Expression of Granzyme B in Human Articular Chondrocytes

KIWAMU HORIUCHI, SEIJI SAITO, RYOHEI SASAKI, TAISUKE TOMATSU, and YOSHIAKI TOYAMA

ABSTRACT. ***Objective.*** To investigate the expression of granzyme B (GrB) in normal and rheumatoid arthritis (RA) articular cartilage, and to analyze the relationship between the expression of GrB and apoptotic chondrocytes in RA cartilage.

Methods. Normal cartilage samples were obtained from 9 resected joints and RA cartilage samples were obtained from 12 patients with RA during joint replacement surgery. Cartilage sections were analyzed by immunohistochemistry for the presence of GrB, and the mRNA expression of GrB in chondrocytes was analyzed by *in situ* hybridization and nonquantitative and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of perforin (PFN) was also assessed. Apoptotic chondrocytes were detected using TUNEL staining and their morphology was examined using electron microscopy.

Results. The immunohistochemical analyses revealed GrB and PFN expression in normal chondrocytes and a larger number of GrB and PFN-positive chondrocytes in RA cartilage. *In situ* hybridization and RT-PCR confirmed the expression of GrB and PFN mRNA, and semiquantitative RT-PCR showed elevated concentrations of GrB and PFN expression in RA chondrocytes. The distribution of GrB and PFN-positive cells in the RA cartilage samples was similar to that of apoptotic cells.

Conclusion. GrB and PFN expression is present in normal human articular chondrocytes and elevated in RA chondrocytes. The targets and precise functions of GrB expressed in chondrocytes remain to be determined, but GrB may be involved in the remodeling mechanism of matrix macromolecules and the endogenous degradation of RA cartilage. (J Rheumatol 2003;30:1799–810)

Key Indexing Terms:
GRANZYME B CHONDROCYTE RHEUMATOID ARTHRITIS PERFORIN
CARTILAGE MATRIX REMODELING CARTILAGE DEGRADATION

Granzyme B (GrB) is a serine protease that is highly expressed in cytotoxic lymphocytes, such as cytotoxic T cells (CTL) and natural killer (NK) cells. GrB is stored in the granules of activated CTL and NK cells with a number of other cytotoxins, including the pore-forming protein perforin (PFN)[1,2]. When CTL or NK cells recognize and adhere to virus-infected or malignant cells, these granules migrate to the site of cell contact and release their contents into the intercellular space. Cytotoxins are endocytosed by the target cell, and PFN mediates their release into the cytoplasm by disrupting the endocytic vesicles[3,4]. Once released from the endocytic vesicles into the cytoplasm, GrB initiates apoptosis by cleaving caspases[5,6] and Bid, which is a Bcl-2 family member[7-10]. GrB also has substrates in the nucleus[11,12] and is necessary for the rapid fragmentation of target-cell DNA[13].

Several observations suggest that GrB is involved in joint inflammation and destruction in patients with rheumatoid arthritis (RA). Froelich, *et al*[14] and Ronday, *et al*[15] indicated that GrB is capable of degrading the proteoglycan component of cartilage *in vitro*. Further, a significantly higher number of GrB-positive cells (mainly NK cells) were found in RA synovial tissue than in the synovial tissue of patients with osteoarthritis (OA)[16]. Another recent study reported an increased concentration of GrB in the synovial fluid of patients with RA, compared with concentrations in patients with OA and reactive arthritis[17,18]. The concentrations of GrB in the synovial fluid were significantly higher than those in corresponding plasma samples, indicating that GrB is locally produced within the inflamed joint. These observations in synovial fluid correspond with those in rheumatoid synovial tissue, where the presence of GrB has also been reported, and suggest that GrB acts directly on the cartilage matrix, thereby contributing to the destructive process. Further, Ronday, *et al* demonstrated the presence of GrB at the invasive front of the pannus tissue in RA joints[15], where the cartilage-pannus junction is the site of severe cartilage invasion and destruction by enzymes, free oxygen radicals, and nitric oxide[19-21]. These studies indicate that

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exogenous GrB, which is mainly produced by NK cells, is involved in the destruction of articular cartilage in RA joints via an enzymatic mechanism.

Recently, Hirst, *et al* showed that GrB is expressed in human adult testis and placenta[4], although many studies have reported that only cytotoxic lymphocytes are capable of expressing GrB[1-3,5-13,16,22]. The Hirst study also indicated that GrB could exert extracellular effects under physiological conditions and has a PFN-independent role in extracellular matrix remodeling. GrB facilitates the migration of developing germ cells in the testis and is involved in extracellular matrix remodeling during parturition in the placenta. These findings suggest that GrB may be expressed in tissues other than cytotoxic lymphocytes and that it might be involved in processes other than the immune system under physiological conditions.

We investigated GrB expression in normal cartilage and RA cartilage. In addition, we assessed the expression of an apoptosis-related molecule, PFN, and analyzed the relationship between the expression of GrB and apoptotic chondrocytes in RA cartilage. Our results show that GrB and PFN expression is present in normal human articular chondrocytes and elevated in RA chondrocytes. This is the first description of GrB expression in normal and RA chondrocyte, and suggests novel roles for this proteinase in the pathogenesis of RA.

MATERIALS AND METHODS

Patients and materials. Normal human cartilage was obtained from 8 patients who had undergone a hip replacement for a femoral neck fracture and in one patient from a knee joint amputated for distal ischemia. No patient had any history of joint disease. Human RA cartilage was obtained from the knees of 12 patients during joint replacement surgery. All 12 patients satisfied the 1987 American College of Rheumatology criteria for RA[23]. Informed consent was obtained from each patient prior to surgery.

Immunohistochemistry. The polyclonal antibody to human GrB (C-19) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Monoclonal antibodies for human GrB (2C5 and 18.1) were purchased from Santa Cruz Biotechnologies and Pharma Cell (Paris, France). The monoclonal antibody for human PFN was purchased from Calbiochem (San Diego, CA, USA).

Cartilage was fixed in 10% paraformaldehyde and embedded in paraffin. Sections (5 µm) were pretreated with bovine testicular hyaluronidase (0.5 mg/ml) for 30 min at 37°C, and endogenous hydrogen peroxidase activity was quenched in 0.3% hydrogen peroxide. After 30 min blocking in 5% normal goat and rabbit serum, the sections were incubated for 14 h at 4°C with anti-GrB antibody (C-19 at a dilution of 1:90, 18.1 at dilution 1:30, 2C5 at dilution 1:60) and mouse anti-PFN antibody (at dilution 1:40). The presence and distribution of GrB was determined by 2 methods: (1) A biotinylated rabbit anti-goat IgG (Chemicon, Temecula, CA, USA) or a biotinylated goat anti-mouse IgG (Biosource, Camarillo, CA, USA) was used as the secondary antibody. The avidin-biotin-peroxidase complex method (Vector, Burlingame, CA, USA) and diaminobenzidine tetrahydrochloride (DAB) were used to develop the color, followed by hematoxylin counterstaining. (2) The other method was visualization with rhodamine (TRITC). The reaction of GrB antibody was detected with a rhodamine-labeled second antibody (at dilution of 1:40; Dako, Gilustrup, Denmark), each for 2 h.

