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Identification and Temporal Kinetics of Elastinolytic Metalloproteinases in Skin Wounds

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Abstract

An important role in the differentiation of acute wound healing as compared to the chronic one is attributed to proteases. The aim of this work was the study of the expression kinetics, the activity and the role of elastinolytic MMP_s (gelatinases, stromelysins, metalloelastase) in the process of delayed healing of burned skin. Biopsies of burned skin, harvested from children, at different stages of healing, were used. In chronic wounds, gelatinase A (MMP-2) was initially over-expressed. Its active form was repressed in time, in favour of the latent form. The MMP-9 level increased gradually, reaching a peak at 2 weeks after the wounding, when the normal healing process started. Absent for 8 weeks, stromelysin appeared as a band at 57 kDa. In conclusion, among elastinolytic enzymes, MMP-2 participates at the long-time remodeling of chronic burned skin wounds, while MMP-3 and MMP-9 have important roles in cell proliferation and migration, during the granulation tissue formation.

Keywords: wound healing, metalloproteases, connective tissue remodeleng, burned skin

Introduction

Daily human activities can lead to accidents and different types of wounds. The most complex and difficult to treat is the profound extended burn [1]. It was shown that an important role in the difference between acute and chronic wound healing is played by proteases. Matrix metalloproteases (MMP_s) are responsible for the degradation and remodelling of the extracellular matrix (ECM). They are enzymes belonging to the endopeptidases class, which act on one or more matrix components [2]. Among the 26 different members of the MMP family, some have the ability to degrade soluble elastin *in vitro*: gelatinases A (MMP-2) and B (MMP-9), stromelysins 1 (MMP-3) and 2 (MMP-10) and macrophage metalloelastase (MMP-12) [3]. Moreover, elastases from other proteolytic families (serine-, thiol- and aspartic-proteases) act on elastic fibers [4]. It was shown that once destroyed, elastin was slowly or not at all remade [5], leading to anaesthetic scars. Although in mice and rats initiation of elastogenesis was observed after one week [6] and in pigs after 6 weeks [7], in humans elastin repair took years and was incomplete [8].

The methods for the analysis of MMP_s with elastinolytic activity are based on the cleavage of synthetic substrates, quantified spectrophotometrically [9, 10] or fluorimetrically

[11]. More specific methods make use of natural substrates, insoluble or soluble elastin [12, 13]. Zymography is one of the most specific and sensitive methods for MMP_s analysis [14].

The aim of this work was to study the kinetics of the expression of elastinolytic MMP_s involved in delayed healing of skin burns by means of a specific and sensitive method.

Materials and Methods

Materials. Burned skin biopsies were obtained from children who suffered 2^{nd} and 3^{rd} degree burns, after different healing times: 3, 6 and 8 weeks. The samples were harvested in physiological serum and immediately transported on ice to the laboratory.

MMP_s extraction. The tissue was cut in small pieces of approximately 1-2 mm^3 and extracted in 10 volumes of Tris-HCl 0.05M buffer, pH 7.5, with 2M guanidium hydrochloride, at 4°C, overnight. The extracts were aliquoted and freezed.

MMP_s analysis. MMP_s from tissue extracts were identified by gelatin-zymography [15]. To the standard acrylamide mixture, denatured type I collagen was added to a final concentration of 1 mg ml^{-1} or 0.5 mg ml^{-1}, respectively. Samples having the same protein concentration were migrated in 7.5% SDS-polyacrylamide gels, for 2 hours. Then, the gel was washed and incubated in Tris-HCl buffer, pH 7.6, with Triton X-100 and stained with Coomassie Brilliant Blue R-250. MMP's activity was visualised as not-coloured bands on the blue background of the gels. Enzyme identification was done on the basis of their molecular weight, as compared to the molecular standards co-migrated on the same gels.

Activation and inhibition of MMP_s. For activation of pro-MMP_s, samples were treated with 1mM 4-aminophenylmercuric acetate (APMA), for 30 minutes, at 37°C and migrated as described above. For inhibition studies, enzymatic extracts were treated with 1mM EDTA or 5mM PMSF, for 30 minutes, at 4°C, before electrophoresis.

Western blot for MMP-3 identification. Samples from the protein extracts were separated by electrophoresis in 7.5% SDS-polyacrylamide gels and transferred on nitrocellulose membrane. This was incubated serially with monoclonal antibody for MMP-3 (Sigma), followed by anti-mouse IgG coupled to peroxidase and with the peroxidase substrate.

Results

Gelatin-zymography of burned skin samples

In this study, we have adapted a method, based on gelatin zymography, for the study of the kinetics of MMP_s from burned skin found in acute and chronic wound healing processes.

