

Molecular aspects of wound healing in diabetes

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Abstract

Previous research studies have clearly shown that failure of foot ulcer healing can eventually lead to amputation. It is therefore important that the diagnosis and treatment of ulcers is both timely and effective. Two major classes of regulators of extracellular matrix (ECM) – growth factors and the group of enzymes termed matrix metalloproteinases (MMPs) – and their effect on healing diabetic wounds are the focus of this study. A number of animal models of wound healing are also discussed, with the aim of developing strategies to improve the rate of tissue repair in diabetes.

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Introduction

The prevalence of diabetes is increasing worldwide and has been forecast to double in the next 20 years. This major increase in morbidity and mortality of diabetes is due to the development of both macro and micro-vascular complications, including failure of the wound healing process. For example, foot ulcers occur in 15% of all patients with diabetes and precede 84% of all lower leg amputations^{1,2}. Each year in Australia alone, approximately 3000 diabetic patients require amputation secondary to foot ulceration³.

The essential components of diabetic foot ulcer treatment are to reduce foot bearing pressure (in the case of neuropathic ulcers) and to increase blood supply (in vascular ulcers). Antibacterial therapy is also important⁴. Yet despite optimised

treatment, and for reasons not completely understood, some ulcers fail to heal. Previous research studies have clearly shown that failure of healing eventually leads to deep-seated infection and amputation. Therefore impaired wound healing is the pivotal event responsible for most of the morbidity (and mortality) of diabetic foot disease.

Consequently, a detailed understanding of the wound healing process in diabetes and how it can be improved is of great importance. However, efforts to develop new therapies are hampered by a lack of knowledge of the molecular mechanisms responsible for the pathologies as well as a lack of suitable models for the study of chronic wounds.

Normal wound healing is characterised by an orderly series of cell and tissue responses that can be grouped into four major phases^{2,5}. The initial injury phase stimulates platelet aggregation and clot formation to attain haemostasis. The resident platelets then release cytokines and growth factors to attract the various cell types utilised in the second phase. In this inflammatory phase of healing, neutrophils recruited from the circulation release chemotactic substances followed by macrophage arrival to suppress bacterial infection and remove dead tissue. In the third phase, characterised by proliferation, the wound bed is gradually replaced by granulation tissue which is produced mainly by fibroblasts laying down extracellular matrix (ECM) assisted by the endothelial cells promoting angiogenesis. During the fourth and final stage of wound healing, the granulation tissue is further remodelled to increase wound tensile strength.

In wounds that occur in diabetes, a persistent inflammatory phase is commonly witnessed at histopathology, associated with a delay in the formation of mature granulation tissue and

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a parallel reduction in wound tensile strength (Figure 1)^{2,6}. Why this persistent inflammation occurs is unclear, although continual bacterial infection^{1,2,7} and increased formation of advanced glycation end-products may play a role⁸.

Regulators of ECM

Two major classes of regulators of ECM – growth factors and the group of enzymes termed matrix metalloproteinases (MMPs)^{9,10} – have been the focus of our studies investigating the effects of diabetes on wound healing.

Growth factors and impaired wound healing in diabetes

Growth factors play an important role in regulating wound healing. One of their major functions in promoting the progression of wound healing is in switching the early inflammatory phase to the later phase of granulation tissue formation. Non-healing wounds often show defects in the type and amount of growth factors. Some growth factors, particularly inflammatory growth factors such as TNF-β, IL-β and IL-6 are reported to be increased in chronic venous ulcers and in non-healing burns.

Prolonged inflammation is associated with increased neutrophil infiltration and increased protease activity (discussed in detail below). The end result is ongoing tissue destruction rather than repair. Decreases in growth factors responsible for tissue repair such as platelet derived growth factor (PDGF) and transforming growth factor-β (TGF-β)

