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Metalloproteinase production by rabbit articular cartilage: comparison of the effects of interleukin-1α in vitro and in vivo

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Abstract To assess the effects of interleukin-1 on intact articular cartilage in vitro, explants from young and adult rabbits were cultured with interleukin-1 and the distributions of the matrix metalloproteinases and tissue inhibitor of metalloproteinases (TIMP-1) were investigated by indirect immunofluorescence microscopy. One to 2-week-old cartilage chondrocytes synthesized collagenase in response to pure or crude interleukin-1 (monocyte conditioned medium), with subarticular cells most responsive. Collagenase synthesis was not stimulated in adult articular chondrocytes when explants were treated with either pure or crude interleukin-1. Stromelysin, gelatinase and TIMP-1 could not be demonstrated within any zone of the cartilage, indicating that their synthesis was not stimulated by either pure or crude interleukin-1. The addition of fibroblast growth factors, either alone or in combination with interleukin-1, did not modify these responses. These results contrast markedly with observations on cultured chondrocyte monolayers, where interleukin-1 treatment induces near co-ordinate expression of metalloproteinases. To assess the effects of interleukin-1 in vivo, it was injected into adult rabbit knee joint spaces and the articular cartilage subsequently analysed for evidence of altered metalloproteinase production by immunocytochemistry. No significant increase in metalloproteinase or TIMP-1 synthesis by chondrocytes was detected, although the cartilage matrix showed a marked loss of toluidine blue metachromasia. We conclude that metalloproteinases are not involved in the rapid loss of proteoglycan from cartilage matrix in these situations.

Key words Interleukin-1 · Metalloproteinase
Collagenase · Cartilage · Arthritis

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Introduction

The matrix metalloproteinases (MMPs) have diverse specificities but together can degrade all the macromolecular components of connective tissues [25]. This family of proteinases can be divided into subgroups, the major ones having at least two separate gene products. The collagenases specifically cleave interstitial collagens types I, II, and III at a single locus; gelatinases cleave denatured collagens and type IV collagen; stromelysins are more general proteinases, cleaving proteoglycan core protein, fibronectin and type IV collagen. The synthesis of these proteinases by connective tissue cells, such as chondrocytes and fibroblasts, can be induced by culturing the cells in vitro in the presence of interleukin-1 (IL-1; [23, 27, 38]). The effect of IL-1 on chondrocytes in vitro can be potentiated by fibroblast growth factors (FGFs; [8, 35]). These data have led to the concept that the erosion of articular cartilage that occurs in rheumatoid arthritis could be due to high levels of IL-1 in the inflamed joints, causing articular cartilage chondrocytes to synthesize and secrete increased amounts of MMPs, which locally degrade the cartilage matrix.

To gain evidence for this view of cartilage breakdown, several groups have injected IL-1 directly into rabbit joints. A single intra-articular injection of either highly purified porcine IL-1α or β, or human IL-1 or recombinant human IL-1α or β (rHuIL-1α or β) into New Zealand White rabbits gave a dose-dependent loss of proteoglycan from the cartilage at 24 h, with a marked increase in concentration of glycosaminoglycan in the synovial fluid [18, 34]. Using Old English rabbits and a partially purified porcine synovial IL-1 preparation, the maximal proteoglycan loss after a single injection was after 3 days [10]. After 3 intra-articular injections of this material at 3-day intervals all joint cartilages showed extensive loss of proteoglycan, and the decrease continued for a further week [33]. In all these studies cartilage metachromasia, assessed histologically with either safranin O or toluidine blue, mirrored the proteoglycan changes. In the study of Page Thomas et al. [33] no his-

tological evidence of collagen breakdown was observed, and the proteoglycan was gradually replaced over the ensuing 3–4 weeks. Recently McDonnell et al. [22] demonstrated a direct correlation between the accumulation of stromelysin in the synovial fluid of rabbits injected with IL-1 and the appearance of proteoglycan within those fluids. These workers subsequently reported that stromelysin mRNA and protein levels were induced in both the synovium and cartilage of IL-1 injected joints but not in contralateral control joints [21]. However, IL-1 was not a sufficient stimulus to induce collagenase mRNA expression in cartilage in this in vivo model [42].

To extend the above studies on the effects of IL-1 on intact articular cartilage rather than isolated chondrocytes, we cultured articular cartilage explants from young and adult rabbits with IL-1 and monitored by indirect immunofluorescence microscopy the distribution within the explants of the MMPs collagenase, stromelysin and gelatinase, and their inhibitor, TIMP-1. FGFs were also studied alone and together with IL-1 in an attempt to modify the response of the articular cartilage chondrocytes in vitro. In addition, for comparative purposes, we injected IL-1 in vivo into rabbit knee joints and analysed the articular cartilage subsequently for MMP production to gain evidence for induction of MMP synthesis.

Materials and methods

Reagents

Pig blood mononuclear cell conditioned medium (MCM), used as a source of crude IL-1, and pure porcine IL-1α were prepared as described by Saklatvala et al. [38]. rHuIL-1α and rHuIL-1β were a gift either from Roche (New Jersey, USA) or from Dr. J. Saklatvala. Acidic and basic FGFs were purchased from Biogenesis (Bournemouth, UK). All other reagents were from Sigma.

