Human granzyme B mediates cartilage proteoglycan degradation and is expressed at the invasive front of the synovium in rheumatoid arthritis

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Abstract

Objective. To investigate the cartilage-degrading capacity of granzyme B and the presence of granzyme B-positive cells at sites of erosion in the rheumatoid synovium.

Methods. Granzyme B was added to [3H]proline-[35S]sulphate-labelled cartilage matrices and to cartilage explants. Proteoglycan degradation was assessed by the release of 35S and glycosaminoglycans into the medium and collagen degradation was assessed by the release of 3H and hydroxyproline and by measuring the fraction of denatured collagen. Granzyme B expression was studied at the invasive front of the synovium by immunohistochemistry.

Results. Granzyme B induced loss of both newly synthesized, radiolabelled proteoglycans in cartilage matrices and resident proteoglycans of the cartilage explants. No effect on collagen degradation was found. Granzyme B-positive cells were present throughout the synovium and at the invasive front.

Conclusion. The presence of granzyme B-positive cells at the invasive front of the synovium together with its ability to degrade articular proteoglycans supports the view that granzyme B may contribute to joint destruction in rheumatoid arthritis.

Key words: Granzyme B, Cartilage destruction, Rheumatoid arthritis.

Proteolytic degradation of articular cartilage and bone is a characteristic feature of rheumatoid arthritis (RA). Cartilage destruction at the invasive front of the inflamed synovial tissue (pannus) has been attributed mainly to serine proteinases and matrix metalloproteinases produced by fibroblast-like synoviocytes, macrophages, chondrocytes and polymorphonuclear cells [1].

Recently, a set of serine proteinases, called granzymes, has been identified [2, 3]. Granzymes, which include granzyme A, a proteinase with trypsin-like activity, and granzyme B, a proteinase which specifically cleaves behind aspartic acid residues, are soluble cytolytic proteinases able to induce apoptosis in target cells in the presence of perforin [4, 5]. If the lysosome-like granules of activated cytotoxic lymphocytes and natural killer cells are released from the cells, granzymes may also exert extracellular effects [6, 7]. Granzyme A can stimulate the production of interleukin (IL)-6 and IL-8 by fibroblasts and epithelial cells as well as that of IL-6, IL-8 and tumour necrosis factor α by monocytes [8, 9]. Furthermore, these enzymes may be involved in the remodelling of the extracellular matrix, as illustrated by the capacity of granzyme A to degrade basement membrane type IV collagen [10–14]. Several

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observations suggest a role of granzymes in joint inflammation and destruction in patients with RA. In the synovial fluid of patients with RA, lymphocytes have been shown to express granzyme A messenger RNA [15, 16]. Recently, increased concentrations of soluble granzyme B were found in the synovial fluid of RA patients when compared with patients suffering from osteoarthritis or reactive arthritis [17]. The levels of soluble granzyme B were significantly higher in synovial fluid than in corresponding plasma samples, indicating local production within the inflamed joint. These observations in synovial fluid are in line with those in rheumatoid synovial tissue, where the presence of granzyme A [18–20] and granzyme B [19, 20] has been reported. The number of granzyme B-positive cells, mainly natural killer cells, was found to be specifically elevated in patients with RA and the degree of expression correlated positively with parameters of arthritis activity [20].

To further investigate the potential role of granzymes in the pathophysiology of joint destruction, we assessed the capacity of granzyme B to degrade newly synthesized and resident proteoglycans and collagen in bovine articular cartilage. In addition, we investigated the presence of granzyme B-containing cells at a characteristic site of joint destruction in RA: the pannus–hard tissue junctions of the metacarpophalangeal joints.

Materials and methods

Production of radiolabelled cartilage matrix

In this study we used alginate beads to culture chondrocytes, as described previously [21]. The chondrocytes were obtained from bovine metacarpophalangeal articular cartilage, isolated by collagenase digestion, embedded in alginate and cultured in Dulbecco’s Modified Eagle Medium, 2% glutamic acid (DMEM Glutamax medium; Gibco Life Technologies, Grand Island, New York, USA) supplemented with 10% fetal calf serum (v/v), 50 μg/ml ascorbic acid, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% carbon dioxide. After 4 weeks of culture, a cartilage matrix is produced that contains proteoglycan aggregates incorporated in a three-dimensional network of collagen type II and collagen cross-links [22]. To radiolabel the newly formed proteoglycans and collagen fibrils in the cartilage matrix, [35S]sulphate and [3H]proline (Amersham International, Amersham, UK) were added to the culture medium. After 4 days of culture, the culture medium was changed twice weekly with fresh medium containing 0.25 μCi/ml [35S]sulphate and [3H]proline. After 28 days of culture, the alginate gel was suspended in 55 mM sodium citrate to dissolve the beads and release the synthesized matrix. The solution was centrifuged (750 g, 6 min) and the matrix pellet was suspended in DMEM and plated in 24-well plates. The matrix was coated for 3 days at 4°C, fixed with methanol and air-dried. Plates were stored at −20°C until use.

