Granzyme B Induces Apoptosis of Chondrocytes with Natural Killer Cell-like Cytotoxicity in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Granzyme B, an apoptosis-inducing factor, is expressed in natural killer (NK) cells, an important factor in innate immunity. We previously reported that granzyme B is expressed in arthritic cartilage and chondrocytes, and suggested that granzyme B expression is related to apoptosis distribution. We have now investigated whether granzyme B directly induces apoptosis in chondrocytes and whether chondrocytes possess NK cell-like function.

Methods. Chondrocytes included the human C-28/12 chondrocyte cell line, normal chondrocytes, and rheumatoid arthritis (RA) chondrocytes. Apoptosis was analyzed by ELISA and TUNEL after C-28/12 cells were incubated with active granzyme B. NK cell markers were examined in chondrocytes by FACS and immunohistochemistry. Chondrocytes with or without Z-AAD-CMK, a known granzyme B inhibitor, were stimulated with PHA (20 μg/ml), followed by coculture with K562 cells in order to test chondrocyte cytotoxity.

Results. Granzyme B was successfully introduced into C-28/12 chondrocytes, and was confirmed to dose-dependently induce apoptosis. Immunohistochemically, chondrocytes expressed the surface antigens of NK cells and exhibited cytotoxicity against K562 cells, which served as an indicator of cytotoxicity. Z-AAD-CMK inhibited cytotoxicity against K562 cells in a dose-dependent manner, thus confirming that chondrocyte cytotoxicity against K562 cells is dependent on granzyme B.

Conclusion. Our findings indicate that chondrocytes possess NK cell-like activity related to innate immunity, and that apoptosis is induced in these cells by granzyme B. Our findings suggest that inflammation activates granzyme B, which participates in the destruction of RA-affected joints. (First Release Sept 1 2008; J Rheumatol 2008;35:1932–43)

Key Indexing Terms:

GRANZYME B	CHONDROCYTE	APOPTOSIS
CYTOTOXIC ACTIVITY	NATURAL KILLER CELL	ARTHRITIS

Granzyme B is part of a family of structurally related serine proteases expressed in cytotoxic lymphocytes such as natural killer (NK) cells, NK T cells, and cytotoxic T lymphocytes[1,2], and acts to bring about the death of target cells after cosecretion with perforin. Perforin is critical for allowing granzyme B to access substrates within the target cell, after which granzyme B is able to enter target cells and form cytotoxic immunological synapses[3-5].

NK cells are a key part of innate immunity due to their ability to secrete cytokines and mediate cytotoxic activity, and act as an important first line of defense. Preformed granules that contain mature granzyme B are constitutively expressed in NK cells; thus, NK cells are always armed with functional granzyme B. Granzyme B is therefore an important factor involved in innate immunity. Indeed, granzyme B is used to monitor cytotoxicity as an alternative to Cr^{51} [6,7]. NK cells, or NK-like cells, have also been identified in target organs of patients with autoimmune disease[8].

There is substantial evidence that rheumatoid arthritis (RA) has an autoimmune component. The disease affects multiple joints, consistent with the process being systemic[9], and is associated with the production of autoantibodies such as rheumatoid factor (RF) and the formation of immune complexes[10]. Although RA has elements characteristic of an autoimmune disease, the specific self-antigens responsible for inducing or perpetuating this inflammatory response remain unidentified.

Recent advances suggest that joint inflammation may result from activation of an innate immune response induced by a diverse range of pathogens[11,12]. Serum from patients with autoimmune diseases harbors autoantibodies that react with proteolytic fragments, resulting from granzyme B cleavage of intracellular proteins[13]. However, granzyme B is also present extracellularly during inflammatory episodes, as shown by detection of this protease in plasma and synovial fluid from individuals with inflammatory disease[14,15]. The increased concentrations of soluble granzyme B in var-

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ious diseases are considered to reflect cell-mediated cytotoxicity induced by the release of granules from cytotoxic lymphocytes[14].

The expression of granzyme B was originally believed to be limited to cytotoxic lymphocytes. Recently, however, granzyme B has been found in neutrophils[16], basophils[17], and the synovial membrane of joints affected by RA[18,19]. We have also clearly demonstrated higher expression of granzyme B and perforin in hyaline cartilage samples and chondrocytes from RA-affected joints compared with cartilage samples or chondrocytes from joints affected by osteoarthritis (OA) using immunohistochemistry, semiquantitative reverse transcription-polymerase chain reaction, and *in situ* hybridization[20]. Moreover, the distribution of granzyme B-positive cells was similar to that of apoptotic cells[20]; apoptosis promotes the progressive destruction of cartilage in patients with RA or OA[21-23]. In addition, granzyme B is a matrix proteinase that reacts in the neutral region involved in the destruction of articular cartilage with the degradation of aggrecan and fibronectin, which are major components of cartilage[24,25], particularly in RA cartilage[26]. However, no study has shown that granzyme B actually induces apoptosis in cartilage.

Recent studies on the role of toll-like receptor (TLR) suggest that innate immunity causes inflammatory reactions to destroy cartilage components[27-29]. Thus, we hypothesized that overlapping functions exist in the regulation of processes involving innate immunity, inflammation, and cartilage destruction. Thus, evaluating cell-mediated cytotoxicity by granzyme B in chondrocytes will provide a direct measure of their innate immune function.

We first investigated whether apoptosis occurred in RA samples. We then investigated whether granzyme B induced apoptosis in human chondrocytes by using a chondrocyte cell line. Finally, we focused on whether granzyme B is related to innate immunity or cytotoxicity in human chondrocytes. Our findings demonstrated that granzyme B actually induces apoptosis in chondrocytes, which also showed NK cell-like activity.

MATERIALS AND METHODS

Synovium or cartilage from patients with RA (n = 15, 13 women, 2 men, age 49–78 yrs, mean 64.8 yrs) or OA (n = 4 women, 0 men, age 56–78 yrs, mean 65.5 yrs) and femoral head cartilage from patients with hip fractures (n = 4, 3 women, 1 man, age 67–93 yrs, mean 74.4 yrs) were obtained during implant arthroplasty or at the time of endoprosthetic replacement for acute transcervical fractures, with written informed consent, in accord with the ethical requirements of our university. Patients fulfilled the American College of Rheumatology criteria for the diagnoses of RA and OA[30,31]. Primary cultures of chondrocytes were prepared as follows. Cartilage from patients with RA and femoral head from patients with hip fractures was cut aseptically and then minced into small pieces. These pieces were digested with clostridial collagenase 1 (Sigma, St. Louis, MO, USA), 1 mg/ml in phosphate buffered saline (PBS). Collagenase digestion was carried out at 37°C for 18 h with stirring. After digestion, cells were cultured in 24-well microplates at a density of 1×10^5 cells/well in humidified 5% CO_2/95% air at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 units/ml). Cells were maintained in an atmosphere of 5% CO_2 in air at 37°C for 48 h and were used in further analyses.

We used the human C-28/12 chondrocyte cell line derived from human costal cartilage (kindly provided by Dr. M.B. Goldring, Harvard University, Boston, USA)[32,33]. C-28/12 cells are used extensively as a reproducible *in vitro* model to study a variety of chondrocyte functions, including apoptosis induction experiments requiring large numbers of cells[34,35]. For experiments, 1×10^6 cells were seeded in 25-cm^2 flasks and cultivated in humidified 5% CO_2/95% air at 37°C in DMEM containing 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 units/ml).