In *normal healing wounds*, a prominent band at 57 kDa, probably of latent MMP-3 and two faint bands at 41 kDa (active MMP-1) and 28 kDa (active MMP-3) were visible, at 2 weeks after the wounding (fig. 1a). Moreover, MMP-3 was identified in extracts obtained from the skin around the wound (fig. 1b).

To confirm the belonging of the enzymes to the MMP class, the samples were first treated, with a metalloprotease inhibitor, EDTA, and a serine-protease inhibitor, PMSF, and, then, electrophoresed. In the first case, all the proteolytic bands disappeared (fig. 1c), while in the second case they were still present, thus indicating that the enzymes were indeed MMP. We also evaluated the influence of the substrate concentration on the method's sensitivity. MMP activity was examined on polyacrylamide gels with 0.5 mg ml^{-1} and 1 mg ml^{-1} gelatin, respectively. At 1mg ml^{-1}, the lysis bands were sharper. However, on 0.5 mg ml^{-1} gelatin gels,

an additional band was observed, with slower mobility, belonging, probably, to MMP-9 (fig. 1e).

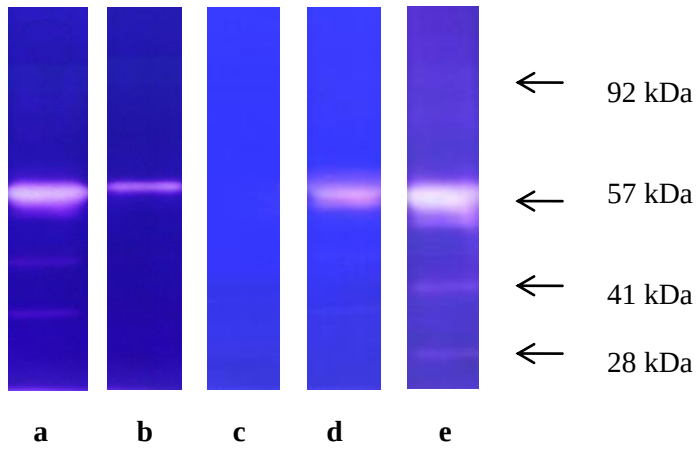

Fig. 1 – Zymographic profile of MMP_s in acute wound healing. The extracts were from: the burned area (a), around the burned area (b), the burned area treated with EDTA (c) and PMSF (d), the burned area on 0.5 mg/ml gelatin substrate (e).

In *chronic burned wounds*, among MMP_s with elastinolytic activity, at 3 weeks after the wounding, the band corresponding to the active form of gelatinase A was well defined (MMP-2, 62 kDa) (fig. 2a). During the healing process, this active form was repressed in favour of the latent form (72 kDa), found in greater quantities at 6 weeks (fig. 2b) and 8 weeks (fig. 2c) after the wounding. Gelatinase B (MMP-9, 92 kDa), in latent form, was absent at 3 weeks after the wounding (fig. 2a), but it was observed after 6 weeks (fig. 2b) and reached a peak after 2 months from the wounding, simultaneously to the start of the normal wound healing process. Stromelysine (MMP-3) was absent until 8 weeks after the wounding, when it appeared as a band at 57 kDa (fig. 2c).

To demonstrate the presence of the latent form of gelatinases, after 8 weeks from the wounding, the zymographic profile, after *ex vivo* activation of the extract with APMA, was analyzed. When the active forms appeared, there were extra bands at 83 kDa (active MMP-9) and at 62 kDa (active MMP-2) (fig. 2d).

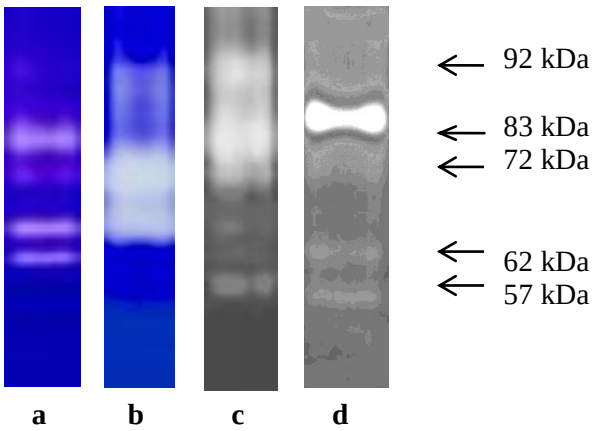

Fig. 2 – Zymographic profile of MMP_s in chronic skin wounds. The samples were harvested at 3 (a), 6 (b) and 8 weeks (c) after the wounding. The MMP_s from the latest extract activated with APMA (d).

MMP-3 identification by immunoblot

While the gelatinases were identified only during the prolonged healing process, stromelysine was present in both wound types, at different stages.

