Release and Activation of Matrix Metalloproteinase-9 During In Vitro Mechanical Compression in Hypertrophic Scars

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Objective: To investigate induction of matrix metalloproteinases (MMPs) during mechanical compression of hypertrophic scars. Mechanical pressure blocks hypertrophy induced on extracellular matrix in scars by a mechanism that involves MMP-2 (gelatinase A) and MMP-9 (gelatinase B).

Design: We assayed conditioned media obtained from normotrophic and hypertrophic scars during 24 hours of in vitro mechanical compression using gelatin zymography.

Setting: Scars from various areas of the bodies of hospitalized patients.

Patients: We obtained 3 normotrophic and 7 hypertrophic biopsy specimens from 10 patients (5 men and 5 women).

Intervention: In vitro compression at a pressure of 35 mm Hg/cm² for 24 hours.

Main Outcome Measures: Vitality of scars was analyzed by means of lactic dehydrogenase test; medium samples were collected for zymographic analysis of MMP activity.

Results: We found MMP-2 in basal (uncompressed) samples from normotrophic and hypertrophic scars. Mechanical compression induced MMP-9 release and activation (range, 86.7%-78.7%) in hypertrophic scars after 4 hours.

Conclusion: Production, release, and activation of MMP-9 in hypertrophic scars could be an effector mechanism responsible for hypertrophy regression induced by mechanical compression.

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Hypertrophic scars (HSs) result from alterations due to skin injuries, especially burns, in the normal processes of cutaneous wound healing. Hypertrophic scars are characterized by excessive deposition of fibroblast-derived extracellular matrix (ECM) proteins, especially collagen, in the derma during a long period by means of persistent inflammation and fibrosis. The last phase of normal wound healing consists of a systematic dissolution of granulation tissue and its replacement with a more organized and elastic ECM. This process seems to be altered in HSs.

The major effectors of ECM degradation and remodeling belong to a family of structurally related enzymes called matrix metalloproteinases (MMPs). The MMPs are secreted in an inactive proform requiring activation by means of proteolytic cleavage. Their activity is zinc-dependent and is inhibited by members of the family of tissue inhibitors of metalloproteinases. The activity of MMPs is regulated at multiple levels, and resident cells of a tissue typically do not produce MMPs unless they are needed for remodeling.

During cutaneous wound repair, the activity of MMP-2 (gelatinase A; 72-kd type IV collagenase) and MMP-9 (gelatinase B, 92-kd type IV collagenase) persists after wound closure and seems to be important in the remodeling process.

Hypertrophy after burning is still treated mostly by means of elastocompression, using elastic garments. The overall effect of pressure is an acceleration of the remodeling process that, in the absence of pressure bandaging, can occur several years after the formation of the HS. The mechanisms responsible for hypertrophy remission after such a technique are still poorly understood, although the interval of therapeutically useful pressure has been identified as ranging from 10 to 35 mm Hg/m².

To our knowledge, no studies have investigated the role of MMPs in the regression process induced in HS by elastocompression. In this in vitro study, we investigated the presence of MMP-2 and...
PATIENTS AND METHODS

We used 10 burn scars taken from different areas of the body. The biopsy specimens (rectangular sections of 1 × 0.8 cm) were obtained after informed consent from 10 patients (5 men and 5 women; mean ± SEM age, 27 ± 13 years) undergoing surgery.

We assessed the clinical stage of each scar on the basis of macroscopic observation before surgery and later by means of histological findings.

We examined NSs (n=3) obtained from patients whose lesions underwent optimal healing, and HSs (n=7). Every sample was weighed and divided into 2 subspecimens, one to undergo compression and the other to serve as a control sample. In vitro compression was performed using an electromechanical load transducer (Instron 5564; Instron Corporation, Canton, Mass). The specimens destined for compression were placed in an organ chamber containing 20 mL of serum-free RPMI 1641 medium plus penicillin (100 U/mL) and streptomycin (100 µg/mL), inserted into a thermostatic bath at 37°C, oxygenated, and compressed using a metal punch. A pressure equal to 35 mm Hg was applied to the section surface (skin side) for 24 hours. Control samples were maintained in serum-free RPMI 1641 plus antibiotics at 37°C in an atmosphere of 5% carbon dioxide.

