

Matrix metalloproteinase-7–dependent release of tumor necrosis factor- α in a model of herniated disc resorption

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Received for publication April 14, 1999, and accepted in revised form October 20, 1999.

Herniated disc (HD), one of the major causes of low back pain, is often resolved spontaneously without surgical intervention. Resorption is associated with a marked increase in infiltrating macrophages, and the matrix metalloproteinases (MMP) MMP-3 and MMP-7 have been implicated in this phenomenon. We developed a murine organ culture model in which intact intervertebral discs were cocultured with peritoneal macrophages to investigate the role of MMPs in HD resorption. Using macrophages isolated from MMP-null mice, we report that macrophage-produced MMP-7 was required for proteoglycan degradation, loss of wet weight, and macrophage infiltration of cocultured discs. The inability of MMP-7–deficient macrophages to infiltrate discs could not be attributed to a defect in macrophage migration. MMP-7 was required for the release of the cytokine TNF- α from peritoneal macrophages. The generation of soluble TNF- α was essential for the induction of MMP-3 in disc cocultures, which in turn is required for the generation of a macrophage chemoattractant and subsequent macrophage infiltration. TNF- α release from macrophages was necessary but insufficient for disc resorption, which required macrophage infiltration. We conclude that there is extensive communication between macrophages and chondrocytes in HD resorption and that an essential component of this communication is the requirement for MMPs to release soluble bioactive factors.

J. Clin. Invest. **105**:143–150 (2000).

Introduction

Low back pain (LBP) affects from 60 to 80% of Americans in their lifetimes (1). Herniated disc (HD), the protrusion of a degenerated disc into the spinal canal or neural foramina involving compression of nerve tissues (2), is responsible for symptoms in up to 40% of all LBP patients (1). Although this condition results in almost 280,000 operations in the US each year (3), approximately half of the patients with back pain recover spontaneously within 6 weeks (4). Magnetic resonance imaging (MRI) analysis of HD patients revealed that an obvious and progressive decrease in the size of HD occurred most commonly in HDs that were prominently exposed to the epidural space (5) and correlated with abundant vascularization, as determined by the accumulation of the contrast agent gadolinium-diethylenetriamine pentaacetic acid (GD-DTPA) (6). The intervertebral disc, which is normally nourished only by diffusion from the vertebrae, becomes vascularized after exposure to the spinal canal, and surgical samples from patients with HD display granulation tissue with prominent macrophage and mild T-lymphocyte infiltration that is not observed in healthy intervertebral disc (7). Thus, natural resorption of HD appears to

occur by a vascularization-mediated process and correlates with prominent macrophage infiltration.

The matrix metalloproteinases (MMPs) belong to the metzincin family of zinc-dependent proteases and are known to degrade the various components of the extracellular matrix (ECM). MMP-3 (stromelysin-1, EC 3.4.24.17) and MMP-7 (matrilysin, EC 3.4.24.23), in particular, can degrade cartilage proteoglycans such as aggrecan (8, 9). Both of these MMPs are strongly expressed in human surgical samples of HD, whereas normal-appearing discs lack significant expression of these MMPs (10–12). We therefore hypothesized that these MMPs are upregulated when infiltrating macrophages encounter chondrocytes in the intervertebral disc and are responsible for HD resorption.

We have reported the development of a coculture system of chondrocytes and macrophages to examine the role of MMPs in the resorption process (13). We demonstrated that the addition of macrophages to disc cultures resulted in the marked upregulation of MMP-3 in the disc chondrocytes, the generation of a macrophage chemoattractant, and resorption of the disc as demonstrated by a loss in wet weight (13). Using discs isolated from MMP-null mice, we determined that the produc-

tion of MMP-3 by the chondrocytes was required for macrophage infiltration and disc resorption. The current study examines the role of macrophage MMP-3 and MMP-7 in the process of HD resorption using the coculture model. Surprisingly, the production of MMP-7 by macrophages was found to be required for macrophage infiltration into disc tissue through a mechanism involving the release of soluble TNF- α .

Methods

The experimental protocols were approved by the Institutional Animal Care and Use Committee, Vanderbilt University Medical Center.

Mice. Homozygous wild-type 129/Sv, MMP-3-null 129SvEv, and MMP-7-null 129SvEv mice were used in these studies. MMP-3-null and MMP-7-null mice were generated by homologous recombination in embryonic stem cells, as described (14, 15).

