].

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MATRIX METALLOPROTEINASE STRUCTURE AND THEIR ACTIVATION

Matrix metalloproteinases (MMPs) are members of a larger family of enzymes that all require a zinc ion (the 'metallo' part of their name) in their active site for their catalytic activity to occur. They are produced as inactive precursor proteins, known as zymogens, which require cleavage to form an active enzyme[5]. Historically, MMP family members were categorised by their extracellular substrate preferences and separated into categories such as the collagenases and gelatinases (Table 1). However, these enzymes often have multiple ECM and non-ECM substrates, for example, collagenase I substrates include collagen I-VI, VII and X, fibronectin, tumour necrosis factor and insulin-like growth factor binding proteins (Table 1). This has led to their more recent reclassification into one of eight classes according to their domain organisation (Figure 1)[6].

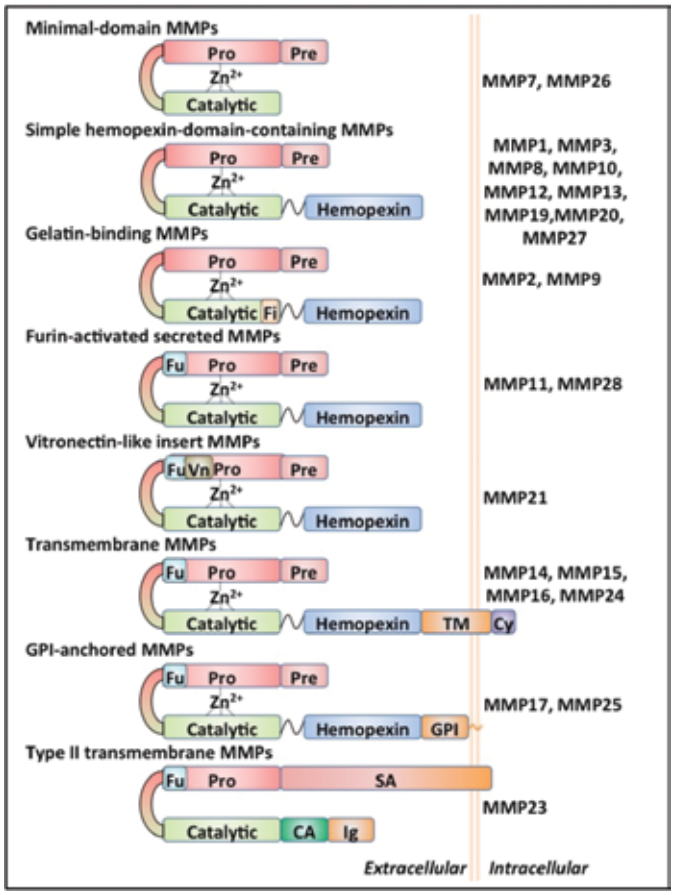

Figure 1: Classification of MMPs according to their domain structure. Pro, pro-domain; Pre, N-terminal signal sequence, Zn2+, zinc; Fi, a gelatine binding domain that resembles collagen-binding repeats in fibronectin; Fu, furin; TM, transmembrane domain; Vn, vitronectin-like; SA, signal anchor; GPI, glycosylphophatidylinositol anchoring sequence; CA, cysteine array; Ig, immunoglobulin.

The 23 human MMPs share a number of common features (Figure 1) including a pro-domain of about 80 amino acids containing a cysteine consensus sequence (PRCXXPD), which encompasses a free thiol group[6]. The pro-domain folds, via a linker region of variable length, over the catalytic domain and is bonded to the zinc in the active site via its cysteine residue (Figure 2). Through this coupling the pro-domain inhibits enzymatic activity by blocking the entrance of a catalytically important water molecule into the active site (Figure 2). To activate the catalytic activity this interaction needs to be disrupted by a mechanism known as the cysteine switch (Figure 2). This occurs either through (i) the cleavage of the N-terminal pro-peptide blocking the active zinc by serine proteinases or other MMPs (*in vivo*), or (ii) via a conformational change of the protein, which is performed by chaotropic agents such as SDS or after chemical modification of the cysteine in the pro-domain by reactive oxygen species (*in vitro*)[5-8]. Typically activation of the enzyme occurs by cleavage of this pro-peptide after its delivery to the cell surface and in some cases after its secretion. MMPs, like the MT-MMPs, which have a furin protease cleavage site, can also be activated within the cell[9].

