

# Cartilage Destruction Is Partly Induced by the Internal Proteolytic Enzymes and Apoptotic Phenomenon of Chondrocytes in Relapsing Polychondritis

NAOHISA OUCHI, MIWA UZUKI, AKIHISA KAMATAKI, YASUHIRO MIURA, and TAKASHI SAWAI

**ABSTRACT. Objective.** We analyzed 9 cases by immunohistochemical studies in order to elucidate the mechanisms of cartilage destruction in relapsing polychondritis (RP), which often involves the external auricle and respiratory tract through immunological disorder.

**Methods.** Cartilage tissues were obtained during surgical operations. Cell species in the granulation tissues, especially near the cartilage, were identified by cell-surface markers [CD3, CD4, CD8, CD20, CD45 (LCA), and CD68]. The proteolytic enzymes expressed in the cells in the perichondral granulation and in chondrocytes themselves were analyzed by immunohistochemical studies using anti-matrix metalloproteinase (MMP) -1, -3, -8, -9, and -13, and cathepsin D, K, L, and elastase antibodies. Apoptosis and nitric oxide (NO), an apoptosis-related factor, were also examined using ApopTag and antinitrotyrosine antibody, respectively.

**Results.** Among cell species that infiltrated in perichondral granulation, LCA, CD68 (monocytes/macrophages), and CD4 cells were dominant in number; MMP-8, MMP-9, and elastase were expressed only in the perichondral granulation; whereas MMP-3 and cathepsin K and L were detected in both chondrocytes and granulations. Out of 9 cases examined, 6 revealed apoptotic cells in excess of 50% of chondrocytes. There was a strong correlation between the number of apoptotic cells and the number of MMP-3-positive ( $r = 0.83$ ) and cathepsin K-positive cells ( $r = 0.92$ ). Abundant NO-expressing cells were observed in the chondrocytes in degenerated cartilage, similar to apoptosis.

**Conclusion.** Cartilage destruction in polychondritis is induced not only by perichondral inflammation, but also by intrinsic factors expressed in chondrocytes themselves, including certain kinds of proteolytic enzymes and apoptosis. (J Rheumatol First Release Jan 15 2011; doi:10.3899/jrheum.101044)

## Key Indexing Terms:

RELAPSING POLYCHONDRITIS      CARTILAGE      GRANULATION      APOPTOSIS

Relapsing polychondritis (RP) is a rare disease characterized by inflammation of the systemic cartilage tissues and a pattern of repeated remission and recurrence. The chondritis appears in the external auricle, the nose, the respiratory tract, and joints<sup>1</sup>. In addition, inflammation also involves organs consisting of certain kinds of proteoglycans such as the eye<sup>2</sup>, heart<sup>3</sup>, and inner ear<sup>4</sup>. Among these, the most common clinical manifestation is auricular chondritis<sup>5</sup>. Subsequent loss of the auricular cartilage may result in a floppy appearance or cauliflower ear. The most serious complication of RP is involvement of the cartilage of the respi-

ratory tract<sup>6</sup>. In severe cases, there may be extensive inflammation and edema of the laryngeal cartilage and tracheal cartilage, causing narrowing of the airways<sup>7</sup>. The 5- and 10-year probabilities for survival after diagnosis of relapsing polychondritis are 74% and 55%, respectively<sup>8</sup>. The most widely used diagnostic criteria are those established by McAdam, *et al* in 1976<sup>1</sup> and modified by Damiani, *et al* in 1979<sup>9</sup>. The pathogenesis of RP is unknown, but it has been suggested that it is a form of autoimmune disease<sup>10</sup>. Approximately 30% of patients with RP exhibit complications characteristic of autoimmune disease, including rheumatoid arthritis (RA)<sup>11</sup>, systemic lupus erythematosus<sup>12</sup>, anticardiolipin antibody syndrome<sup>13</sup>, and Sjögren's syndrome<sup>14</sup>. Type II, IX, and XI collagens and matrilin-1, a cartilage matrix protein, were suggested as antigens<sup>15,16,17</sup>. Cells responsible for cartilage destruction in ear lesions are neutrophils, lymphocytes, monocytes/macrophages, and plasma cells in perichondral granulation. These infiltrate the tissues adjacent to the cartilage and cause diffuse basophilic loss suggesting depletion of proteoglycan from cartilage. However, precise cytochemical analysis of cells involved in the destruction of cartilage in RP has not been reported.

From the Department of Pathology and Department of Orthopaedic Surgery, Iwate Medical University, Morioka; and Department of Pathology, Toho University, Ota-ku, Japan.

