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Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients

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Abstract

Aims/hypothesis. The molecular factors that cause an acute wound in diabetic patients to become chronic have not yet been established. Wound healing is known to require a balance between the accumulation of collagenous and non-collagenous extracellular matrix components and their remodelling by matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs). Our aim was to assess if the concentrations of MMPs and TIMPs were different between acute and chronic wounds in diabetic patients by analysing biopsy samples.
Methods. A 5 mm punch biopsy was taken from 20 diabetic foot ulcers of patients before initiating treatment and from traumatic wounds of 12 non-diabetic patients 2 days after injury. The concentrations of MMP-1, MMP-2_{pro}, MMP-2_{active}, MMP-8, MMP-9 and TIMP-2 were measured in detergent extracts of the biopsy homogenates using ELISAs and gelatine-zymography.
Results. The concentration of MMP-1 was increased 65-fold in biopsies of diabetic foot ulcers compared with the concentrations measured in biopsies of traumatic wounds. Similarly, MMP-2_{pro} were increased threefold, sixfold for MMP-2_{active}, twofold for MMP-8 and 14-fold for MMP-9 compared to average concentrations in biopsies of traumatic wounds. Furthermore, the expression of TIMP-2 was reduced twofold in diabetic wounds compared with lesions of non-diabetic patients.
Conclusion/interpretation. The combination of increased concentrations of MMPs with decreased concentrations of TIMP-2 in chronic diabetic foot ulcers compared with healing wounds in normal patients suggests that the increased proteolytic environment contributes to the failure of diabetic wounds to heal. New treatment strategies for healing chronic diabetic foot ulcers could be directed towards reducing concentrations of MMPs and increasing levels of TIMPs. [Diabetologia (2002) 45:1011–1016]

Keywords Diabetes mellitus, matrix metalloproteinases, wound healing.

Diabetic foot ulcers cause considerable morbidity and mortality, and also pose a major problem in health care because they are among the most costly types of chronic wounds to care for [1, 2, 3]. The use of protocols for prevention and treatment of diabetic foot ulcers has improved the situation but the amputation rate, which is estimated at 28 000 amputations per year in Germany, remains high [4, 5, 6]. Thus, the care for pressure ulcers still remains a considerable clinical problem [7, 8].

Several studies have focused on characterizing the molecular environments of acute and chronic wounds in an attempt to understand the processes that change an acute healing wound into a chronic one [9, 10, 11, 12, 13, 14, 15]. The data from these studies have led to the hypothesis that diabetic foot injuries often fail

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Abbrevations. MMP, Matrix metalloproteinase; TIMP-2, tissue inhibitor of matrix metalloproteinases-2.

to heal because persistently high concentrations of pro-inflammatory cytokines in the wound induce high concentrations of proteases, which degrade multiple growth factors, receptors and matrix proteins that are essential for wound healing [16, 17, 18, 19, 20, 21].

Among the proteases that have been consistently reported to be increased in chronic wounds are the matrix metalloproteinases (MMPs). The MMP family of proteases are zinc-dependent endopeptidases that can degrade essentially all extracellular matrix (ECM) components. MMPs are produced by several different types of cells in skin including fibroblasts, keratinocytes, macrophages, endothelial cells, mast cells, and eosinophils. MMP activity is specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs) [22, 23, 24]. In general, MMPs are not constitutively expressed in skin but are induced temporarily in response to exogenous signals such as cytokines, growth factors, cell-matrix interactions and altered cell-to-cell contacts. MMPs also play important roles in various normal physiologic situations, including morphogenesis of tissues during embryological development, angiogenesis, parturition, ovulation and remodelling of scar tissue by proteolytically restructuring the extracellular matrix. However, MMPs can also contribute to pathological conditions that are characterized by the excessive breakdown of ECM components such as in rheumatoid arthritis, tumour cell invasion and metastasis [24, 25].

In normal wound healing, MMPs seem to be involved in various processes. In the first phase of wound repair, MMPs participate in the removal of devitalised tissue. During the repair phase, MMP activities are necessary for angiogenesis, for contraction of wound matrix, for migration of fibroblasts, and for keratinocyte migration and epithelialisation. During the final phase of wound healing, MMPs participate in the remodelling of newly synthesised connective tissue. In summary, spatially and temporally controlled expression of several distinct MMPs is necessary for normal wound healing and tissue repair [14, 26, 27, 28, 29].

