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Temporal Study of the Activity of Matrix Metalloproteinases and Their Endogenous Inhibitors During Wound Healing

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Abstract The restoration of functional connective tissue is a major goal of the wound healing process. This regenerative event requires the deposition and accumulation of collagenous and noncollagenous matrix molecules as well as the remodelling of extracellular matrix (ECM) by matrix metalloproteinases (MMPs). In this study, we have utilized substrate gel electrophoresis, radiometric enzyme assays, and Western blot analyses to determine the temporal pattern of appearance and activity of active and latent MMPs and their inhibitors during the entire healing process in a partial thickness wound model. Through the use of substrate gel electrophoresis, we studied the appearance of proteolytic bands whose molecular weight was consistent with their being members of the MMP family of enzymes. Proteolytic bands whose molecular weight is consistent with both the active and latent forms of MMP-2 (72 kDa, Type IV gelatinase) were detected in wound fluid of days 1–7 after wounding. The number of active MMP-2 species detectable in wound fluid was greatest during days 4–6 after wounding. The most prominent proteolytic band detected each day migrated with a molecular weight consistent with it being the latent form of MMP-9 (92 kDa, Type V pro-collagenase). In contrast to MMP-2, the active form of this enzyme was never detected. The presence of MMP-1 (interstitial collagenase) was detected by immunoblot in the wound fluid from days 1–6 post-injury. Using a radiometric enzyme assay for collagenase inhibitory activity we have also determined the time course of activity of endogenous matrix metalloproteinase inhibitors. We have correlated these data to the known cellular events occurring in the wound during this time period as well. This study establishes a prototypical pattern of MMP appearance in normal wound healing. It may also provide potential intervention sites for the therapeutic use of inhibitors of aberrant MMP activities which characterize chronic wounds.

Key words: wound healing, metalloproteinase, metalloproteinase inhibitor, connective tissue remodeling, chronic wounds

Wound healing is a complex process which requires tight regulation of regenerative and degenerative processes [Buckley-Sturrock et al., 1989]. Successful wound healing is characterized by the regeneration of normal connective tissue which exhibits proper vascular and extracellular organization and function. This restoration event is a dynamic process which involves the deposition and accumulation of key collagenous and noncollagenous matrix molecules as well as the tightly regulated remodelling of the extracellular matrix (ECM) by matrix metalloproteinases (MMP).

The MMPs are members of a multigene family of metal-dependent enzymes. The activity of these enzymes is responsible for connective tissue remodelling and is considered to be the rate-limiting step in ECM degradation [for review see Woessner, 1991; Docherty and Murphy, 1990; Matrisian, 1990]. The delicate balance between the activity of MMPs and their endogenous inhibitors (tissue inhibitors of metalloproteinases, TIMP) is responsible for the establishment and maintenance of a stable extracellular matrix architecture [Shapiro et al., 1992]. The MMPs are classified on the basis of

Abbreviations used: APMA, aminophenyl mercuric acetate; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

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their substrate specificity and include interstitial collagenase (Type I collagenase, MMP-1), neutrophil collagenase (MMP-8), the 72-kDa gelatinase (Type IV collagenase, MMP-2), the 92 kDa gelatinase (Type V, MMP-9), stromelysin (MMP-3), uterine metalloproteinase (MMP-7, Pump-1) [Woessner, 1991], and the recently reported membrane-type matrix metalloproteinase (MT-MMP) [Sato et al., 1994]. Control of the activity of MMPs is complex and occurs at the level of transcription, at the level of activation of the enzyme from its latent to its active form, and at the level of its inhibition by endogenous TIMPs. Collagenase activity catalyze the turnover and restructuring of matrix components which is a significant event in wound repair, and this restructuring has been suggested to be critical to cell movement in the ECM after wounding as well [Buckley-Sturrock et al., 1989].