N. Ouchi, MD, PhD, Assistant Professor, Department of Pathology, Department of Orthopaedic Surgery, School of Medicine, Iwate Medical University; M. Uzuki, MD, PhD, Assistant Professor, Department of Pathology, Toho University; A. Kamataki, PhD, Assistant Professor; Y. Miura, MD, PhD, Assistant Professor; T. Sawai, MD, PhD, Professor, Department of Pathology, School of Medicine, Iwate Medical University.

Address correspondence to Prof. T. Sawai, Department of Pathology, School of Medicine, Iwate Medical University, Uchimarui 19-1, Morioka, Iwate 020-8505, Japan. E-mail: tsawai@iwate-med.ac.jp

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In order to elucidate the mechanisms of cartilage destruction in polycondritis, we carried out immunohistochemical analysis of the inflammatory cell species comprising granulation tissues, especially near the cartilage, and of the expression of proteolytic enzymes contained in cells in perichondral granulation and in chondrocytes themselves. We also examined the expression of apoptosis of chondrocytes, recently suggested as an important factor in cartilage destruction, by TUNEL assay (terminal deoxynucleotide transferase-mediated dUTP nick end-labeling).

## MATERIALS AND METHODS

**Samples.** Cartilage tissues of 7 external auricles and 2 respiratory tracts were obtained from 8 patients with RP as defined by the McAdam or Damiani-modified criteria. Tissues were taken with the consent of the patients. The tissues were fixed in 4% paraformaldehyde in phosphate buffered saline solution (PBS) for 24 h, and then embedded in paraffin. Sections 3- $\mu$ m thick were prepared and stored until use.

**Hematoxylin-eosin stain and toluidine blue.** Two sections were subjected to hematoxylin-eosin (HE) staining and toluidine blue staining for ordinary observation and demonstration of proteoglycan depletion from cartilage.

**Immunohistochemistry and histochemistry.** Immunohistochemical study was performed using paraffin sections. Antibodies used were as follows: monoclonal antibodies to CD3 (Nichirei, Tokyo, Japan), CD4 (Nichirei), CD8 (Nichirei), CD20 (Nichirei), CD68 (Dako, Glostrup, Denmark), LCA (Nichirei), MMP-1 (Kanebo, Tokyo, Japan), MMP-8 (Fuji Chemical, Tokyo, Japan), and MMP-9 (Kanebo); polyclonal antibodies to MMP-3 (Kirkinenn, *et al*) and elastase (Leica Microsystems, Wetzlar, Germany) produced in sheep, and to MMP-13 (Fuji Chemical), cathepsin D (Leica Microsystems), cathepsin L (Santa Cruz Biotechnology, Santa Cruz, CA, USA), nitrotyrosine (Upstate Biotechnology, Lake Placid, NY, USA), IgG (Dako) and IgM (Dako) produced in rabbit, and goat anti-cathepsin K (Santa Cruz Biotechnology). Nitric oxide (NO)-related apoptosis was demonstrated by immunohistochemistry using antinitrotyrosine antibody. Immunohistochemical procedures were as follows. After deparaffinization in xylene and in 100% ethanol, sections were treated with 1% hydrogen peroxide in methanol for 30 min, followed by rinsing with PBS for 15 min. They were covered with 10% normal rabbit serum (CD3, CD4, CD8, CD20, CD68, LCA, MMP-8, and elastase), 10% normal horse serum (MMP-1, MMP-9, and cathepsin K), and 10% normal goat serum (MMP-13, cathepsin L, IgG and IgM) in PBS for 30 min at room temperature. The excess normal serum was removed and the sections were covered with the primary antibodies overnight at 4°C. After 24 h the sections were rinsed with PBS and covered with the secondary antibodies for 30 min at room temperature. The sections were then covered with avidin-biotin-antiperoxidase (MMP-1, -3, -9, cathepsin K, and elastase) or streptavidin-biotin-antiperoxidase (CD3, CD4, CD8, CD20, CD68, LCA, IgG, IgM, MMP-8, MMP-13, cathepsin D and cathepsin L) for 30 min at room temperature and rinsed in PBS. Antigenic sites on sections were exposed to 3-3' diaminobenzidine tetrahydrochloride and counterstained with methyl green. The number of inflammatory cells positive for each CD antigen was counted per 400-mm<sup>2</sup> area in 20 microscopic fields of perichondral regions. The positive cells of each proteolytic enzyme were also counted and compared by number. NO was demonstrated by application of the microwave method using rabbit antinitrotyrosine antibody. NO-related apoptosis was demonstrated by immunohistochemistry using antinitrotyrosine antibody.