After a biotinylated goat anti-mouse IgG (Biosource) was used as the secondary antibody, the presence and distribution of PFN was determined using the avidin-biotin-peroxidase complex method, followed by hematoxylin counterstaining. Isotype control sections were prepared under identical conditions replacing the primary GrB and PFN-detecting antibody with a purified normal goat and mouse IgG control antibody.

In situ hybridization. Riboprobes were prepared as follows. Oligonucleotide primers JT300PstI and JT301BamHI were designed to amplify a nucleotide fragment 747b–873b of GrB gene, and were used to screen the human lymph node cDNA library (Clontech, San Jose, CA, USA). The fragment obtained was subcloned into the PstI/BamHI sites of pSPT18 and pSPT19 (Roche). GAPDH gene (119 bp) was also subcloned into the pstI/BamHI sites of pSPT18 and pSPT19 (Roche) and used as positive control. Digoxigenin-labelled RNA probes for both the GrB fragment and GAPDH were generated following the methods outlined in the DIG RNA labeling kit (Roche).

In situ hybridization was performed as follows. Normal human cartilage tissue was preserved in 10% EDTA for 50 days at 4°C, followed by fixation in 4% paraformaldehyde. Sections 7 µm thick were cut from the tissue and mounted on 3-aminopropyl-triethoxysilane coated glass slides. The slides were immersed in ethanol 3 times at intervals of 5 min and in DEPC-treated water and washed with PBS. Then the slides were immersed in 50% formamide/2 × SSC for prehybridization. Then 50 µl of hybridization mix, containing 50% formamide, 2 × SSC, 1 µg/µl of tRNA, 1 µg/µl of salmon sperm DNA, 1 µg/µl of BSA, 10% dextran sulfate, and the riboprobe at a concentration of 1 ng/µl was added onto each section and covered with parafilm and incubated at 42°C for 16 h. The incubated slides were washed in 2 × SSC at 42°C for 20 min to remove parafilm (2 changes of 20 min each), and then incubated in 20 µg/ml RNase A in NTE buffer at 37°C for 30 min. Subsequently the slides were transferred into NTE buffer and washed in 0.1 × SSC at 42°C for 20 min (3 changes of 20 min each). Digoxygenin-labeled probes were detected according to the methods outlined in the DIG nucleic acid detection kit (Roche).

Chondrocyte isolation. Chondrocytes were released from the cartilage by the modification of a published method[24]. Briefly, to ensure that only cartilage tissue was used, cartilage specimens were examined thoroughly, both macroscopically and microscopically (in the immunohistochemical experiment). Only those specimens with no pannus were processed further. This consisted of 2 mg/ml pronase for 1 h followed by 1 mg/ml collagenase (type IV; Sigma-Aldrich, St. Louis, MO, USA) for 6 h at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Norwalk, CT, USA) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin; Gibco BRL). The digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were used for RNA extraction.

Cytotoxic lymphocyte isolation. Human peripheral blood mononuclear cells (PBMC) were freshly isolated from heparinized venous blood from the same 12 patients with RA at the time of operation by Ficoll density gradient centrifugation. Isolated PBMC were used for RNA extraction of GrB and PFN because only NK cells and CTL are known to express GrB and PFN in PBMC.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for GrB and PFN. Total RNA was extracted from the chondrocytes using Trizol Reagent (Gibco BRL) according to the manufacturer's instructions. First-strand cDNA was generated from 5 µg of the total RNA from each sample using a First-strand cDNA Synthesis Kit for RT-PCR (Boehringer Mannheim, Indianapolis, IN, USA). Based on a published method[25], PCR amplification was performed using target-specific primers (for GrB: sense 5'-GGG-GAA-GCT-CCA-TAA-ATG-TCA-CCT-3' and antisense 5'-TAC-ACA-CAA-GAG-GGC-CTC-CAG-AGT-3', producing a 431 bp band; for PFN: sense 5'-CGG-CTC-ACA-CTC-ACA-GG-3' and antisense 5'-CTG-CCG-TGG-ATG-CCT-ATG-3', producing a 369 bp band) and Takara Taq™ (Takara, Kyoto, Japan). NK cells and CTL, which are known to express GrB and PFN, were used as positive controls[26]. The PCR conditions were as follows: an initial denaturation step at 94°C for 5 min followed by denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and

extension at 72°C for 1 min (30 cycles), and a final extension at 72°C for 7 min (Program Control System, ASTEC). The resulting PCR products were run on 1.5% agarose gels (3.0% agarose gels for ß-actin) and visualized with ethidium bromide for size verification. Sequencing of the PCR products was performed using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Norwalk, CT, USA) and a Big Dye Terminator Mix (Perkin Elmer). PCR for ß-actin was performed on each sample as a control using the primers 5'-CAA-GAG-ATG-GCC-ACG-GCT-GCT-3' and 5'-TCC-TTC-TGC-ATC-CTG-TCG-GCA-3', which produce a 275 bp band. Each RT-PCR reaction was also run using a sample containing water instead of RNA, which served as the negative control.

Semiquantitative RT-PCR experiments. Semiquantitative PCR experiments for GrB and PFN were performed as follows. First, the concentration of the cDNA sample was adjusted prior to the quantitative PCR experiments for GrB and PFN using a Human ß-actin Competitive PCR Set (Takara). The highly concentrated cDNA samples were then diluted proportionally. The amount of GrB and PFN mRNA was normalized against 10 ng total RNA for ß-actin. DNA internal standards (i.e., mimics) with the same primer template sequence as the target cDNA but containing a completely different, smaller intervening sequence were prepared using the Competitive DNA Construction Kit (Takara) according to the manufacturer's instructions. The amplification efficiencies of the DNA mimics were similar to that of the target cDNA. Under the same RT-PCR conditions as described above, aliquots of sample cDNA mixed with 10-fold serial dilutions of the DNA mimics were coamplified as templates in the presence of the primers used in the nonquantitative RT-PCR experiment. However, only 28 cycles of denaturation, annealing, and extension were performed because the target cDNA and DNA mimics displayed a logarithmically linear product formation (band density) in the range of 24–30 PCR cycles. The PCR products were then analyzed using a published method[27]. Briefly, the PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed. These photographs were then scanned and the resulting images were analyzed using Adobe Photoshop and US National Institutes of Health Image 1.62 software to quantify the mRNA concentrations.

In situ detection of apoptosis. *In situ* detection of apoptosis was performed according to the terminal dUTP nick-end labeling (TUNEL) method[28,29] using an ApopTag kit (Oncor, Gaithersburg, MD, USA) and hematoxylin and eosin (H&E) staining. Briefly, cartilage sections (5 µm) were digested with proteinase K (Sigma, St. Louis, MO, USA), and endogenous peroxidase was inactivated by immersing the sections in 0.3% hydrogen peroxide. DNA fragments were then tailed with digoxigenin-nucleotide by terminal deoxynucleotidyl transferase and incubated with a peroxidase conjugated anti-digoxigenin antibody. The reaction was visualized using DAB, and the sections were counterstained with methyl green. Doublestaining for GrB and TUNEL was performed by first staining the sections for GrB, as described, followed by TUNEL staining with FITC conjugated anti-digoxigenin. Fluorescent signals in the ApopTag assay and expression of GrB marker were detected using a confocal laser microscope (Bio-Rad MRC 1024, Hemel, Hempstead, UK). In the H&E stained sections, an apoptotic chondrocyte was defined as a cell with a condensed, pyknotic nucleus and either shrunken, deeply eosinophilic cytoplasm or a fragmented nucleus and cytoplasm[30].