Disc/macrophage cocultures. The intervertebral disc/peritoneal macrophage coculture system was described previously (13). Briefly, activated murine macrophages were obtained by intraperitoneal administration of 3% thioglycollate medium (Difco Laboratories, Detroit, Michigan, USA). For some experiments, red fluorescent cell linkers (PKH26; Sigma Chemical Co., St. Louis, Missouri, USA) were injected into the peritoneum 3 days after thioglycollate administration according to the manufacturer's instructions. Cells were harvested by peritoneal lavage 4 days after thioglycollate treatment. Coccygeal intervertebral disc tissues were obtained using a dissecting microscope after skin and soft tissues were removed. Whole intervertebral disc tissues (5 discs/35-mm dish) and peritoneal macrophages (10^6 /mL) were cocultured in 5 mL of Opti-MEM (GIBCO BRL, Grand Island, New York, USA) containing 50 μ g/mL gentamycin and 0.25 μ g/mL Fungizone (GIBCO BRL) for 2 days in a humidified environment of 5% CO₂ at 37°C. For some experiments, recombinant

murine TNF- α (R&D Systems, Minneapolis, Minnesota, USA), MMP-7 (1 ng/mL; Chemicon International, Temecula, California, USA), goat anti-mouse TNF- α (1 ng/mL; R&D Systems), anti-mouse IL-1 β (2 μ g/mL; R&D systems), or control goat IgG (1 ng/mL; Sigma) was added to the culture medium. Coculture media was clarified by centrifugation and processed for determination of MMP-3 or TNF- α protein. Tissue was embedded in OCT compound, snap-frozen, and 6- μ m cryosections were examined with a fluorescent microscope.

To measure degradation of disc tissue quantitatively, intervertebral discs (1 disc per well of 24-well tissue-culture plate; Corning-Costar, Cambridge, Massachusetts, USA) were cultured with or without peritoneal macrophages (10^6 /mL) for 3 days in 1 mL Opti-MEM with 50 μ g/mL gentamycin, and 0.25 μ g/mL Fungizone (GIBCO BRL). Before and after completion of incubation, samples were removed from the culture medium, patted briefly with sterile gauze to remove surface water, and their wet weight was measured with an autobalance (CAHN 28 automatic electrobalance, CAHN Instrument Co., Paramount, California, USA). Samples were then fixed in 4% paraformaldehyde, paraffin embedded, and sections stained with 0.25% Safranin O as an indicator of proteoglycan content in tissue sections (16).

Western blotting. Following 2 days in culture, serum-free conditioned media were collected from single cultures and cocultures and total protein was quantitated using Bio-Rad assays (Bio-Rad Laboratories, Hercules, California, USA). Cell media were diluted 1:1 with Laemmli sample buffer. Protein (22.5 μ g) from each sample was subjected to SDS-PAGE (10 or 15% gel) under reducing conditions, and the proteins were transferred onto nitrocellulose membranes (Nitro ME; MSI, Westborough, Massachusetts, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 0.15 M NaCl, 0.01 M Tris, pH 8.0) containing 0.05% Tween-20 (TBS-T) for 1 hour, followed by incubation with a 1:5000 dilution of anti-MMP-3 antibody (17). Recombinant rat MMP-3 was used as positive control (17). Membranes were then washed with TBS-T and incubated sequentially with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, California, USA) for 1 hour, washed again, and incubated with peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) for 1 hour. Immunoreactive proteins were identified by chemiluminescence with a solution of 2.5 mM luminol (Sigma), 0.4 mM *p*-coumaric acid (Sigma), and H₂O₂ in 50 mM TRIS (pH 6.5).

Migration assay. Migration activity of peritoneal macrophages was examined by a modified Boyden chamber as described previously (13). Macrophages (1.2×10^6 cells/mL) were suspended in serum-free DMEM containing 200 μ g/mL BSA or Opti-MEM and added to the upper wells. DMEM with 10% FBS, 200 mg/mL BSA, or conditioned media derived from macrophage and disc tissue was added to the lower chamber as indi-

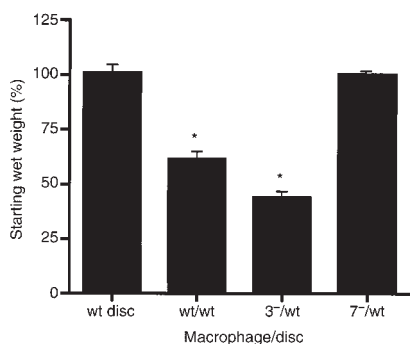
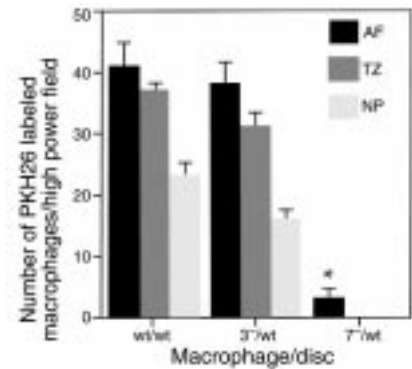


Figure 1

Role of macrophage MMPs in in vitro disc resorption. Discs from wild-type (/wt) mice were cocultured with macrophages from wild-type (wt/), MMP-3-null (3-), and MMP-7-null (7-) mice. Wet weights were determined at day 0 and day 3, and the percent of starting wet weight was calculated for each individual disc. Values presented represent the mean and SD of 3-5 discs/group. *Significant difference from starting wet weight ($P < 0.05$).