Immunohistochemistry for granzyme B and perforin. For immunostaining of synovium and cartilage, we used polyclonal antibodies against granzyme B (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA)[36] or monoclonal anti-granzyme B (GrB7; Sigma)[37], and monoclonal antibody against perforin (Calbiochem, EMD Biosciences, La Jolla, CA, USA). For granzyme B staining, cartilage and synovial tissues from patients with RA and OA were fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were cut into 5-μm sections. Slides were then treated with 0.3% H_2O_2 in PBS to quench endogenous peroxidase. For demasking of granzyme B, sample sections were subjected to three 5-min heating cycles in citrate buffer using a microwave oven before staining. Immunoperoxidase staining was performed using the Dako EnVion System (Dako Japan, Kyoto, Japan). Negative controls contained no primary antibody. Stained slides were examined microscopically. The percentage of granzyme B- and perforin-positive cells in the cartilage was determined in 10 high-power fields (40×), using the following semiquantitative criteria: 0 = negative; 1+ = less than 25% positive staining; ++ = 25–50% positive staining; and +++ = greater than 50% positive staining[38].

Determination and quantification of apoptosis. Apoptosis was examined by the TdT-mediated dUTP-biotin nick-end labeling (TUNEL) method (Apoptosis in situ Detection Kit, Wako, Osaka, Japan) or staining with annexin-V (Annexin V Apoptosis Detection Kit, MBL, Nagoya, Japan). Synovium and cartilage were fixed for 2 h at 4°C in cold 4% paraformaldehyde prepared in 0.1 M phosphate buffer (pH 7.4). C-28/12 chondrocytes were fixed for 30 min at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Sections (20 μm) were treated with 0.3% hydrogen peroxide and protein blocking solution. After permeabilization (0.1% Triton X 100 in 0.1% sodium citrate for 2 min at 4°C on ice), tissue sections were washed in PBS and incubated with labeling solution for 1 h at 37°C. TUNEL-stained structures were detected using an Apoptosis in situ Detection Kit (Wako) according to the manufacturer's protocol. DAB was used as a substrate. The percentage of apoptotic cells in the cartilage was determined in 10 high-power fields (40×), and was classified using the semiquantitative criteria described above.

Annexin-V can be used as a sensitive probe for phosphatidylserine on the outer layer of the cell membrane, and can therefore detect apoptotic cells. Staining of cartilage tissues and primary articular chondrocytes from RA patients was performed with a FITC-labeled, Annexin-V Apoptosis Detection Kit (MBL) exploiting the Rapid protocol, which allows direct binding of FITC-labeled annexin-V in tissue culture media. Cartilage tissues and primary chondrocytes from RA patients were washed with PBS and incubated with 150 μl of Annexin-V in Hepes buffer (10 mM Hepes/NaOH, pH 7.4; 140 mM NaCl; 5 mM $CaCl_2$) for 15 min at room temperature. After 3 rinses with PBS, slides were mounted and analyzed under a fluorescence microscope (TE2000-E, Nikon, Tokyo, Japan) equipped with a FITC filter.

Immunohistochemistry and flow cytometry for NK cell markers. Primary chondrocytes from RA and the femoral head and C-28/12 chondrocytes were used. A staining kit (Simple stain MAX-PO kit, Nichirei, Tokyo, Japan; or Dako Envision Kit Universal) was used for immunostaining of CD4 (Abcam Plc, Cambridge, UK), CD8 (Zymed Laboratories, South San Francisco, CA, USA), CD14 (Zymed), CD16 (Southern Biotech, Birmingham, AL, USA), CD56 (Lab Vision, Fremont, CA, USA), CD94

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(Ancell, Bayport, MN, USA), CD161 (Ancell), and KIR2DL1 (CD158a; Santa Cruz Biotechnology). Sections were counterstained with hematoxylin. Negative controls contained no primary antibody but an equivalent dilution of serum or IgG protein.

For phenotypic analysis of CD16 and CD56 by flow cytometry, 0.25×10^6 C-28/12 cells/250 µl medium were added to 3 tubes and 5 µl of isotype control (IOTest IgG2a-FITC, Cyto-Stat MsIgG1-RD1, Beckman Coulter, Fullerton, CA, USA) and monoclonal antibodies CD16FITC (Beckman Coulter) and CD56 phycoerythrin (BD Biosciences, San Jose, CA, USA) were added to the respective tubes. Tubes were incubated 15 min at 4°C in the dark and then washed with cold PBS at 1200 rpm for 5 min. Stained cells were fixed with 0.5 ml 1% paraformaldehyde and kept covered with foil at 4°C until further analysis on a flow cytometer (BD Biosciences).

Induction of apoptosis in chondrocytes. In order to introduce activated granzyme B (human, natural, Kamiya Biomedical, Seattle, WA, USA) into C-28/12 chondrocytes, BioPorter Protein Delivery Reagent (Genlantis, San Diego, CA, USA) was used. BioPorter is composed of a 2:1 mixture of a cationic lipid, TFA-DODAPL, and a neutral lipid, DOPE[39]. C-28/12 chondrocytes were seeded into 24-well culture dishes for 24 h before incubation with BioPorter, which was prepared according to the manufacturer's protocol. Briefly, chloroform was used to dissolve the dried BioPorter reagent, then the tubes were left open in a hood to evaporate the chloroform. Activated granzyme B protein solution was diluted at 10 to 70 µg/ml in PBS (10 mM Hepes, 150 mM NaCl, pH 7.0), and then transferred into a subconfluent population of chondrocytes using BioPorter protein delivery reagent. The preparation was incubated 4 h in serum-free medium, followed by washing twice with PBS, and medium was then replaced with serum-containing medium. The culture was then incubated an additional 24 h at 37°C before apoptosis assessment. To detect chondrocyte apoptosis, an ELISA (ApoStrand ELISA Apoptosis Detection Kit, Biomol International, Plymouth Meeting, PA, USA) was used as specified by the manufacturer. Briefly, chondrocytes were treated with formamide, which denatures DNA in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis[40]. Single-strand DNA in apoptotic cells was then detected using a monoclonal antibody. The amount of apoptotic expression was then determined using a plate reader at an absorbance of 405 nm.

After granzyme B (40 µg/ml) was introduced into C-28/12 chondrocytes, samples for transmission electron microscopy were fixed immediately after culture in a solution of 50 mM sodium cacodylate, 2% (v/v) gluteraldehyde, 7% (w/v) ruthenium hexamine trichloride for 6 h at room temperature, then overnight at 4°C. Fixed samples were washed with a solution of 65 mM NaCl, 100 mM sodium cacodylate (pH 7.4) and stored in 70% ethanol before embedding in Epon 812 and polymerization at 60°C. Samples stained with uranyl acetate in methanol were evaluated under a transmission electron microscope (JEM-1200 EX, JEOL, Tokyo, Japan).

