COMPARATIVE IMMUNOLOCALIZATION STUDIES OF COLLAGENASE 1 AND COLLAGENASE 3 PRODUCTION IN THE RHEUMATOID LESION, AND BY HUMAN CHONDROCYTES AND SYNOVIOCYTES IN VITRO

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SUMMARY
The degradation of fibrillar type II collagen is a major feature of cartilage destruction in rheumatoid arthritis (RA). Since collagenase 3 is produced by chondrocytes and preferentially degrades type II cartilage collagen, it seemed likely that this enzyme would have a prominent role in the destruction of rheumatoid joints. Using immunolocalization techniques, we have examined and compared the production and distributions of collagenase 1 and collagenase 3 in cells and tissues derived from rheumatoid knee arthroplasties. Primary cultures of chondrocytes stimulated with interleukin-1β showed that most of the cells produced collagenase 1, whereas only a minority (≤5–10%) produced collagenase 3; a few chondrocytes demonstrated the co-ordinate production of both enzymes. Primary cultures of rheumatoid synoviocytes produced collagenase 1, but not collagenase 3. Both enzymes were demonstrated in the rheumatoid lesion. Collagenase 1 was more commonly observed in both synovium and cartilage (22 of the 28 specimens), was especially prominent at cartilage erosion sites, and most of the positive specimens demonstrated extracellular enzyme. By contrast, collagenase 3 was observed less frequently (7/28 specimens) and was produced by relatively few chondrocytes and synovial cells, this usually being much less than that observed for chondrocytes of osteoarthritic cartilage. These observations suggest different regulatory mechanisms for the production of collagenases 1 and 3 in the rheumatoid lesion, and demonstrate that the distribution and production of collagenase 1 are far more prevalent than those for collagenase 3.

KEY WORDS: Collagenase 1, Collagenase 3, Immunolocalization, Rheumatoid lesion, Synoviocytes, Chondrocytes.
Chondrocyte cell cultures

Macroscopically normal articular cartilage sampled from rheumatoid arthroplasty material was enzymically digested as previously described [12]. One millilitre of isolated human articular chondrocyte (HAC) suspensions containing $\sim10^4$ cells/ml of Dulbecco's modified Eagles medium (DMEM) + 20% fetal calf serum (FCS; Gibco Life Technologies, Paisley) was applied to the HAC and ASC coverslips, followed by FITC-conjugated swine anti-rabbit IgG and Texas red-conjugated sheep anti-mouse IgG (Amersham, UK) as described previously [14].

Rheumatoid synovial cell cultures

Rheumatoid synovial tissue was enzymically dissociated as described previously [13]. One millilitre of a cell suspension containing $10^4$ cells/ml of DMEM + 10% FCS was added to each well of a 12 well culture dish containing a sterile 19-mm-diameter glass coverslip and cultured as previously described [13, 14]. After 48 h, DMEM + 0.25% lactalbumin hydrolysate (LH) containing interleukin-1$\beta$ (IL-1$\beta$) at a final concentration of 0.5 ng/ml was added to half the wells, whilst the rest received fresh DMEM + 0.25% LH. After 16 h, monensin (which inhibits secretion) was added to all the wells at a final concentration of 5 $\mu$M and after a further 6 h coverslips were fixed for 10 min in 70% ethanol, air dried and stored desiccated at 4°C until required for immunolocalization.

Synovial cell cultures

Primary ASC cultures, comprising a mixture of both synovial fibroblasts and macrophages (CD68 positive), demonstrated variable collagenase 1 and mouse monoclonal anti-collagenase 3 in TBS was applied to the HAC and ASC coverslips, followed by FITC-conjugated swine anti-rabbit IgG and Texas red-conjugated sheep anti-mouse IgG (Amersham, UK) as described previously [14].

Tissue sections. Five micrometre tissue sections of cartilage–pannus junctions or osteoarthritic cartilage were cut, dewaxed, rehydrated and examined for the presence of collagenases 1 and 3 using the primary antibodies described above. Tissue sections were first incubated for 30 min in 10% (v/v) serum of the species in which biotinylated antibody was raised. Primary antibodies to collagenases 1 and 3 were applied to sections for 2 h at 20°C. After three washes in TBS, biotinylated swine anti-rabbit IgG or biotinylated goat anti-mouse IgG (both from Dako), diluted 1:300, were applied to the sections for 45 min at 20°C. After washing (3 × TBS), avidin–biotin complex (Dako) conjugated to alkaline phosphatase (AP) was applied to the sections for 45 min at 20°C and was shown to be specific for the relevant collagenase. Specimens were described as ‘positive’ for intra- and/or extracellular staining relative to controls as determined by two independent assessors.