**TUNEL assay.** The sections were deparaffinized in xylene followed by 100% ethanol, then permeated with 20  $\mu$ g/ml of proteinase K for 15 min, and incubated with terminal deoxynucleotidyl transferase (TdT) enzyme and reaction buffer (ApoTag kit, InterGen, Purchase, NY, USA) for 60 min at 37°C. They were then covered with antidigoxigenin peroxidase for

30 min at room temperature, exposed to 3-3' diaminobenzidine tetrahydrochloride, and counterstained with methyl green.

The number of chondrocytes undergoing apoptosis in 400-mm<sup>2</sup> areas in 20 randomly-chosen fields was counted, and the mean number of positive cells per field was calculated. The relationship between the number of apoptotic cells and positive cells expressing proteolytic enzymes such as MMP-3 and cathepsin K was then examined.

## RESULTS

**Patients.** Out of 8 cases meeting the McAdam or Damiani-modified criteria for polycondritis, 4 were male and 4 were female, ranging in age from 34 to 80 years, with an average age of 65.1 years.

**Histopathology of cartilage and granulation around the cartilage.** The histopathology was highly characteristic of relapsing polycondritis. Acidophilic (pink) coloration of the cartilage matrix with proteoglycan-depleted cartilage [in contrast to the basophilic (blue) hue of normal cartilage] was seen by routine hematoxylin and eosin staining (Figure 1a). Focal or diffuse infiltration by predominantly mononuclear inflammatory cells, with occasional polymorphonuclear leukocytes and plasma cells in the perichondral tissues, was associated with dissolution of cartilage from its periphery inward (Figure 1b). Fibroblastic granulation tissue frequently coexists and leads to partial sequestration of the cartilage matrix. There was no evidence of necrotizing angitis or thrombosis.

**Cell features.** Immunohistochemical staining revealed that LCA-positive cells including macrophages/monocytes, granulocytes, and lymphocytes infiltrated diffusely in the granulation tissue around the cartilage. Among the inflammatory cells, LCA-, CD68-, CD3-, and CD4-positive cells were dominant. Related to CD3-positive T lymphocytes, CD4-positive cells comprising lymphocytes (helper) and histiocytic cells increased significantly, while CD8-positive (cytotoxic) lymphocytes were less abundant (Figures 2 and 3).

**Expression of proteolytic enzymes in perichondral granulation and cartilage.** MMP-1 was present in perichondral granulation tissue and less so in chondrocytes. MMP-3 was diffusely expressed from pericellular regions to matrix in cartilage, but not so conspicuous in perichondral granulation. MMP-8 was only present in granulocytes in perichondral granulation. MMP-9 was present in granulocytes and spindle cells in perichondral granulation, and was not present in cartilage. Limited presence of MMP-13 was noted both in chondrocytes and in perichondral granulation. Cathepsins D, K, and in particular L were expressed both in chondrocytes and in perichondral granulation. Elastase was present only in perichondral granulation tissue. From these expression patterns, proteolytic enzymes expressed in RP were divided into 3 groups: the first group of enzymes such as MMP-9, MMP-8, and elastase was expressed only in the perichondral granulation and was not seen in cartilage; the second group was moderately present in both chondrocytes and perichondral granulation; the

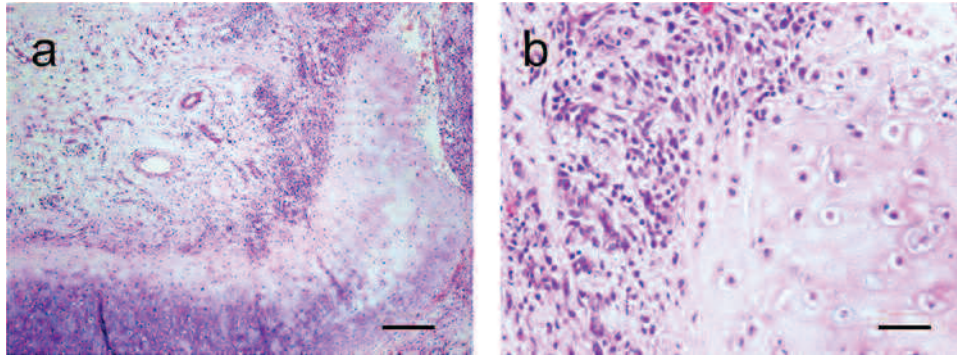


Figure 1. Histological feature of polychondritis of the left auricle. Granulation tissues coexist with partial sequestration of cartilage matrix (a; H&E stain, original magnification  $\times 6.6$ ). Acidophilic (pink) coloration of degenerated cartilage (b). Infiltration of inflammatory cells composed of mononuclear inflammatory cells with occasional polymorphonuclear leukocytes and plasma cells in perichondral tissues (H&E stain, original magnification  $\times 33$ ).

