Original Article

Localization of cathepsins G and L in spontaneous resorption of intervertebral discs in a rat experimental model

W. Meng[1], K. Yonenobu[2], K. Ariga[1], T. Nakase[1], S. Okuda[3], K. Obata[1], H. Yoshikawa[1]

[1]Department of Orthopaedic Surgery, Osaka University Medical School, Osaka, Japan, [2]Department of Orthopaedic Surgery, Osaka-Minami National Hospital, Osaka, Japan, [3]Department of Orthopaedic Surgery, Osaka Rosai Hospital, Osaka, Japan,

Abstract

To determine the involvement of cathepsins G and L in the mechanism of spontaneous resorption of herniated intervertebral discs, localization of these cathepsins in this process was examined immunohistochemically using a rat model of autologous transplantation of coccygeal discs. Rat coccygeal discs were resected and autotransplanted into the subcutaneous space of the skin of the back. Paraffin-embedded sections of intervertebral disc tissue, harvested at various post-transplantational periods, were immunohistochemically stained with antibodies for cathepsin G, cathepsin L, MMP-1, MMP-3 and ED-2. The number of positive cells was counted in each part of the transplanted discs. Immunolocalization of cathepsins G and L in various types of disc cells was first observed early in the post-transplantation period. From two days after the operation, histology showed invasion by granulation tissue, with many macrophages, in all sections. Subsequently, the number of macrophages in granulation tissue was observed to increase, along with a gradual increase in the percentage of cells positive for MMP-1 and MMP-3. In addition to the ability of cathepsins G and L to degrade major extracellular matrix components of intervertebral discs, cathepsin G is capable of activating latent pro-MMPs. The up-regulation of cathepsins G and L in the intervertebral disc tissue in this spontaneous resorption model suggests that these proteinases may be involved in degradation of extracellular matrix, leading to the natural resorption of herniated discs.

Keywords: Disc Herniation, Natural Resorption, Matrix Metalloproteinase

Introduction

Intervertebral disc herniation is responsible for symptoms in a significant fraction of patients with sciatica and low back pain conditions which affect a great number of people[1-3]. Although disc herniation results in a huge number of operations, reports on the natural course of disc herniation show that the related symptoms are often relieved spontaneously[4,5]. Recently, observation of the spontaneous regression of herniated discs has been made possible by the development of imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI)[6-10]. The abundant extracellular matrix (ECM) of an intervertebral disc must be degraded before spontaneous regression can take place. Reports describing mechanisms of spontaneous resorption of herniated intervertebral discs suggest that matrix-degrading enzymes are involved in the resorption process[11-13].

Matrix metalloproteinases (MMPs), the metzincin family of zinc-dependent proteinases, are the proteinases whose functions in intervertebral discs have been most vigorously investigated, because they are able to degrade major ECM components of intervertebral discs at neutral pH[14,15]. In recent investigations, it was proved that various MMPs, including MMP-1 and MMP-3, are expressed in herniated or degenerated intervertebral discs[12,13,16-19]. In the disc resorption process depending on the interaction between disc cells and macrophages, MMP-3 and MMP-7 are reported to play an essential role in the cascade of degradation of disc matrix[20,21].

In addition to MMPs, serine proteinases and cysteine proteinases have been investigated as possible components of the process of degradation of ECM of intervertebral discs. The presence of serine proteinases, serine proteinase inhibitors and cysteine proteinase inhibitors suggests that expression of inhibitors is involved in the regulation of ECM degradation of intervertebral discs[22-25]. Cathepsin G, a chymotrypsin-like neutral serine protease, and cathepsin L, a cysteine protease, are capable of degrading major ECM

components of intervertebral discs[15,26-31]. Furthermore, cathepsin G, like other serine proteases, can activate several latent pro-MMPs, including MMP-1 and MMP-3[32,33]. Cathepsins G and L have been reported to be involved in the degeneration of intervertebral discs[22,34]. These findings suggest that cathepsins G and L may contribute to the process of natural resorption of herniated intervertebral discs.