Although studies to date have focused on the deposition, accumulation, and strength of ECM components, such as collagen, during wound healing, relatively little is known about the kinetics of appearance and activity levels of the enzymes which regulate that deposition and accumulation [Porras-Reyes et al., 1991]. Using a wound healing model which provides the opportunity to study the daily differences in the wound healing process over the entire time course of healing, we have determined the presence and relative abundance of matrix metalloproteinases and their inhibitors involved in the wound healing process. Elucidation of the pattern of appearance and activity of these MMPs under normal wound healing conditions may facilitate our understanding and treatment of chronic wounds which have been described as exhibiting deregulated MMP activities [Grinnell et al., 1992; Saarialho-Kere et al., 1992; Hennessey et al., 1990; Hasebe et al., 1987; Wysocki et al., 1991, 1993].

MATERIALS AND METHODS

Wound Model

Wound fluid from partial thickness wounds was collected daily as described by Breuing et al., [1992]. Briefly, medium partial thickness excisional wounds (15 × 15 × 1.2 mm, 30 wounds per pig) were created on the backs of four female Yorkshire pigs (3–4 months old; 45–50 kg). Each wound was covered with a separate liquid-tight vinyl chamber (P.A. Medical Corp., Columbia, TN) filled with 1.2 ml normal saline containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The base of the chamber has a central opening the same size and shape as the wounds created. The solution was exchanged every 24 h, pooled daily, centrifuged, and filtered on 0.45 μm filters. All wounds were confined within longitudinal, 12-cm-wide paraspinal stripes between the crest of the shoulders and the coccygeal tuberosity with thickness of intact epidermis ranging from 0.04–0.07 mm and thickness of intact dermis ranging from 2.3–3.2 mm. Uniformity of the created wounds was verified histologically.

All pigs utilized in these experiments were fed a standard porcine diet and were housed at 20–23°C in an atmosphere of about 65% humidity with a light cycle of 12 h on/12 h off. After wounding, they were kept separately in custom-made, smooth-sided, stainless steel cages to minimize subsequent wound trauma and disruption of applied chambers. Pigs were suspended in a Panepinto sling (Charles River Laboratories, Wilmington, MA) for wounding, biopsy, and daily chamber replacement.

Substrate Gel Electrophoresis

Proteinase activity was visualized using SDS-polyacrylamide gels copolymerized with gelatin as described previously [Herron et al., 1986]. This method permits the simultaneous detection of the different types of MMPs present in the fluid as well as their relative activity levels. As described by Herron and coworkers [1986], Type I gelatin (Difco, Detroit, MI) was added to the standard Laemmli acrylamide polymerization mixture at a final concentration of 1 mg/ml. Depending on the experiment (see legends for details) wound fluid from each day was mixed with sample buffer (10% SDS, 4% sucrose, 0.25 M Tris-HCl [ICN, Cleveland, OH], pH 6.8, and 0.1% bromophenol blue); equivalent volumes of wound fluid diluted in substrate buffer were loaded into wells of a 4% acrylamide Laemmli stacking gel on a Mini-Protean II apparatus (Bio-Rad, Richmond, CA) and run as previously described [Laemmli, 1970]. Following electrophoresis, gels were incubated in 2.5% Triton X-100 with gentle shaking for 30 min at ambient temperature with one change of detergent solution. Gels were then rinsed and incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl buffer, pH 8, 5 mM $CaCl_2$) (Sigma, St. Louis, MO) and 0.02% NaN_3 (Sigma). Following incubation, the gels were stained for 15–30 min in 0.5% Coomassie Blue R-250 in acetic acid:

isopropyl alcohol:water (1:3:6), followed by destaining in water, and photography. Proteolytic activity appears as clear zones, demonstrating lysis of the substrate in the gels, against a background of the dark-stained gel. In order to verify that the proteolytic activities that were detected were, in fact, metalloproteinase activities, samples were electrophoresed as described above and the gels then incubated in substrate buffer in the presence or absence of 1,10-phenanthroline (10 mM) (Sigma), an inhibitor of MMPs. To distinguish latent from active forms of the MMPs, samples were exposed to 4-aminophenylmercuric acetate (APMA) (1 mM) (Sigma) as previously described [Herron et al., 1986]. Treatment of MMPs for 30 min at 37°C results in the activation of proforms of MMPs to the lower molecular weight, active form of the enzymes. Molecular weights of latent and active species were determined using an IS-1000 Digital Imaging System (Alpha Innotech Corp San Leandro, CA). Organomercurial compounds have been reported to cause autoactivation of MMPs by the removal of the amino-terminal propeptide [Stetler-Stevenson et al., 1989; Springman et al., 1990; Nagase et al., 1990; Mallya and Van Wart, 1990]. Unless otherwise indicated all reagents were obtained from Bio-Rad.