The MMP catalytic domain contains three conserved histidines (HEXGHXXGXXH) that further coordinate the zinc ion (Figure 2). Substrate specificity is then dictated by minor differences in the catalytic domain and further coordinated by a third region, known as the hemopexin-like domain, which is approximately 200 amino acids in length and is present in all MMPs except for MMP7, MMP23 and MMP26 (Figure 1)[7]. Additionally, MMP23 also has an immunoglobulin-like domain after the catalytic site and a unique cysteine-rich domain, while MMP2 and MMP9 have three repeats of a fibronectin type II motif in their metalloproteinase domain (Figure 1).

With the exception of MMP23, all MMPs have an N-terminal signal peptide whose role is to target the protein to the endoplasmic reticulum (ER) and Golgi complex for transport to the cell surface to be either incorporated into the plasma membrane or to be secreted depending on the MMP (Figure 1). Those MMPs (MT-MMPs) that are not secreted but anchored in the plasma membrane do so either because they have a transmembrane domain, a glycosylphosphatidylinositol (GPI) domain or a signal anchor in their sequence (Figure 1)[10].

Table 1: Classification of MMPs according to their substrate specificity. Agg, aggrecan; Dec, decorin; EL, elastin; Fib, fibrillin; FBN, fibrin; FN, fibronectin; Gel, gelatin; IGFBPs, insulin-like growth factor binding protein; LN, laminin; PG, proteoglycan-linked protein; TGF, transforming growth factor; TNFα, tumor necrosis factor alpha; VN, nectin.

Class	Common name(s)	MMP#	ECM substrates	Non-ECM substrates
Collagenases	Collagenase 1, Interstitial, Fibroblast, Tissue C	MMP1	Col I, II, III, VI, VII, X, Agg, FN, Gel, LN, PG, VN	IGFBPs, CXCL12, pro-TNFα
	Collagenase 2, Neutrophil C	MMP8	Col I, II, III, V, VII, VIII, Agg, EL, FN, Gel, LN	CXCL5
	Collagenase 3	MMP13	Col I, II, III, IV, VII, IX, X, XIV, Agg, FN, Gel, Fib	CXCL12
Gelatinases	Gelatinase A, 72-kDa gelatinase	MMP2	Col I, III, IV, V, VII, X, XI, XIV, Agg, EL, FN, Gel, LN, PG, VN, Dec, Fib,	CCL7, CXCL12, pro-TNFα, IGFBPs, pro-TGFβ, pro-IL-1β
	Gelatinase B, 92-kDa gelatinase	MMP9	Col IV, V, VII, X, XIV, Agg, EL, Gel, LN, PG, VN, Dec, Fib, FBN,	pro-TNFα, pro-IL-1β, IL-8, IL-2Rα, CXCL7, CXCL8, CXCL1, CXCL12
Stromelysins	Stromelysin 1, Transin 1	MMP3	Col III, IV, IX, X, XI, Agg, EL, FN, Gel, LN, PG, VN, Dec, Fib	CXCL12, E-cadherin, pro-TGFβ1, pro-TNFα, IGFBPs, pro-IL-1β
	Stromelysin 2, Transin 2	MMP10	Col III, IV, V, IX, X, Agg, EL, FN, Gel, LN, PG	
	Stromelysin 3	MMP11		IGFBPs
Matrilysins	Matrylisin, Matrin, PUMP-1	MMP7	Col IV, X, Agg, EL, FN, Gel, LN, PG, VN, Dec, Fib	pro-TNFα, E-cadherin, β4-Integrin
	Matrylisin-2, Endometase	MMP26	Col IV, FN, Gel	
Membrane-type (MT)	MT1-MMP	MMP14	Col I, II, III, Agg, FN, gel	CXCL12, CD44, pro-TNFα/TGFβ
	MT2-MMP	MMP15	Agg, FN, Gel, LN, FBN	
	MT3-MMP	MMP16	Col III, FN, FBN, VN, LN, Gel	
	MT4-MMP	MMP17	Fib, FBN	Pro-TNFα
	MT5-MMP	MMP24	Gel	
	MT6-MMP, Leukolysin	MMP25	Col IV, Gel, FN	
Other	Enamelysin	MMP20	Agg	
	Epilysin	MMP28		
	Metalloelastase	MMP12	Col I, IV, Agg, EL, FN, Gel, LN, PG, LN, Fib	Latent TNF
	RASI 1	MMP19	Col IV, I, FN, Gel, LN	
	Xenopus MMP	MMP21		
	MMP23b/CA-MMP	MMP23	Gel	
		MMP27		