Cytotoxic activity of chondrocytes. Our study was designed to test the hypothesis that chondrocytes have NK cell activity. Thus, C-28/12 chondrocytes and primary articular chondrocytes from the femoral head were stimulated with phytohemagglutinin (PHA), a granzyme B activator[41], and cytotoxicity was examined using K562 cells as targets. The NKtest Kit (Orpegen Pharma, Heidelberg, Germany) and the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MMT) assay[42] were used to determine whether the chondrocytes were cytotoxic to K562 cells. Specifically, 1×10^4 chondrocytes from the femoral head and C/28 were cultured in a culture plate for 24 h, then stimulated with 20 µg/ml PHA-L (Roche, Basel, Switzerland) in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% glutamine, and gentamicin for 0 (control), 1, 2, or 4 hours. After incubation was finished with PHA, cultures were washed with PBS 5 times to remove PHA. Next, 1×10^5 floating K562 cells were mixed with adherent chondrocytes. Cells were cocultured for 24 or 48 h in C/28 cells, or for 48 h in primary chondrocytes from the femoral head. Cell viability was determined by MMT assay, and the formazan pigment generated by viable cells was measured with a plate reader at 545 nm.

Whether these cytotoxic effects depend on granzyme B was tested using the cell-permeable, synthetic peptide-based granzyme B inhibitor Z-Val-Ala-Asp(OMe)-fluoromethylketone (Z-AAD-CMK; Kamiya Biomedical, Seattle, WA, USA), previously shown to block human and murine granzyme B[43]. C-28/12 cells (1×10^4 cells) were pretreated for 30 min at 37°C with Z-AAD-CMK at concentrations of 0 to 100 µM. Control cells were treated with an equivalent amount of vehicle (DMSO). Following incubation, cytotoxic activity was examined as follows; 1×10^4 C-28/12 cells were stimulated with 20 µg/ml PHA-L for 4 h, then 1×10^5 floating K562 cells were mixed with adherent chondrocytes. After coculture for 48 h, cell viability was determined by MMT assay.

Statistical analysis. The Wilcoxon rank-sum test was used to analyze results of cytotoxicity tests. Significance was set at $p < 0.05$. Statistical analyses were conducted using SPSS 11.0 J (SAS, Tokyo, Japan).

RESULTS

Annexin-V immunostaining for apoptosis in articular tissue. First, the presence of apoptosis in RA-affected cartilage and primary chondrocytes cultivated from RA patients was examined. Annexin-V staining confirmed apoptosis in the RA-affected cartilage (Figure 1B) and primary chondrocytes from RA patients (Figure 1D).

Immunohistochemistry of granzyme B, perforin, and apoptosis in cartilage. Articular tissues from the OA and RA patients, obtained during implant arthroplasty, were immunostained for granzyme B and perforin. Apoptosis was detected by TUNEL. Cells positive for granzyme B (Figure 2D, 2E, 2F), perforin (Figure 2G, 2H, 2I), and apoptosis (Figure 2J, 2K, 2L) were confirmed in all cartilage tissues from the OA or RA patients (Table 1). However, granzyme B-positive cells were distributed over an extensive area, from the outer layer of the cartilage to the tidemark, in RA patients (Figure 2E, 2F) when compared to OA patients (Figure 2D). Staining for perforin and apoptosis was weak when compared to granzyme B staining in both OA and RA specimens (Figure 2G-2L, Table 1). In cartilage from the RA patients where tissue destruction was greatest, the surface tangential layer of cartilage disappeared and remained only in the middle transitional and deep zones, where granzyme B-positive cells formed many clusters. The tidemark of the deep zone of cartilage destroyed by the invasion of blood vessels from the subchondral bone area, and the cells within and outside the lumens of these invading vessels, were strongly stained (Figure 2M).

Immunohistochemistry of granzyme B, perforin, and apoptosis in RA synovium. Accentuated granzyme B and perforin expression and apoptosis were also noted in RA synovial tissues, particularly in the pannus region invading the superficial layer of cartilage tissues (Figure 3C, 3D, 3E, 3F).

Localization of granzyme B and perforin and the expression of apoptosis in cartilage sections are summarized in Table 1. RA cartilage samples were often tightly covered with synovial membrane and were markedly thinner, and the same samples exhibited disorganization of cartilage architecture, with only the middle or deep zone remaining on the subchondral bone. In these cases, quantification of positive cells in the superficial layer or the middle transitional zone

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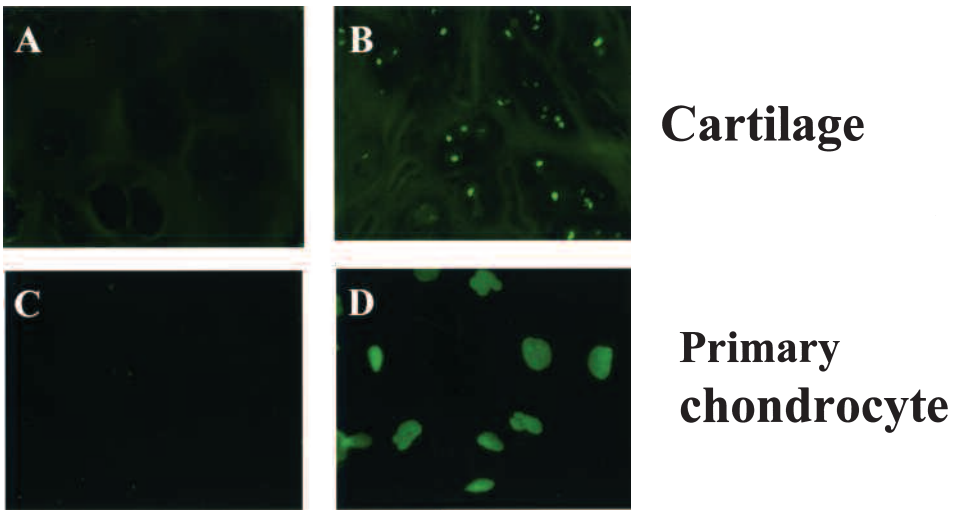

Figure 1. Presence of apoptosis in RA-affected cartilage and primary RA chondrocytes was confirmed by annexin-V staining. (A) Negative control of RA-affected cartilage. Untreated cartilage was used as a control. (B) Apoptosis in RA-affected cartilage was confirmed. (C) Negative control of RA-affected primary chondrocytes. Untreated cells were used as a control. (D) Apoptosis in RA-affected primary chondrocytes was confirmed. Original magnification A, B ×100; C, D ×200.

of cartilage tissue layer was not possible. Our studies showed that granzyme B staining from the superficial zone to the deep zone of cartilage, and its localization, coincided with that of perforin and apoptotic cell death in cartilage.

Expression of apoptosis caused by introduction of granzyme B. When granzyme B was introduced into C-28/12 chondrocytes, the rate of apoptosis increased dose-dependently in the range of 10 to 40 μg/ml, by ApoStrand ELISA. On the other hand, when the concentration of granzyme B exceeded 40 μg/ml, the development of apoptosis decreased dose-dependently (Figure 4A). On analysis of apoptosis using the TUNEL method, staining was most pronounced at 40 μg/ml, with gradual attenuation as the concentration of granzyme B increased beyond 40 μg/ml (Figure 4B). This phenomenon can be explained by the increase in concentration of granzyme B, which caused a greater number of nonviable chondrocytes, resulting in reduced TUNEL staining. This explanation also applies to the lower rate of apoptosis detection by ELISA. As shown by ApoStrand ELISA and TUNEL staining, delivery of granzyme B results in cell death.