The purpose of this study was to clarify the roles of cathepsins G and L in the mechanism of spontaneous resorption of intervertebral discs. Cathepsins G and L were immunolocalized on various types of cells in disc tissue in an experimental rat disc resorption model.

Materials and methods

Animal model

The rat experimental disc resorption model was prepared as previously described, with some modification. Twenty-one Wistar rats (12-week-old males) were anesthetized with intraperitoneal injection of pentobarbital. From each rat, three coccygeal intervertebral discs (6th, 7th and 8th) were resected with caudal endplate cartilage. The discs were then separately transplanted autologously into the subcutaneous space of the skin of the back. Transplanted discs were harvested at 1, 2, 3, 7, 14 and 21 days after the operation. Nine discs, from 3 rats, were resected but not transplanted (day 0 group).

Tissue processing

Tissue specimens were prepared for histological evaluation and immunohistochemistry. All samples were fixed in 10% neutral buffered formalin at room temperature for 24 hours. They were then dehydrated and embedded in paraffin according to the standard method. From the resulting blocks, 3-4 μm-thick paraffin sections were cut, using a microtome, and mounted on L-polylysine-coated slides for hematoxylin and eosin staining and subsequent immunohistochemistry.

Antibodies

Mouse monoclonal antibody against rat macrophage (ED2) was purchased from Biosource (Camarilo, CA, USA), and goat polyclonal antibodies against rat cathepsin G and cathepsin L were purchased from Santa Cruz Biotechnology (Delaware, CA, USA). Rabbit polyclonal antibodies against rat MMP-1 and MMP-3 were purchased from CHEMICOM (Tamecula, CA, USA).

Immunohistochemical staining

Immunohistochemical staining was performed by the streptoavidin-peroxidase technique, using histofine SAB-PO kits (Nichirei, Tokyo, Japan) according to the method recommended by the manufacturer. Briefly, tissue sections were deparaffinized, and rehydrated and placed in 3% H_2O_2 in methanol to block endogenous peroxidase. After washing, the sections were blocked with 10% normal serum from the same species as the secondary antibody (to minimize background staining), followed by incubation with primary antibody under the following conditions: anti-cathepsin G (1:200), 12 hours at 4 °C; anti-cathepsin L (1:50) and anti-MMP-1 (1:50), 1 hour at room temperature; anti-MMP-3 (1:800) and anti-rat macrophage (ED2, 1:100), 18 hours at 4 °C. Normal serum from the same species as the primary antibody was used as a control for the primary antibody. After washing in phosphate buffered saline (PBS, pH 7.2), the sections were incubated with secondary antibody for 20 minutes at room temperature in a humid chamber, and then incubated with peroxidase-conjugated streptoavidin (Nichirei) for 20 minutes at room temperature in a humid chamber and washed in PBS. Substrate reagent (3,3′ diaminobenzidine tetrahydrochloride, Dojindo, Tokyo, Japan) was then added. Counterstaining was performed with hematoxylin, and the sections were then mounted.
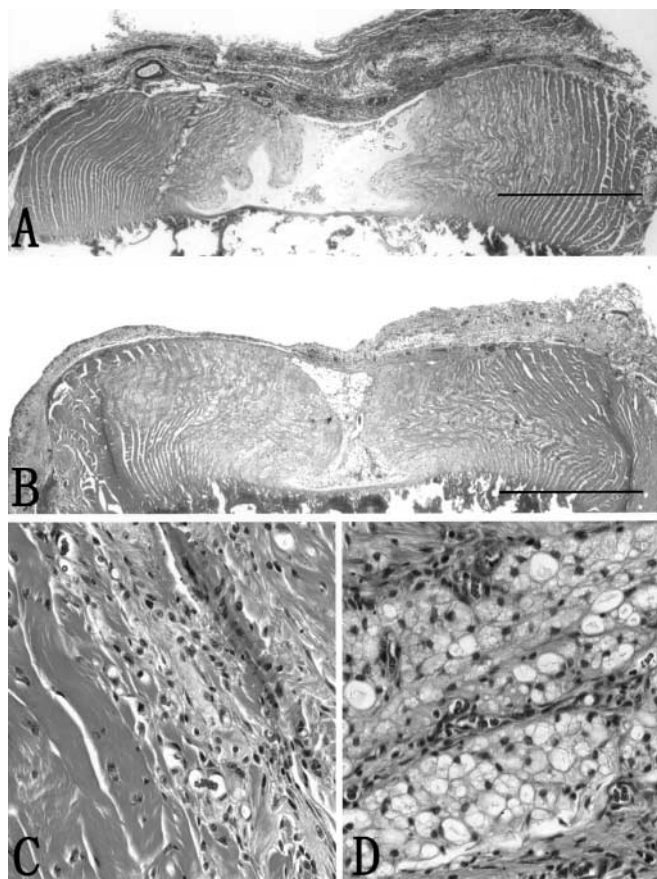