MMPS DEGRADE ECM AND NON-ECM COMPONENTS

Once activated, MMPs can degrade a range of ECM components such as collagen and fibronectin as well as specific non-ECM proteins housed within its matrix such as insulin growth factor binding protein (Table 1 and Figure 1)[6]. Given the diversity of their substrates, MMPs can influence a number of cellular processes. Firstly, MMPs degrade damaged collagen and other broken ECM components from sites of injury. Damaged collagen is unable to form proper fibrils with newly synthesised collagen, which would result in a disorganised and weakened ECM. Secondly, degradation of the ECM reduces the physical barriers that impede the migration of immune cells into the injured tissue. By altering the ECM microenvironment, MMPs also control behaviour such as cell adhesion and migration, which alter depending on ratios of individual ECM components. Within the matrix, MMPs can alter the activity of specific non-matrix molecules such as cytokines, chemokines and surface receptors. This occurs through a number of mechanisms including degradation to completely remove these mediators, proteolytic release them from the ECM, cleavage to release/activate the biologically active part of proteins, or by altering the activity of inhibitors[9]. Once active, MMPs can also alter the catalytic activity of other MMPs not only by cleavage of their pro-domains but also by degradation of inhibitors or other proteases[8].

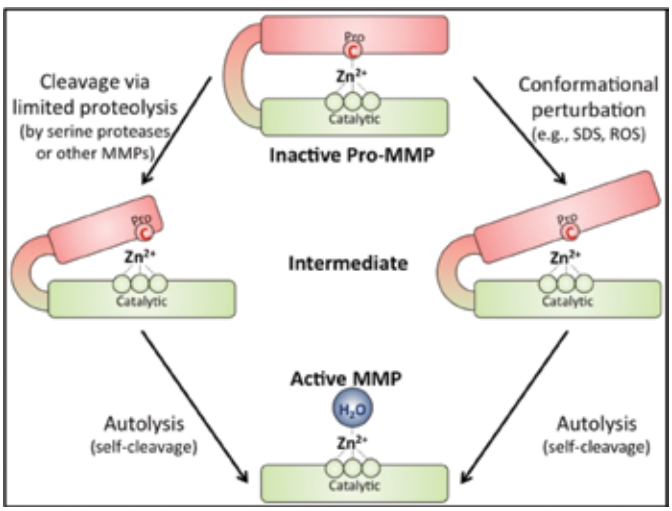

Figure 2: Mechanisms of pro-MMP activation. MMPs are held in the inactive state through the conserved cysteine in the pro-domain interacting with the catalytic cleft bound zinc ion, thus preventing substrates binding to the catalytic site. Limited proteolysis or conformational perturbation of the MMP structure releases the cysteine-zinc interaction (cysteine switch). Finally, autolysis of the pro-domain irreversibly frees the catalytic site for interaction with substrates or inhibitors.