Figure 2

Quantitative analysis of macrophage infiltration in MMP-null organ cultures. Macrophages from wild-type or MMP-null mice were cocultured with wild-type disc tissue. The genotype of the mice donating macrophages/disc tissue is indicated. wt = wild-type; 3⁻ = MMP-3-null, 7⁻ = MMP-7-null tissue. In a representative experiment, whole disc tissue was divided into 3 regions: nucleus pulposus (NP; inner gelatinous region), annulus fibrosus (AF; outer layer), and transition zone (TZ; intermediate layer), and the number of PKH26-labeled red fluorescent macrophages in 3 high-power fields in each region was determined. Data were analyzed with the Kruskal-Wallis test and were presented as mean ± SD. *Statistically significant difference ($P < 0.05$) compared with wild-type.



cated. After incubation for 5 hours at 37°C, the number of cells in 6 high-power fields (×400) migrating through the 8-μm pores was counted. Statistical analysis of the data was performed using the Mann-Whitney test. A P value of less than 0.05 was considered significant.

Quantification of TNF-α. Soluble TNF-α was measured in cell-culture supernatants with a sandwich ELISA and specific murine TNF-α antibodies (R&D Systems). For some experiments, 2-hour conditioned media from macrophages that had been treated with 0 or 100 ng/mL rMMP-7 in the presence or absence of 10 μg/mL cycloheximide (Sigma) were used as samples. Assays were performed according to the manufacturer's protocol and read at 450 nm with a microplate reader. Samples and standards were run in duplicate. The assay was linear between 5.1 pg/mL and 1.98 ng/mL. All values are expressed as the mean and SD. Statistical analysis was performed with the Mann-Whitney test and P less than 0.05 was considered statistically significant.

Results

Macrophage MMP-7 is required for disc resorption. Macrophages have been reported to produce multiple MMPs, including MMP-3 and MMP-7 (18-22). In a murine cellular culture model of HD resorption, the coculture of thioglycollate-elaborated macrophages and chondrocytes encased in alginate beads resulted in a marked induction of both MMP-3 and MMP-7 protein in coculture medium (13). RT-PCR revealed that mRNA for both of these enzymes was produced by the macrophages (13). To determine if macrophage elaboration of either of these MMPs was required for disc resorption, macrophages from wild-type, MMP-3-null, and MMP-7-null mice were isolated and placed in coculture with intervertebral discs from wild-type mice. Disc resorption was measured by determining the wet weight of the tissue before and 3 days after coculture with macrophages. Discs cocultured with wild-type macrophages demonstrated a $38.7 \pm 3.2\%$ decrease in wet weight (Figure 1). Macrophages from MMP-3-null mice showed a similar $55.2 \pm 2.1\%$ reduction in wet weight over the 3-day coculture period. In contrast, discs cocultured with macrophages from MMP-7-null mice demonstrated no resorption during this time period and retained their original weight, similar to that observed with discs cultured in the absence of macrophages. Thus,

macrophage MMP-7, but not MMP-3, is required for disc resorption in the coculture model.

The role of MMP-7 in macrophage infiltration and migration. We have observed previously a positive correlation between disc resorption and disc infiltration by macrophages (13). To determine if the requirement for macrophage MMP-7 for disc resorption was also related to an effect on macrophage infiltration, peritoneal macrophages isolated from wild-type, MMP-3-null, and MMP-7-null mice were labeled with fluorescent markers and cocultured with wild-type discs. Wild-type and MMP-3^{-/-} macrophages attached to the surface of the discs and invaded the outer annulus fibrosus, the inter-