An important mechanism for the regulation of the activity of MMPs is via binding to members of the family of proteins referred to as TIMPs (TIMP-1 to TIMP-4). TIMPs are relatively small proteins (21 000 to 29 000 M_r) yet they have several biological functions, including inhibition of active MMPs, stimulation of cell division, binding to ECM, inhibition of angiogenesis and induction of apoptosis [30, 31]. Most likely, the balance between MMP and TIMP concentrations plays a crucial role in successful wound healing [28, 32].

To assess the role of proteases and inhibitors in diabetic foot ulcers, we compared the concentrations of five MMPs and TIMP-2 in biopsies of chronic diabetic foot ulcers with the concentrations measured in biopsies of traumatic wounds from healthy persons in the early phase of wound healing.

Materials and methods

Recruitment of patients. Each patient gave their written informed consent to participate in the study protocol, which was approved by the Human Studies Committee of the University of Magdeburg. Twelve patients with traumatic lesions referred to the Department of Surgery of the University of Magdeburg Medical School were recruited. Twenty diabetic patients with chronic ulcers on the plantar surface of the foot were recruited from referrals to the Diabetology Clinic. The mean age of the diabetic patients was 65 years ± 11 years (range: 39 to 83 years) and was higher than the mean of 36±21 years in the healthy injured group (range 18 to 89 years). HbA_{1c} concentrations were higher in the diabetic group (7.8±2%) than in the healthy traumatic injury patients (5.1±0.4%). The mean duration of diabetes mellitus was 15 years ± 5.7 years. All participants were clinically free from macrovascular disease in the lower limbs. Peripheral foot pulses were palpable and the ankle-to-brachial blood pressure ratio was greater than 0.8.

Harvesting of tissue biopsies. A 5 mm diameter biopsy was taken from the center of the diabetic foot ulcer or the traumatic wound, immediately frozen in liquid nitrogen and stored at –80°C. The biopsies of patients with diabetic foot lesions were taken during the first visit. The biopsies of the traumatic injury group (incision or contusion wounds of the extremities) of non-diabetic patients were taken 2 days after the injury during the granulation phase of healing.

Measurement of MMP-1, MMP-8 and TIMP-2 by ELISA. Concentrations of MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase) and TIMP-2 in the biopsies were measured by using commercial ELISAs according to the manufacture's protocols (Amersham Pharmacia Biotech, Piscataway N.J., USA). Briefly, biopsies were homogenized in phosphate buffered saline (PBS) (PBS) containing 1% (V/V) Triton X-100 at a constant ratio of 2 ml of buffer per 1g of tissue (wet weight) using 15 ml frosted glass-on-glass Duall vessels. The tissue was then homogenized in a 15 ml frosted glass-on-glass Duall vessel [33].

Homogenates were centrifuged at 12000×g for 5 min at 4°C and the supernatant solutions were collected. The range of the MMP-1 ELISA is 6 to 100 ng/ml and detects total MMP-1 protein (proMMP-1, active MMP-1, and MMP-1/TIMP-1 complex). The range of the MMP-8 ELISA is 1 to 32 ng/ml and detects pro and active MMP-8 (100%), active MMP-8/TIMP-1 complex (50%), and active MMP-8/TIMP-2 complex (30%). The range of the TIMP-1 ELISA is 3 to 50 ng/ml and is specific for total TIMP-1 (free and complexed TIMP-1). All standards and samples were assayed in duplicate. The MMP-1, MMP-8 and TIMP-2 concentrations for each biopsy were calculated from the best fit curve of the standard curve, and were expressed as ng/ml of homogenate supernatant solution.

Measurement of MMP-2 and MMP-9 by gelatin zymography. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) were measured in biopsy homogenates by quantitative gelatin zymography using minor variations of a previously described procedure [34]. Briefly, equal volumes of the supernatant solutions from the tissue homogenates and 2× sample buffer containing SDS were incubated for 10 min at room temperature, then 12 µl of each sample were loaded into the wells of 10% polyacrylamide Tris-glycine gels containing 0.1% gelatin substrate (Invitrogen, Carlsbad, Calif., USA). Gels were run under non-denaturing conditions at 125 volts at 4°C for 2 h, then the gels were renatured in zymogram renaturing buffer containing 0.25% Triton X-100 for 1 h at 37°C followed by development