On electron microscopy, untreated C-28/12 cells appeared normal. These cells filled their lacunae and contained readily identifiable intracytoplasmic organelles, and a distinct and dense pericellular matrix. Electron microscopy of the C-28/12 cells treated with granzyme B transfer demonstrated the characteristic hallmarks of apoptosis. Specifically, apoptotic chondrocytes showed nuclear compaction, volume shrinkage, vacuolization of the cytoplasm, and cell fragmentation. Many of these cells also showed at least partial separation from the surrounding territorial matrix (Figure 4C).

Expression of NK cell-surface antigens on chondrocytes. C-28/12 cells and primary chondrocytes from the femoral heads of patients with hip fractures or those from RA patients, positive for CD16, CD56, CD94, CD161, and KIR2DL1, which are all surface markers for NK cells, were detected (Figure 5A, panels b, e, h, j, and l for C-28/12 cells; Figure 5B, panels b, e, h, k, and n for the femoral head; Figure 5B, panels c, f, i, l, and o for RA). However, the T cell markers CD4 and CD8 were not detected in C-28/12 cells (Figure 5A, panels n and p). Expression of CD16 and CD56 was detected on C-28/12 chondrocytes by flow cytometry. The percentage of CD56+ and CD56+CD16+ chondrocytes was 34% and 28%, respectively. It appeared that the C-28/12 chondrocytes had a high percentage of CD56+ cells and CD16+CD56+ cells, while CD16+ cells were relatively scarce (Figure 5C).

Cytotoxic activity of chondrocytes. C-28/12 chondrocytes stimulated with PHA (20 μg/ml) for 0, 1, 2, or 4 h decreased the number of viable K562 cells compared to controls. In particular, after 48 h of coculture with K562 cells, cytotoxicity against K562 cells was 28.5% ($p < 0.01$). Cytotoxicity was lowest after 24 h of coculture with K562 cells (Figure 6A), and when primary chondrocytes from the femoral head were stimulated with PHA (20 μg/ml) after 48 h of coculture with K562 cells, cytotoxicity against K562 cells was 16.3% ($p < 0.05$; Figure 6B).

The dependence of the cytotoxic effects of C-28/12 cells on granzyme B was tested using a highly specific synthetic peptide that irreversibly inhibits granzyme B activity, ZVAD-FMK. ZVAD-FMK blocked this activity in a dose-dependent manner from 0 to 100 μM. At 100 μM, ZVAD-FMK blocked the cytotoxic activity by about 38% ($p < 0.05$; Figure 6C).

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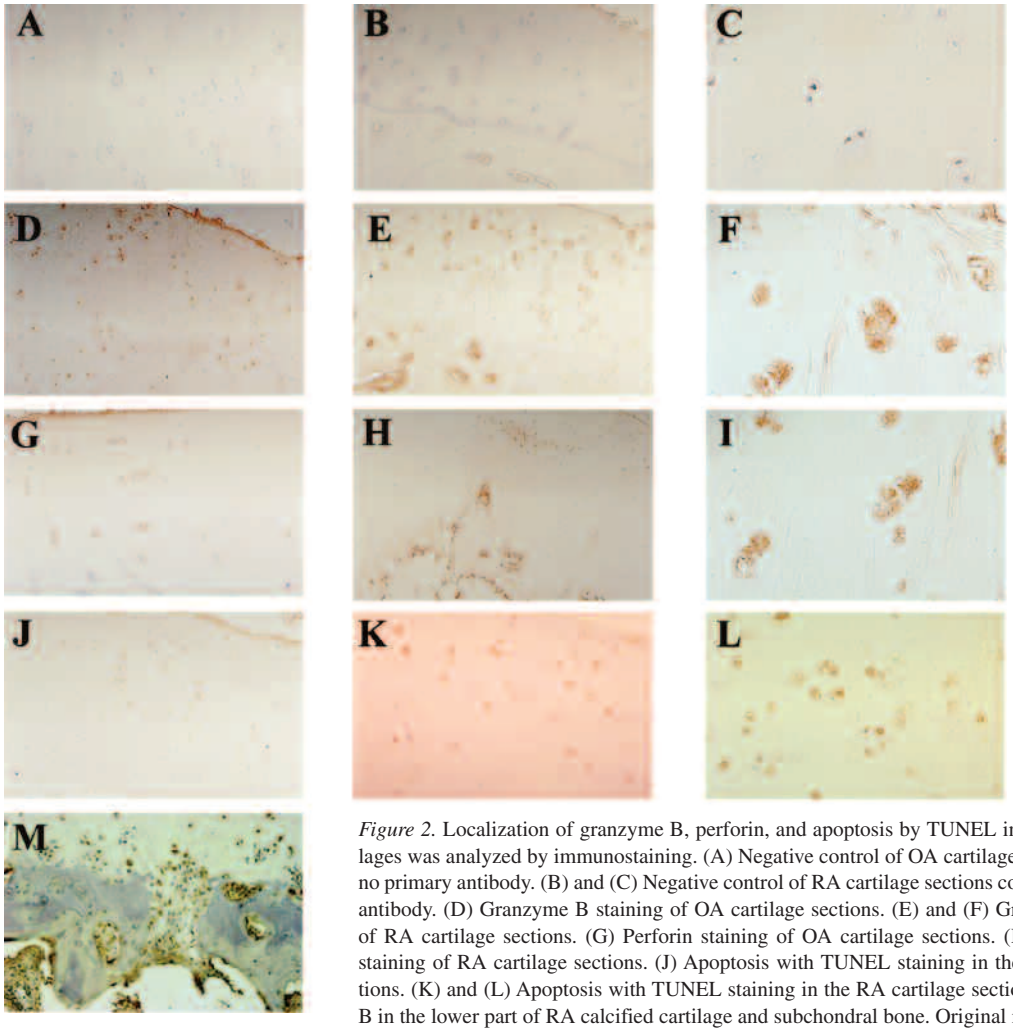

Figure 2. Localization of granzyme B, perforin, and apoptosis by TUNEL in OA and RA cartilages was analyzed by immunostaining. (A) Negative control of OA cartilage sections contained no primary antibody. (B) and (C) Negative control of RA cartilage sections contained no primary antibody. (D) Granzyme B staining of OA cartilage sections. (E) and (F) Granzyme B staining of RA cartilage sections. (G) Perforin staining of OA cartilage sections. (H) and (I) Perforin staining of RA cartilage sections. (J) Apoptosis with TUNEL staining in the OA cartilage sections. (K) and (L) Apoptosis with TUNEL staining in the RA cartilage sections. (M) Granzyme B in the lower part of RA calcified cartilage and subchondral bone. Original magnification A, B, D, E, G, H, J, K ×100; C, F, I, L ×400.