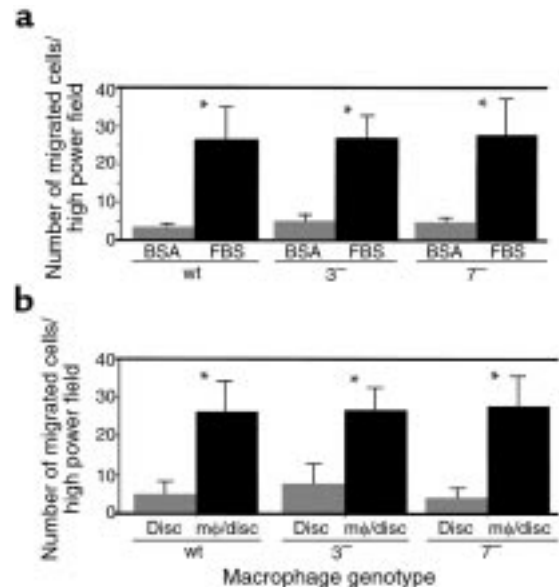


Figure 3

Quantitative analysis of macrophage migratory activity. Wild-type (wt), MMP-3-null (3⁻), and MMP-7-null (7⁻) macrophages were placed in the top well of a modified Boyden chamber, and the number of macrophages migrating through the filter was counted in 6 random ×400 high-power fields for each well. (a) BSA (200 μg/mL) in DMEM or FBS (10%) in DMEM was placed in the lower chamber. (b) Conditioned medium from intervertebral discs from wild-type mice (disc) or discs cocultured with wild-type macrophages (mφ/disc) was placed in the lower chamber. Data from 2 experiments performed in triplicate were analyzed with the Mann-Whitney test and presented as mean ± SD. *Statistically significant differences ($P < 0.05$) compared with control medium.

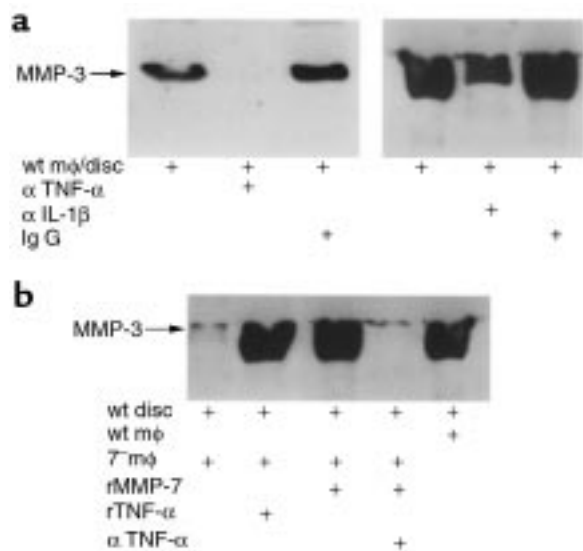


Figure 4 Role of MMP-7 in TNF- α induction of MMP-3. MMP-3 in the conditioned medium from 2-day cocultures of discs from wild-type mice (wt disc) and macrophages from wt (wt m ϕ) or MMP-7-null (7-m ϕ) mice was analyzed by Western blotting. (a) Anti-murine TNF- α antibody (α TNF- α , 1 ng/mL), anti-murine IL-1 β (α IL-1 β , 2 μ g/mL) or control Ig G (1 ng/mL) was added to the cultures as indicated. (b) Recombinant murine TNF- α (rTNF α , 1 ng/mL), anti-murine TNF- α antibody (α TNF- α , 1 ng/mL), or recombinant MMP-7 (rMMP-7, 1 ng/mL) was added to the medium. Arrow indicates the migration of recombinant rat MMP-3.

mediate transitional zone, and the inner nucleus pulposus layers (Figure 2). In contrast, the majority of the MMP-7^{-/-} macrophages remained on the outside of the disc, with no infiltration into the transitional zone or nucleus pulposus and a 96% reduction in the number of cells that penetrated the annulus fibrosus. These results suggested a potential role for MMP-7 in macrophage migration and/or infiltration into disc cartilage.

To examine the migratory ability of MMP-7-null macrophages, we used a modified Boyden chamber assay to determine the percentage of cells capable of migrating through 8- μ m pores to the distant side of a polycarbonate filter. Wild-type macrophages showed very little migratory ability toward serum-free DMEM with BSA, but showed an 8-fold increase in the number of cells migrating toward medium containing 10% FBS (Figure 3a). MMP-7-null and MMP-3-null macrophages showed no statistical difference in their migratory ability compared with wild-type macrophages (5.3-fold and 5.7-fold, respectively, Figure 3a). We have previously demonstrated that macrophage/disc cocultures produce a potent macrophage chemoattractant (13). Similar to the results obtained with FBS, there was no difference in the ability of wild-type, MMP-3-null, and MMP-7-null macrophages to respond to the chemoattractant activity produced by conditioned medium from macrophage/disc cocultures (Figure 3b).