Electron microscopy. Cartilage specimens from one normal sample and 3 RA patients were cut in 0.1 × 0.1 cm pieces and fixed in 2.5% glutaraldehyde solution overnight. After rinsing, the samples were postfixed in 1% osmium tetroxide, dehydrated through a graded series of ethanol, and embedded in epoxy resin. Semi-thin sections (1 µm) were stained with toluidine blue, and areas showing the most cells with nuclear condensation were chosen for cutting into ultra-thin sections. Sections were counterstained with lead citrate-uranyl acetate and examined with a transmission electron microscope (JEM-1200EX, JEOL, Tokyo, Japan).

Microscopic evaluation. GrB and PFN-positive cells, TUNEL-positive cells, and apoptotic cells in H&E staining in the cartilage samples were quantified using a published method[30,31]. Briefly, normal and RA sections in which the cartilage layer was preserved were divided into 2 zones, superficial and deep, and each section was divided into 6 microscopic fields (40× magnification; Olympus, Tokyo, Japan) with 3 fields in each zone. The percentage of positive chondrocytes within these defined fields was separately calculated at 400× magnification for the entire thickness of cartilage and for each layer of cartilage.

Statistical analysis. Data are expressed as the means ± standard error of the mean (SEM). Group comparisons were performed using the Mann-Whitney U test for independent samples of nonparametric data. P values < 0.05 were considered to be significant.

RESULTS

Clinical features. Clinical and demographic data are presented in Table 1. The mean age was 51.7 ± 4.8 years in the RA group and 70.2 ± 2.8 years in the control group. The mean disease duration at the time of operation was 47.5 ± 6.6 months in the RA group. The mean number of swollen joints was 6.9 ± 1.1 in the RA group. Rheumatoid factor was detected in all the patients with RA. Joint erosions were present in all patients in the RA group and absent in all patients in the control group.

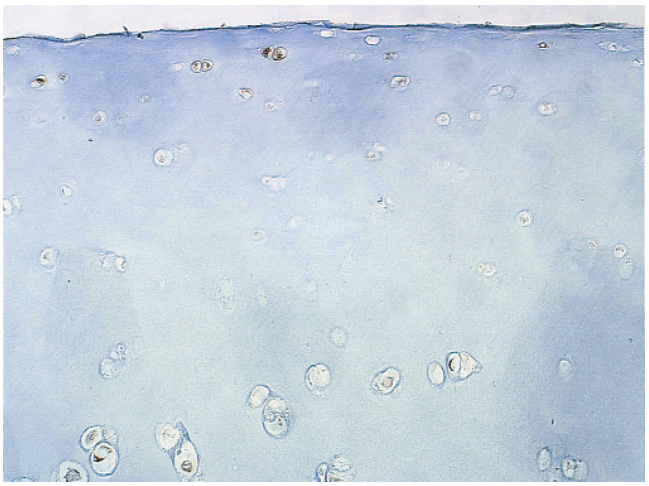
All RA patients were under the care of rheumatologists and were receiving systemic antirheumatic medications. The preoperative medical treatment for patients was as follows. Two out of the 12 patients were managed with 2 disease modifying antirheumatic drugs. Specifically, the total number of patients taking methotrexate was 6; penicillamine, 3; auranofin, 2; bucillamine, 2; and salazosulfapyridine, one. Three of the 12 patients were treated with steroids, and all 12 patients also took nonsteroidal antiinflammatory drugs, such as indomethacin. No attempt was made to standardize any patient's medical treatment before the operation.

Immunoreactivity of GrB and PFN in chondrocytes. To investigate the *in situ* expression of GrB and PFN in cartilage, tissue sections of normal cartilage and RA cartilage were stained with the antibody against each protein. Normal cartilage contained fewer GrB and PFN-positive cells in both the superficial and deep zones than the RA cartilage (Figures 1A, 1B, 1E and Tables 2, 3). The staining of GrB

Table 1. Clinical data for the study patients. The 9 patients (4 men, 5 women) with normal cartilage had a mean ± SEM age of 70.2 ± 2.8 years. Except for sex, values are mean ± SEM.

	RA, n = 12
Sex, M:F	2:10
Age, yrs	51.7 ± 4.8
Disease duration, mo	47.5 ± 6.6
Swollen joints, n	6.9 ± 1.1
Morning stiffness, by VAS	4.8 ± 1.0
CRP, mg/dl	4.2 ± 1.3
ESR, mm/h	60.4 ± 5.6

VAS: visual analog scale (0–10), CRP: C-reactive protein, ESR: erythrocyte sedimentation rate.



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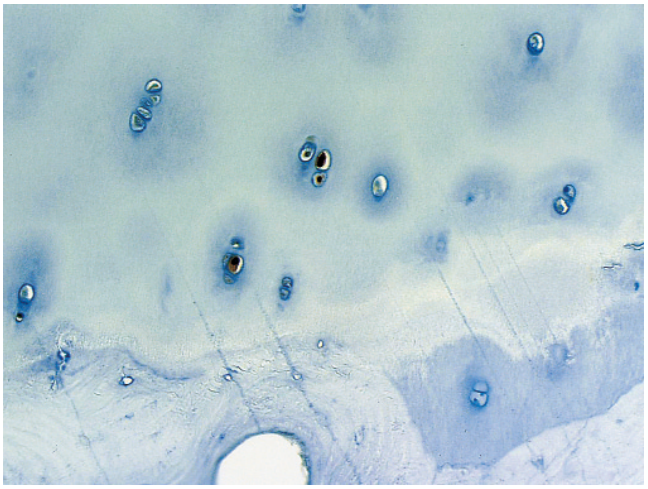

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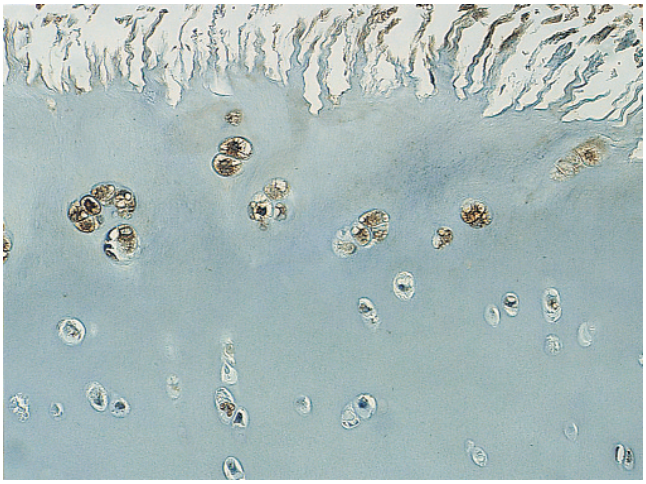