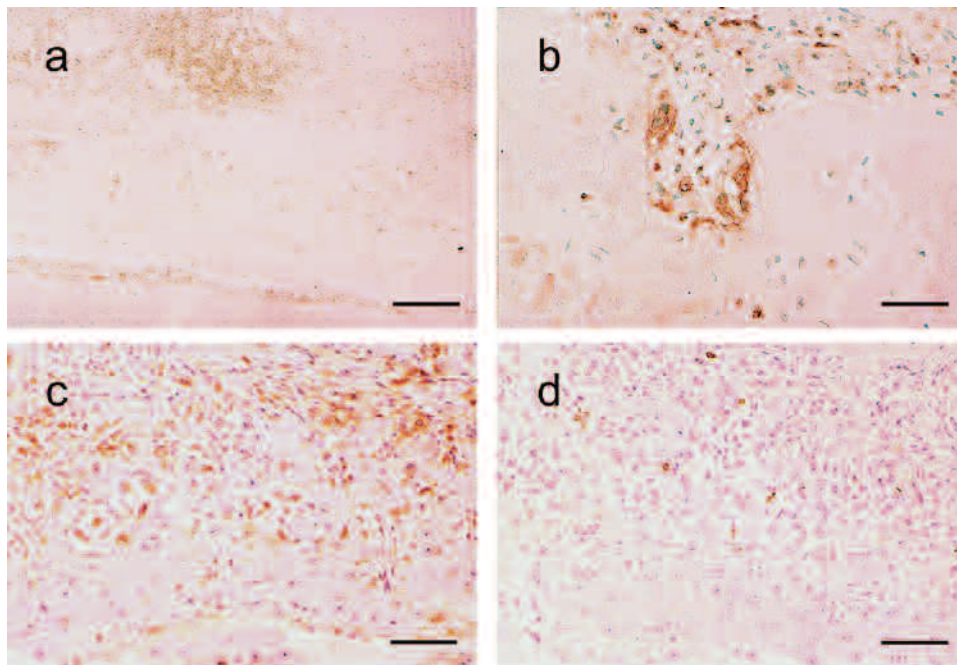


Figure 2. Panel of inflammatory cells in perichondritis, in the perichondral region, demonstrated by immunohistochemistry. LCA-positive (a), CD68-positive (b), and CD4-positive cells (c) were abundant compared with CD8+ and CD20+ (d). (Immunohistochemistry; a, original magnification  $\times 6.6$ ; b, c, d  $\times 13.2$ .)

third group comprised enzymes such as cathepsins K and L, which were expressed abundantly in chondrocytes, although detected both in cartilage and in perichondral regions (Figure 4). The ratio of MMP and other proteolytic enzymes in and around the deteriorated cartilage is shown in Figure 5.

*Expression of apoptotic phenomenon in chondrocytes.* Out of 9 cases in which the expression of apoptosis in chondrocytes was examined by TUNEL assay, 6 cases showed a significant apoptotic phenomenon in polychondritis (Figure 6). The ratio of TUNEL-positive cells to all chondrocytes

counted was from 15% to 98%, with a mean of 61.7% (Table 1). Positive cells were more prominent in the peripheral region than in the inner region of deteriorating cartilage.

*Correlation between the apoptotic phenomenon and expression of proteolytic enzymes.* There was a significant positive correlation between the number of apoptotic cells and MMP-3 ( $r = 0.86$ ) and cathepsin K ( $r = 0.92$ ), respectively, in cartilage (Figures 7 and 8).

*Correlation between the apoptotic phenomenon and expression of nitric oxide.* NO was present in many chondrocytes in deteriorated cartilage (Figure 9).

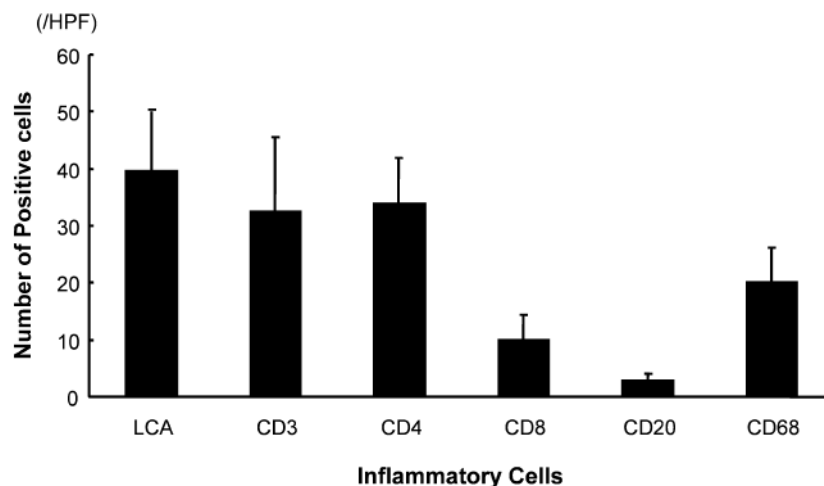


Figure 3. Comparison of inflammatory cells in perichondral granulation tissue. CD68+ and CD4+ cells were dominant compared with CD20+ and CD8+ cells.