Macrophage MMP-7 is required for the release of TNF- α and

induction of chondrocytic MMP-3. The inability of MMP-7-null macrophages to infiltrate disc tissue was reminiscent of results observed previously with wild-type macrophages and MMP-3-null discs. Using an identical experimental design, the infiltration of macrophages into cocultured disc tissue was effectively inhibited when the disc chondrocytes were unable to produce MMP-3 (13). We have demonstrated that an important role of chondrocytic MMP-3 is to release a macrophage chemoattractant. This observation suggested the possibility that there was an association between macrophage MMP-7 and the release of the MMP-3-dependent macrophage chemoattractant. We demonstrated previously that coculturing with macrophages is required for the induction of chondrocytic MMP-3 (13). To determine if macrophage MMP-7 was required for the induction of MMP-3 in disc cocultures, conditioned medium from wild-type and MMP-7-null macrophages cocultured with wild-type discs was analyzed for MMP-3 protein by Western blotting. Coculturing with wild-type macrophages resulted in the induction of MMP-3 protein, whereas no MMP-3 was observed following coculture with MMP-7-null macrophages (Figure 4b, last 2 lanes). Thus, the production of MMP-7 by macrophages was required for the induction of disc MMP-3 in cocultures. Previous studies indicated that both macrophages and alginate-encased chondrocytes produce MMP-3 mRNA, but the most dramatic effect of coculture on MMP-3 appears to occur in chondrocytes (13).

A potential mechanism for MMP-3 induction by coculture with macrophages is through macrophage-produced inflammatory cytokines. Both TNF- α and IL-1 β have been shown to be produced by activated macrophages (23), and both have been shown to induce MMP-3 (24, 25). To determine if either of these cytokines was required for MMP-3 induction in cocultures, conditioned medium from cocultures of peritoneal macrophages and whole disc tissue in the presence of antibodies that neutralize the activity of murine TNF- α and IL-1 β was collected. MMP-3 expression in coculture media was strongly inhibited by the TNF- α neutralizing antibody but was maintained in the presence of a control IgG (Figure 4a). In contrast, neutralizing antibodies to IL-1 β had a very modest effect on the induction of MMP-3 (Figure 4a). Therefore, we conclude that macrophage TNF- α plays an important role in upregulating MMP-3 expression in our coculture model.

To determine if the inability of MMP-7-null macrophages to upregulate MMP-3 is related to a reduction in macrophage-derived, soluble TNF- α , MMP-7-null macrophages were cocultured with wild-type discs, and the conditioned medium was analyzed for the induction of MMP-3 by Western blotting. As described previously, MMP-7-null macrophages induced no MMP-3, in contrast to the strong induction of MMP-3 protein by wild-type macrophages (Figure 4b). Adding recombinant MMP-7 to the cocultures restored the induction of MMP-3 protein. Interesting-

ly, adding recombinant TNF- α to the coculture gave equivalent induction of MMP-3 protein. Finally, the restoration of MMP-3 production by rMMP-7 was blocked by a neutralizing antibody to TNF- α . We conclude that the MMP-7 production by macrophages was required for the induction of MMP-3 in cocultures and that this effect was mediated by a pathway that involved soluble TNF- α (sTNF- α).

MMPs, including MMP-7, have been shown to be capable of processing TNF- α to its soluble form (26). To determine if MMP-7 had an effect on the release of sTNF- α , TNF- α was measured by ELISA in conditioned medium from cocultures of discs and macrophages isolated from wild-type and MMP-7-null mice. Soluble TNF- α was easily detectable in the conditioned medium of wild-type macrophages/disc cocultures, whereas less than 2% of this amount of TNF- α was present in the conditioned medium of MMP-7-null macrophage cocultures (Figure 5a). Adding recombinant MMP-7 to MMP-7-null macrophage cocultures restored the amount of sTNF- α to a level similar to that seen with the wild-type macrophages, indicating that the TNF- α was being produced and was available on the cell surface but was not released into the conditioned medium in the absence of MMP-7. The effect of MMP-7 on levels of soluble and cell-surface TNF- α was also examined in isolated macrophages alone. Because the ELISA assay recognizes only sTNF- α , cell-surface TNF- α was measured by treating washed macrophages with MMP-7 for 2 hours and determining the amount of sTNF- α produced. Wild-type macrophages produced approximately 1.0 ng/mL of sTNF- α over a 2-hour time period (Figure 5b). This represented the majority of the TNF- α produced, because the addition of recombinant MMP-7 to release available cell-surface TNF- α resulted in only a slight elevation in total sTNF- α levels. The amount of cell-surface, MMP-7-cleavable TNF- α in MMP-7-null macrophages was identical to that in wild-type macrophages, although the majority of this material was cell-surface associated as determined by the lack of sTNF- α in the absence of exogenous MMP-7. This effect was not altered in the presence of the protein synthesis inhibitor cycloheximide, further indicating that the effect of MMP-7 was not on the synthesis of TNF- α but on its release from the cell surface.