EXPRESSION AND REGULATION OF MMPS

Due to their role in degrading both ECM and some non-ECM components, it is crucial to control MMP protease activity in order to avoid unwanted and excess tissue damage[7]. Accordingly, the catalytic activity of MMPs is regulated at numerous levels including gene expression, localisation, zymogen activation and by their specific endogenous inhibitors, which tend to keep the MMPs inactive in the ECM. The MMPs are typically expressed at low levels in the skin but the expression of specific MMPs, such as MMP9, is upregulated during wound healing and high levels are often found in chronic wound fluid (Table 2 and discussed later). Within wounds their gene transcription can be induced in keratinocytes, fibroblasts, endothelial cells and inflammatory cells by cytokines, hormones, and cell contact with the ECM or other cells. Furthermore, their expression can be regulated by epigenetic modifications or mRNA (de)stabilisation[6].

The location of an MMP also regulates its activity. MMPs are typically active either at the cell surface as membrane-anchored proteins with the catalytic site facing the extracellular milieu or secreted into the extracellular milieu where its substrates are located. Newly synthesised MMPs must be recruited to the cell surface for them to be activated and also secreted. This serves as an additional level of regulation

and the process is highly controlled, requiring distinct intracellular trafficking molecules, such as the SNAREs and Rabs, for delivery to the cell surface. Moreover, the surface localisation is often polarised with MT-MMPs being concentrated on membrane protrusions at the leading edge of cells, which are thought to be important for migration and invasion. For example, MT1-MMP, MMP2 and MMP9 are often found located on invadopodia, which are actin-rich protrusions in the cell membrane that are necessary for the migratory/invasive nature of cells[11]. In addition to the membrane-anchored MMPs many secreted MMPs are often localised to the cell surface after secretion where they bind to surface receptors such as the adhesion receptors or cell surface proteoglycans. This too serves to maintain a high local concentration of enzyme close to the cell, targeting their catalytic activity to areas in close contact with the cell surface. Examples include the binding of MMP1 and MMP2 to the adhesion receptors α2β1 integrin and αVβ3 integrin respectively, the binding of MMP9 with the adhesion receptor CD44 and binding of MMP7 to the tetraspanin CD151 and to surface proteoglycans.

A number of endogenous MMP-specific inhibitors exist to regulate their function and are known as the tissue inhibitors of metalloproteinases (TIMPs). There are four known TIMPs, designated TIMP1, TIMP2, TIMP3 and TIMP4. These inhibitors are relatively small at 21-28 kDa and are cysteine-rich proteins that bind MMPs reversibly in a 1:1 ratio[7,9]. Non-specific endogenous inhibitors also exists such as plasma proteinase inhibitors like α2-macroglobulin that act by entrapping the proteinase within the macroglobulin, which is then mopped up by receptor-mediated endocytosis. For ECM remodelling there needs to be a precise balance between MMPs and their TIMPs to allow ECM remodelling to occur without causing excessive degradation.

MMPS IN ACUTE AND CHRONIC WOUNDS

During the wound healing process MMPs play numerous important roles at multiple stages of tissue repair including the inflammatory, proliferative and remodelling phases. Throughout the inflammatory phase immune cells, principally neutrophils and macrophages, secrete large amounts of MMPs to degrade damaged ECM components. This not only helps to clean the wound of dead and damaged tissue, but also ensures the proper interaction of newly synthesised collagen with undamaged ECM at the wound edge. Secretion of MMPs from these immune cells also supports their own migration through uninjured tissue into the wound. Throughout the proliferative phase, MMPs regulate many essential processes such as the release of biologically active proteins that control cell proliferation. Their activity also facilitates the migration and adhesion of fibroblasts, endothelial cells and keratinocytes into the site of injury to form granulation tissue and re-epithelialise the wound. The degradation of capillary basement membrane by MMPs also facilitates the restoration of the vascular network. Later in the remodelling phase scar contraction by myofibroblasts and the remodelling of the initial scar tissue is also dependent on ECM cleavage through MMPs[12,13].