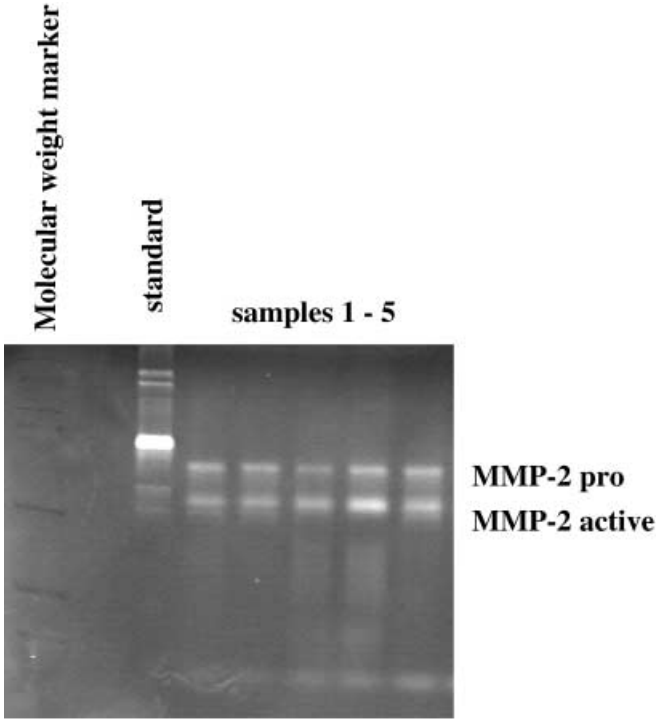

Fig. 1. Zymography of MMP-2 pro + active

in zymogram developing buffer (Invitrogen) at 37°C for 36 h. Gels were then fixed in 12.5% tricholoracetic acid for 10 min then stained with Rapid Coomassie Stain (Diversified Biotech, Boston, Mass., USA) for 30 min. MMP activities were detected by the presence of a clear band against a blue background, indicating proteolytic degradation of the gelatin substrate in that area by the MMP enzyme (Fig. 1). Purified standards of pro-MMP-9, pro-MMP-2 and active MMP-2 (Chemicon International, Hofheim, Germany) were included. Gels were photographed with a digital camera and the relative pixel density of each band was measured using an image analysis software (E.A.S.Y. 429 K, Herolab, Wiesloch, Germany). Concentrations of pro and activated MMP-2 and MMP-9 in samples were calculated from standard curves generated with recombinant pro and activated MMP-2 and MMP-9 (Oncogene Research Products, Cambridge, Mass., USA). Concentrations of pro and activated MMP-2 were expressed as pg/ml and MMP-9 as ng/ml of homogenate supernatant.

Statistical methods. Results were expressed as means ± SD and a Student's *t* test was used for statistical analyses. If parameters were not equally distributed, a nonparametric equivalent to the Student's *t* test, the Mann-Whitney U or the Wilcoxon Test, was used. The statistical software program SPSS 9.0 was used for calculations.

Results

There were significant differences in the concentrations of MMPs and TIMP-2 in biopsies from diabetic foot ulcers compared with non-diabetic traumatic wounds (Fig. 2). Specifically, the average concentration of MMP-1 was increased 65-fold ($p<0.001$) in biopsies of the chronic diabetic foot ulcers