Medium samples were taken from both subspecimens (1 mL) at the beginning of the constant compression (pressure stabilized after 5-10 minutes at 35 mm Hg; time 0), and at 0.5, 1, 2, 4, and 24 hours. Before storing, we immediately analyzed aliquots of all samples to check the vitality of the scar tissue by using a commercial kit with a sensitivity of 3 to 5 U/L to evaluate the amount of lactate dehydrogenase (LDH) (LDH-optimized UV method; Sigma-Aldrich Corp, St Louis, Mo). We obtained the readings at 340 nm using a spectrophotometer (Beckman DU-68; Beckman, Milan, Italy).

The samples were stored at −80°C and used for measurements of MMP activity by means of zymography. Latent and active gelatinases were detected by means of zymogram analysis using a combination of sodium dodecyl sulfate and polyacrylamide gels copolymerized with 0.2% gelatin.

These enzymes become dissociated from tissue inhibitors of metalloproteinases by the presence of sodium dodecyl sulfate during electrophoresis. Removal of sodium dodecyl sulfate after electrophoresis allows the proenzymes to reassociate in an active or partially active conformation, which permits their detection and the detection of lower-molecular-weight-activated forms. In brief, conditioned media from control and compressed samples were mixed with sample buffer and underwent electrophoresis directly without boiling or reduction. After electrophoresis, sodium dodecyl sulfate was extracted from the polyacrylamide gel using Triton X-100 (Sigma, Milan, Italy), and the gel was incubated in a solution of 0.05M Tris (pH, 7.5) containing 5mM calcium chloride and 5mM zinc chloride at 37°C overnight. Gels were stained with Coomassie brilliant blue R-250 and then destained. Proenzyme and active gelatinase were detected as clear bands against the blue background of the stained gelatin. Positive controls for gelatinase A and B (Chemicon International, Inc, Temecula, Calif) were used to distinguish the 2 enzymes from their activated forms. Unconditioned medium samples were used as a negative control.

We performed a densitometric analysis of the bands seen on gels using NIH Image 1.62 software (National Institutes of Health, Bethesda, Md). We used SPSS software for Windows (SPSS Inc, Chicago, Ill) for the statistical analysis of data. The t test was used for data; P<.05 indicated statistical significance.

MMP-9 in culture media conditioned with HSs and normotrophic scars (NSs) and the effect of mechanical compression on MMP expression and activation.

RESULTS

We used gelatin zymography to monitor the activity of MMP-2 and MMP-9 in RPMI 1641 medium that was conditioned for 24 hours with biopsy specimens obtained from burn scars. In the basal (uncompressed) condition, pro–MMP-2 was present at a barely detectable level in NS and at significantly higher levels in HS (Figure 1A). The analysis of conditioned media obtained from compressed specimens showed that pro–MMP-2 presence was almost undetectable in NS and HS, with only 1 exception in an HS that already showed a strong MMP-2 activity in the uncompressed sample. In the same samples, the presence of pro–MMP-9 and MMP-9 activities was observed mainly in HS (Figures 1 and 2B). Compression also induced the release of a small amount of pro–MMP-9 and MMP-9 in NS (Figures 1 and 2B). The mean percentage of activation, calculated as a percentage of the activated form divided by the total MMP-9 activity (proenzyme and activated form) for all 7 HSs examined, was 82.6% (range, 78.7%-86.7%). The expression and activation of MMP-9 induced by the mechanical compression in HS biopsy specimens was also time dependent (Figure 3). The
active form of MMP-9 in compressed specimens was detectable starting from 4 hours, and its presence increased by 1200% after 24 hours. The MMP-9 mean percentage of activation after 4 hours was 86.1% (range, 82.2%-88.4%), slightly but not significantly higher than the 24-hour value. The mean (±SE) release of LDH measured after 24 hours in basal NS and HS samples (controls) was 35.9±7.7 and 38.0±11.1 U/L, respectively. Compression caused a statistically insignificant increase in LDH release (NS, 44.8±17.2; HS, 51.1±8.1), indicating that no significant tissue damage occurred.