Culture methods

Articular cartilage was obtained from femoral condyles of either young (1–2 weeks old) or adult (5 months old) New Zealand White rabbits. It was either removed as small chips (300 mg cartilage per 3 cm^2 dish per 2 ml medium) or full thickness explants (2 pieces per stainless steel grid with 1 ml medium) as stated. In later experiments where the anatomical site was defined, cartilage was removed from the medial and lateral condyles and both supracondylar ridges by cutting as deeply as possible. The culture medium was Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated normal rabbit serum (NIRS, Burroughs Wellcome, Beckenham, UK) which contained negligible inhibitory activity against collagenase. Cultures were maintained at 37° C in 5% CO_2/air and those destined for immunolocalization of MMPs and TIMP-1 were treated with monensin (5 μM) for 6 h immediately prior to freezing the tissue in liquid nitrogen for 90 s [36]. In addition, some cartilage was frozen directly ex vivo. In experiments in which the effects of FGFs were studied, the culture medium was either DMEM without serum, or Iscove's medium (Flow; with the additions, bovine serum albumin, 40 mg/ml; transferrin, 3 mg/ml; soya bean lipid, 2 mg/ml) without serum.

Rabbit chondrocytes were prepared from 1-week-old rabbits as described previously [16, 40]. First passage cells were plated at 1.2×10^5 cells per well in 24-well plates (Flow) with DMEM with 10% fetal bovine serum (FBS) and grown to confluence. Acidic FGF (aFGF, 100 ng/ml, with heparin 20 μg/ml) and basic FGF (bFGF, 100 ng/ml) were then added to the chondrocyte cultures either separately or together with rHuIL-1α (2 ng/ml and 10 ng/ml), and incubated for 48 h.

Enzyme assays

Culture media from explant or chondrocyte cultures were assayed for collagenase, stromelysin and gelatinase activities in the presence of 0.7 mM 4-aminophenylmercuric acetate using either [^{14}C]-collagen, [^{14}C]-casein or heat denatured [^{14}C]-collagen as substrates [26]. One unit of enzyme degrades 1 μg of substrate/min at 35° C or 37° C (gelatinase, stromelysin).

Antibodies

Polyclonal antisera to the rabbit MMPs and TIMP-1 were raised in sheep. They have been fully characterized; collagenase [16], stromelysin [27], gelatinase A and B [28], TIMP-1 [13] have been used in previous immunolocalization studies in the rabbit [2, 17]. Pooled normal sheep serum was used as control. Immunoglobulins (IgG) were prepared from all sera as previously described [15]. The second antibody used for the indirect immunofluorescence was a monovalent Fab' preparation, labelled with fluorescein isothiocyanate, of a porcine antibody raised to sheep Fab' fragments (pig-FITC; [15]).

Immunolocalization of MMPs and TIMP-1

The MMPs and TIMP-1 are usually secreted in vivo in small amounts and are not stored intracellularly. To increase sensitivity so as to locate precisely the cellular source of these proteins, tissues were incubated with the ionophore monensin, which blocks translocation of secreted protein while allowing synthesis to continue [15, 29]. This results in intracellular accumulation of antigen in the Golgi apparatus and secretory vesicles of cells, which can then be immunolocalized by indirect immunofluorescence.

Frozen sections (7 μm) were cut on a cryostat and taken onto glass slides. The sections were fixed (4% freshly prepared formaldehyde, 30 min), permeabilized (0.1% Triton X-100, 5 min) and then incubated for 30 min at room temperature with IgG preparations of either the antisera or normal sheep serum (all at 50 μg/ml). Fifteen sections were cut at each location in all samples, and the antibodies assigned to sets of five (e.g. anticollagenase on nos. 2, 7 and 12) to limit variation in tissue histology as far as possible. The sections were washed in phosphate buffered saline (PBS, 3× 5 min) after each of the above steps. The sections were incubated for 30 min with the pig-FITC second antibody, washed, and counterstained with methyl green (1 mg/ml, 2 min) to stain nuclei red. Sections were mounted in Citifluor (University of Kent, Canterbury) and viewed by fluorescence microscopy on a Zeiss Photomicroscope III with epifluorescence and wide band FITC filter. Photographs were taken either on Ektachrome 400 ASA film uprated during processing to 1600 ASA or on Agfachrome 1000 RS film uprated during processing to 2000 ASA. Subsequently the coverslips were removed and sections stained either with Harris' haematoxylin and eosin or with toluidine blue to assess cartilage matrix metachromasia. These were photographed on Kodak Ektachrome 50 film.

Injection of IL-1 into rabbit knee joints in vivo

To study the effect of IL-1 in vivo three different protocols were used because methodologies of previous studies had varied [10, 18, 21, 33, 34].

Method 1

A 5-month-old rabbit (Old English, weight 1.9 kg) was given 3 injections into the knee joints at 3-day intervals. The left knee re-

ceived the vehicle alone (0.4 ml of 0.01 mg/ml Healonid, sodium hyaluronate in PBS, Pharmacia) and the right knee received 20 ng porcine IL-1α in vehicle at each injection. The rabbit was killed 3 days later. This injection schedule was chosen to give maximum glycosaminoglycan release from the articular cartilage [33]. The preparation of porcine IL-1α used had not been tested for presence of endotoxin but injection of endotoxin into rabbit knee joints was reported to result in polymorphonuclear leucocyte infiltration but no loss of glycosaminoglycan ([34]; P. Page Thomas, personal communication). Cartilage was removed from both supracondylar ridges, the intercondylar notch, medial and lateral condyles, and patella from each knee. The synovium and fat pad adjacent to the patella were also removed and cut in half. Cartilage samples from the right supracondylar ridge, right condyle and half the synovium were frozen directly. Cartilage samples from the left supracondylar ridge and condyle, the intercondylar notch, patella and remaining half of the synovium were cultured in DMEM with 10% FBS and 5 μM monensin for 6 h before freezing. Frozen sections (7 μm) were cut and stained as described above.