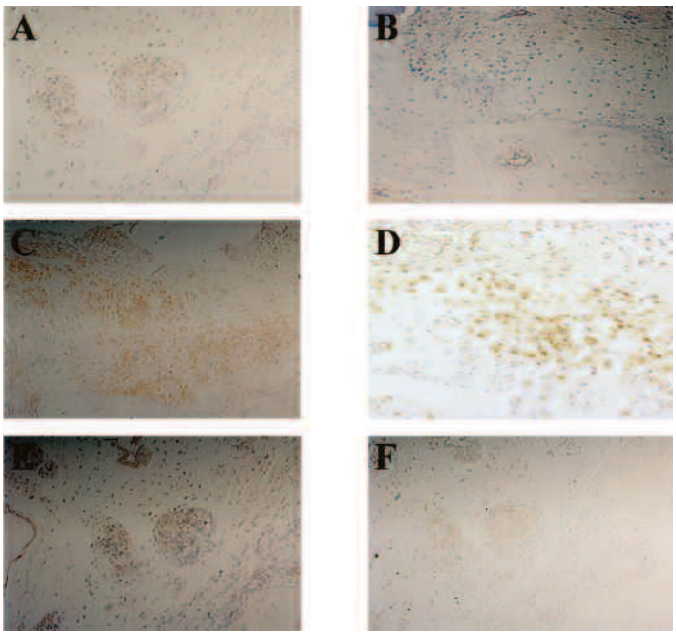

Figure 3. Localization of granzyme B- and perforin-positive cells at the RA synovium or pannus-cartilage tissue junction was analyzed by immunostaining. Granzyme B antibody-positive cells were seen in both the synovium (C) and pannus-cartilage tissue junction (D). (A) Negative control of RA synovial sections contained no primary antibody. (B) Negative control of the pannus-cartilage tissue junction sections from RA patients contained no primary antibody. (C) Granzyme B in synovial tissue sections. (D) Granzyme B in pannus-cartilage tissue junction sections from RA patients. (E) Perforin in synovial tissue sections. (F) Perforin in the pannus-cartilage tissue junction sections from RA patients. Original magnification ×100.

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Table 1. Localization of granzyme B and perforin, and expression of apoptosis in cartilage sections. Quantification of positive cells was performed over several areas in each section. The percentage of positive cells was evaluated in 10 high-power fields (40×) in 3 consecutive tissue sections, using the following semi-quantitative criteria: 0 = negative, + = less than 25% positive staining; ++ = 25–50% positive staining; and +++ = greater than 50% positive staining.

	Granzyme B-positive Cells				Perforin-positive Cells				Apoptotic-positive Cells			
	Super	Middle	Deep	Calcified	Super	Middle	Deep	Calcified	Super	Middle	Deep	Calcified
RA 1	ND	+	+	++	ND	+	+	0	ND	+	+	0
2	+	++	+	0	+	+	+	0	+	++	+	0
3	ND	++	++	++	ND	+	+	+	ND	+	+	+
4	ND	ND	++	++	ND	ND	+	0	ND	ND	+	+
5	++	+	+	++	++	+	+	+	+	+	+	+
6	ND	ND	++	++	ND	ND	+	+	ND	ND	+	+
7	+	+	+	0	+	+	0	0	+	+	0	0
8	0	+	+	0	0	+	0	0	0	+	+	0
9	ND	ND	+	++	ND	ND	+	0	ND	ND	+	+
10	ND	+	+	0	ND	+	+	0	ND	+	+	0
11	ND	+	+	0	ND	+	+	0	ND	+	+	0
12	+	+	+	+	+	+	+	+	0	+	+	+
OA 1	+	+	0	0	+	+	0	0	+	+	0	0
2	0	+	0	0	0	+	0	0	0	+	0	0
3	+	+	0	0	+	+	0	0	+	0	0	0
4	0	+	+	0	0	+	0	0	0	+	+	0

Super: superficial tangential zone; Middle: middle transitional zone; Deep: deep zone; Calcified: calcified cartilage; ND: not detected.

DISCUSSION

Apoptosis of chondrocytes is believed to be one of the factors responsible for the progression of OA and RA. The results of several studies have suggested that chondrocyte apoptosis is induced by a number of factors, such as Fas L and tumor necrosis factor, and that apoptosis is a decisive factor in cartilage destruction[44,45]. However, although many studies have indicated that granzyme B induces apoptosis in lymphocytes and other blood cells, no reports have confirmed whether granzyme B induces apoptosis in cartilage. This is the first report to demonstrate that granzyme B induces chondrocyte apoptosis.

Immunostaining of cartilaginous tissue showed numerous granzyme B-positive cells among the chondrocytes in the pannus lesion, and even in vascular tissue that infiltrates to the tidemark, which forms a boundary separating the cartilaginous tissue and subchondral tissue from the calcified cartilage layer. As granzyme B was present in the part of the cartilage where tissue damage was progressing, it appears to be one of the factors responsible for cartilage destruction. Granzyme B is also reported to be involved in the destruction of cartilage-forming connective tissue components, such as aggrecan and fibronectin[25,26,46]. Further, granzyme B is involved not only in induction of apoptosis, but also acts as a matrix proteinase; it participates directly in the destruction of the cartilaginous tissue substrate. Therefore, unlike other apoptosis-inducing factors or serine-type proteolytic enzymes, granzyme B apparently has 2 unique activities, apoptosis and destruction of cartilaginous substrate.

Tak, *et al* have stated that granzyme B is expressed in RA-affected synovial tissue, even in the pannus section, but they did not discuss granzyme B expression in the blood vessels that invade the cartilaginous tissue or subchondral regions. These studies led to the hypothesis that cytotoxic lymphocytes, such as NK cells and cytotoxic T lymphocytes, that are transported by the fine vasculature in the synovial membrane are responsible for the expression of granzyme B in synovial tissue; however, they did not discuss the possibility that cells other than lymphocytes may express granzyme B[19,26]. However, this study and previous reports[20] clearly show that granzyme B is present in cartilaginous tissues and chondrocytes, and is particularly notable in damaged cartilaginous sections.

Recently, granzyme B has been used as a marker of cell toxicity[10,11]. Thus, based on the hypothesis that granzyme B expressed in chondrocytes is involved in innate immunity, chondrocytes may express lymphocyte markers. However, few studies have examined lymphocytic markers expressed in chondrocytes. Malejczyk, *et al* examined rat epiphyseal chondrocytes for lymphocyte surface markers, and reported staining for CD4 (w3/25), but not for pan T (OX19), B cells (HIS14), or CD8 (T8)[47]. Bujía, *et al*[48] showed that the isolated chondrocytes always gave negative reactions for anti-CD4 antibody on immunostaining, while no reports have mentioned CD8-positive chondrocytes. In our study, chondrocytes were not stained for CD4 or CD8; therefore, we conclude that T cells are absent among C-28/12 chondrocytes. On the other hand, CD16, CD56, CD94, CD161, and KIR2DL1, characteristic surface antigens in NK cells, were present in C-28/12 cells and primary articular chondrocytes from the femoral head after hip fracture or RA. Studies have indicated that monocyte/macrophage surface antigens CD14 and CD68 are present in chondrocytes, and that chondrocytes show monocyte/macrophage-like functions[49-51].