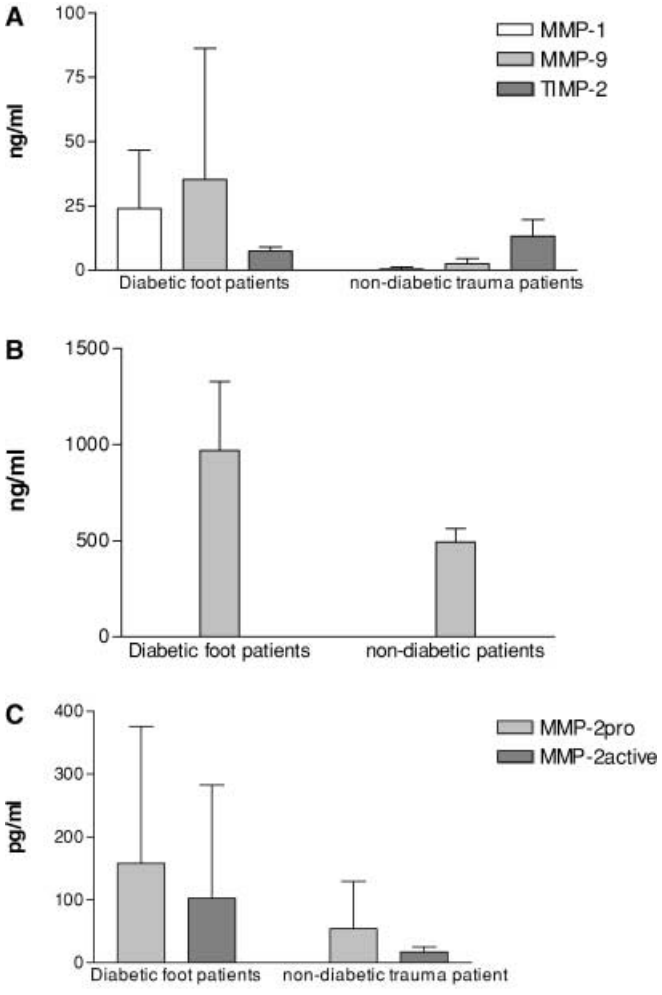

Fig. 2A–C. Expression of A MMP-1, MMP-9 and tissue inhibitor of proteinase-2 (TIMP-2); B MMP-8; C MMP-2 pro + active in diabetic foot lesions and healthy control subjects after an injury (data given as mean values and error bars as SD)

(24±23 ng/ml) compared with the average concentration measured in the biopsies of the traumatic wounds (0.4±0.8 ng/ml). Similarly, the average concentration of pro-MMP-2 in diabetic ulcers (158 pg/ml±218) was increased threefold ($p=0.041$) compared with traumatic injuries (54 pg/ml±75). The average concentration of active MMP-2 in diabetic ulcers (103 pg/ml±180) was increased sixfold ($p=0.033$) and the average concentration of MMP-8 was increased twofold ($p<0.002$). The average concentration of MMP-9 was increased 14-fold ($p=0.027$) in diabetic ulcers compared with average concentration in biopsies of the traumatic wounds (35.2±51 ng/ml for diabetic patients vs. 2.5±2 ng/ml for non-diabetic patients). In contrast to increased concentrations of MMP in diabetic wounds, the concentrations of TIMP-2 were lower ($p<0.007$) in the chronic diabetic foot ulcers (7.4±1.6 ng/ml) than in non-diabetic traumatic wounds (13.2±6.4 ng/ml).

Discussion

Proteolytic degradation of ECM is essential for repair and remodelling of cutaneous wounds [35]. As all chronic wounds begin as acute wounds, it is still not known at what point the healing sequence is interrupted whereby normal acute wound healing fails to occur [13, 15]. One of the first major processes in wound healing is inflammation, and this phase is regulated by several pro-inflammatory cytokines which are potent inducers of MMP synthesis in fibroblasts and inflammatory cells [29, 36, 37]. MMPs can be involved in various tasks during wound repair such as the removal of devitalised tissue, the regulation of keratinocyte migration and the angiogenesis [27]. During maturation MMPs are involved in the process of remodelling of newly synthesized connective tissue and regulate the activity of growth factors [22]. Spatially and temporally controlled expression of several distinct MMPs seems to be associated with normal wound healing and ulcer repair [22, 28, 38]. Several studies have shown this influence in different types of wounds, including acute normally healing human experimental [27, 39] and burn wounds [30] as well as venous stasis and pressure ulcers [38, 40, 41].

It is likely that the balance between protease and inhibitor concentrations plays a crucial part in successful wound healing. The persistence of increased concentrations of proteases during the wound healing process seems to contribute to the failure of the acute wound to heal. Possible, high concentrations of pro-inflammatory cytokines and proteases act as a positive feedback loop involving inflammatory cells releasing cytokines, which stimulate wound cells to secrete proteases that destroy tissue and prevent the wound from closing [17, 34]. Compared with acute wounds, fluid from non-healing venous and pressure ulcers contains high concentrations of activated gelatinases and low concentrations of MMP inhibitors [40, 41]. Accordingly the expression of TIMPs by keratinocytes is reduced in chronic wounds [28, 42, 43]. In chronic venous ulceration no qualitative differences were found in the expression of different MMPs (MMP-1, -3, -10) [44]. Nevertheless it has been suggested that decreased inhibitors of MMP and increased proteases (MMP) leading to excessive proteolysis retards the healing of venous ulcers [44].