Figure 1. Typical appearance of transplanted discs. Photomicrographs show typical histological patterns of autotransplanted discs. Hematoxylin & eosin staining. (A) A whole disc two days after transplantation (×15). (B) A whole disc at seven days after transplantation (×15). (C) Outer annulus fibrosus at seven days after transplantation (×200). (D) Nucleus pulposus at seven days after transplantation (×200). Scale bars represent 1mm in (A) and (B).
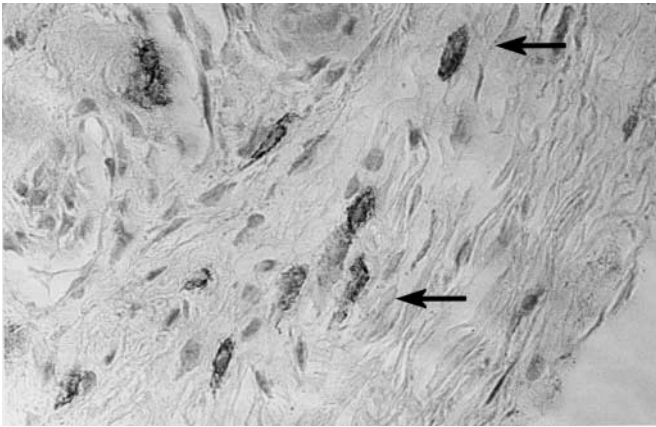
Figure 2. Typical appearance of immunoreactive cells for ED-2. Photomicrograph shows the image obtained from immunohistochemical assay for ED-2. ED-2-positive cells can be seen in the surrounding granulation tissue at 14 days after transplantation (×200). Arrows indicate positive cells.

Figure 3. The number of ED-2-positive macrophages (mean ± standard error) in granulation tissue at each time point. Marked increase began at 7 days after transplantation. * P<0.01.

Cell count

The autotransplanted disc specimen was divided into three parts: annulus fibrosus, nucleus pulposus and surrounding granulation tissue. The total number of cells and the number of cells positive for each antibody in annulus fibrosus and granulation tissue were determined by counting cells in five randomly selected fields under high magnification (×400). The positivity rate for each part was then calculated as the number of positive cells divided by the total number of cells.

Statistical analysis was performed using the unpaired Student's t-test. A 95% confidence interval (p<0.05) was considered statistically significant.

Results

Histological findings.

The first significant observation of granulation tissue and neovascularization surrounding discs occurred at two days after transplantation (Fig. 1A), and the amount of this tissue increased markedly from 7 days post-transplantation (Fig. 1B). In the annulus fibrosus, inflammatory cells were first observed at two days after transplantation, and thereafter the lamellar structure of the annulus fibrosus was gradually lost and replaced with granulation tissue from the outer margin (Fig. 1C). The margin of the nucleus pulposus became unclear at two days after transplantation. Invasion by fibrous granulation tissue and blood vessels caused the nucleus pulposus to become partitioned at 7 days post-transplantation (Fig. 1D), and thereafter the nucleus pulposus was gradually replaced.