Method 2

Two New Zealand White rabbits (weight, 2.8–3.0 kg) were used. rHuIL-1α (100 ng in 0.5 ml sterile pyrogen free saline) was injected into the left knee joint spaces and the right knees received an equal volume of vehicle. The animals were killed 24 h after injections. Cartilage and patella with adjacent synovium were removed as described above and cultured in DMEM with 10% FBS and monensin (5 μM) for either 6 or 24 h before freezing.

Method 3

Two hundred nanograms of rHuIL-1β (in 1 ml pyrogen free saline) was injected into the left knee space of a New Zealand White rabbit (weight, 3.5 kg) and vehicle alone injected into the right knee joint. After 9 h, monensin (5 μM in 1 ml pyrogen-free saline) was injected into both knee joint spaces. The rabbit was killed 12 h after the IL-1 injection, cartilage and synovium were removed as before and frozen directly without culture.

Table 1 Total collagenase activities in culture media from full thickness explants (A) and small chips of articular cartilage (B), treated for varying times with additions. Articular cartilage samples from knee joints of 18 New Zealand white rabbits (1–2 weeks old) were removed and cultured either as full thickness explants on stainless steel grids or as small chips. The culture media were changed daily for the first 4 days, then on day 7: at each medium change one full-thickness explant and one small chip from each treatment were removed for immunolocalization of collagenase. The culture media removed at these times were assayed in duplicate for collagenase activity as described in Materials and methods. The number in parentheses in (A) denotes the number of media samples assayed to give the mean values ±SEM (*Dex* dexamethasone)

Treatment	Collagenase activity U/ml				
	Day 1	Day 2	Day 3	Day 4	Day 7
A. Full-thickness explants					
	(5)	(4)	(3)	(2)	(1)
Control	0	0.22 ±0.08	0.31 ±0.12	2.00 ±0.01	1.00
1% MCM	0.92 ±0.04	4.00 ±0.37	4.13 ±0.56	6.55 ±1.36	10.44
1% MCM +Dex. 200 nM	0.05 ±0.02	0.14 ±0.04	1.05 ±0.07	1.98 ±0.13	5.26
IL-1α, 2 ng/ml porcine	0.19 ±0.02	1.07 ±0.08	1.52 ±0.15	2.97 ±0.27	4.05
IL-1α+Dex. 200 nM	0	0	0.19 ±0.05	0.62 ±0.04	2.98
B. Small chips					
Control	0	0	0	0.09	Contam
1% MCM	0.58	1.07	0.92	1.77	4.13
1% MCM+Dex. 200 nM	0	0	0	0.27	0.37
IL-1α, 2 ng/ml porcine	0.46	0.81	1.28	1.55	1.50
IL-1α+Dex. 200 nM	0	0	0	0	0

Results

The effects of MCM and IL-1 on collagenase synthesis by explants of young rabbit articular cartilage

Initially experiments were carried out to determine whether chondrocytes in articular cartilage from 1- to 2-week-old rabbits synthesized collagenase in culture, and whether the collagenase could be immunolocalized within chondrocytes by immunofluorescence microscopy. Articular cartilage from knee joints of 18 young rabbits was removed and cultured either as a full thickness explant or as small chips. The culture media removed on days 1, 2, 3, 4 and 7 were assayed for collagenase activity and the results are shown in Table 1. Both full thickness explants and small chips of cartilage demonstrated an increase in collagenase synthesis when treated with either 1% MCM or pure porcine IL-1α, although the latter was less effective. Dexamethasone, when included with either MCM or IL-1α, inhibited the increase in collagenase activity in the medium, in agreement with published work [20], but was less effective on full thickness explants than on small chips.

When sections of cartilage cultured for 48 h (plus 6 h culture with monensin) were stained with anti-collagenase IgG and examined by fluorescence microscopy, chondrocytes in control medium were always predominantly negative (data not shown). However, with both full thickness explants and small chips incubated with either MCM or porcine IL-1α some chondrocytes had intracellular green fluorescence, indicating synthesis of collagenase (Fig. 1a–c), and areas of extracellular matrix were also fluorescent (Fig. 1a, c). Adjacent control sections stained with normal sheep serum IgG were all negative (not shown). There was a discrete distribution of positive cells, more clearly discernible with full thickness explants than chips, whose orientation clearly varied. Subarticular and hypertrophic chondrocytes responded to both MCM and IL-1α (Fig. 1a, b) whereas articular cells were often negative (Fig. 1a). Connective tissue overlying the cartilage in these 1- to 2-week-old rabbit joints, which is difficult to remove completely, had random positive cells and fluorescence on adjacent extracellular matrix (Fig. 1c). When dexamethasone was included with both MCM and IL-1α the chondrocytes