Radiometric Enzyme Assay for Collagenase Inhibitory Activity

Collagenase inhibitor activities were determined according to a modification [Moses et al., 1990; Murray et al., 1986] of the method of Johnson-Wint [1980]. To test for the presence of collagenase inhibitors, appropriately diluted wound fluid samples (100 μl) were combined with 100 μl of bovine corneal collagenase prepared as previously described by us [Moses et al., 1990], and then added to wells containing radiolabelled rat tail tendon (Type I) collagen prepared as described previously [Murray et al., 1986]. After incubation with the radiolabelled collagen at 37°C for 2.5 h, the supernates which contained the soluble radiolabelled collagen were transferred to scintillation vials and then counted in a Beckman (Fullerton, CA) model LS 3801 scintillation counter.

Western Blot Analysis

Wound fluid samples from each day were electrophoresed on SDS-15% polyacrylamide gels and transferred electrophoretically to nitrocellulose sheets [Towbin et al., 1979]. The nitrocellulose sheets were incubated with a monoclonal antibody (H18G8) [Werb et al., 1989] which recognizes MMP-1 (interstitial collagenase) kindly provided by Dr. Zena Werb. Cross-reactivity was visualized by successive incubations with a conjugate of goat anti-rabbit IgG and alkaline phosphatase and enzyme substrate.

RESULTS

Substrate Gel Electrophoresis

Metalloproteinase species in pig wound fluid from a partial thickness wound were analyzed. In this in vivo model using the chamber system in a liquid environment, an early fibrinous clot fills out the excised portion of skin. By day 4 after wounding, epithelial migration can be detected starting from the epidermis of the wound margin and dermal appendages. New connective tissue develops on the top of the wound bed, upon which epithelial migration takes place. By days 6/7 after wounding, the wounds have been reepithelialized and the new connective tissue has been richly vascularized. After this period, fibroblasts, which had been the predominant cell during this time period, begin to decrease [Breuing et al., 1992].

The presence of MMP species in wound fluid samples collected 1 h after wounding and daily until the wound was healed was first detected by SDS-substrate gel analysis. As shown in Figure 1 (lane 1), no detectable proteolytic activity was observed in the wound fluid collected 1 h after wounding, even at doses 7× as high as that tested from days 1–10 after wounding. The most prominent proteolytic band detected in wound fluid migrated at an approximate Mr of 94 kDa. This porcine gelatinase activity corresponds to the reported size of the pro MMP-9 (92 kDa collagenase) [Herron et al., 1986]. Relative to the other zones of lysis (discussed below) the band observed at approximately 94 kDa is the most prominent each day. These results were observed in three separate sets of wound fluid from the different animals. To determine whether this band observed in the wound fluid samples was indeed the precursor form of this gelatinase, we treated the wound fluid with 1 mM aminophenyl-mercuric acetate (APMA). APMA treatment results in the activation of proforms of MMPs by proteolytic modification resulting in an observable decrease in Mr. After APMA treatment of wound fluid samples, a de-