## DISCUSSION

Relapsing polychondritis is complicated by various diseases, in particular by autoimmune diseases such as arthritis<sup>18</sup>, vasculitis<sup>19</sup>, conjunctivitis<sup>20</sup>, cardiac valvular disorder<sup>21</sup>, and certain autoimmune-associated diseases such as myelodysplastic disease<sup>22</sup>. The etiology of RP is still unknown, but hypergammaglobulinemia, especially anti-type II, type IX<sup>15,16</sup>, and matrilin-1 antibodies<sup>17</sup>, has been reported in some patients. IgG concentration in our cases was fairly high, from 2394 to 2930 mg/dl (mean 2826.5 ± 129.2 mg/dl). However, only one case showed slight elevation of anti-type II collagen antibody. It was reported that the titer of anti-type II collagen antibody was high only at disease onset and not at the time of disease flare<sup>23</sup>. We did not detect any relationship between the immunoglobulin deposits in the inflamed tissues and cartilage destruction (data not shown), which may partly account for the duration of illness and the inflammatory stage.

Immunohistochemical staining showed many LCA-positive cells infiltrated in the granulation around the cartilage. In addition, CD68-positive cells were significantly increased. In more distant surrounding tissues, CD3-positive cells, CD4-positive lymphocytes (helper), and spindle cells were significantly increased, while CD8- and CD20-positive cells and cytotoxic lymphocytes were not found in large numbers (Figure 3). Compared with the synovial tissues of RA, LCA- and CD68-positive cells were similarly prominent, and CD4- and CD8-positive cells less prominent. In RA synovial tissues, CD68- and CD4-positive cells, including spindle-shaped histiocytic cells, are the major cellular components associated with the cartilage and bone destruction, together with chondroclasts and osteoclasts, and express MMP-9 and cathepsin D, K, and L. Therefore, it is suggested that similar cells in RP may function in cartilage

and bone destruction. The histological characteristics of cell features differ from the synovial tissues, which are characterized by significant lymphocytic infiltration, but are similar to the granulation tissue called pannus, which plays an important role in destruction of cartilage and bone in RA. The MMP family consists of collagen-degrading enzymes including the collagenases (MMP-1, MMP-8, MMP-13), gelatinase A (MMP-2), gelatinase B (MMP-9), stromelysin (MMP-3), and a membrane-type MMP (MMP-14). Cathepsin D, K, and L and elastase are also able to degrade cartilage in RA and osteoarthritis (OA). We demonstrated that many kinds of cells expressed the various proteolytic enzymes, especially MMP-9 in granulation surrounding cartilage tissue and MMP-3 in both granulation and cartilage. MMP-9 is a major secretion product expressed from stimulated polymorphonuclear leukocytes and macrophages, and the active form of this gelatinase cleaves denatured collagens, fibronectin, elastin, and collagens of types IV, V, VII, and XI and is inhibited by tissue inhibitor of metalloproteinase-1<sup>24</sup>. High levels of MMP-9 have been detected in human rheumatoid synovial fluid, and MMP-9 was remarkably highly expressed in the synoviocytes of the lining layer and in macrophages in inflamed synovial tissue<sup>25</sup>. MMP-9 was considered to have a strong capacity to degrade the extracellular matrix of the articular cartilage in RA<sup>25</sup>. From strong expression of MMP-9 in macrophages and neutrophils within the perichondral granulation in this study, we consider that MMP-9 may participate in the destruction of cartilage not only in RA but also in RP. MMP3 causes deterioration in a wide variety of connective tissues and plasma protein substrates, including proteoglycan; collagen of types IV, V, VII, IX; and denatured type I collagen, laminin, fibronectin, elastin,  $\alpha$ 1-proteinase inhibitor, immunoglobulin, and substance P. In addition, it has a significant function