Immunohistochemical staining

Most of the ED2-positive macrophages were observed in the surrounding granulation tissue (Fig. 2). They began to appear
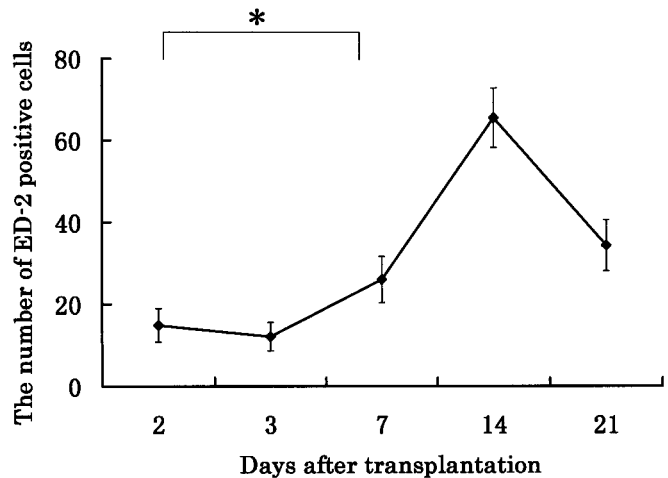
at two days post-transplantation, and the number of positive cells increased gradually until two weeks post-transplantation (Fig. 3). A marked increase in ED2-positive cells was first observed at 7 days after transplantation. Cells positive for other antibodies were localized in the annulus fibrosus, nucleus pulposus and granulation tissue (Fig. 4A-D).

In the annulus fibrosus, many disc fibrochondrocytes began to exhibit positive staining for cathepsin G at one day post-transplantation. In this period, invasion by macrophages (Fig. 3) and expression of other proteinases were not marked (Fig. 5). The positivity rate for cathepsin G decreased gradually thereafter. Marked expression of cathepsin L was first observed at two days post-transplantation, and the positivity rate decreased gradually thereafter. These two cathepsins reached their peak positivity rates before marked infiltration by macrophages. Significant up-regulation of MMP-1 and MMP-3 in fibro-
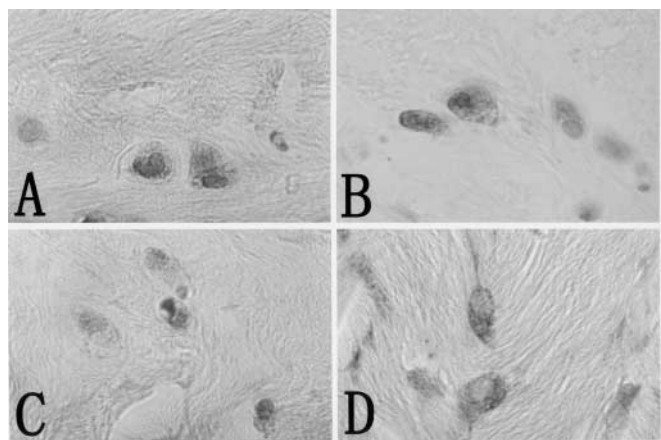

Figure 4. Typical appearance of immunoreactive cells for proteinases. Photomicrographs show the image obtained from immunohistochemical assay for proteinases in annulus fibrosus (×200). (A) Cathepsin G-positive cells. (B) Cathepsin L-positive cells. (C) MMP-1-positive cells. (D) MMP-3-positive cells.

chondrocytes of the annulus fibrosus was first seen two days after the operation, and expression gradually increased thereafter (Fig. 5B). A marked increase in the rate of positivity for MMPs was observed at 1 week after transplantation. Although the MMP-3-positivity rate continued to increase, the MMP-1-positivity rate decreased thereafter. The patterns of change in expression of MMP-1 and MMP-3 were similar to the pattern of change in macrophage infiltration.