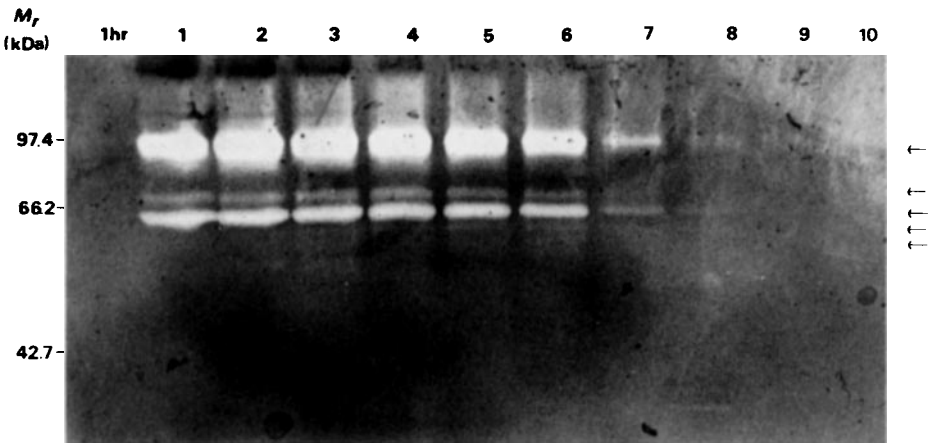

Fig. 1. SDS-substrate gel electrophoresis of wound fluid (0.33 μl) collected from partial thickness wounds 1 h after wounding and daily for 10 days. MMP activities are observed as zones of clearance in a polyacrylamide gel impregnated with gelatin and stained with Coomassie R-250. The *left lane* shows the relative molecular mass of the protein standards. The *arrows* in the *right lane* indicate the location of proteolytic zones. This is a representative zymogram of three separate sets of wound fluid from three different animals.

crease in molecular weight (approximately 4 kDa) of the band at 94 kDa was observed, suggesting that this form of the enzyme was a latent one (Fig. 2).

A second band of proteolytic activity was observed at an apparent Mr of approximately 72 kDa (Fig. 1). Comparison of the Mr of this gelatinase activity with that of known MMPs suggest that it corresponds to the reported size of the latent form of MMP-2 (72 kDa gelatinase) [Herron et al., 1986; Stetler-Stevenson et al., 1989; Howard et al., 1991]. After activation with APMA, a molecular weight reduction of approximately 6 kDa was observed, consistent with this gelatinase being the latent form of this Type IV collagenase species [Howard et al., 1991] (Fig. 2).

In order to better visualize the various activated forms of MMP-2, to varying amounts of wound fluid from each day (dose curves) were tested in this zymography system until optimal resolution of the active forms was achieved (Fig. 3). Three active forms of MMP-2 were observed. Each of these different active forms exhibited different patterns of appearance. A lytic zone at approximately 66 kDa was detected at a relatively constant level of intensity throughout days 1–6 after wounding (Fig. 3). A decrease in the intensity of this zone was observed in day 7 wound fluid, with the band completely absent in days 9 and 10 (Fig. 1). This active form of MMP-2 produced the most prominent band of the three active forms and was significantly more intense than the latent enzyme on all days.

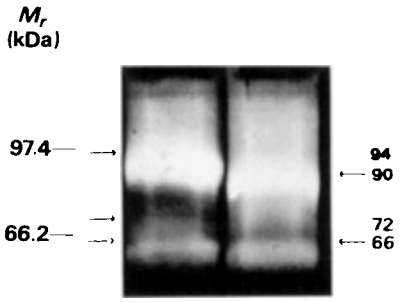

Fig. 2. SDS-substrate gel electrophoresis of wound fluid before (*left lane*) and after (*right lane*) treatment with 4-aminophenyl- mercuric acetate (APMA). Decreases in relative molecular mass for proteolytic species at 94 and 72 kDa were detected and quantitated using an IS-1000 Digital Imaging System (Alpha Innotech Corp.). Day 2 wound fluid is depicted here as the representative example.

The 64 kDa activated form was detected in the wound fluid of days 3 through 6, its appearance peaking on days 4 and 5 (Fig. 3). A third proteolytic band was observed at 62 kDa in fluid throughout days 2 through 6, disappearing by day 7 (Fig. 3). We note that the intensity of all of the MMP-2 forms is less than that of the MMP-9 species in wound fluid of each day tested.

When zymography was performed using the metalloproteinase inhibitor 1,10-phenanthroline (10 mM), total disappearance of all of the proteolytic bands was observed (data not shown) resulting in a blue-stained gel without zones of clearance, indicating that all of the proteolytic activities detected are of the metalloproteinase class [Herron et al., 1986].