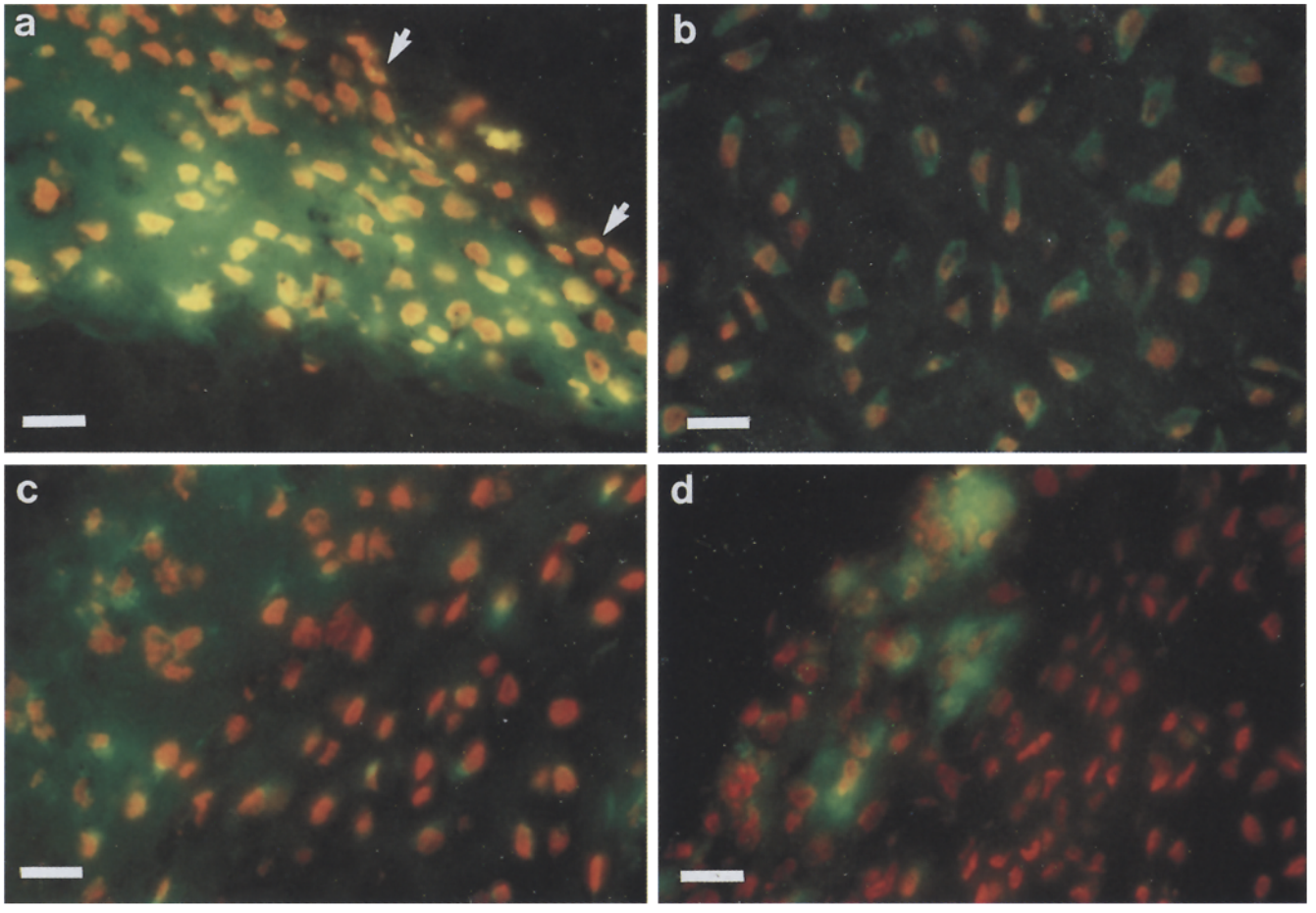

Fig. 1a–d Immunolocalization of collagenase in full thickness explants and small chips of young rabbit articular cartilage cultured with various additions. Full-thickness explants and small chips of articular cartilage were cultured for 48 h, with monensin added for the last 6 h. Frozen sections were cut, fixed, permeabilized and stained with anti-collagenase IgG followed by pig-FITC as described in Materials and methods. Nuclei were counterstained red with methyl green. Sections were viewed by fluorescence microscopy. *Bars* 20 μm. **a** Edge of a full-thickness explant cultured in 1% mononuclear cell conditioned medium (MCM). Chondrocytes and cartilage matrix are *bright green* indicating presence of collagenase, while articular surface cells *(arrows)* have only nuclear counterstain, strongly suggesting that they are not synthesizing collagenase. **b** Full-thickness explant cultured in porcine interleukin (IL)-1α. Hypertrophic chondrocytes have weak but clearly positive juxtanuclear fluorescence indicating synthesis of collagenase. **c** A small chip of cartilage cultured in 1% MCM. Some chondrocytes have positive juxtanuclear fluorescence while others are negative. Connective tissue overlying cartilage has fluorescence on the extracellular matrix and positive cells. **d** Full-thickness explant cultured in 1% MCM with dexamethasone. The articular and subarticular chondrocytes *(bottom right)* are negative but overlying soft connective tissue has positive cells and adjacent extracellular matrix staining, indicating that these cells continue to synthesize collagenase in the presence of dexamethasone

were negative, whereas the overlying connective tissue still had both positive cells and staining on adjacent extracellular matrix, suggesting that collagenase synthesis by these cells continued in the presence of dexamethasone (Fig. 1d). Sections of cartilage cultured for only 24 h in either MCM or IL-1α had a few weakly positive cells in areas corresponding to the distribution outlined above but the pattern was less distinct. Sections from tissue frozen ex vivo stained with anti-collagenase IgG were completely negative.