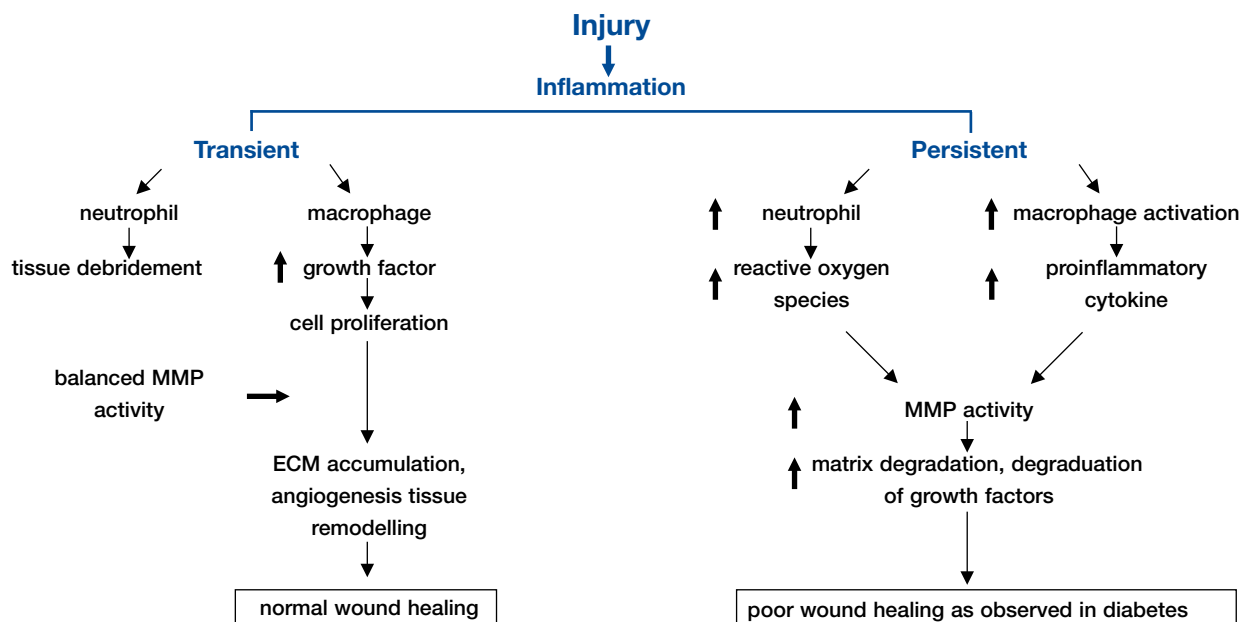
have also been documented in diabetic wounds¹¹⁻¹³. For some growth factors, the defect appears to be at a receptor level rather than in growth factor concentration^{4,14}.

More recently, there has been considerable interest in connective tissue growth factor (CTGF), a downstream modulator of TGF-β. The relationship between CTGF and TGF-β is complex. CTGF gene and protein levels are potentially upregulated by TGF-β; in turn, CTGF potentiates the bioactivity of TGF-β by facilitating its binding to the type 2 TGF-β receptor¹⁵. Like TGF-β, the gene and protein levels of CTGF normally rise during wound healing to promote wound healing. However, its levels in chronic wounds in diabetic subjects have not been reported and are the subject of ongoing studies in our laboratory. How growth factors become unbalanced in chronic wounds is not yet clear. Conceptually, their gene expression may be reduced, they may be bound to protein macromolecules and prevented from migration to the wound by fibrin deposits¹⁶ or degraded by proteases. Understanding these processes will assist in the targeting of potential therapies.

MMPs and impaired wound healing in diabetes

For wounds to heal, ECM not only needs to be laid down but must also be progressively degraded and remodelled in a regulated manner to form mature wound tissue. The enzymes primarily involved in the degradative arm of this process are the MMPs. They comprise a family of some 24 distinct but structurally related enzymes. They are involved

Figure 1. A schematic representation the steps of the wound healing process in normally healing and chronic wounds.



in tissue re-organisation, inflammation and remodelling and each has a different but sometimes overlapping spectrum of substrates. When acting together, they can degrade almost all ECM components¹⁷.

MMPs can be divided into four subgroups based on substrate specificity – interstitial collagenases, stromelysins, type IV collagenases and membrane type-MMPs (MT-MMPs) (Table 1). MMPs are secreted in latent forms, which need to be cleaved to become biologically active. MMP activities are themselves tightly regulated by their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which bind to the active enzyme with high affinity. Four TIMPs (TIMP 1-4) have been identified.

MMP activity in some chronic wounds of a number of different subtypes is increased by some 30 fold when compared with acute healing wounds⁶. Similar to growth factors, alterations in the balance in MMPs and other protein degrading enzymes are documented in delayed wound healing, including diabetic ulcers^{10, 18-20}.

In patients with diabetes, alterations in MMP expression and activation are consistent with a pattern associated with increased degradation of newly formed ECM^{6, 21}. For example, in our studies of chronic non-healing diabetic ulcers, sustained activation of MMP-2 and MMP-9 is observed (Figure 2). Similar studies of tissue obtained from neuropathic ulcers in diabetic patients have shown a consistent increase in the concentration of MMPs compared with tissue obtained from traumatic wounds. These changes would result in increased tissue breakdown, and an associated reduction in wound strength.

The abnormality of MMPs in diabetes may also be due to underlying changes in the functioning of cells involved

in wound healing. For example, fibroblasts taken from unwounded skin in patients with diabetes show increased MMP-2 and MMP-3 compared with fibroblasts from non-diabetic control subjects²¹.