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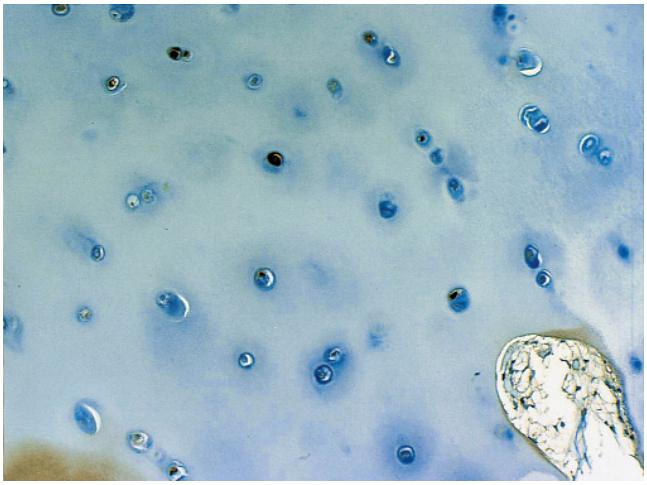

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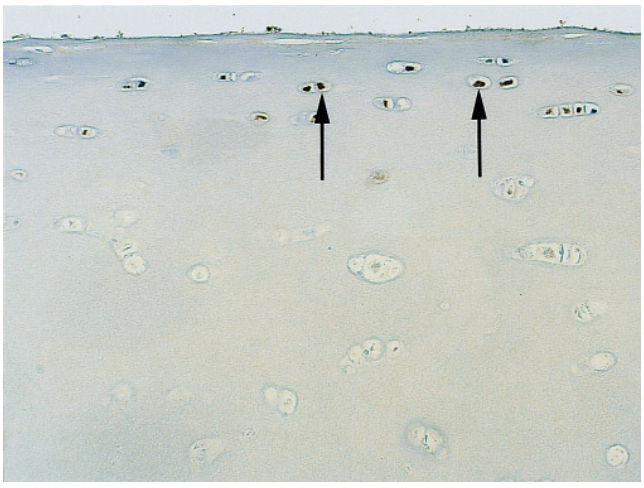

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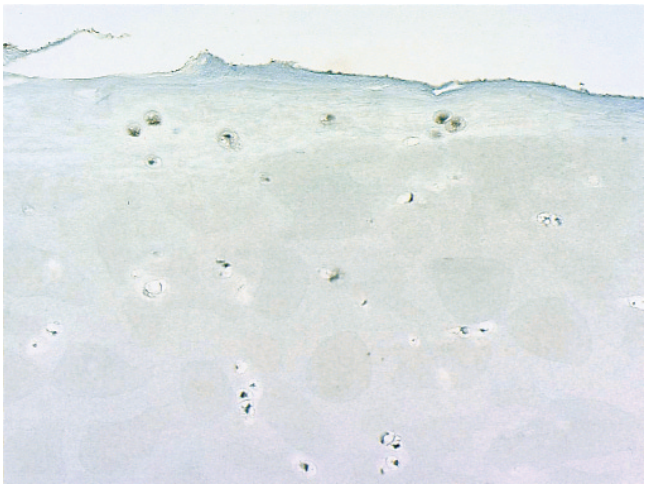

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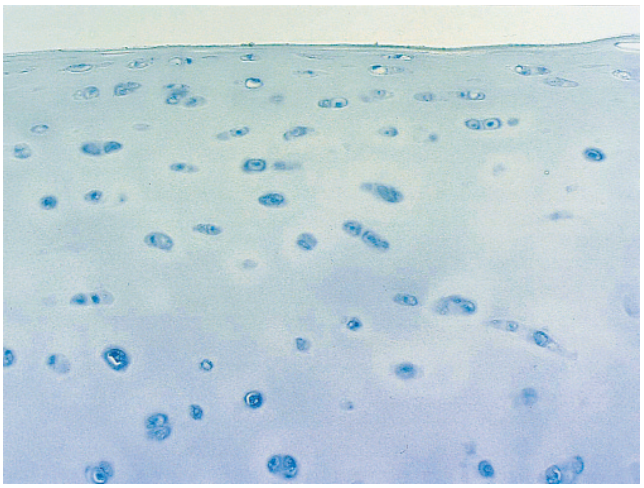

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Figure 1. Immunohistochemical expression of granzyme B (GrB) and perforin (PFN) positive chondrocytes in normal and RA cartilage. Paraffin sections of cartilage samples were incubated with anti-GrB or anti-PFN antibody and stained by the immunoperoxidase method, followed by hematoxylin counterstaining. (A) Superficial zone of normal cartilage shows a small number of GrB-positive cells (antibody: C-19). (B) Deep zone of normal cartilage, showing a tendency for more pronounced staining for GrB (antibody: C-19) compared with the superficial zone. (C) Superficial zone of RA cartilage shows GrB-positive cells (antibody: C-19) predominantly in the superficial zone. (D) Deep zone of RA cartilage (antibody: C-19). (E) Normal cartilage, showing a small number of PFN-positive cells (arrows) in the superficial zone. (F) RA cartilage, showing PFN positive cells predominantly in the superficial zone. (G) Isotype matched negative control shows negative staining. Results are representative of 9 normal and 12 RA cartilage samples (original magnifications ×200).

Table 2. Percentages of granzyme B (GrB)-positive, and perforin (PFN)-positive cells in normal and RA chondrocytes. Values are the mean ± standard error of the mean (SEM).

	Antibody	Normal Cartilage, n = 9	RA Cartilage, n = 12	p
GrB-positive cells	C-19	10.9 ± 1.38	21.4 ± 3.91	0.04
	18.1	11.5 ± 1.91	23.0 ± 3.71	0.01
	2C5	11.1 ± 1.39	18.9 ± 2.70	0.02
PFN-positive cells		7.0 ± 1.41	11.1 ± 1.49	0.02

Mann-Whitney U test was performed to compare number of GrB and PFN-positive cells between normal and RA samples.

Table 3. The percentages of granzyme B (GrB)-positive cells, perforin (PFN)-positive cells, apoptotic cells by H&E staining, and TUNEL-positive cells compared by zones. Values are the mean ± standard error of the mean (SEM). Mann-Whitney U test was performed to compare the total (superficial and deep) number of positive cells between normal and RA samples.