Degradation of newly synthesized cartilage matrix by granzyme B

Radiolabelled cartilage matrices were slowly thawed at room temperature in DMEM and incubated for 24 h at 37°C with 1 ml natural human granzyme B at respectively 0.4 and 0.04 μg/ml in phosphate-buffered saline (PBS) with 0.05%(v/v) Tween-20 (Janssen Chimica, Geel, Belgium). Granzyme B was obtained from Evanston Hospital Corporation, Evanston, Illinois, USA (lot 10.5.95, 32 units/μl, isolated from human T-cell line YT-Indy and purified using HPLC; specific activity 16.8 units/μg). Depletion of newly synthesized proteoglycans and collagen was followed by the release of 35S and 3H respectively. The release of radioactivity into the medium and the remaining radioactivity in the matrix were measured with a liquid scintillation analyser (Tri-Carb 1900 CA; Packard, Meriden, Connecticut, USA). The percentage of matrix degradation was calculated as: ([d.p.m. (medium)]/[d.p.m. (medium) + d.p.m. (matrix)]) × 100.

Culture of bovine articular cartilage explants

Bovine metacarpophalangeal joints were acquired from a local abattoir immediately after the cows (1–2 yr old) had been killed. Articular cartilage was removed aseptically from the joint, washed twice with PBS and prepared as slices with a wet weight of approximately 30 mg (dry weight approximately 10 mg, consisting of 60–70% collagen type II; proteoglycans accounted for a large part of the remainder [23]). The cartilage slices were incubated in DMEM Glutamax at 37°C in a humidified atmosphere of 5% carbon dioxide. Each culture consisted of one piece of cartilage in 0.5 ml of medium.

Degradation of cartilage explants

Bovine articular cartilage explants were incubated with 1 ml of 0.4 and 0.04 μg/ml human granzyme B for 15 and 90 h respectively. Proteoglycan loss was followed by measuring the release of sulphated glycosaminoglycans (GAG) in the culture medium using a commercially available assay (Blyscan; Biocolor, Belfast, UK), and expressed as μg per mg cartilage. Collagen degradation was assessed by measuring hydroxyproline release into the culture medium by HPLC [24] (expressed as pmol per mg cartilage) and by measurement of the fraction of denatured collagen in the cartilage explants, as described previously [25].

Immunohistochemistry

The demonstration of granzyme B-containing cells at the invasive front of the pannus tissue requires the preservation of the osteochondrosynovial transition zone, for which the decalcification of tissue needs to be avoided. Therefore, we embedded metacarpophalangeal joints in plastic before 3-μm sections were cut and incubated with antibody specific for human granzyme B. Human metacarpophalangeal joints from three patients with RA were obtained at joint replacement. Slices of 3 mm were embedded in polymethylacrylate [26].
tissue was fixed by overnight incubation in acetone at a constant temperature between −15 °C and −19 °C. Subsequently, the tissues were impregnated by constant rotation for 6 h at 4 °C in moulding cup trays (Polysciences, Warrington, Pennsylvania, USA). To this end, 90 mg of benzyloperoxide containing 20–25% water (Merck, Darmstadt, Germany) was dissolved in 20 ml of 2-hydroxyethylmethacrylate and 20 ml of 2-hydroxypropylmethacrylate followed by addition of 1 ml of a mixture of 6.25% (v/v) N,N-dimethylaniline (Merck) and 93.75% (v/v) polyethylene glycol 400 (Fluka, Buchs, Switzerland). The polymerization mixture was stirred for 5 min at room temperature before addition to the tissue. Sections (3 μm) were cut on a motor-driven Reichert-Jung 2050 microtome, harvested on water containing 0.05% (v/v) ammonia and dried overnight at room temperature. Sections were incubated with monoclonal antibodies specific for recombinant human granzyme B [27] for 60 min. In control sections, the primary antibody was omitted or irrelevant antibodies were applied (isotype-matched anti-human immunodeficiency virus antibody, a gift from TNO, Rijswijk, The Netherlands). Subsequently, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark) was added, followed by incubation with biotinylated tyramide [28] and addition of streptavidin–HRP (Zymed, San Francisco, California, USA); each incubation step lasted 30 min. HRP activity was detected with hydrogen peroxide as substrate and aminoethylcarbazole (Sigma, St Louis, Missouri, USA). Slides were counterstained with Mayer’s Hämalaunlösung (Merck) and, after washing with distilled water, mounted in Kaiser’s glycerol gelatine (Merck). The sections were washed between all steps with PBS and all incubations were carried out at room temperature.