How diabetes causes these changes is not yet clear. One possible mechanism is via effects of proinflammatory and profibrotic cytokines, known to regulate MMP expression. Most MMP genes have TGF- β inhibitory elements in their promoter regions and their expression is decreased by TGF- β ²². In contrast, TIMP expression is increased by TGF- β ²².

From these findings, it is clear that TGF- β plays an important role in the regulation of MMP activities at both transcriptional and post translational levels. However, the direct effects of CTGF on MMP activity have not been extensively studied. We have recently reported that CTGF upregulates the expression of mesangial cell TIMP-1²³. Whether CTGF plays a role in regulation of TIMP expression in wounds in diabetes is the subject of ongoing studies in our laboratory.

Models of wound healing

To define the precise effects of diabetes on growth factor and MMP expression in wounds more precisely, our research group has used a number of animal models.

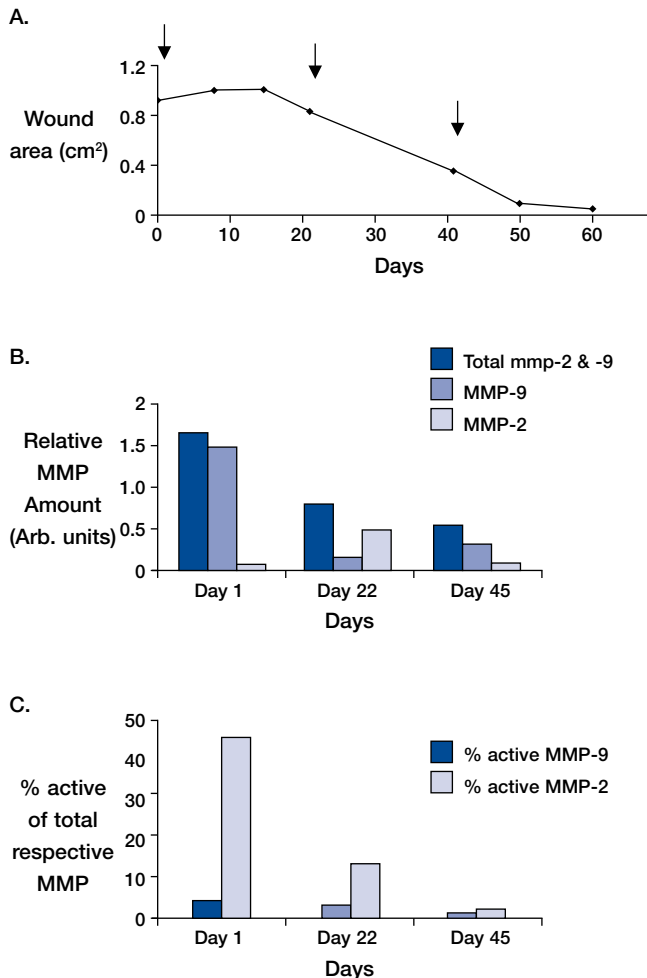
Rodent studies

The tensile strength of linear wounds created surgically in rodents was reduced by diabetes, to a degree curvilinearly related to the severity of hyperglycaemia²⁴. In a subsequent study, it was found that, in diabetes, granulation tissue formed in response to wounding was deficient in collagen, a substance that underpins the structural strength of wounds²⁵. More recently, we have developed a full thickness skin wound

Table 1. The major types of MMPs present in wounds and their substrates.

Type of MMP	MMP designation	Predominant cell type	Substrate
Interstitial collagenases	MMP-1 MMP-8	Fibroblasts, keratinocytes endothelial cells Stored in neutrophils	Type I, II and III collagen
Type IV collagenases/ or gelatinases	MMP-2 MMP-9	Fibroblasts, keratinocytes and endothelial cells Leukocytes and possibly keratinocytes	Type IV, V and VI collagen, fibronectin and gelatin
Stromelysins	MMP-3 MMP-7 MMP-10	Fibroblasts, keratinocytes endothelial cells	Proteoglycans, laminin, elastin fibronectin and Type III, IV, V collagen
Membrane-type type metalloproteinases	MMP-14	Fibroblasts endothelial cells	MMP-2

Figure 2. Representative profile of MMP activities in samples of wound fluid obtained from a diabetic ulcer over time.
 A Change in wound area over time, arrows depict time of wound fluid sampling.
 B Relative amounts of total MMP-2 and MMP-9 (filled bar) MMP-9 (hatched bar) and MMP-2 (non-filled bar) in wound fluid sample at each of the time points sampled.
 C Amount of MMP-9 or MMP-2 in active form expressed as a % of the respective total MMP.



model which has the advantage that enough tissue is available to allow the gene and protein levels of various factors to be studied.