	RA Cartilage, n = 12		Normal Cartilage, n = 9		
	Superficial Zone	Deep Zone	Superficial Zone	Deep Zone	p
GrB-positive cells (Antibody: C-19)	29.7 ± 4.26	14.0 ± 3.19	11.3 ± 1.79	10.3 ± 1.24	0.04
PFN-positive cells	18.1 ± 2.41	6.3 ± 0.96	9.1 ± 1.87	5.7 ± 1.54	0.02
Apoptotic cells* by H&E staining	6.73 ± 0.81	2.78 ± 0.56	1.74 ± 0.26	1.29 ± 0.22	< 0.01
TUNEL-positive cells	31.8 ± 3.52	15.2 ± 2.42	6.21 ± 0.97	2.98 ± 0.79	< 0.01

* An apoptotic chondrocyte was defined as a cell with a condensed, pyknotic nucleus and either shrunken, deeply eosinophilic cytoplasm or a fragmented nucleus and cytoplasm.

had a tendency to be more intense in the deep zone when the deep zone was compared to the superficial zone in every section. Indeed, the RA cartilage contained significantly higher numbers of GrB and PFN-positive cells (Figures 1C, 1D, 1F and Table 2). GrB and PFN-positive cells were predominantly observed in the superficial zone of the RA cartilage and to a lesser extent in the deep zone (Table 3). The percentage of GrB positive chondrocytes was significantly higher than that of PFN in RA cartilage. Since the expression of GrB was thought to be restricted to CTL and NK cells[32,33], we confirmed our immunohistochemistry findings using 3 other antibodies to human GrB (both monoclonal and polyclonal); all 3 antibodies revealed the presence of GrB in chondrocytes from normal and RA cartilage samples (Table 2). Isotype matched controls revealed only low levels of background staining (Figure 1G).

Analysis of GrB and PFN transcripts in chondrocytes. To eliminate the possibility that the GrB antibodies were recognizing a highly homologous but uncharacterized serine proteinase in the chondrocytes, we examined whether GrB transcripts could be detected by *in situ* hybridization using riboprobes, or by RT-PCR performed on RNA samples extracted from RA and normal chondrocytes.

In situ hybridization was performed using probes specific

for GrB on normal human cartilage. The GrB antisense probe indicates the presence of GrB mRNA in chondrocytes (Figure 2A), which were predominantly observed in the deep zone of the normal cartilage and to a lesser extent in the superficial zone. A section hybridized with the GrB sense probe showed no staining (Figure 2B) and indicated the specificity of the antisense hybridization. *In situ* hybridization confirmed results of the immunohistochemistry, localizing GrB in chondrocytes within the cartilage, especially in the deep zone of normal cartilage.

The expected single band (431 bp) for GrB was observed in all 12 RA and 9 normal cartilage specimens (Figure 3A), and the single band (369 bp) for PFN was observed in 12 RA and 8 normal cartilage specimens (Figure 3B). Nucleotide sequencing of the PCR amplification products confirmed the identity of the amplified bands. The positive sample controls obtained from cytotoxic lymphocytes (NK cells and CTL) were always positive, whereas the negative controls were always negative.

To determine the levels of GrB and PFN mRNA expression, total RNA from the chondrocytes and cytotoxic lymphocytes was characterized using semiquantitative RT-PCR analysis. The levels of GrB mRNA were $0.61 \times 10^6 \pm 0.23 \times 10^6$ mRNA copies per ng of total RNA in normal chondrocyte samples, $9.65 \times 10^6 \pm 2.23 \times 10^6$ mRNA copies in RA chondrocyte samples, and $139 \times 10^6 \pm 16.8 \times 10^6$ mRNA copies in RA cytotoxic lymphocytes. The levels of PFN were $0.22 \times 10^6 \pm 0.05 \times 10^6$ mRNA copies per ng of total RNA in normal chondrocyte samples, $0.85 \times 10^6 \pm 0.23 \times 10^6$ mRNA copies in RA chondrocyte samples, and $103 \times 10^6 \pm 19.7 \times 10^6$ mRNA copies in RA cytotoxic lymphocytes. GrB and PFN transcript levels in the RA chondrocytes were significantly upregulated, compared to the levels observed in normal chondrocytes (Figures 4A, 4B, Table 4). GrB and PFN transcript levels in RA cytotoxic lymphocytes were significantly elevated compared to levels observed in the RA chondrocytes.

In situ detection of chondrocyte apoptosis. Cartilage sections were stained using the TUNEL and H&E techniques to detect the presence of apoptotic chondrocytes. RA cartilage contained apoptotic cells in both the superficial and deep zones, but more apoptotic cells were observed in the superficial zone (Figure 5A). The distribution of apoptotic cells in the RA cartilage was similar to that of GrB and PFN-positive cells (Table 3). In the RA cartilage, areas with a high frequency of apoptotic cells corresponded with clusters of chondrocytes. In contrast, normal cartilage contained very low numbers of apoptotic cells, almost all of which were located in the surface zone (Figure 5B). The specificity of the staining was indicated by the omission of TdT in the TUNEL staining. Apoptotic chondrocytes were morphologically confirmed by H&E staining (Figure 5C). As previously described[30], our data also showed that the frequency of apoptotic cells was lower in the H&E stained sections than in TUNEL stained sections (Table 3). This observation may suggest the presence of false positives in TUNEL staining, which occasionally occurs in necrotic cells. However, the distribution of apoptotic cells was similar for both staining methods, and a certain percentage of apoptotic cells in H&E staining and TUNEL-positive cells should indicate true chondrocyte apoptosis. Double-immunofluorescence staining for TUNEL and GrB was performed to examine the relationship between the location of the TUNEL-positive and GrB-positive cells. As shown in

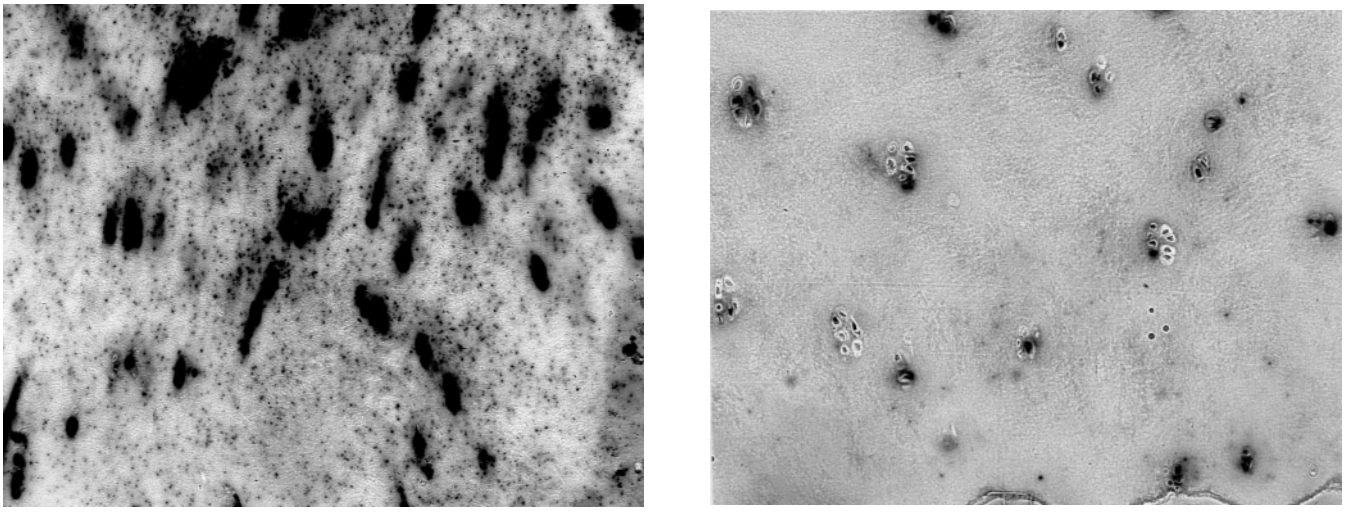


Figure 2. In situ hybridization analysis of normal articular cartilage using GrB riboprobes. (A) Deep zone of normal cartilage: the GrB antisense probe indicates the presence GrB mRNA in chondrocytes. Positive chondrocytes were predominantly observed in the deep zone and to a lesser extent in the superficial zone. (B) A section hybridized with GrB sense probe shows no staining, indicating the specificity of the antisense hybridization (original magnification ×200).