Controls

Control incubations included treatments with buffer only, or similar dilutions of non-immune sera substituted for the primary antibody. All consistently showed negative results. Conventional epitope unmasking techniques of trypsinization (0.1 mg/ml in 0.1% CaCl$_2$ in TBS for 20 min at 37°C) or microwave treatment (5 min at 850 W) failed to increase the extent of collagenase 3 staining to that described.

Photography

All cells and tissue sections were examined on a Zeiss Photomicroscope III; dual localization was visible through individual filter sets for FITC and Texas red, and micrographs were taken using Kodak Ektachrome 100 T colour reversal film.

RESULTS

Chondrocyte cultures

Primary HAC without IL-1$\beta$ stimulation showed that $\sim$20% of cells were immunostained for collagenase 1, whereas $<5\%$ were positive for collagenase 3. By contrast, HAC cultures treated with IL-1$\beta$ showed that up to 80% of the cells were positive for collagenase 1, compared to only 5–10% which demonstrated collagenase 3. Dual localization techniques were used to determine whether individual cells were producing one or both of these enzymes under in vitro conditions. Although the majority of cells were found to produce only one enzyme (Fig. 1A and B), some chondrocytes were shown to produce both the collagenases, as judged by intracellular immunostaining (Fig. 1C and D).

Rheumatoid synovial cells

Primary ASC cultures, comprising a mixture of both synovial fibroblasts and macrophages (CD68 positive), demonstrated variable collagenase 1...
immunostaining: whereas from 10 to 60% of the fibroblastic cells were positive for collagenase 1 production, the macrophages were devoid of enzyme. Collagenase 3, however, was consistently absent from these cell cultures. Subcultures of rheumatoid synovial fibroblasts (passage numbers 1–4) were examined for immunodetection of collagenases 1 and 3, after treatment with IL-1β. Whereas >95% of the fibroblasts were shown to be positive for collagenase 1, no evidence was found for collagenase 3 production (Fig. 1E and F).

Rheumatoid tissues
Collagenase 3 was demonstrated by immunostaining in seven of the 28 rheumatoid tissue specimens. Chondrocytes were occasionally found to contain the enzyme (Fig. 2), and a few positive cells were observed at the cartilage–pannus junction of one specimen, but the tissue origin of these cells is uncertain (Fig. 2B). Tissue sections were generally negative for collagenase 3 and weak extracellular staining was seen in only one specimen. In contrast to these results for collagenase 3, collagenase 1 was localized both extracellularly and intracellularly in a majority of the specimens examined. Several showed localized areas of extracellular staining at the cartilage–pannus junction and within the synovial tissue. Cells in the synovium often showed intracellular staining, and four of the 28 specimens showed evidence of chondrocytes producing collagenase 1 (Fig. 2). However,
Fig. 2. (Caption on next page.)
it was often noted that individual chondrocytes exhibited quite different collagenase phenotypes within any one area, with some cells strongly stained for collagenase 1 or collagenase 3 adjacent to chondrocytes negative for enzyme (Fig. 2C and D). Assessment of the distributions of both enzymes in synovial tissues showed a preponderance of cells stained for collagenase 1 rather than collagenase 3 (Fig. 2E and F). Table I provides an overall assessment of the relative frequency and distribution sites for the two collagenases compiled from the immunohistochemical observations of the 28 rheumatoid specimens.

In contrast to the observations of collagenase 3 in ‘rheumatoid’ cartilage, the immunostaining for this enzyme was much more common in the chondrocytes of eight different specimens of osteoarthritic cartilage (Fig. 2G and H). Whilst five OA cartilage specimens showed ~60% of chondrocytes positively stained, the remainder showed relatively little collagenase 3 staining.

**DISCUSSION**

The matrix metalloproteinases are known to degrade various components of the cartilage matrix [16] and several have been demonstrated in rheumatoid tissues, either by immunolocalization [17] or by *in situ* hybridization [18, 19]. Whereas a role for collagenase 1 and stromelysin 1 in cartilage destruction of rheumatoid joints has been substantiated by their demonstration at cartilage erosion sites [8, 20], the contribution of collagenase 3 (MMP-13) remains obscure [1]. Collagenase 3 has been shown to have a broad spectrum of activity against extracellular matrix proteins; this includes collagenolytic as well as gelatinolytic activity [6], degradation of cartilage aggrecan [21], and an ability to degrade cartilage type II collagen preferentially [4, 6]. By contrast, the more specific fibroblast collagenase 1, preferentially degrades collagens type III and I, with type II collagen being degraded approximately five times more slowly than type I [22]. Since collagenase 3 is characteristically produced by activated chondrocytes *in vitro* [4, 5], and by chondrocytes of osteoarthritic cartilage [3, 23], it seemed likely that this enzyme would play an important role in cartilage destruction associated with the rheumatoid lesion. However, our immunohistochemical findings have demonstrated that collagenase 3 was produced in only a minority of the rheumatoid specimens examined, in contrast to the higher frequencies observed for osteoarthritic cartilage and the wider and more frequent distributions for collagenase 1.