The rodent models have an advantage over human studies in that factors such as the glycaemic control, wound size and infection status can be systematically controlled. However, unlike the situation in humans, rodent wounds heal partly by contraction rather than by granulation tissue formation and epithelialisation, limiting its applicability to the study of ulcers in diabetes.

Baboon studies

Over the past 15 years, we have established and maintained a colony of Type 1 diabetic baboons²⁶. They are used for the study of the development of chronic diabetic complications, in particular kidney disease. More recently, we have developed a model to study granulation tissue formation in these baboons. Nylon mesh drums are surgically placed subcutaneously in the limb of the baboons and are then removed after several weeks.

Our data show a persistent inflammatory wound phenotype in the diabetic baboons at various time points. Analysis of wound fluid MMPs by zymography²⁷ showed that expression of both MMP-9 and MMP-2 (Figure 3). Moreover, compared with non-diabetic animals, the relative concentrations of the biologically active forms of MMP-9 and MMP-2 are increased in the diabetic baboons. Skin biopsy material from the control of these baboons can also be used to study the possible pathogenic effects of diabetes on fibroblast and keratinocyte growth.

This primate model is closer to the situation in humans and allows the interaction of growth factors, MMPs and potential interventions to be studied. In addition, this experimental system has the advantage over rodent studies in that the wound tissue will be obtained from primates with duration of diabetes (10-15 years), similar to that seen in humans with long-term diabetes when complications begin to emerge.

Conclusion

Overall, the major focus of our studies is to examine the expression profiles of growth factors and MMPs obtained in each of the above models of wound healing in diabetes. Hopefully they will facilitate the development of strategies to improve the rate of tissue repair in diabetes.

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Figure 3. Profile of MMP-9 and MMP-2 in wound fluid obtained from wound chambers implanted in the thigh of six control (filled bar) and eight diabetic baboons (non-filled bar) for 2 or 4 weeks.

A Total MMP-9.

B Total MMP-2.

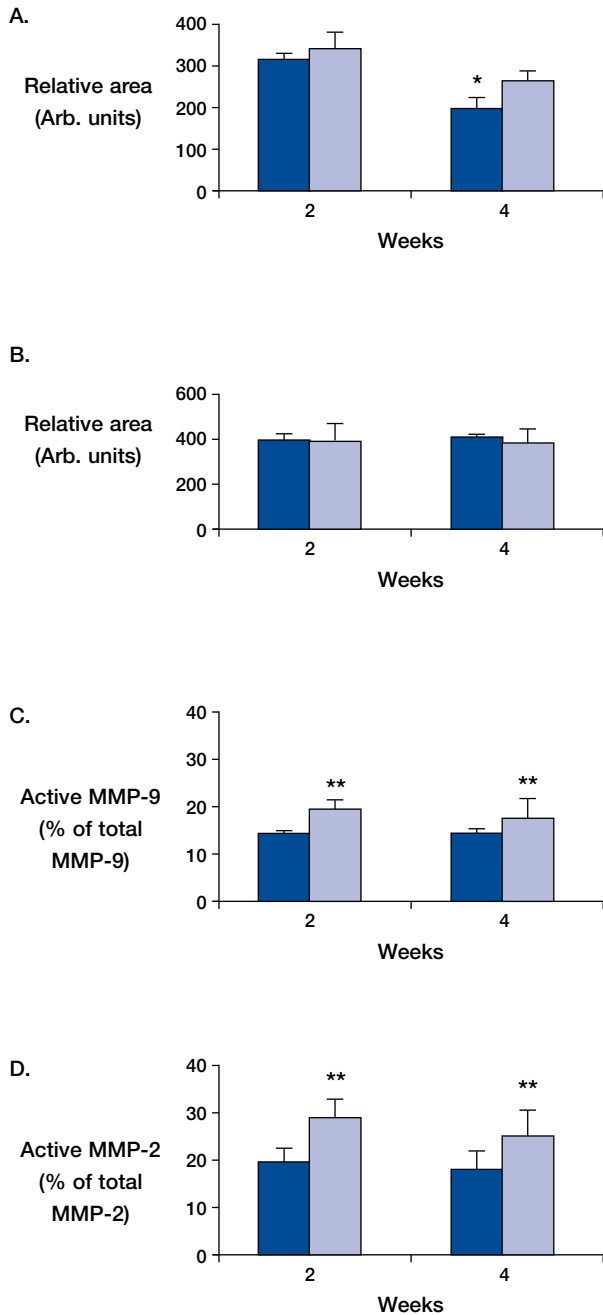
C The amount of active MMP-9 expressed as a % of its respective total MMP.

D The amount of active MMP-2 expressed as a % of its respective total MMP.

Results are expressed as mean ± SD and compared by ANOVA using Duncans Multiple Range Test.

* $p < 0.05$ different from 2 week control.

** $p < 0.05$ different from respective control.



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