These results demonstrate that young rabbit cartilage synthesizes collagenase in culture in response to both MCM and IL-1α; that collagenase can be assayed in the culture medium and immunolocalized at 48 h within the cells; and that collagenase-secreting cells have a discrete pattern of distribution.

Distribution of collagenase, stromelysin, gelatinase and TIMP-1 in explanted young rabbit cartilage

To investigate in detail the distribution of cells responsive to MCM and IL-1α in the young rabbit cartilage explants and to determine whether the pattern was the same for all three MMPs and TIMP-1, the above experiments were repeated taking full-thickness explants from defined sites in the joint (see Materials and methods). The culture media removed at 48 h were assayed for collagenase and stromelysin and the results are shown in Table 2. Only low levels of both enzyme activities were found in the medium, reflecting both the reduction in total weight of cartilage used and the early time point (cf. Table 1).

Table 2 Collagenase and stromelysin activities in culture media removed on day 2 from full-thickness explants taken from defined sites in the young rabbit knee. Full-thickness cartilage explants were taken from defined sites in the joint (see Materials and methods). From each rabbit four pairs of explants could be prepared, consisting of cartilage from the supra condylar ridge and the adjacent condyle from the two knee joints. To minimize variation between animals, the pairs of explants were allocated to a control and three experimental treatments. Four rabbits were used and explants were cultured for 48 h. The media were assayed (duplicate samples) for collagenase and stromelysin and the average values are shown for the number of samples given in parentheses

Treatment	Collagenase U/ml	Stromelysin U/ml
Control (4)	0	0
1% MCM (3)	0.58	0.23
1% MCM+Dexamethasone 200 nM (3)	0.35	0.13
IL-1α, porcine 2 ng/ml (3)	0.15	0.06
IL-1α+Dexamethasone 200 nM (3)	0	0.02

Chondrocytes in both the condyles and supracondylar ridges cultured in control medium and monensin and stained with the antisera to MMPs and TIMP-1 were negative. Chondrocytes of both explants cultured in MCM and monensin, however, showed a distinct pattern of distribution as illustrated in Fig. 2. Sections stained with anti-collagenase IgG showed that articular cells had little or no immunofluorescence, suggesting that they were unresponsive to MCM (Fig. 2a), whereas the subarticular and hypertrophic chondrocytes had intracellular immunofluorescence indicating synthesis of collagenase (Fig. 2a–c). The subarticular chondrocytes had the brightest intracellular staining and fluorescence was also present on inter-territorial matrix in this region (Fig. 2b). Adjacent sections stained with anti-stromelysin IgG (Fig. 2d), anti-TIMP-1 IgG (Fig. 2e) and anti-gelatinase A and B IgG (not shown) had no staining in any of the chondrocyte zones indicating that, unlike in chondrocyte monolayers [27], chondrocytes in intact articular cartilage exposed to MCM do not necessarily synthesize MMPs co-ordinately. In all explants where the section included bone, cells secreting gelatinase were observed within bone lacunae (Fig. 2f). None of the treatments altered the distribution or intensity of this staining.

Chondrocytes in explants cultured with IL-1α revealed the same patterns of fluorescence as described above for MCM, but fewer cells in each zone were positive for collagenase and the staining intensity was weaker, particularly in the subarticular zone where there was intracellular fluorescence but no matrix staining. As with MCM, the chondrocytes were negative for stromelysin, gelatinase and TIMP-1. Explants cultured with MCM and dexamethasone and stained with anti-collagenase IgG contained fewer positive chondrocytes than the MCM-treated explants. Positive cells were less bright and located mainly in the subarticular and hypertrophic zones. No chondrocytes stained in sections incubated with either the anti-stromelysin, or anti-gelatinase or anti-TIMP-1 IgGs.

Where connective tissue was attached to the articular cartilage explants, occasional positive cells were seen staining for collagenase (Fig. 2a, arrow), stromelysin and gelatinase (not shown). Cells synthesizing MMPs were also seen in connective tissue overlying explants treated with dexamethasone, confirming the results of the previous experiment (Fig. 1d) that dexamethasone did not inhibit synthesis in these cells. MMPs synthesized by the connective tissue cells as well as the chondrocytes both contribute to enzyme activity assayed in the culture medium, less that retained by the explant matrix. This may explain the variation in the assay results and the incomplete inhibition by dexamethasone. It also underlined the importance of immunocytochemical studies to identify correctly the cellular origins of these proteinases.

Effect of fibroblast growth factors on the cartilage response to IL-1

It has been reported [7, 35] that the effect of IL-1 on chondrocytes in vitro is potentiated by addition of FGFs, with the production of increased amounts of collagenase and neutral proteinase activity in the culture medium. The question therefore arose whether FGFs, alone or with IL-1, would also stimulate chondrocytes within articular cartilage explants in culture to synthesize stromelysin and restore the near coordinate synthesis of collagenase and stromelysin seen with isolated chondrocytes cultured with IL-1 [27]. Consequently experiments were made to (1) repeat the work of Chandrasekhar and Harvey [7] to check that in our laboratory FGFs also potentiated the effects of rHuIL-1α on chondrocyte monolayers; (2) find a culture medium without serum that would support growth of cartilage explants in culture; and (3) repeat the previous experiment with rHuIL-1α on cartilage explants using a culture medium without serum, to which FGFs could be added.