Chronic wounds are thought to be stuck in the inflammatory phase of repair and they have a high immune cell burden, particularly neutrophils and macrophages[14,15]. These immune cells release huge levels of MMPs as well as other inflammatory mediators. This results in uncontrolled matrix degradation, depletion of growth factors and activation of cytokines such as TNF, therefore prolonging inflammation and impairing epithelisation and healing. This leads to a vicious cycle of recruitment of immune cells and increased ECM degradation, which further fuels inflammation and tissue destruction. Studies have identified distinct MMPs and TIMP profiles in wound fluid from different types of chronic wounds, with elevated levels of specific MMPs and a reduction in some TIMPs (Table 2)[16-20].

Table 2: Alterations in MMP and TIMP levels in venous leg ulcers, diabetic foot ulcers, pressure injuries and mixed ulcers.

	Elevated levels	Reduced levels	Reference
Venous Leg ulcers	MMP1, MMP2, MMP3, MMP8, MMP9, MMP12, MMP13	TIMP1, TIMP2	16, 17
Diabetic foot ulcers	MMP1, MMP2, MMP8, MMP9	TIMP2	18
Pressure injuries	MMP9	TIMP1	19
Ulcers (mixed group)	MMP8, MMP26		20

To date it appears that neutrophil and macrophage-derived MMP9 is one of the prime proteases accountable for ECM degradation in non-healing wounds and its levels correlate with severity of the ulcer[21,22]. MMP9's ability to degrade collagen more effectively than any other MMP might be why high levels of MMP9 impact so highly on healing outcomes[23]. Furthermore, cleavage of fibronectin, another MMP9 substrate, changes the biological properties of this ECM component and it can, therefore, affect MMP activity, cell migration and proliferation in such a way as to increase infiltration of inflammatory cells, prolonging inflammation[24,25]. The ratio of MMP9 and TIMP1 in the wound fluid is believed to be a good predictor of healing outcomes, with their ratio lowering as pressure injuries heal[19]. The significance of high MMP9 levels in wounds is highlighted by data from a murine wound model that showed the application of high levels of recombinant human MMP9 to acute wounds led to decreased type-IV collagen in the basement membrane, decreased epithelial migration and delayed wound healing[26]. Tissue from healed areas of burn wounds and healing leg ulcers show reduced MMP9 expression and activity after its temporal elevation[27]. Thus, although the transient and local expression of MMP9 in acute wounds is necessary to promote the healing process, high levels are detrimental

Table 3: Properties of healing and chronic wounds (adapted from Stechmiller et al., 2010).

Healing wounds	Chronic wounds
High mitogenic activity	Low mitogenic activity
Low level of cytokines	High level of cytokines
Low level of proteases	High level of proteases
High level of growth factors	Low level of growth factors
Competent fibroblasts	Senescent cells
Functional angiogenesis	Suppression of angiogenesis

to the healing process[26]. The role of MMP2 in chronic wounds is more controversial in that high levels have been found in venous leg ulcers and diabetic foot ulcers[16], whereas other studies in pressure injuries found low levels of MMP2[19]. It has been suggested that low levels of MMP2 are due to the reduced number of MMP2 secreting fibroblasts in chronic wounds[28].

CURRENT AND FUTURE THERAPIES TO ALTER MMP LEVELS IN CHRONIC WOUNDS

A number of different wound healing strategies that reduce MMP in wounds have been employed. Studies have shown that the use of compression bandages for venous leg ulcers can lead to a reduction in certain MMPs, namely MMP3, MMP8 and MMP9[16,29]. Pro-MMP9 levels have also been found to be lowered in chronic wounds treated with topical negative pressure treatment for 10 days and the ratio of MMP9 to TIMP1 is reduced in wound fluid from these patients, suggesting it might improve healing outcomes[30]. In light of the overactivity of MMPs and their detrimental role in wounds, highly absorbent foam dressings have been used to soak up wound fluid[31,32]. This removes molecules such as the MMPs and also pro-inflammatory cytokines that can strongly up-regulate MMP9 expression. These absorbent dressing can also have molecules incorporated in them that are believed to have protease-modulating properties. For example, incorporation of polyphosphate in highly absorbent foam has been shown to reduce the activity of MMP2 and MMP9 *in vitro*[33]. Studies have also suggested that absorbent dressings containing oxidised regenerated collagen might improve MMP9/TIMP ratios and thus healing in diabetic foot ulcers[32].