Calculations and statistical analysis
All experiments were performed in triplicate. The results are given as the mean and standard error of the mean. Differences between levels of degradation were calculated with two-sided Student’s t-test for independent samples.

Results

Incubation with granzyme B results in release of newly synthesized proteoglycans of the chondrocyte extracellular matrix and leaves newly synthesized collagen unaffected

Incubation with 0.4 μg/ml granzyme B induced the release of the [35S]sulphate-labelled matrix components after 24 h. Release of approximately 30% above the background level of 35S from the matrix was observed. Granzyme B at 0.04 μg/ml did not induce degradation of [35S]sulphate-labelled matrix (Fig. 1, black bars).

In contrast, granzyme B did not induce the release of [3H]proline-labelled collagen after 24 h of incubation at any of the concentrations tested (Fig. 1, hatched bars).

**Granzyme B degrades resident proteoglycans and not collagen of cartilage explants**

Bovine articular cartilage explants were incubated with 1 ml of 0.04 and 0.4 μg/ml granzyme B for 15 and 90 h. Fifteen hours of incubation with 0.4 μg/ml granzyme B resulted in a significant increase in GAG release, to 3.3 μg/mg cartilage (Fig. 2, black bars). After 90 h of incubation, 0.04 and 0.4 μg/ml granzyme B increased GAG release to 6.1 and 11.7 μg/mg cartilage respectively (Fig. 2, black bars).

No collagen degradation could be detected after 90 h of incubation with either concentration of granzyme B; hydroxyproline release did not rise above the background level (Fig. 3, hatched bars) and no increase in the amount of denatured collagen was found in the explants (Fig. 3, black bars).

**Localization of granzyme B-positive cells at the pannus–hard tissue junction**

Immunohistological analysis of plastic-embedded sections of metacarpophalangeal joints from patients with RA yielded granzyme B-positive cells throughout the synovium (Fig. 4A) and also, although not in massive numbers, at the invasive front of the pannus tissue (Fig. 4B). Granzyme B was found in granules both inside and outside the cells (Fig. 4A). Staining was

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**Calculation of activity**

% degradation = [(activity at time t - background activity) / (maximum activity - background activity)] * 100

**Graph 1**

- [35S]sulphate
- [3H]proline

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**Fig. 1.** Degradation of [35S]sulphate-labelled and [3H]proline-labelled cartilage matrix cultured in alginate beads for 4 weeks after incubation with 0.04 and 0.4 μg/ml human granzyme B for 24 h (black and hatched bars respectively). The background levels (bg) indicate the spontaneous release of 35S and 3H into the medium without addition of granzyme B. Results are mean and standard error of the mean of radioactivity released into the culture medium as a percentage of total radioactivity (n = 3). *P < 0.05 (Student’s t-test for independent samples).
negative when the primary antibody was omitted or irrelevant antibodies were applied as controls.

Discussion

RA is a progressive, destructive disease affecting the metacarpophalangeal and metatarsophalangeal joints most frequently. Within the joint, the pannus tissue overgrows and invades the underlying cartilage and bone. This tissue destruction involves both damage to the collagen fibrillar network and loss of proteoglycans [29, 30]. Cartilage degradation results primarily from the action of the extracellular proteolytic enzymes produced by many cell types in response to microenvironmental factors. Several classes of proteinases are responsible for tissue destruction in RA. Much emphasis has been placed on serine proteinases and matrix metalloproteinases [30-35]. Recently, granymes have also been suggested to play a role in the degradation of extracellular matrix [11, 17, 36]. Granymes are serine proteinases that are found in granules in cytotoxic T cells and natural killer cells [19, 20]. Levels of granyme A and B are specifically increased in the synovial fluids of RA patients compared with those of patients with osteoarthritis or reactive arthritis [17]. In RA synovial tissue, a significantly higher number of granyme

**Fig. 2.** Release of GAGs from bovine articular cartilage explants after incubation with 0.04 and 0.4 μg/ml human granzyme B for 15 and 90 h (black and shaded bars respectively). Results are given as the mean and standard error of the mean GAG (μg) released in the culture medium per mg cartilage (n = 3). *P < 0.05, **P < 0.01 compared with background levels (bg) at the same time point; #P < 0.05 compared with levels with 0.04 μg/ml (Student’s t-test for independent samples).