The effects of FGFs on collagenase and stromelysin synthesis by chondrocyte monolayers are shown in Fig. 3. The IL-1-induced stimulation of both enzymes was potentiated, particularly when aFGF and bFGF were used together, but FGFs in the absence of IL-1 additions had no effect. Note that the units of enzyme activities assayed are considerably higher than those obtained from full-thickness explants (Table 2).

To find a culture medium without serum that would support growth of cartilage explants in culture, articular cartilage explants were excised as before but cultured for 48 h in either serum-free DMEM or Iscove's medium with additions. Those in serum-free DMEM did not respond well to either MCM or rHuIL-1α, and immunolocalization on sections from these explants confirmed that there was considerable chondrocyte necrosis. However, cartilage explants cultured for 48 h in Iscove's medium without serum showed significant MMP release into the culture medium with both MCM and rHuIL-1α treat-

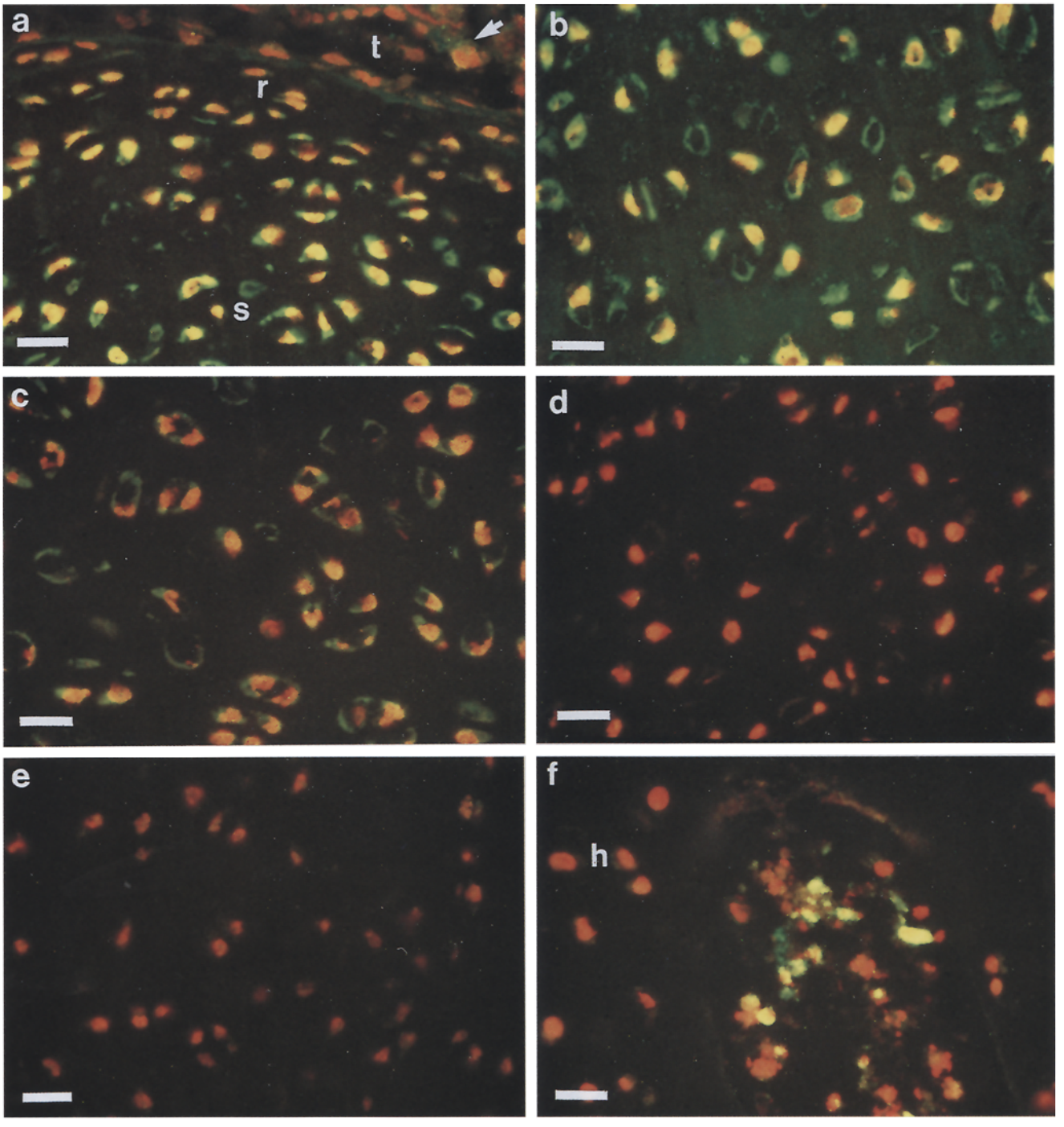

Fig. 2a–f Distribution of collagenase, stromelysin, gelatinase and TIMP-1 in young rabbit articular cartilage explants determined by immunolocalization. Cartilage explants from the condyle of a young rabbit were cultured for 48 h, then monensin was added to fresh medium for a further 6 h before freezing the tissue for immunolocalization. Frozen sections were cut, fixed, permeabilized and stained with antiserum IgG followed by pig-FITC. Nuclei were counterstained red and sections viewed by fluorescence microscopy. *Bars* 20 μm. **a** Condyle cultured with MCM for 48 h and stained with anti-collagenase IgG. Section shows overlying connective tissue (*t*) with one weakly positive cell *(arrow)*, flattened articular cells (*r*) with little or no staining and subarticular chondrocytes (*s*) with weak positive intracellular enzyme. **b** As in **a,** but deeper into the subarticular zone showing chondrocytes with intense intracellular staining for collagenase and inter-territorial matrix staining, indicating that these cells are strongly responsive to MCM. **c** As in **a,** but showing hypertrophic chondrocytes with weak intracellular collagenase staining and no matrix fluorescence. **d** Adjacent section to **a–c** stained with anti-stromelysin IgG. The subarticular chondrocytes have no intracellular green fluorescence, nor is there fluorescence of the matrix. **e** Adjacent section to **d** stained with anti-TIMP-1 IgG. Subarticular chondrocytes are again negative. **f** Condyle cultured for 48 h in control medium with monensin added for a further 6 h, and the frozen section stained with the anti-gelatinase IgG. Cells in a bone lacuna are strongly positive, whereas hypertrophic chondrocytes (*h*) are negative