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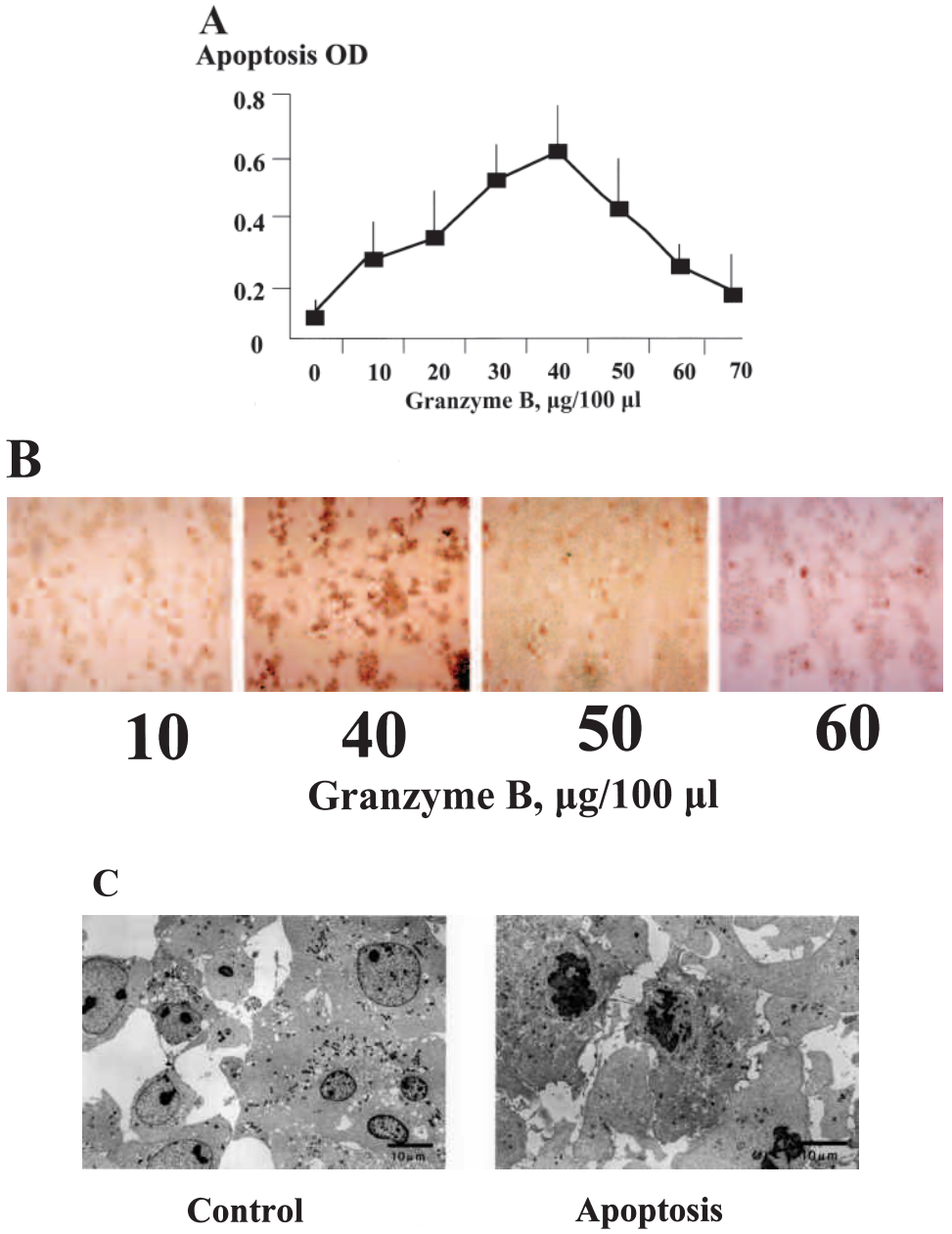

Figure 4. Apoptosis was induced by introduction of granzyme B with BioPorter protein delivery reagent. (A) When granzyme B was introduced into cultured C-28/12 chondrocytes, the rate of apoptosis increased dose-dependently in the range of 10 to 40 µg/ml. However, when granzyme B concentration exceeded 40 µg/ml, the rate of apoptosis decreased dose-dependently. (B) TUNEL staining is most pronounced at 40 µg/ml, with gradual attenuation as the concentration of granzyme B increases beyond 40 µg/ml. (C) On electron microscopy, untreated C-28/12 chondrocytes of nuclei and cytoplasm appear normal (control). Electron microscopy of C-28/12 chondrocytes treated with granzyme B show the characteristic hallmarks of apoptosis. Original magnification ×10,000.

Because chondrocytes have NK cell-surface antigens, they may also have NK function; therefore, we investigated their cytotoxicity. Following PHA stimulation, chondrocytes exhibited cytotoxicity against K562 cells. This chondrocyte cytotoxicity was partially dependent on the effects on granzyme B.

The expression of TLR is important in innate immunity, and the expression of TLR-2, -3, -9, -7, -8, and -4 on NK cells has been reported[52]. TLR-1, -2, -4, and -5 are expressed in chondrocytes[53,54]. A recent report describes that TLR-1 to -9 are present at the messenger RNA level in human articular chondrocytes[55]. After exposure to inflammatory cytokines or lipopolysaccharide, TLR-2 and TLR-4 levels increase in OA-affected chondrocytes, and this augments the levels of matrix metalloproteinase, nitric oxide, and prostaglandin E_2, all of which are related to joint

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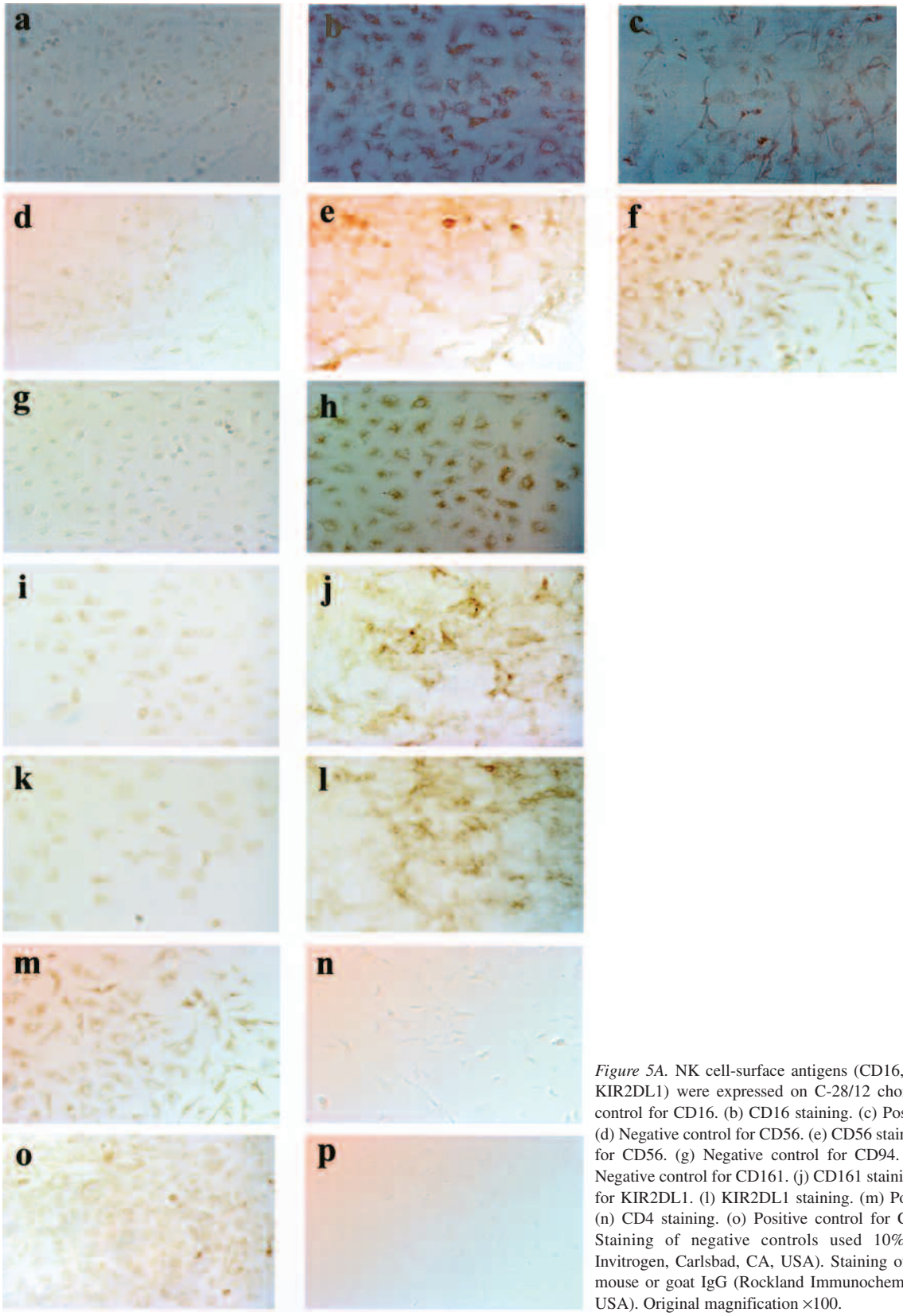