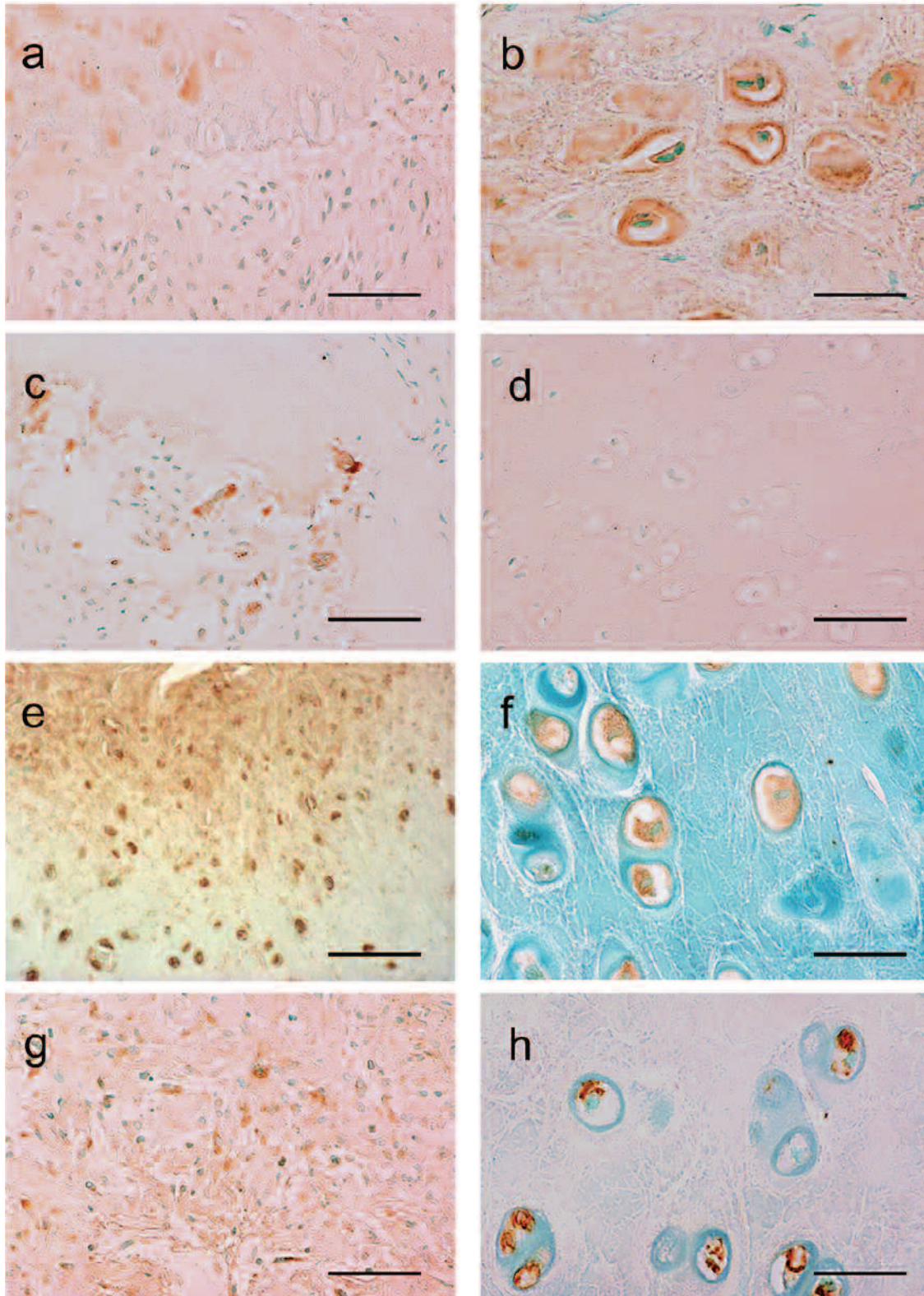
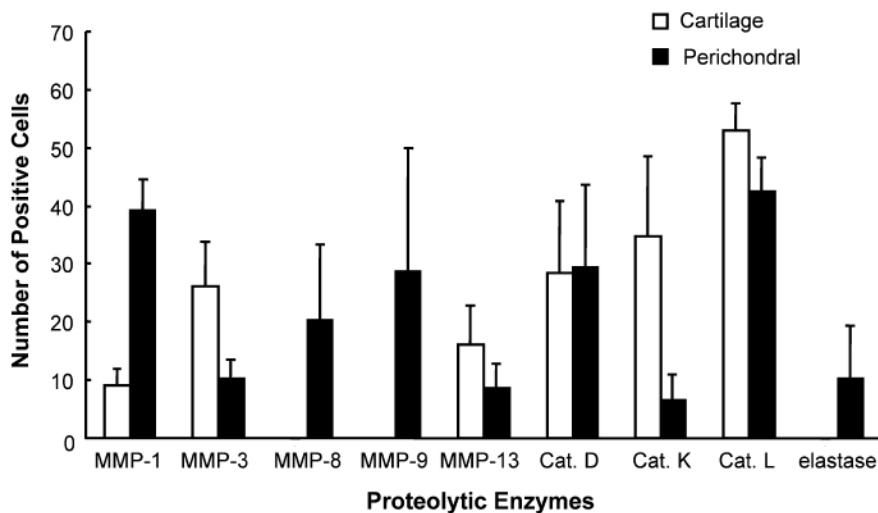


Figure 4. Panel of proteolytic enzymes in cartilage. MMP-3 (a, b), cathepsin K (e, f), and cathepsin L (g, h) were expressed in cytoplasm of chondrocytes and granulation tissues around the cartilage. MMP-9 (c, d), however, was not detected in chondrocytes in any severe inflammatory conditions (b, d). (Immunohistochemistry; a, c, e, g, original magnification  $\times 13.2$ ; d,  $\times 33$ ; b, f, h,  $\times 66$ ).