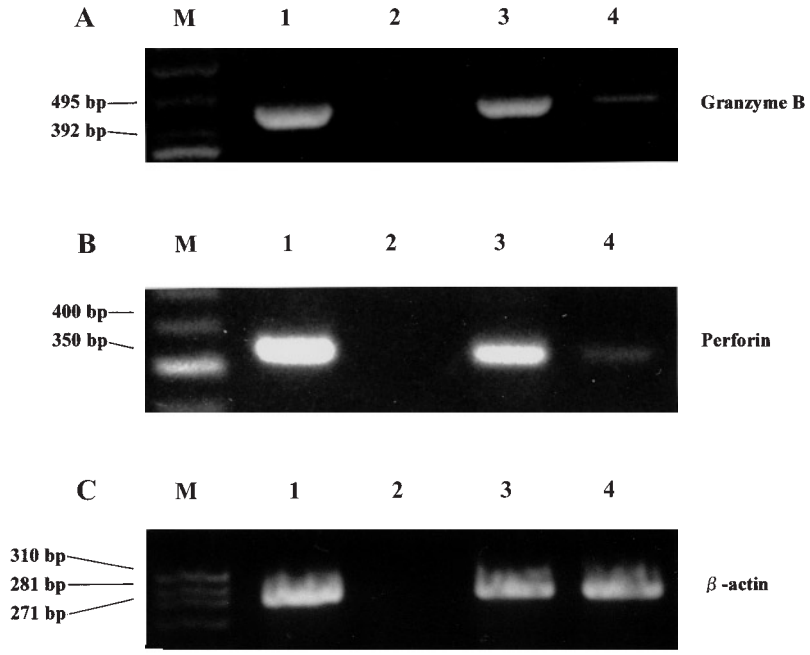


Figure 3. Representative RT-PCR results showing amplified (A) GrB bands (431 bp); (B) PFN bands (369 bp); and (C) ß-actin bands (275 bp) from normal and RA chondrocytes. Lane M, marker, indicated in bp; Lane 1, positive control; Lane 2, negative control; Lane 3, RA chondrocytes; Lane 4, normal chondrocytes.

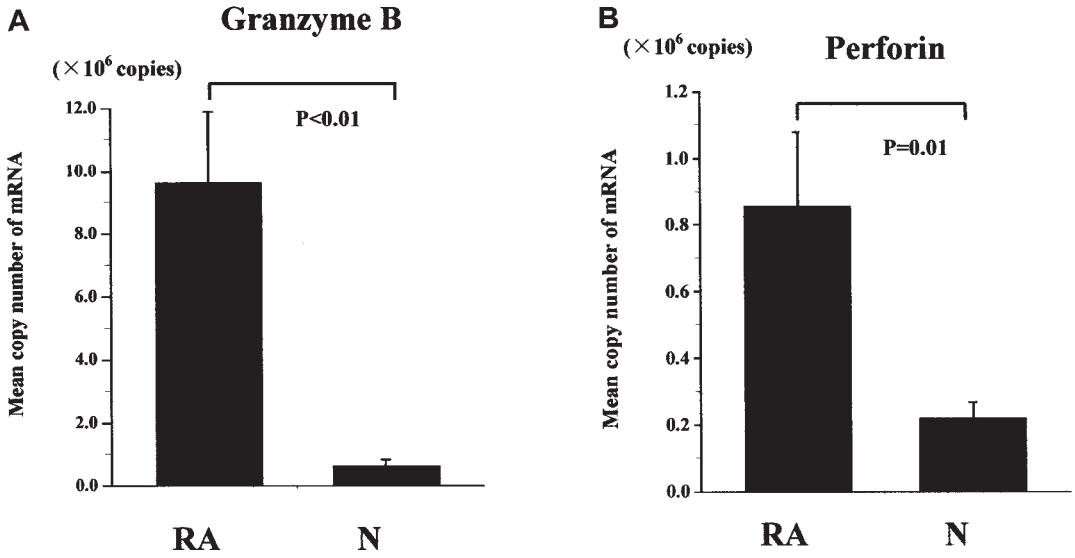


Figure 4. Semiquantitative RT-PCR for GrB and PFN in chondrocytes. Results derived from 3 independent experiments, densitometrically analyzed 3 times each. Results are expressed as mean GrB and PFN mRNA copies ± SEM in normal (N) and RA chondrocytes per nanogram (ng) total RNA. P values by Mann-Whitney U test for independent samples of nonparametric data.

Figure 5D, some chondrocyte clusters exhibit TUNEL and GrB-positive chondrocytes that are in contact with GrB-positive chondrocytes. GrB staining was observed in the cytoplasm and TUNEL staining was observed in the nucleus.

We further confirmed chondrocyte apoptosis by an ultrastructural study. The results of electron microscopy of a representative chondrocyte in normal cartilage are shown in Figure 6A. The nucleus appeared large and round, with minimal chromatin. The cytoplasmic membrane was intact

Table 4. Quantitative measurement of granzyme B (GrB) and perforin (PFN) mRNA expressed in normal and RA chondrocytes. Results are expressed as mean GrB and PFN mRNA copies per nanogram total RNA. Mann-Whitney U test comparing mRNA measurements between RA and normal samples.

	GrB	PFN ($\times 10^6$ copies)
Normal cartilage		
Patient		
1	0.03	0.00
2	0.61	0.23
3	0.04	0.24
4	0.26	0.17
5	0.91	0.54
6	0.07	0.11
7	0.76	0.33
8	2.20	0.09
9	0.61	0.25
Mean	0.61	0.22
SEM	0.23	0.05
RA cartilage		
Patient		
1	9.05	0.31
2	3.85	0.15
3	2.45	0.20
4	6.12	0.38
5	9.84	2.74
6	3.31	1.15
7	1.29	0.58
8	12.42	1.20
9	7.00	0.19
10	18.62	1.34
11	27.96	1.61
12	13.84	0.41
Mean	9.65	0.85
SEM	2.23	0.23

SEM: Standard error of the mean.

and did not show surface ruffling or blebbing. In contrast, an apoptotic chondrocyte from RA cartilage had nuclear change that included chromatin condensation and irregular nucleus. The cytoplasmic membrane was irregular, and the cytoplasm was shrunken, consistent with apoptosis (Figure 6B).