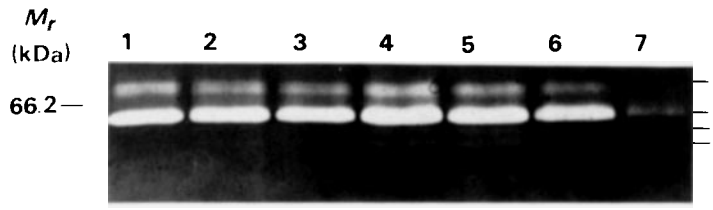

Fig. 3. In order to better visualize all forms of MMP-2, various amounts of wound fluid from each day post-wounding were tested until optimal resolution was achieved. A band of proteolytic activity was observed at an apparent Mr of 68–72 kDa and corresponds to the latent form of MMP-2. Three active forms of MMP-2 were observed which exhibit different patterns of appearance and activity. These activities migrate at approximately 66, 64, and 62 kDa.

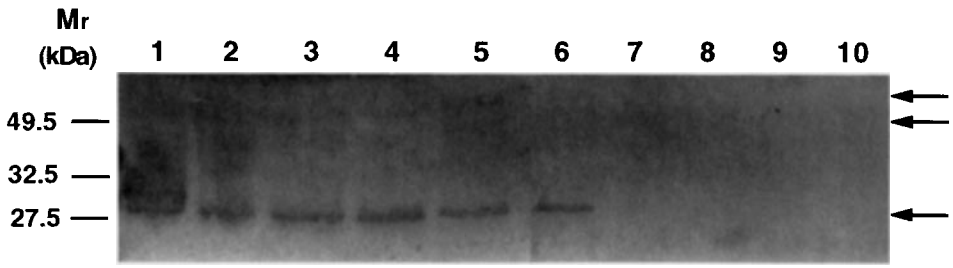

Fig. 4. Western blot analysis of wound fluid using a monoclonal antibody (H18G8) which recognizes MMP-1. One immunoreactive band at approximately 27 kDa was detected in wound fluid from days 1–6 after wounding. Two faint immunoreactive bands at approximately 45 and 49.5 kDa were also detected in fluid from days 3–5 post-injury.

Collagenase Western Blot Analysis

To determine the time course of appearance of MMP-1 (interstitial collagenase) in these wound fluid samples, Western blot analysis was conducted using a monoclonal antibody (H18G8) which recognizes interstitial collagenase [Werb et al., 1989]. Interestingly, the strongest immunoreactivity was detected as a single band migrating at an approximate molecular weight of 28 kDa (Fig. 4), consistent with the reported Mr of fibroblast interstitial collagenase (MMP-1) [Woessner, 1991]. Alternatively, this immunoreactive protein may represent another MMP which also cross-reacts with the antibody used. Two fainter immunoreactive bands corresponding to a previously reported porcine MMP-1 [Chen et al., 1992] were also observed at approximately 45 and 49 kDa.

Radiometric Enzyme Assay for Collagenase Inhibitory Activity

Samples of wound fluid from each day were assayed using a radiometric enzyme assay for collagenase inhibition as previously described by us [Moses et al., 1990; Murray et al., 1986]. Potent anti-collagenase activity was detected in wound fluid samples from the earlier days of the time course (Fig. 5).

DISCUSSION

In this study, we have utilized substrate gel electrophoresis, quantitative radiometric enzyme assays, and immunoblot analyses to determine the temporal pattern of appearance of active and latent MMPs in a partial thickness wound healing model. This system provides a unique opportunity to study the kinetics of appearance of the MMPs whose activity is an absolute requirement for the remodelling of the extracellular matrix during wound healing. Although much has been learned regarding the temporal pattern of appearance of the cellular components involved in the wound healing process, the time course of appearance and disappearance of MMP activity has remained relatively unknown despite their importance in wound repair [Porras-Reyes et al., 1991]. Substrate gel electrophoresis is particularly suited to this type of temporal study. Wound fluid represents a complicated mixture containing both zymogens and active forms of several different MMPs which are not as readily visualized and detected using radiometric assays alone [Lefebvre et al., 1991]. Large numbers and different forms of these enzymes can be efficiently and accurately detected using a zymography system. Substrate gel analysis also provides the