To determine and compare the kinetics of MMP-3, we have performed electrophoresis of the enzymes on polyacrylamide gel and, then, we have analyzed them by Western blot, using a specific monoclonal antibody to stromelysine. We observed a single band migrating at approximately 57 kDa, corresponding to the molecular mass of MMP-3, appearing in the normal healing wound after 2 weeks, and in the chronic wound after 8 weeks (fig. 3).

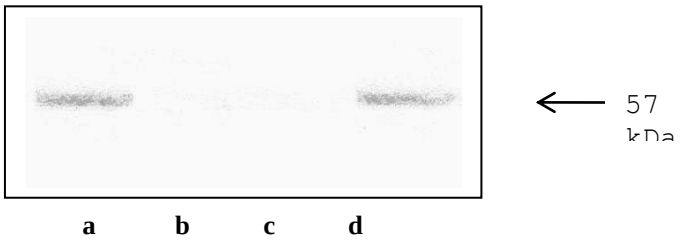

Fig. 3 – MMP-3 identification in samples from the acute healing wound (a) and the chronic wound after 3 (b), 6 (c) and 8 (d) weeks.

Discussions

The wound healing is an interactive, dynamic process, in which the tissue destruction initiates a reconstruction process for the restoration of the tissue's structure and functions. It is made up of 3 phases which overlap in time: the inflammation, the granulation tissue formation and the ECM remodeling. In normal cases it lasts 2 weeks, unlike the case of dermal chronic wounds which heal in several months [16]. In acute wounds, proteolysis is essential for allowing the migration of cells in the wound, the remodeling of the granulation tissue and the reconstruction of the dermis normal architecture. In dermal chronic wounds an aberrant spatial and temporal expression of proteins and an excessive activation of them take place [17]. MMP expression was not detected in normal skin *in vivo* [18]. After the wounding, more MMP_s are temporarily expressed during the healing process. Although the events involving cellular components are known, the expression, repression and activity of MMP_s remain relatively unknown despite their importance in wound healing [19].

In this research, we have adapted a method based on gelatin-polyacrylamide electrophoresis for the identification of the types of MMP_s with elastinolytic activity, in active or latent form, extracted from human burned skin. This technique allows the study of the kinetics of MMP_s, whose activity is essential for ECM remodeling during the healing process. The zymographic system is adequate for the temporal study of different MMP_s, present in skin, in both zymogene and active forms. Using a 0.5 mg/ml substrate concentration, we detected these MMP efficiently and accurate, and we analyzed the different MMP species on the basis of their molecular mass and activation state. This method was completed by immunoblot analysis to determine the appearance time of MMP-3 in wounds with prolonged healing.

In the acute healing process, MMP-3 in latent and active form, together with small quantities of the MMP-9 proform, were identified by comparison to the molecular mass of the markers co-migrated in gel. The expression and activity of a certain MMP is limited to a specific zone in the wound and to a particular stage of the repair process. We observed that MMP-3 is found in high quantities in latent form, thus it did not have an essential role in the initial stages of the process. The faint lysis band, observed at 92 kDa, corresponded to gelatinase B (MMP-9). It was shown that it could cleave types IV and V collagens, fibronectin and gelatin. Also, gelatinase B had a high affinity for the components of the elastic fibers from human skin [20] and could specifically degrade insoluble elastin, thus being similar to serine-proteases [21].

In chronic burns, the analysis of the observed bands was more difficult. The infiltration of the polymorphonuclear cells contributed to the differentiation and raising of the MMP level in the wound. Previous studies showed that neutrophils participated to the activation of gelatinase A present in the matrix [22, 23] Macrophages, although being able to express a variety of MMP_s, produced only a small number of enzymes in cutaneous wounds [24, 25]. Gelatinase A (MMP-2) was not only reactive towards elastin, but as well towards fibronectin, denatured collagens, components of the basal membrane. Its presence in both pro-enzymatic and active form could be correlated with the high turn-over rate of collagen types [26]. During the studied healing process, a gradual repression of the active form of MMP-2, together with the increase in MMP-9 level were observed. MMP-9 was expressed stronger in the healing phase and acted to remove the connective tissue destroyed by the collagenases, thus helping to the restoration of the normal structure and function of the tissue. We confirmed, this way, that gelatinases have different roles in the healing process of cutaneous wounds.

Previous studies showed that the appearance of MMP-3 in the chronic wound corresponded to the migration of fibroblasts in the wound and to the lowering of the macrophage number, which determined the deposition of the granulation tissue [27, 28]. It was shown that fibroblasts secreted MMP_s in a different way compared to the macrophages, from both quantitative and qualitative points of view [29]. The presence of stromelysine 1 together to MMP-9, at 8 weeks after the wounding, indicated the starting of the normal wound healing process.

In conclusion, among the MMP_s with demonstrated elastinolytic activity, MMP-2 participates at the long-time remodeling process of chronic burned wounds, while MMP-3 and MMP-9 have important roles in cell proliferation and migration, during the formation of the granulation tissue.

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