In granulation tissue, expression of cathepsins G and L, MMP-1 and MMP-3 was also observed in inflammatory cells and fibroblast-like cells. Expression of cathepsin G, MMP-1 and MMP-3 was first observed at two days post-transplantation, and increased until 7 or 14 days after transplantation (Fig. 6). These patterns of increase were similar to the pattern of change in macrophage infiltration. The majority of cells in the granulation tissue showed positive staining for cathepsin L from two to three days post-transplantation, and the percentage of positive cells decreased gradually thereafter.

Discussion

In this study, we found marked up-regulation of cathepsins G and L in autotransplanted intervertebral discs in the early post-transplantion period in a rat spontaneous disc resorption model. Marked increases in invasion by macrophages and expression of MMP-1 and MMP-3 followed marked expression of the cathepsins. Type I and II collagen and aggrecan are major components of the matrix of intervertebral discs[37,38]. The proteinases evaluated in this study are capable of degrading these matrix proteins[15,26,27,29,30]. Therefore, our results provide direct evidence of the involvement of cathepsins G and L, MMP-1 and MMP-3 in the spontaneous disc resorption process, and they suggest that these proteinases function as matrix-degrading enzymes. In the present study, cathepsins G and L were abundant in various types of cells in intervertebral discs which were eventually resorbed.

Previous studies suggested that MMPs are responsible for ECM degradation involved in degeneration of intervertebral discs and the spontaneous resorption process[12,13,16-21]. In the disc resorption process, infiltration by inflammatory cells such as macrophages is thought to be essential. The macrophage-dependent disc resorption process was analyzed in detail in previous studies[20,21]. MMP-3 and MMP-7 are reportedly essential factors in macrophage-dependent spontaneous disc resorption[20,21]. TNF-α derived from macrophages was also proved to be essential in secretion of MMP-3 by disc cells[21]. In the present study, MMP-3 expression increased with increasing infiltration by macrophages, a result consistent with those of previous reports. The roles played by MMPs in the
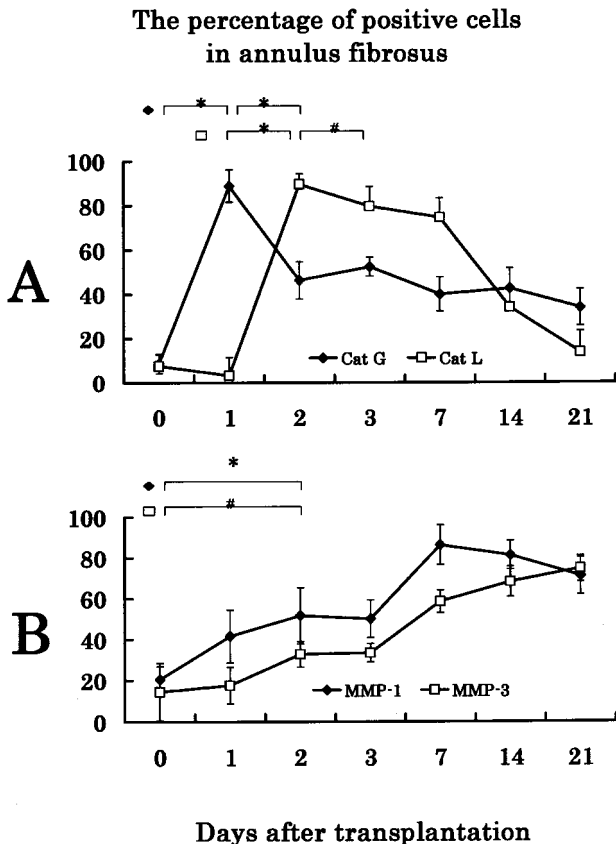