Being able to target one, a combination of or all of the MMPs thought to play a role in inhibiting the healing of chronic wounds (Table 2) would be the ideal, yet many of the inhibitors produced to date are not selective. The development of selective MMP inhibitors has been proven to be a difficult approach as MMPs share very similar structures around and within their active sites[4,8]. Broad-spectrum MMP inhibitors, such as the small non-peptidic inhibitor GM6001 (Galardin), that commonly contain a chelating group binding the zinc ion in the catalytic domain have been developed. GM6001 has been shown to have some benefits in the healing of corneal ulcers, although in skin wounds it also negatively impacts on keratinocyte migration, myofibroblast formation and scar contraction[34-36]. One of the disadvantages of these types of non-selective MMP inhibitors is that they have poor bioavailability, necessitating frequent administration. They also target MMPs that are beneficial during the wound healing process, as well as those that are detrimental, leading to limited efficacy. Similarly, topically applied TIMPs have been used to inhibit MMP activity but again due to their broad inhibitory nature they have not proven to be very successful[37]. Thus, broad spectrum MMP inhibitors are not suitable as a longer term therapeutic[4].

An exception to this is the tetracyclines, which have shown to be promising in the clinic in terms of improving chronic wound healing outcomes. When used at sub-antimicrobial doses, doxycycline has been found to inhibit collagenase activity through its ability to bind the zinc and calcium cations in the catalytic domain of the MMPs[13,41-

[44]. Doxycycline has shown some promising results in diabetic patients with foot ulcers receiving topical doxycycline and in patients with venous leg ulcers receiving oral doxycycline[45,46]. In both cases an improvement in healing rate was seen after treatment with low doses of doxycycline[45,46]. Studies in patients with periodontitis or corneal ulcers have confirmed this enhanced healing with low doses of doxycline[13,42,47]. Doxycycline has been approved for use in the US by the FDA in periodontitis but does not yet appear to be approved for this use in Australia[38].

More selective MMP inhibitors have been designed with two currently under evaluation or recently finished Phase II clinical trials[38,39]. AZD1236, a MMP9 and MMP12 inhibitor, has just undergone a phase II clinical trial for chronic obstructive pulmonary disease[39]. The treatment was well tolerated and whether its MMP inhibitor activity could be of benefit in terms of chronic wound treatment is yet to be tested[39]. A second Phase II clinical trial is currently under way in refractory hepatitis C patients with the MMP inhibitor CTS-1027 (N-hydroxy-4-([4-(4-chlorophenoxy) benzenesulfonyl] methyl)-2, 3, 5, 6-tetrahydropyran-4-carboxamide). This compound inhibits MPP2, MPP3, MMP8, MMP9, MMP12, MMP13 and MMP14 activity while having no effect on MMP1 (or MMP7), as inhibition of MMP1 activity has been associated with muscular and skeletal side effects[38]. The results of this study have yet to be published, although studies using a pre-clinical model of liver fibrogenesis have shown that CTS-1027 is of benefit in reducing fibrosis. Whether this would have the same effect on wounds or if it could be of benefit in chronic wound healing is unknown[40]. The aminopeptidase N/CD13 inhibitor actinonin has been found to inhibit MMP14-mediated cleavage of pro-MMP2 and activation but again no studies have been conducted in wound models[48]. New targeted approaches include function-blocking antibodies to MMPs, such as DX-2400, an MMP14 inhibitor that blocks the processing of MMP2 to its active form, which in animal studies reduces tumour progression[49]. Its effect on chronic wounds has not been tested.

The above studies show that broad-spectrum MMP inhibitors have been mostly ineffective so far as MMPs share very similar structures around and within their active sites and MMPs have other important physiological functions. While more specific inhibitors have been developed their impact on wound healing is not yet known. Being able to specifically regulate MMP activity on other levels might also lead to better therapeutic treatments, such as the targeting of mediators that alter MMP expression or targeting the secretory mechanisms.

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