DISCUSSION

Our study confirms the presence of GrB in human articular chondrocytes, in contradiction to the current consensus that GrB is confined to NK cells and cytotoxic lymphocytes. Normal cartilage consists of only one type of cell, the chondrocyte, and the extracellular matrix. Articular cartilage does not contain blood vessels, lymphatic vessels, or nerves[34]. Therefore, the GrB-positive cells in the normal cartilage samples must be chondrocytes. The presence of GrB mRNA was confirmed by *in situ* hybridization and RT-PCR. These findings suggest that GrB expression occurs in the chondrocytes of human articular cartilage. The view that GrB was present only in cytotoxic lymphocytes arose because the original analysis of GrB mRNA expression was performed using human peripheral blood lymphocytes[32]. Subsequent immunohistochemical analyses have also focused exclusively on the tissues of the immune system[35–38]. A comprehensive investigation of human GrB expression has not apparently been performed, so its presence in nonimmune tissues and possible additional physiological functions has not been considered[4].

GrB-positive chondrocytes were observed throughout the entire zone of normal cartilage, with a tendency to more pronounced staining occurring in the deep zone. *In situ* hybridization confirmed the results of the immunohistochemistry at the transcript level, localizing GrB more pronouncedly in the deep zone of the normal cartilage. These findings suggest that GrB has some role in chondrocyte metabolism, especially in the deep zone, under physiological conditions. Throughout life, chondrocytes are required for the turnover in matrix macromolecules that enables articular cartilage to be maintained. Continuous internal remodeling occurs as the chondrocytes replace matrix macromolecules lost through degradation. Enzymes such as matrix metalloproteases produced by the chondrocytes are responsible for the degradation of the matrix macromolecules, and chondrocytes respond to the presence of fragmented matrix molecules by increasing their synthetic activity to replace the degraded components of the macromolecular framework[39]. Exocytosed GrB may be involved in these remodeling mechanisms because of its proteoglycanase activity. In the deep zone of cartilage, the matrix synthesized by the chondrocytes has the highest concentration of proteoglycan and a lower concentration of collagen and water relative to the other cartilage zones[39]. GrB has proteoglycan-degrading activity and does not have any collagenolytic activity[14,15]. These observations are consistent with our findings and support our hypothesis that GrB is involved in the matrix remodeling of cartilage. In addition, the percentage of GrB-positive chondrocytes was significantly higher than that of PFN-positive chondrocytes in RA cartilage (as determined by immunohistochemistry). The distribution of GrB-positive cells was similar to that of PFN-positive cells in serial cartilage sections. In the immune system, GrB and PFN are simultaneously expressed in the cytoplasmic granules of cytotoxic lymphocytes. Our findings suggest that some chondrocytes express GrB but do not express PFN, and that GrB has PFN-independent nonimmune functions *in vivo*, as described above.

To further investigate the potential role of GrB in the pathophysiology of joint destruction in RA, the expression of GrB in RA cartilage was examined. Previous studies have shown that exogenous GrB degrades the surface of articular cartilage via synovial fluid and the cartilage-pannus junction[14-17]. However, little is known about the effects of endogenous GrB, produced by chondrocytes, on RA carti-

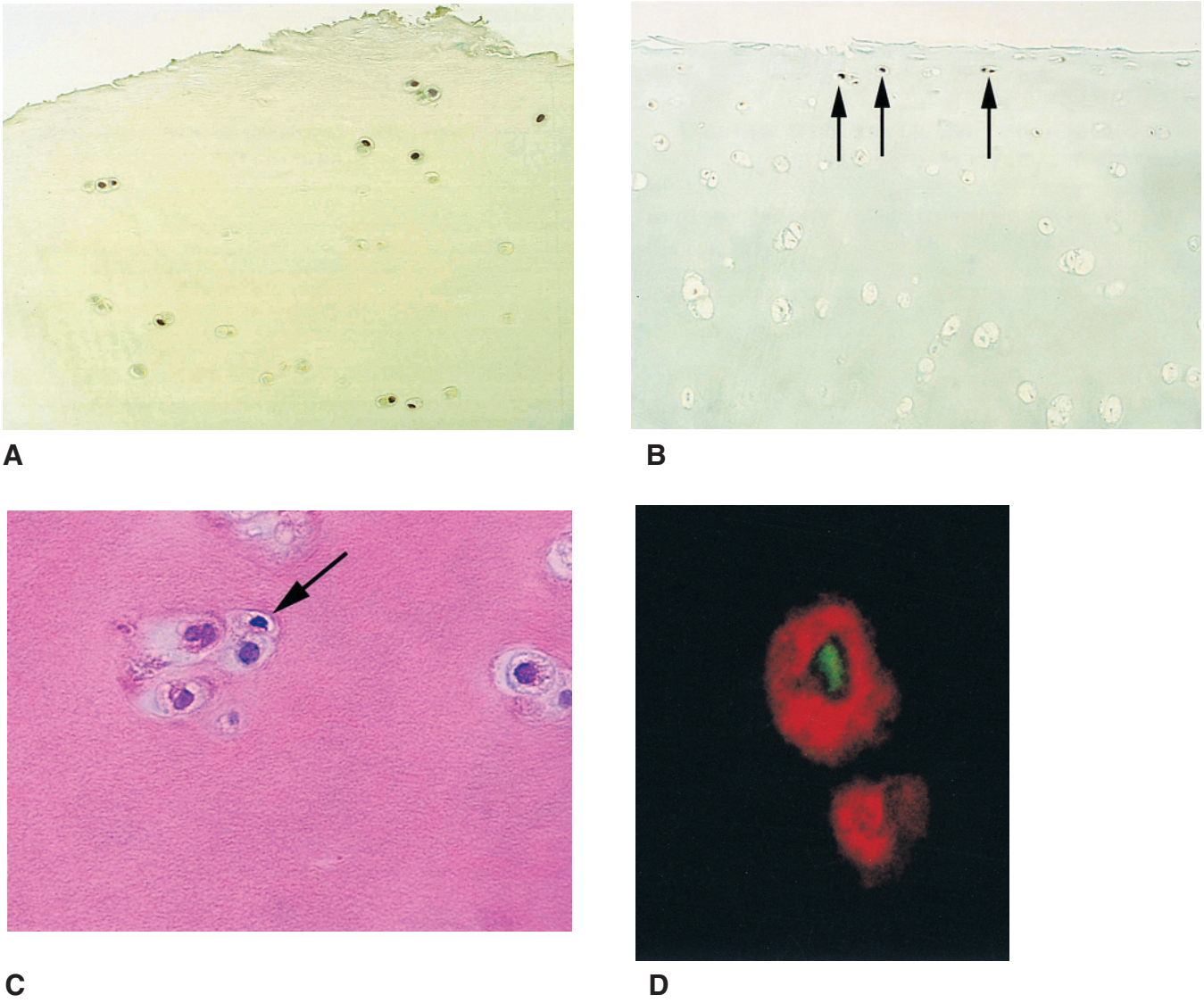
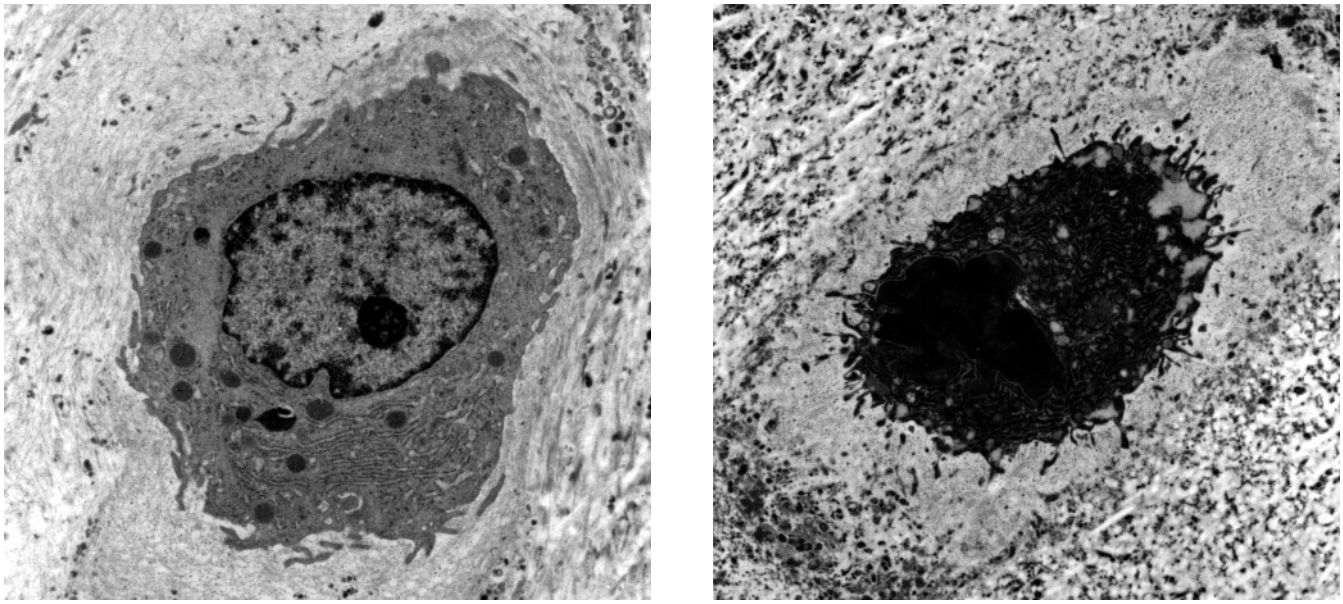