Disc resorption requires matrixlysin-dependent release of TNF- α and macrophage infiltration. We demonstrated that macrophage MMP-7 was required for the release of TNF- α and for macrophage infiltration of disc tissue. To determine if either of these events is related to the requirement for MMP-7 for disc resorption, wild-type and MMP-7-null macrophages were cocultured with wild-type discs in the presence of TNF- α or TNF- α -neutralizing antibodies and disc resorption was measured by the loss of wet weight. As demonstrated previously, discs cultured with MMP-7-null macrophages showed no evidence of resorption, whereas wild-type macrophages induced a 48.8 \pm 5.5% loss of wet weight over a 3-day time period

(Figure 6). TNF- α was required for disc resorption as indicated by the absence of resorption in the presence of neutralizing antibodies to TNF- α . The inability of MMP-7-deficient macrophages to induce resorption was rescued by the addition of recombinant TNF- α , indicating that disc resorption required MMP-7-dependent release of TNF- α . To determine if macrophages were required for disc resorption solely as a source of TNF- α or if macrophage infiltration per se was also an important component of disc resorption, rTNF- α was added directly to wild-type discs in the absence of macrophages. Although rTNF- α was sufficient to induce MMP-3 in the discs, it was not sufficient to induce disc resorption as measured by a loss of wet weight (Figure 6, right-hand column; see also ref. 13). Thus, in vitro disc resorption required macrophage infiltration.

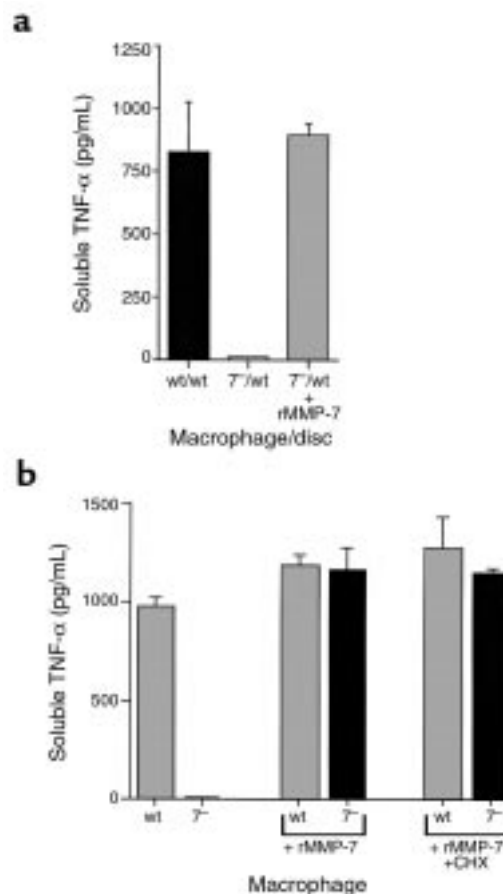


Figure 5 Analysis of soluble and cell-surface TNF- α levels. (a) Forty-eight-hour conditioned medium from cocultures of wild-type discs (/wt) and wild-type (wt/-) or MMP-7-null (7⁻/) macrophages was analyzed for soluble TNF- α levels by ELISA. The 7⁻/wt cocultures were incubated in the absence or presence of recombinant MMP-7 (rMMP-7, 100 ng/mL) for the duration of the coculture period. (b) Two-hour conditioned medium from wild-type (wt) or MMP-7-null (7⁻) macrophages either in the absence (to measure soluble TNF- α) or presence (to measure cell-surface TNF- α) of recombinant MMP-7 (100 ng/mL). Where indicated, 10 μ g/mL cycloheximide (CHX) was added for the duration of the conditioning. Results are mean and SD of duplicate samples from 2 separate experiments.