**Figure 5.** Comparison of proteolytic enzymes in cartilage and perichondral granulation demonstrated by immunohistochemistry. MMP-1, 8, and 9 and cathepsins D, K, and L were abundantly expressed in the perichondral region, whereas MMP-3 and cathepsin K and L were prominent in cartilage. Among proteolytic enzymes, MMP-9 and MMP-8 were expressed only in inflammatory cells in perichondral granulation and never in chondrocytes.

**Table 1.** Ratio of apoptotic cells to total chondrocytes. Apoptotic chondrocytes as a percentage of total chondrocytes in deteriorated cartilage ranged from 15% to 98% (average 61.7%). Six in 9 cases of relapsing poly-chondritis revealed more than 50% apoptotic cells.

	Case								
	1	2	3	4	5	6	7	8	9
Apoptosis-positive cells (%)	79	89	98	85	52	66	31	40	15

in the multienzyme cascade involved in activating procollagenase. It is well known that MMP-3 is present in RA cartilage and plays a role in joint destruction. We demonstrated that MMP-3 was very strongly expressed in the chondrocytes of deteriorated cartilage in RP. Thus, MMP-3 appears to be one of the potent proteolytic enzymes produced by the chondrocytes themselves in cartilaginous destruction in RP. MMP-1 and cathepsin D were also expressed but less strongly so in the granulation tissue, whereas cathepsin K and cathepsin L were expressed in the chondrocytes.

In this study, the cysteine proteases, cathepsin K and cathepsin L, were the most strongly expressed, together with MMP, and were positive in chondrocytes and perichondral granulation tissue. Cathepsin K cleaves the native type I and type II collagens close to the N-terminus of its triple helix<sup>26</sup>. Cathepsin L shows very high activity among all cysteine proteinases. Cathepsins K and L are abundantly produced from osteoclasts and osteoclast-like cells, and may participate in osteoclast-mediated bone resorption in RA. Our results suggest that cathepsins K and L are involved in cartilage destruction in RP.

There were many kinds of cells that were positive for LCA antibody, but it is difficult to discriminate between cell species and characterization without employing immunohis-

tochemical methods. Neutrophilic granules contain many kinds of proteolytic enzymes such as elastase, cathepsin G, nonspecific collagenase, and lysozyme. Elastase is a basic serine protease with a molecular weight of about 30 kDa<sup>27</sup>; it has an elastolytic activity with a broad substrate specificity, and digests most proteins. We demonstrated immunohistochemically that elastase-positive cells were present in perichondral granulation tissue. There were many cases in which fairly large numbers of neutrophils infiltrated around the degenerated cartilage. In those cases elastase was significantly expressed in the neutrophils within the granulation tissue surrounding the cartilage. Our results indicated that elastase plays a major role in cartilage destruction in the early stage. Therefore, cartilage destruction may proceed in the early stage as well as in the chronic stage.

The TUNEL assay is relatively specific for apoptosis associated with determination of cell death. The apoptotic phenomenon in chondrocytes has been noted in poly-chondritis and OA, well known as a degenerative disease of cartilage<sup>28</sup>. These results suggest cartilage destruction is induced not only by inflammatory perichondral granulation tissue but also by intrinsic factors in chondrocytes. Generally, apoptosis of chondrocytes is a complex process induced by many factors in autoimmunity and other related

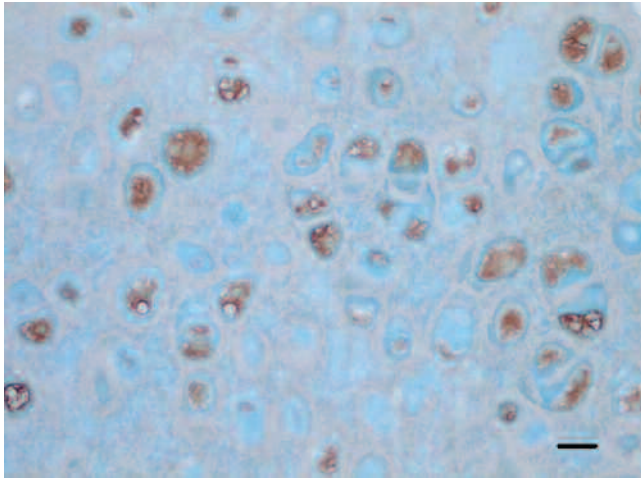


Figure 6. Apoptosis of chondrocytes related to cartilage degeneration demonstrated by TUNEL assay. Many apoptotic cells were detected in RP chondrocytes although the grade of expression differed between cases (ApopTag; original magnification  $\times 33$ ).