Almost all studies on collagenase 3 expression and production in arthritic joint tissue or cells have been based on mRNA analyses or enzyme production monitored by Western blotting techniques. Such studies have shown that human chondrocytes express mRNA for collagenase 3, this being upregulated by the pro-inflammatory cytokines IL-1β and tumour necrosis factor alpha (TNF-α) [3, 5, 23], and that more collagenase 3 protein is produced by chondrocytes from osteoarthritic cartilage [3, 4]. Whereas some studies have found little evidence for collagenase 3 expression by synoviocytes [3, 5], Wernicke *et al.* [11] showed that mRNA for collagenase 3 could be demonstrated in a proportion of arthritic synovial membranes, in parallel with mRNA for collagenase 1 and stromelysin 1. To date, only one abstract [24] has reported immunolocalization studies

**TABLE I**

Summary of immunohistochemical observations on the distributions of collagenase 3 and collagenase 1 in rheumatoid lesions (cartilage–pannus junctions)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>No. of specimens* positively stained</th>
<th>Cartilage Junction</th>
<th>Synovium†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intra-cellular</td>
<td>Extra-cellular</td>
</tr>
<tr>
<td>Collagenase 3</td>
<td>7/28</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Collagenase 1</td>
<td>22/28</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

*Specimens defined as positive represented tissue sections which contained significant staining as determined by two independent assessors.†Synovial tissue remote from cartilage interface.
of collagenase 3 in a limited number of arthritic tissues, claiming lower levels in cartilage from RA compared to osteoarthritis; our findings for osteoarthritic cartilage are in accord with those data.

Our observations from cell cultures confirm earlier studies [3,5,23] that the pro-inflammatory cytokines upregulate collagenase 3 production by chondrocytes, but do not induce collagenase 3 production by synoviocytes [3,5]. By contrast, collagenase 1 is markedly stimulated by these cytokines in both chondrocyte and synoviocyte cultures. Our observations have shown a relative paucity of collagenase 3 in ‘rheumatoid’ cartilage specimens and its occasional production by a few synoviocytes. Collagenase 3 production was invariably restricted to single or very small groups of cells, observations in contrast to the more frequent staining of osteoarthritic chondrocytes. The stimulatory factors responsible for this collagenase 3 response by some cells in vivo remain unclear, but it would seem from the rheumatoid tissues that the full stimulatory requirements are dissimilar to those which regulate collagenase 1 production. As yet, we have no evidence for the coordinate production of both enzymes by individual cells of the rheumatoid lesion, although this has been demonstrated by activated ‘rheumatoid’ chondrocytes in vitro.

The reports of high levels of collagenase 3 expression in ‘osteoarthritic’ chondrocytes are in accord with the prime role for these cells in this degenerative cartilage disease, and are supported by the observations of osteoarthritic cartilage sections in this study. By contrast, histological studies of RA tissues have shown that chondrocyte-mediated chondrolysis is relatively infrequent, the invasive synovial tissue providing most of the degradative processes involved in cartilage erosion [25–27]. We conclude that collagenase 1 production appears to be more prevalent than that for collagenase 3 in the rheumatoid lesion, and that these visual immunolocalization observations provide a new perspective on the previous biochemically based studies of collagenase 1 and 3 expression in rheumatoid tissues and cells.

Acknowledgements

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References


19. Firestein GS, Paine MM. Stromelysin and tissue inhibitor of metalloproteinases gene expression in...

NOTE ADDED IN PROOF
While this paper was ‘in press’ we became aware of the publication by Lindy et al. [28] which examined the distribution of collagenase-3 in six specimens of rheumatoïd synovium and seven specimens of osteoarthritic synovium. Lindy et al. reported a very high incidence of positive staining for collagenase 3, viz. 86% of rheumatoid synovial lining and endothelial cells, and a similar frequency for stained lining and endothelial cells in osteoarthritic synovial tissues. These observations are in direct contrast to the data presented here, where we found no evidence for collagenase 3-positive endothelial cells, and only five of 28 rheumatoid synovial specimens showed relatively few cells (<5%) stained for collagenase 3. At present the reasons for these major differences between the two papers remain uncertain, but may relate to different antibody preparations, specificities and the methodology for immunostaining.