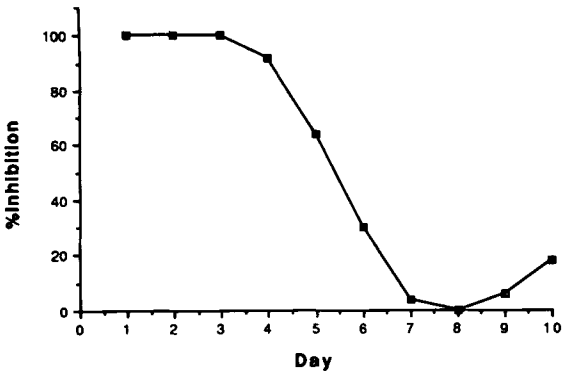

Fig. 5. Wound fluid (50 μl) from days 1–10 following injury was tested in a radiometric collagen film assay for collagenase inhibitory activity. In this assay, 1 U of collagenase produced 10% cleavage of collagen in 2.5 h at 37°C. Collagenase inhibition is defined as the percent decrease in this collagenase activity. All samples were assayed in duplicate. This figure is representative of three different sets of wound fluid from three different animals.

opportunity to detect MMP species based on their molecular weight, to determine their activation state (latent or active) and to detect their abundance relative to each other in complex biological fluids. Complementing this assay system with quantitative radiometric enzyme assays and immunoblot analyses has enabled us to determine the time course of appearance of the key MMPs and their inhibitors during wound healing.

One hour wound fluid samples which showed no detectable proteolytic activity in all of the assays were used as negative controls in this study. As shown in Figure 1, the most prominent proteolytic band detected by zymography in wound fluid from the partial thickness wounds probably represents the latent form of MMP-9 (92 kDa Type V pro-collagenase). We never detected the active form of this enzyme in any of the wound fluid samples. MMP-9 degrades Types IV and V collagens and Types I and V gelatin as its matrix substrates [Woessner, 1991]. The pattern of appearance of this MMP species is consistent with the time course of appearance reported for different cell types into the wound, in particular the macrophages which have been shown to play a key role in wound repair via their influence on matrix turnover [Shapiro et al., 1992; Whalen and Zetter, 1992]. It is known that macrophages are attracted to a wound site during the early acute phase of wound healing. They predominate at the wound site by day 3, after which the granulation tissue phase begins. The granulation period is marked by the migration of capillary sprouts and myofibroblasts into the wound after the macrophages [Whalen and Zetter, 1992]. These inflammatory cells secrete a number of MMPs, in particular a 92 kDa which cleaves Types IV and V collagen [Welgus et al., 1990; Collier et al., 1988]. Resident macrophages such as the alveolar macrophage also produce an interstitial collagenase identical to that of fibroblasts but in higher quantity (approximately 25% more) [Shapiro et al., 1992]. Macrophages have also been shown to produce very little 72 kDa enzyme, producing instead the 92 kDa which is only rarely produced by fibroblasts [Shapiro et al., 1992]. It is important to note that interstitial collagenase, stromelysin, and the 72 kDa gelatinase, MMP-2, are constitutively secreted by a number of cell types, including fibroblasts in a proenzyme form and are subject to activation extracellularly [He et al., 1989]. We never observed active MMP-9 in any of our wound fluid samples. Since no activation system is present within these wounds to activate this enzyme species [Welgus et al., 1990], we speculate that, at least in this wound repair system, MMP-9 may not be a principal MMP in the healing process.