Since the average age of the diabetic patients was higher than the average age of the acute wound patients, it is important to assess whether changes in expression MMP and TIMP with age contributed to the differences observed between acute and diabetic wounds. A previous study [16] showed that in normal skin MMP-2 immunostaining increased with increasing age, but no age or sex-associated changes were observed for MMP-1, MMP-3, or MMP-9 immunostaining. Thus, the 65-fold increase in the average concentration of MMP-1, and the 14-fold increase in the average concentration of MMP-9 measured in the diabetic ulcers cannot be due to age related changes, but only to differences in the expression of these proteins in acute wounds and diabetic ulcers. Furthermore, normal skin from aged persons (60 to 92 years old) contained approximately twofold higher concentrations of pro-MMP-2 than skin from young persons (20 to 30 years old), and no active MMP-2 was detected in either aged or young non-wounded skin [16]. Thus, the threefold increase in average concentration of pro-MMP-2 and the sixfold increase in active MMP-2 measured in the diabetic wounds compared with acute wounds is too large to be explained by increased concentrations observed in healthy persons as they age.

Concentrations of TIMP mRNA and protein in normal and wounded skin of healthy young and aged persons were also studied [16]. Concentrations of TIMP-2 mRNA was about threefold higher in non-wounded skin of young persons compared to aged persons. Three days after injury, concentrations of TIMP-2 mRNA doubled in wound tissue from young persons but did not change in aged persons. In our study, concentrations of TIMP-2 protein were approximately threefold lower in the diabetic ulcers than in the acute wounds. Thus, the lower concentrations of TIMP-2 protein could be caused primarily by differences in TIMP-2 synthesis as skin normally ages. Nevertheless, it is likely that the ratio of MMP to TIMP is more important than the absolute concentration of either.

Biopsies and fluids collected from chronic pressure ulcers have shown high concentrations of pro-inflammatory cytokines, high concentrations of active proteases, especially matrix metalloproteinases (MMPs), and low concentrations of growth factor activity in chronic pressure ulcers [17]. In contrast, fluids from healing skin wounds contained low concentrations of pro-inflammatory cytokines, low concentrations of active proteases, and high concentrations of growth factor activity. These data also showed that as a chronic wound begins to shift towards a healing wound, the pattern of cytokine, protease, and growth factor activity shifts back to the concentrations observed in an acute healing wound.

Our data show higher concentrations of MMP (MMP2, MMP 9, MMP 8) and lower concentrations of tissue inhibitors of metallo matrixproteinases (TIMP-2) in diabetic wounds compared with traumatic lesions of healthy control subjects. Compared with normal wound healing of non-diabetic wounds an overexpression of these proteases leads to an abnormal process of wound healing with rapid chronification. Furthermore, we show an imbalance of MMP and their inhibitors (TIMP-2), which could be involved in the pathogenesis of non-healing chronic ulcers. Further studies should measure MMP and TIMP concentrations at several time points during the healing of diabetic ulcers, with the anticipation that the

MMP:TIMP ratio would decrease as healing progresses. In addition, other proteases and their inhibitors, especially the serine protease, neutrophil elastase, and its inhibitor, alpha 1 protease inhibitor, could play important roles in impairing healing of diabetic ulcers.

While our study shows these effects in tissue biopsies, in previous studies wound fluids were taken for analysis. However, the distribution of proteases in wound fluids insufficiently reflect the true expression, because of interfering conditions such as bacterial contamination.

Our data concur with other studies on chronic pressure ulcers and suggest that diabetic foot injuries often fail to heal because of persistently high concentrations of MMPs. Persistent MMP-concentrations and an imbalance of their inhibitors could be evidence for an early chronification of diabetic foot lesions indicating a molecular environment in chronic wounds hostile for cell replication after injury. We thus assume a general malfunction of cellular wound healing processes in diabetic patients. The prolonged treatment period and high costs of treating stage Wagner 2 to 4 diabetic ulcers emphasises the need for a drug or treatment that promotes healing [45]. The basic mechanisms of wound healing in diabetes on the molecular level need to be understood further to develop innovative treatment strategies, such as applying growth factors [46, 47, 48, 49, 50] or proteinase inhibitors [51] to the non-healing diabetic foot ulceration.

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