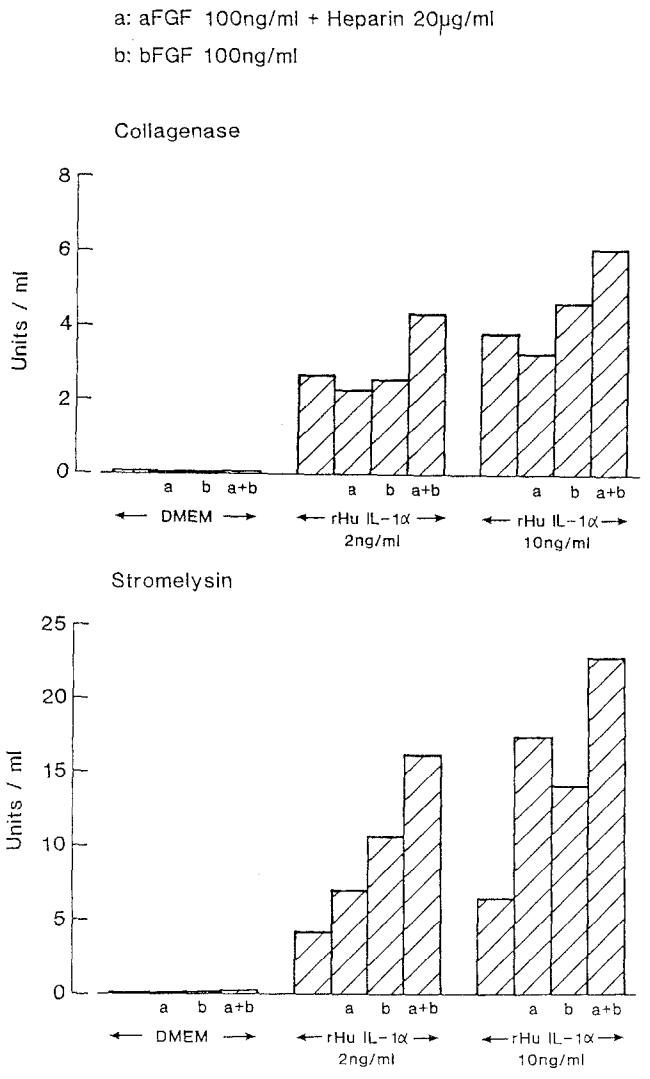

Fig. 3 Fibroblast growth factors (FGFs) potentiate the rHuIL-1α-stimulated collagenase and stromelysin synthesis by rabbit chondrocyte monolayers. Confluent monolayers of rabbit articular chondrocytes were incubated for 48 h in DMEM alone or DMEM with either aFGF (100 ng/ml with heparin 20 μg/ml), bFGF (100 ng/ml) or both, separately and together with rHuIL-1α (2 ng/ml and 10 ng/ml). The culture media were then removed and assayed for collagenase and stromelysin. Each *bar* represents the mean of 2 determinations

ment (Table 3). Immunolocalization demonstrated that after 48 h in either 1% MCM or rHuIL-1α (2 ng or 20 ng/ml) all chondrocytes had intracellular staining for collagenase: the pattern of distribution and intensity was as described above and similar to illustrations in Fig. 2. In adjacent sections stained with antisera to stromelysin, gelatinase and TIMP-1, a few chondrocytes in the upper subarticular zone of each section had weak intracellular immunofluorescence, while the remainder of the subarticular and hypertrophic zones were negative. As previously, any connective tissue present adjacent to the cartilage had positive cells for all MMPs and TIMP-1 in both control and treated explants. Thus, Iscove's medium without serum was able to maintain the articular cartilage explants in culture for 48 h.

To study the effect of FGFs on cartilage in Iscove's medium with and without rHuIL-1α, cartilage was removed from the supracondylar ridge and condyle from young rabbits and cultured for 48 h in the appropriate medium, with monensin added for the last 6 h of the incubation period. Media were assayed for MMP activities and the explants frozen for immunolocalization. The assay results on the culture media are shown in Table 4. As before (cf. Tables 2 and 3) the amounts of all three enzymes in the culture media were much lower relative to those in media harvested from chondrocyte monolayers (Fig. 3), but there was a small increase in all MMP activities when FGFs were included with rHuIL-1α.