Figure 5A. NK cell-surface antigens (CD16, CD56, CD94, CD161, KIR2DL1) were expressed on C-28/12 chondrocytes. (a) Negative control for CD16. (b) CD16 staining. (c) Positive control for CD16. (d) Negative control for CD56. (e) CD56 staining. (f) Positive control for CD56. (g) Negative control for CD94. (h) CD94 staining. (i) Negative control for CD161. (j) CD161 staining. (k) Negative control for KIR2DL1. (l) KIR2DL1 staining. (m) Positive control for CD4. (n) CD4 staining. (o) Positive control for CD8. (p) CD8 staining. Staining of negative controls used 10% goat serum (Gibco, Invitrogen, Carlsbad, CA, USA). Staining of positive controls used mouse or goat IgG (Rockland Immunochemicals, Gilbertsville, PA, USA). Original magnification ×100.

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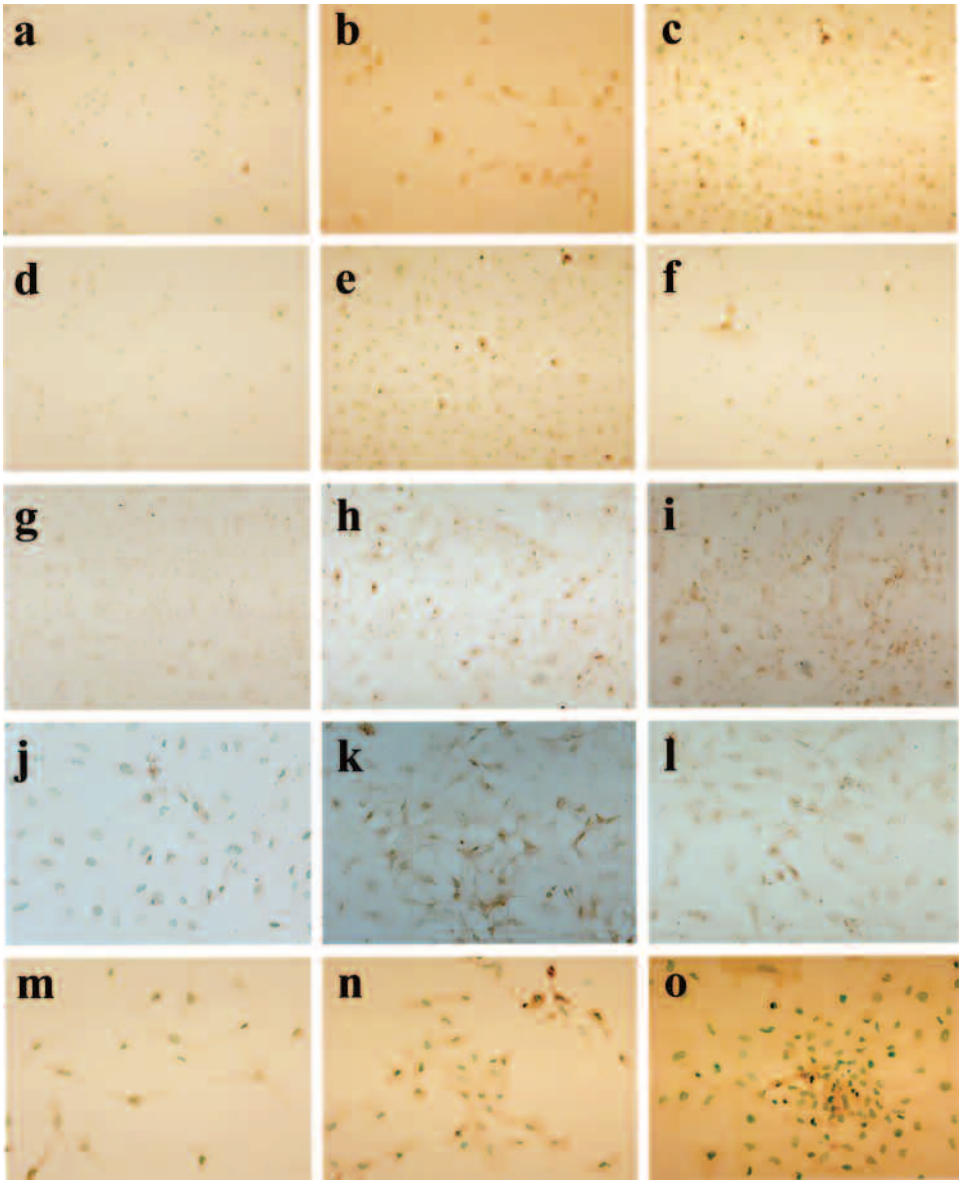

Figure 5B. NK cell-surface antigens (CD16, CD56, CD94, CD161, KIR2DL1) were expressed on primary chondrocytes from the femoral head or RA. (a) Negative control for CD16. (b) CD16 staining for primary chondrocytes from the femoral head. (c) CD16 for primary chondrocytes from RA. (d) Negative control for CD56. (e) CD56 staining for primary chondrocytes from the femoral head. (f) CD56 for primary chondrocytes from RA. (g) Negative control for CD94. (h) CD94 staining for primary chondrocytes from the femoral head. (i) CD94 for primary chondrocytes from RA. (j) Negative control for CD161. (k) CD161 staining for primary chondrocytes from the femoral head. (l) CD161 for primary chondrocytes from RA. (m) Negative control for KIR2DL1. (n) KIR2DL1 staining for primary chondrocytes from the femoral head. (o) KIR2DL1 staining for primary chondrocytes from RA. Original magnification a-i ×100; m, n, o ×200.

destruction[56]. Thus, innate immunity, a major function of NK cells, is believed to be responsible for the formation of inflammatory mediators via the action of TLR and the subsequent development of inflammatory reactions, including antibacterial peptides (human beta-defensin)[57], the alternate pathway of complement activation, mannose-binding lectin, and a number of cytokines[58], whereas cartilage destruction occurs through proteolytic enzymes[26].