Pressure therapy is generally accepted as one of the best noninvasive techniques for preventing and controlling hypertrophic scarring after burn injury. This technique appears to arrest or suppress the production of additional hypertrophic tissues and to enhance the natural remodeling process that occurs long after the initial injury. Although this method is efficient, its mechanisms of action are not known. Knowledge of these mechanisms could result in better clinical treatment of burn injury. Pressure therapy presents some adverse effects when used for extensive burns or in children, and therefore the identification of its effector mechanism(s) could lead to an alternative pharmacological treatment of these patients. A previous study suggested that pressure accelerates the remission phase of the postburn repair process by inducing ECM remodeling and the disappearance of α-smooth muscle α-actin expressing cells. Histological analysis of untreated HS showed concentric nodular collagen fiber heaps in the deep dermis, whereas in HS treated by elastocompression, the remodeling process was evident in the organi-

Figure 2. Representative substrate gelatin zymography of normotrophic (NS) and hypertrophic (HS) scar–conditioned media. Media were conditioned with normotrophic (N1) and hypertrophic (HS1-4) specimens for 24 hours in the basal condition (A) and in the compressed condition (B). MMP indicates matrix metalloproteinase.

Figure 3. Representative substrate gelatin zymography of time-dependent release and activation of matrix metalloproteinase (MMP)-9 in a hypertrophic (HS1) specimen during compression. Medium samples have been collected at different time points (0-24 hours). The appearance of MMP-9 activity is evident after 4 hours of compression. In this sample, MMP-2 activity was also present.

zation of collagen, elastin, and fibrillin deposits. Hypertrophy reduction has also been addressed to an ischemic process characterized by cellular damage and collagen synthesis reduction, but this effect seems to be relevant only for prolonged treatment. In our in vitro model, this effect, if present, was minimized by the oxygenation of the samples throughout the pressure treatment. Moreover, the results of the LDH test showed no significant difference between the viability of basal and compressed samples after 24 hours.

The major effector enzymes involved in the remodeling of the ECM are MMPs. In particular, 2 of these enzymes, MMP-2 and MMP-9, seem to be involved in the remodeling phase of wound healing. In a previous study, MMP-2 activity was significantly elevated in HS, whereas MMP-9 activity was undetectable; however, no studies have reported their possible involvement in the hypertrophy remission induced by elastocompression. In our in vitro model, we observed the presence of pro-MMP-2 in basal conditions in HS-conditioned medium, whereas it was undetectable in samples obtained from NS. When HS samples were compressed, MMP-9 activity appeared in a time-dependent fashion, whereas MMP-2 activity disappeared. At the same time in the NS samples, we found a light, almost undetectable presence of MMP-2 and MMP-9 induced by compression. Matrix metalloproteinase-9 can degrade native type IV and V collagens, elastin, fibronectin, and denatured collagen of all types, and its expression is induced by growth factors such as interleukin 1β and transforming growth factor α. The activation of MMP-9 is believed to be induced by plasmin generated on the cell surface, but no information exists about this system in HS. In a previous study, we observed that in vitro mechanical compression induced release of interleukin 1β and prostaglandin E2 in HS. Thus, in our model, interleukin 1β released by compression may induce MMP-9 synthesis. The presence and activity of tissue inhibitors of metalloproteinases could be checked in this model to understand whether they play a role during the pressure-induced activation of MMP-9.

Given its broad activity against many substrates, the presence of MMP-9 could partially account for the ECM remodeling observed in pressure-treated HS and for the remission of hypertrophy observed in HS treated...
by elastocompression. However, further investigation is necessary to validate this effect of compression in an in vivo model and to identify the upstream events that trigger interleukin 1β release and MMP production, release, and activation.

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