Figure 5. In situ detection of apoptotic chondrocytes. (A) TUNEL staining of RA cartilage showed that TUNEL-positive chondrocytes were more frequently observed in the superficial zone. (B) TUNEL staining of normal cartilage showed very low numbers of apoptotic chondrocytes (arrows), almost all of which are located in the surface zone. (C) H&E staining of RA cartilage; arrow shows an apoptotic chondrocyte characterized by basophilic chromatin condensation, shrinkage of cell volume, and eosinophilic cytoplasm. (D) Photomicrograph showing doublestaining for GrB (red) and TUNEL (green) in RA cartilage. A TUNEL and GrB-positive chondrocyte was in contact with the GrB-positive chondrocyte in the cluster. GrB staining was observed in the cytoplasm and TUNEL staining was observed in the nucleus (original magnification ×200 in A and B; ×600 in C and D).

lage. Our data indicate that elevated levels of GrB are expressed in RA chondrocytes, compared with normal cartilage. If the excess GrB in the RA chondrocytes is exocytosed, this may cause pericellular matrix degradation. These phenomena result in the autolysis of RA cartilage. GrB produced by chondrocytes may also play a role in the enzymatic mechanism of RA cartilage degradation similar to that of matrix metalloproteinases.

Studies have indicated that the mechanism of endogenous cartilage degradation is mediated by chondrocyte apoptosis. In other words, there is a significant increase in apoptotic chondrocytes in RA cartilage compared with normal cartilage[30]. The degree of chondrocyte apoptosis is closely related to cartilage destruction in both RA cartilage and OA cartilage[40,41]. Although there are several reports showing that chondrocyte apoptosis is induced by Fas[42] or nitric oxide[43], controversy still exists about the trigger of induction of apoptosis in RA cartilage chondrocytes. Our data showed the expression of PFN as well as GrB in articular chondrocytes. The simultaneous expression of GrB and PFN in chondrocytes suggests the possibility of apoptosis induction in chondrocytes that are in contact with the GrB



A B

Figure 6. Electron microscopy analysis of normal and RA cartilage. (A) A representative chondrocyte in normal cartilage, showing the nucleus as large and round, with minimal chromatin. The cytoplasmic membrane was intact and showed no surface ruffling or blebbing. (B) An apoptotic chondrocyte from RA cartilage had nuclear change that included chromatin condensation and irregular nucleus. The cytoplasmic membrane was irregular, and the cytoplasm was shrunken, consistent with apoptosis (original magnification ×2500 in A and B).

and PFN-positive chondrocytes. Interestingly, GrB and PFN shared a similar expression pattern to apoptotic chondrocytes in RA cartilage, and some chondrocyte clusters had a tendency to show that TUNEL-positive cells were in contact with GrB-positive cells. Considering that GrB and PFN-expressing cells must be in contact with their target cell for granule mediated (GrB and PFN mediated) apoptosis to occur[1,2], GrB and PFN-expressing chondrocytes may induce the apoptosis of other chondrocytes via this granule mediated pathway in the chondrocyte clusters in which each of the chondrocytes is in contact with the others. In other words, GrB and PFN may be involved in RA cartilage degradation via a chondrocyte apoptotic mechanism.

The interaction between the extracellular matrix and chondrocytes is known to provide an important regulatory function needed to ensure cell survival, and the lack of extracellular matrix bonds on the cell surface may result in chondrocyte apoptosis[44]. From our observations, released GrB may cause pericellular matrix degradation in RA cartilage. Therefore, it is also possible that GrB expression and exocytosis may cause the induction of chondrocyte apoptosis via matrix degradation. The presence of such a pathway would mean that GrB is also involved in an indirect apoptosis induction mechanism, as well as an enzymatic degradation mechanism.

The mechanisms that contribute to elevated levels of GrB and PFN expression from RA chondrocytes are uncertain. In the immune system, the regulation of human NK cell activation is under the control of a network of regulatory signals provided by cytokines[45,46]. That is, the cytotoxic function of the human NK cell can be upregulated by cytokines such as interleukin 2 (IL-2), IL-12, and IL-15[45-47]. Recent studies showed that the expression of IL-12 and IL-15 are highly upregulated in the synovial fluid of RA joints[47,48]. Based on these studies, it may be possible that the regulation of GrB and PFN expression in chondrocytes is also under the control of cytokines from synovial fluid. These problems will be elucidated in future studies.

Our results demonstrate the presence of GrB and PFN expression in human articular chondrocytes, and elevated concentrations of GrB and PFN in RA chondrocytes. The targets and precise function of GrB remain to be determined. However, considering the functions of GrB as reported, they may involve the remodeling mechanism of matrix macromolecules in normal cartilage and the endogenous degradation of RA cartilage.

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