We observed that granzyme B was topographically expressed in RA cartilage and synovium, and this was not reported in our previous studies. Our findings demonstrate that granzyme B is responsible for inducing apoptosis in

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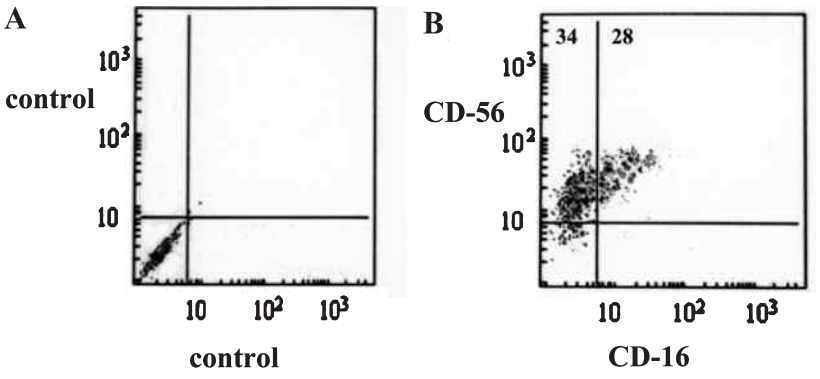

Figure 5C. Expression of CD16 and CD56 detected on C-28/12 chondrocytes by flow cytometry. (a) Negative control for CD16 and CD56. (b) Double-staining of CD56 and CD16. The percentage of CD56+ and CD56+CD16+ chondrocytes among total chondrocytes was 34% and 28%, respectively.

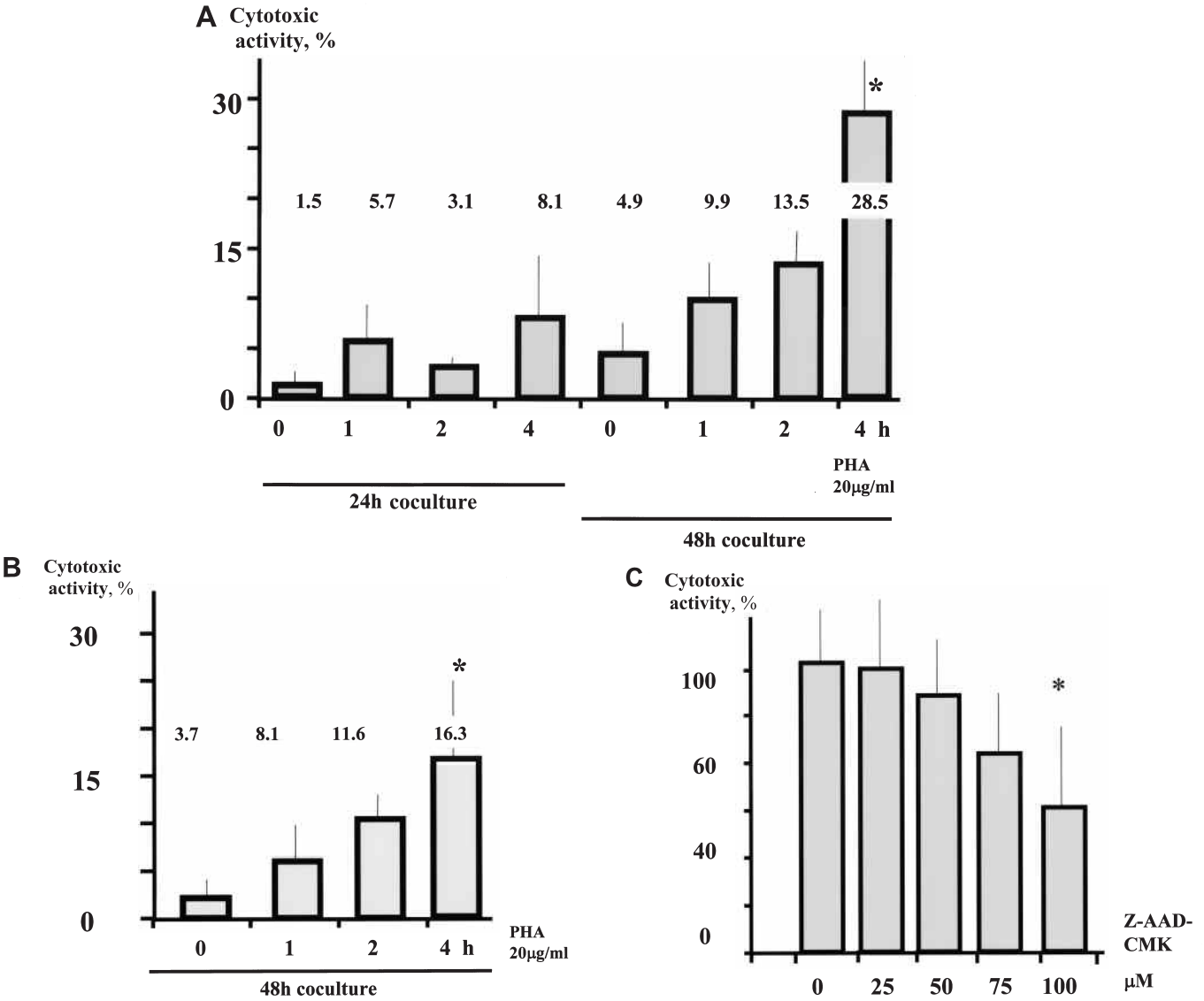

Figure 6. (A) Cytotoxic activity of C-28/12 chondrocytes was examined using K562 cells as targets. C-28/12 cells stimulated with PHA (20 μg/ml) for 1, 2, or 4 h decreased the number of viable K562 cells. When K562 cells were cocultured 48 h with chondrocytes stimulated with PHA for 4 h, cytotoxicity was 28.5% ($p < 0.01$). Cytotoxicity was lowest after coculture for 24 h. Results represent quadruplicate experiments. *$p < 0.01$. (B) Cytotoxic activity of primary chondrocytes was examined using K562 cells as targets. For primary chondrocytes from the femoral head, cytotoxicity was 16.3% ($p < 0.05$) after stimulation with PHA (20 μg/ml) for 4 h following 48 h of coculture with K562 cells. Results represent duplicate experiments. *$p < 0.01$. (C) Dependence of these cytotoxic effects on granzyme B was tested with ZVAD-FMK. ZVAD-FMK blocked the effects of granzyme B in a dose-dependent manner from 0 to 100 μM. At 100 μM ZVAD-FMK blocked the cytotoxic activity by about 38% (*$p < 0.05$).

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chondrocytes. These chondrocytes express NK cell-surface antigens CD16, CD56, CD94, CD161, and KIR2DL1, and show granzyme B-dependent cytotoxicity against K562 cells. This indicates that chondrocytes exhibit NK-cell-like activity related to innate immunity, and when this innate immunity is disrupted by inflammation, NK-cell-like activity may provoke joint destruction via the action of granzyme B.

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