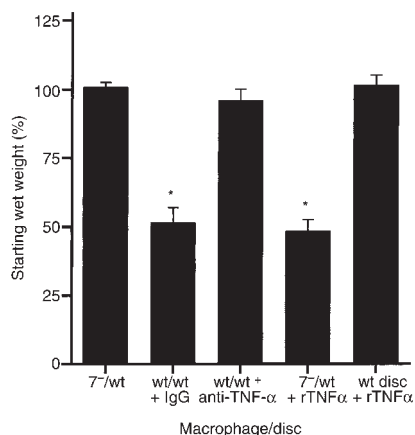


Figure 6 Requirement for TNF- α and macrophage infiltration for disc resorption. Discs from wild-type (/wt) mice were cocultured with macrophages from wild-type (wt/) and MMP-7-null (7-/) mice. Wet weights were determined at day 0 and day 3, and the percent of starting wet weight was calculated for each individual disc. Where indicated, anti-TNF- α (1 ng/mL), a control immunoglobulin (IgG, 1 ng/mL), or recombinant TNF- α (rTNF α , 1 ng/mL) was added to the cocultures. Values presented represent the mean and SD of 3–5 discs/group. *Significant difference from starting wet weight ($P < 0.05$).

Discussion

The natural resorption of HD in patients as monitored by a progressive decrease in disc size correlated directly with the amount of vascularization (5), which was also associated with a prominent infiltration of macrophages (7). We devised an *in vitro* model of HD involving the coculture of murine vertebral discs with peritoneal macrophages and have demonstrated a loss of wet weight and a reduction in proteoglycan staining that mimics HD resorption (13). Using the coculture system and discs or chondrocytes isolated from MMP-null mice, we demonstrated that macrophages stimulated an increase in MMP-3 levels under coculture conditions and that disc MMP-3 was required for resorption, macrophage infiltration, and the generation of a macrophage chemoattractant. We have now extended this work by analyzing the requirement for MMP-3 and MMP-7 produced by activated macrophages in HD resorption using the coculture model.

Macrophage-derived MMP-7, but not MMP-3, was required for disc resorption and macrophage invasion of disc tissue. Metalloproteinases have been implicated previously in macrophage migration and tissue invasion. In particular, metalloelastase (MMP-12) has been shown to be required for macrophage proteolysis and tissue invasion using MMP-12-null mice (27). However, the MMP-7 requirement was specific for disc invasion, because migration in response to either serum or the MMP-3-dependent chondrocytic chemoattractant was not impaired in MMP-7-null macrophages. It is possible that there is a specific role for MMP-7 in macrophages related to a requirement to degrade

matrix material in the discs. However, there is considerable overlap between the known extracellular matrix substrates of MMP-7 and MMP-3 (28), although only the former is required for disc invasion. Although we cannot rule out the possibility that MMP-7 degradation of a specific cartilage substrate contributes to macrophage invasion of disc tissue, we have considerable evidence that the critical role of MMP-7 in macrophages is as a sheddase required for cytokine release and communication with disc chondrocytes.

Our results indicate that the MMP-7 substrate that is ultimately responsible for macrophage invasion and degradation of disc tissue is TNF- α . The presence of TNF- α in macrophage/disc cocultures was required for disc resorption and for the induction of MMP-3 protein as determined by the use of a TNF- α -neutralizing antibody. The cellular source of the TNF- α was primarily the macrophages, because the level of sTNF- α in cocultures was similar to that observed in macrophage cultures alone. However, chondrocytes are known to produce TNF- α (29, 30), and treatment of discs with rMMP-7 resulted in a slight induction of MMP-3 (unpublished data). MMP-7-null macrophages in coculture were deficient in the TNF- α -dependent activities of disc resorption and MMP-3 induction, as well as being incapable of macrophage infiltration. In light of our previous studies indicating an intimate relationship between the release of an MMP-3-dependent chemoattractant from cocultured chondrocytes, macrophage infiltration, and disc resorption, we tested the possibility that MMP-7-mediated release of TNF- α was responsible for MMP-3 induction. The deficiency of MMP-7-null macrophages in inducing MMP-3 protein in cocultures was rescued by the addition of either MMP-7 or TNF- α , and the MMP-7 effect was mediated through a TNF- α -dependent pathway as demonstrated with the neutralizing antibody. Finally, the requirement for macrophage MMP-7 for TNF- α release was shown to be critical for macrophage function in disc resorption by the demonstration that MMP-7-null macrophages supplemented with rTNF- α demonstrate a loss of wet weight equivalent to that observed with wild-type macrophages. We conclude that the release of TNF- α from macrophages is a critical function of macrophage MMP-7 in the coculture model of HD resorption.