As shown in Figure 1, a proteolytic band corresponding to the latent 72 kDa Type IV gelatinase (MMP-2) was observed peaking at day 4 after injury. Matrix substrates of this enzyme include collagens Type IV, V, VII, X, Type I gelatin, fibronectin, and laminin [Woessner, 1991]. Importantly, three active forms of MMP-2 were observed with the two lowest active forms, at approximately 64 and 62 kDa appearing at days 4, 5, 6. These results are consistent with the appearance of fibroblasts into the wound concomittant with a decrease in the number of macrophages [Ross, 1968]. Fibroblasts are known to secrete MMPs in a manner both qualitatively and quantitatively different than that of the macrophages [He et al., 1989; Welgus et al., 1990; Salo et al., 1994]. As macrophages gradually decrease, fibroblasts become more plentiful at the wound site. They first appear near newly functioning capillary loops where they divide and deposit fibrillar collagen [Whalen and Zetter, 1992]. Both deposition and remodelling of collagen have been shown to occur during this period [Buckley-Sturrock et al., 1989]. Capillaries have also been shown to appear at the wound site along with fibroblasts [Whalen and Zetter, 1992] and capillary endothelial cells have also been shown to secrete latent MMP-2 in culture [Herron et al., 1986; Braunhut and Moses, 1994].

If the 72 kDa gelatinase is a secreted product of the EC at the wound site, this would suggest that there are activators of the enzyme in situ that are not present in culture.

Consistent with the pattern of appearance of the active forms of MMP-2 is the decrease in collagenase inhibitory activity as determined by the radiometric enzyme assay (Fig. 5). This assay [Murray et al., 1986; Moses et al., 1990] detects inhibitory activity of the known MIs. These results suggest that collagenase inhibitors are not only present in the wound fluid during the earlier days post-wounding, but also that these MIs may be responsible, at least in part, for the regulation of ECM degradation during early wound healing.

To determine the time course of appearance of MMP-1 (interstitial collagenase) in these wound fluid samples, we conducted immunoblot analysis using a monoclonal antibody directed against interstitial collagenase. Two patterns of immunoreactivity were observed. A strong immunoreactive band was detected at the same intensity in the wound fluid of days 1–6 after wounding at approximately 28 kDa (Fig. 4). This immunoreactive band disappeared by day 6 at which time the wound had become re-epithelialized. Faint immunoreactive bands at approximately 49 and 45 kDa could be detected in wound fluid from days 1–5 after wounding. Chen and coworkers have also reported the presence of a 43 kDa interstitial collagenase activity in the wound fluid of full thickness wounds [Chen et al., 1992]. Type III collagen and other substrates of MMP-1 have been demonstrated to be present at the earliest phase of collagen neosynthesis and deposition in normal wound repair [Whalen and Zetter, 1992]. Since the establishment, remodelling, and maintenance of ECM requires both degradation and synthesis, the consistent presence of MMP-1 at this time point is likely to be an important requirement.

This study is the first to demonstrate the types and levels of MMP and inhibitory activity present during the entire time course of normal wound healing. It has been reported that in chronic wounds, the regulation and control of MMP activity becomes aberrant and MMP levels are significantly higher than in normally healing wounds. For example, a recent report demonstrates that when compared to serum, acute wound fluid (mastectomy fluid) contained a five-to-ten-fold increase in levels of several MMPs including MMP-2 and MMP-9. In chronic wound fluid (leg ulcer fluid), not only were these enzymes increased another five-to-ten-fold over mastectomy fluid, but also both activated enzyme and proenzymes species were detected [Wysocki et al., 1991, 1993]. The authors concluded that the overexpression of MMPs in chronic wounds may interfere with cell-matrix interactions necessary for epithelization and normal wound healing. Data from this and other studies [Grinell et al., 1992; Saarialho-Kee et al., 1992; Hennessey et al., 1990; Hasebe et al., 1987] strongly suggest that at least one strategy for controlling abnormal or chronic wound healing may be at the level of the control of MMP activity. This current report also provides a potential diagnostic approach for screening fluid from abnormally healing wounds since it has been demonstrated that the ability to restructure collagen in a healing wound, a prominent feature of the wound healing process, may be assessed by the measurement of MMP activity. Perhaps even more importantly, this study establishes a prototypical pattern of MMP appearance in normal wound healing against which abnormal/pathological wound repair might be compared.

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