Figure 5. Percentage of cells positive for each proteinase (mean ± standard error) in annulus fibrosus at each time point (by immunohistochemical assay). (A) Cathepsins G and L. (B) MMP-1 and MMP-3. # P<0.05, * P<0.01.
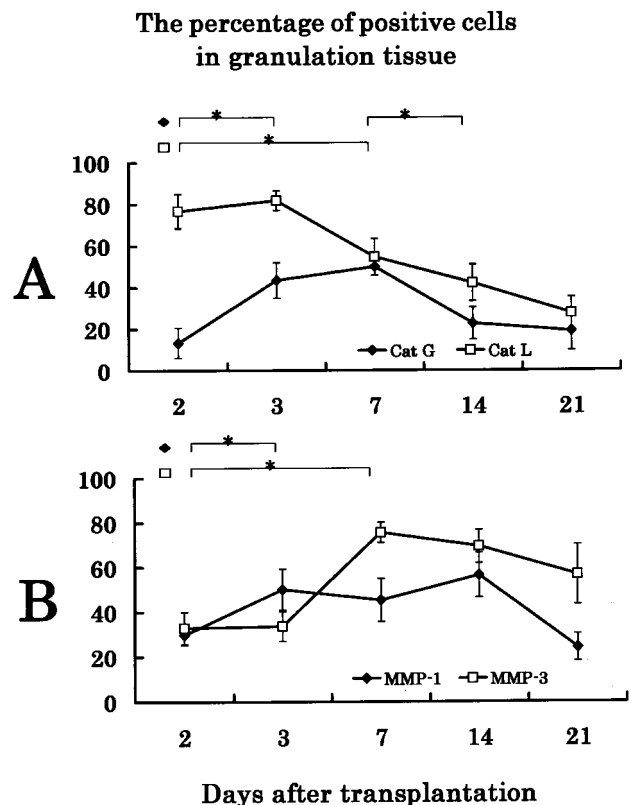

Figure 6. Percentage of cells positive for each proteinase (mean ± standard error) in granulation tissue at each time point (by immunohistochemical assay). (A) Cathepsins G and L. (B) MMP-1 and MMP-3. *P<0.01.

present model may be similar to their roles in the co-culture model[20]. However, these MMPs are synthesized and released as latent proenzymes. Thus, studies of the mechanism of activation of pro-MMPs can provide insights into important aspects of regulatory systems involved in ECM degradation. Latent pro-MMPs reportedly can be proteolytically activated by serine proteinases[39-41]. In addition to its direct activity in degradation of the ECM, cathepsin G is able to activate pro-MMPs in its role as a serine proteinase, and can also degrade tissue inhibitors of metalloproteinase (TIMPs), endogenous inhibitors of MMPs reported to play important roles in regulation of MMPs[32,33,42]. In the present study, the fact that localization of cathepsin G was similar to that of MMP-1 and MMP-3 suggests that cathepsin G might contribute to activation of these MMPs and inactivation of TIMPs, leading to activation of the macrophage-dependent ECM degradation pathway.

Marked expression of cathepsins G and L was first observed early in the post-transplantation period (1 ~ 2 days), but marked infiltration by macrophages occurred later (2 days ~). Unlike MMPs, which exhibit macrophage-dependent or -related-up-regulation, cathepsins seemed to be produced by disc cells independent of macrophage infiltration. In the previous report using the same resorption model as in the present study, the expression of MCP-1 (a chemokine which works as a macrophage chemoattractant) was observed in disc cells early in the post-transplantation period, a pattern similar to that which we observed for cathepsins in the present study[35]. These results suggest that a self-absorption system in disc cells was activated before the start of the macrophage-related resorption process. This system may stimulate macrophage infiltration, and may activate pro-MMPs secreted as a result of induction by macrophages.

Conclusion

Cathepsins G and L were found to be localized in intervertebral disc tissue in a spontaneous resorption model. This finding is the first direct evidence of the involvement of these proteinases in spontaneous resorption of intervertebral discs.

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