The role of MMP-7 in TNF- α release is surprising, for although MMP-7 has been shown to cleave recombinant TNF- α (26), the enzyme identified as the primary TNF- α sheddase is a member of a disintegrin and metalloproteinase (ADAM) family of cell-surface proteins referred to as TNF- α converting enzyme (TACE) (31, 32). TACE activity appears to be unimpaired in MMP-7-null mice, because the levels of plasma sTNF- α are dramatically elevated following *in vivo* administration of LPS (our unpublished data). TACE is not required for MMP-7-mediated shedding of TNF- α , because TACE-deficient monocytes release sTNF- α in response to rMMP-7 (R. Black, personal communication). The

effect of MMP-7 was specific for the release and not the synthesis of TNF- α , because there was no significant effect of MMP-7 on the levels of cell-surface TNF- α in the presence or absence of a protein synthesis inhibitor. TACE and MMP-7 may represent independent mechanisms by which TNF- α is shed from the cell surface, with TACE being involved in inducible release such as in the case of LPS treatment and MMP-7's role in the constitutive release observed in thioglycollate-elaborated peritoneal macrophages. Interestingly, we have recently identified Fas ligand, a member of the TNF- α family of proteins, as a substrate for MMP-7, which is relevant to apoptosis of prostatic epithelial cells during castration-induced involution (33). Thus, specific populations of cells may use MMP-7 to process members of the TNF- α family of cytokines.

A theme that emerges from this work is the complex cross-talk that occurs between different cell types within a tissue and the role MMPs play in this cellular communication (Figure 7). The protrusion of a herniated disc into the spinal column results in contact between inflammatory macrophages and the cartilage tissue. Based on our results, macrophages produce TNF- α and MMP-7, resulting in the release of soluble TNF- α . TNF- α is required for the induction of high levels of MMP-3 in the chondrocytes, which results in the release of a macrophage chemoattractant. The chemoattractant stimulates the infiltration of macrophages, proteoglycan loss, and disc resorption. Our model system does not address the initial phase of neovascularization and macrophage recruitment, and it is not clear if simple contact between circulating cells and disc tissue is sufficient to initiate this cascade of macrophage/chondrocyte interactions. The infiltration of the macrophages is an essential component of disc resorption, and TNF- α induction of MMP-3 activity was not sufficient in itself to induce disc resorption in the absence of macrophages. It is presumed that the abundant proteolytic enzymes associated with differentiated macrophages are responsible for the actual degradation of the cartilage matrix. Neither macrophage MMP-3 nor MMP-7 were required directly for disc resorption, because both MMP-3-null and MMP-7-null macrophages supplemented with exogenous TNF- α efficiently reduced disc wet weight. We speculate that proteases from several proteolytic families act in concert to mediate cartilage destruction during HD resorption.

The contribution of MMPs to processes other than simple matrix destruction has been a recurring theme in recent years, and effects of MMPs on the processing of biological regulators such as growth factors (34), apoptotic factors (26), and cell-surface receptors (35) has been documented. Our studies provide a striking example of the requirement for MMPs in processes such as invasion and connective tissue destruction in which the mechanism of the effect is not directly related to the ability of these enzymes to degrade ECM, but is the result of the processing of bioactive regulatory molecules. Our results further reinforce the notion that

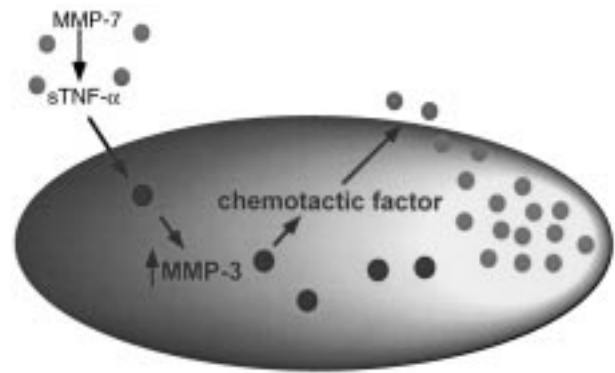


Figure 7

Model of MMP, macrophage, and chondrocyte interactions in HD resorption. Activated macrophages (small gray circles) produced TNF- α , which requires the production of MMP-7 to become soluble (sTNF- α). Soluble TNF- α induces chondrocytes (larger dark circles) to produce MMP-3. MMP-3 production by disc chondrocytes is required for the release of a macrophage chemoattractant and the subsequent macrophage infiltration of the disc. Degradation of the disc (cleared area) is mediated by the infiltration of the macrophages.

the role of MMPs in disease states can extend beyond that of a downstream effector of matrix degradation and contribute a regulatory component to normal and disease processes.

Acknowledgments

The authors thank John Mudgett (Merck Research Laboratories) for MMP-3-null mice, Melodie Henderson for assistance with animal husbandry, and Roy Black (Immunex, Seattle, Washington, USA) for communicating unpublished data and helpful discussions. This work was supported by the Department of Orthopaedics and Rehabilitation, Vanderbilt University, Nashville, Tennessee, USA, and in part by NIH grants R01CA46843 and CA60867 to L.M. Matrisian.

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