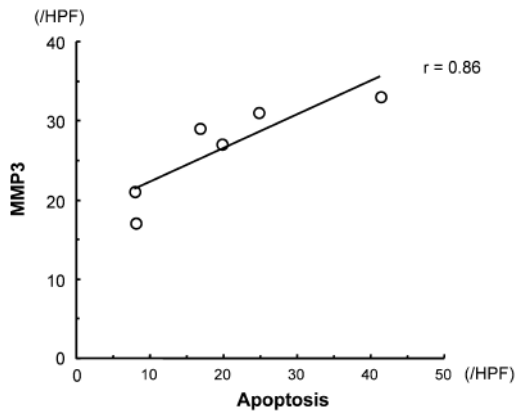


Figure 7. A strong positive correlation was shown between the number of apoptotic cells and MMP-3-positive cells ( $r = 0.86$ ).

diseases such as arteritis, Hashimoto's disease<sup>29</sup>, ulcerative colitis<sup>30</sup>, and myelodysplastic disease<sup>22</sup>. In OA, a large amount of NO serves as a powerful initiator of chondrocyte apoptosis. NO has been implicated in inhibition of neosynthesis of matrix macromolecules of cartilage such as aggrecan through enhancing MMP activity, and in destruction of interleukin 1 (IL-1) receptor antagonist synthesis<sup>31</sup>. Our data revealed a strong positive correlation between numbers of apoptotic cells and numbers of both MMP-3-expressing cells and cathepsin K-expressing cells, suggesting that some interrelationship may be present between apoptosis and the expression of proteolytic enzymes. Experimentally, proteoglycan degradation by ceramide in rabbit cartilage is inhibited by MMP inhibitor<sup>32</sup>, and MMP is induced by IL-1-mediated NO in chondrocytes<sup>33</sup>. Further questions remain concerning the mechanisms of factors leading chondrocytes to apoptosis in RP.

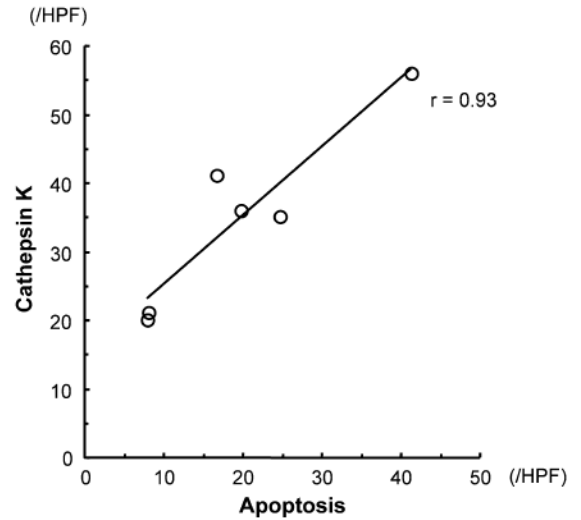


Figure 8. A strong positive correlation was shown between the number of apoptotic cells and cathepsin K-positive cells ( $r = 0.92$ ).

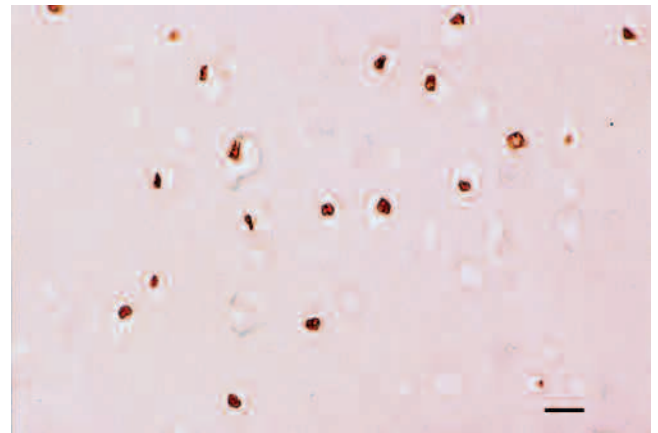


Figure 9. Nitric oxide was expressed in many chondrocytes in deteriorated cartilage. (Immunohistochemistry; original magnification  $\times 33$ ).

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