**Fig. 3.** The hatched bars show the release of hydroxyproline from bovine articular cartilage explants after incubation with 0.04 and 0.4 μg/ml human granzyme B for 90 h. Results are given as the mean and standard error of the mean hydroxyproline (OH-proline; pmol) released into the culture medium per mg cartilage. The black bars show the fraction of denatured collagen after incubation with 0.04 and 0.4 μg/ml human granzyme B in 90 h as the percentage of total collagen (mean and standard error of the mean).

B-positive cells was found compared with synovial tissue of patients with reactive arthritis [36]. When granzyme-containing granules are released from the cells, they may exert extracellular activity, such as proteolysis of extracellular matrix components. Granzyme A was shown to be capable of degrading collagen type IV from the basement membrane [13]. Froelich *et al.* [11] provided evidence for aggrecan-degrading activity of granzyme B. The objectives of the present study were to further explore the cartilage-degrading properties of granzyme B and to investigate the presence of granzyme B at the invasive front of the pannus tissue.

The capacity of granzyme B to degrade articular cartilage matrix was assessed in vitro. The availability of purified human granzyme B provided the opportunity to investigate directly the capacity of this enzyme to degrade an organized, newly synthesized cartilage matrix produced during 4 weeks of chondrocyte culture, and to degrade explants of intact bovine articular cartilage. Incubation with granyme B resulted in the loss of proteoglycans from the newly synthesized cartilage matrix. These results are in line with the findings of Froelich *et al.* [11], indicating that granzyme B is indeed capable of directly degrading newly synthesized proteoglycans. Granzyme B also mediated the release of glycosaminoglycans from whole articular cartilage explants, suggesting the digestion of proteoglycans to protein fragments which are small enough to diffuse out of the tissue.
Granzyme B on hydroxyproline release from the cartilage explants, indicating collagen degradation, was consistent with the lack of effect on \(^{3}H\)proline release from the cartilage matrix. This strongly suggests that \(^{35}S\)sulphate is incorporated mainly in the proteoglycans and that \(^{3}H\)proline is incorporated, probably as \(^{3}H\)hydroxyproline, mainly in the collagen fraction of the cartilage matrix. This allows the use of this sensitive cartilage-degradation model to distinguish between the effects of proteolytic enzymes on proteoglycan and collagen degradation.

To investigate the possibility that granzyme B is involved in cartilage degradation in RA, we investigated the presence of granzyme B at the invasive front of the pannus tissue. Expression of granzyme B has been found in synovial tissue [12]. These granzyme B-positive cells were seen in the synovial lining and in the sublining area, but the presence of the cells at the particular site where erosions developed was not investigated. In the present study we found granzyme B-positive cells throughout the synovial tissue and, although not in massive numbers, at the invasive front of the pannus tissue. We found granzyme B-containing granules both inside and outside the cells. The release of granzyme B-containing granules at the invasive front of the pannus tissue may lead to the active binding of positively charged granzyme B to the anionic extracellular matrix and, in the apparent absence of an endogenous inhibitor, to local concentrations that are effective in degrading proteoglycans. The identity of the granzyme B-positive cells could not be studied directly by double staining for technical reasons, because the use of plastic to embed our sections resulted in non-specific adsorption of certain antibodies. However, because only cytotoxic lymphocytes and natural killer cells are able to express this enzyme [19, 20], the finding of positive staining for granzyme B indicates the presence of these cell types in the invading synovial tissue. The previously demonstrated increased number of these granzyme B-positive cells and their location suggest that cytotoxic lymphocytes and natural killer cells may be involved in matrix degradation in several ways: by cell–cell interaction, by cytokine-mediated stimulation of matrix-degrading synoviocytes, and by the local production of matrix-degrading enzymes, including granzyme B.

The results of the present study confirm that human granzyme B is capable of degrading the proteoglycan component of cartilage. This finding, together with the presence of granzyme B at the invasive front of the pannus tissue and the previously observed increased expression of granzyme B in rheumatoid synovial joints, suggests that granzyme B is involved in the destruction of articular cartilage in RA.

References