The response of cartilage chondrocytes to rHuIL-1α as ascertained by immunolocalization of MMPs and TIMP-1 was again as seen for porcine IL-1α and MCM: subarticular and hypertrophic chondrocytes had intracellular fluorescence indicating collagenase synthesis, but articular cells had little staining (data not shown). The intensity of staining of collagenase within chondrocytes was less than that seen in MCM-treated explants, again as described above for porcine IL-1α. Neither aFGF nor bFGF (alone or in combination) had an effect on the chondrocytes, and when added with rHuIL-1α did not increase the intensity of collagenase staining or stimulate stromelysin, TIMP-1 or gelatinase synthesis by chondrocytes. As before, connective tissue overlying the cartilage contained some cells positive for all MMPs and TIMP-1. We suggest that the small increase in MMP activities was due to increased synthesis by the connective tissue.

Table 3 Metalloproteinase activities in media from young rabbit articular cartilage, maintained in Iscove's medium with additions. Full-thickness cartilage explants were taken from defined sites in the joint as described in Table 2. Four rabbits were used and explants were cultured in Iscove's medium, with additions for 48 h (medium changed at 24 h). The media were assayed (duplicate samples) for collagenase, stromelysin and gelatinase and the average values are shown for the number of samples given in parentheses

Treatment	Collagenase U/ml		Stromelysin U/ml		Gelatinase U/ml	
	24 h	48 h	24 h	48 h	24 h	48 h
Iscove's (4)	0	0	0	0	0	0.03
Iscove's+1% MCM (4)	0.48	0.94	0.27	0.30	0.25	0.26
Iscove's+rHuIL-1α 2 ng/ml (4)	0.28	0.35	0.17	0.17	0.23	0.38
Iscove's+rHuIL-1α 20 ng/ml (4)	0.49	0.93	0.22	0.33	0.46	0.77

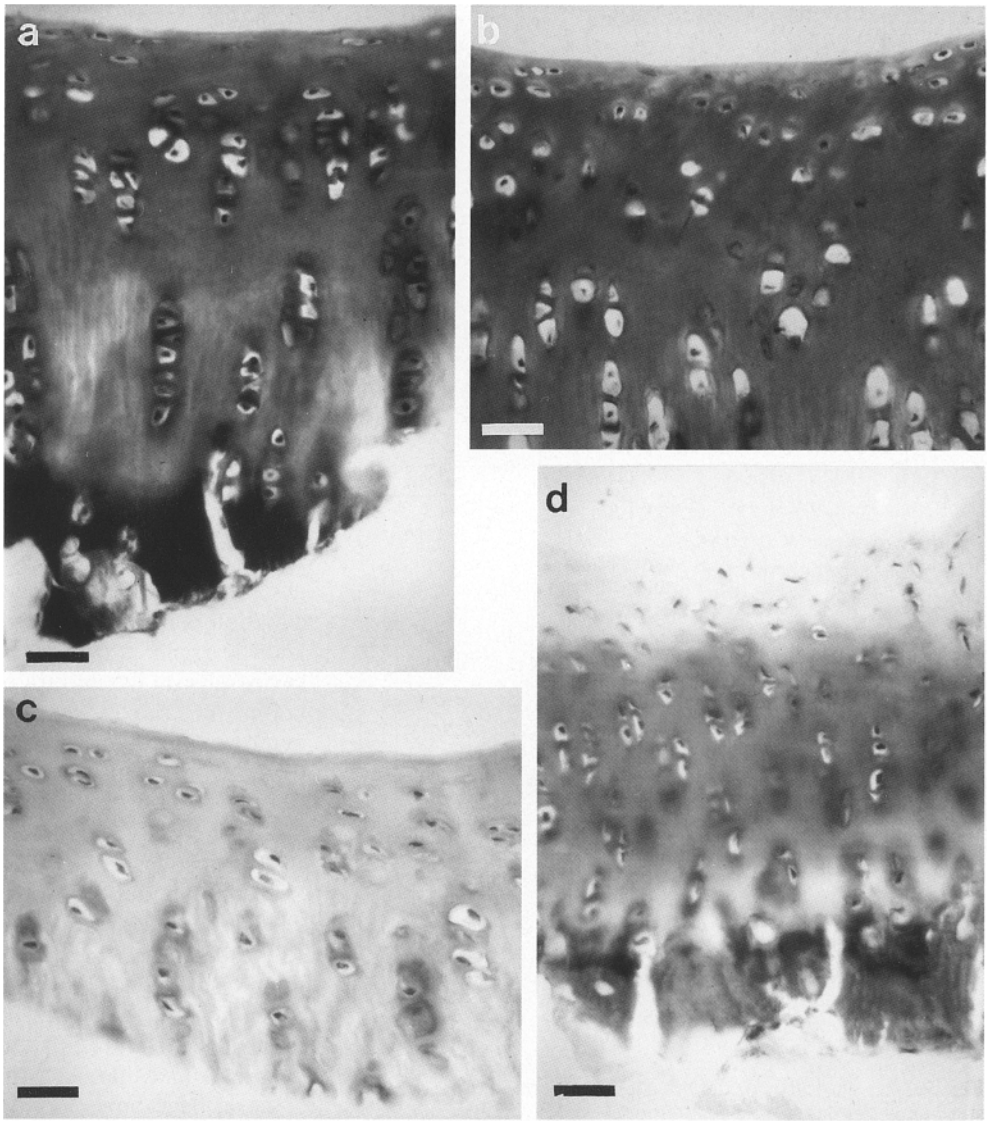

Fig. 4a–d Cartilage metachromasia in control and IL-1-treated adult rabbit joints. Cartilage excised from either control or IL-1-treated joints was frozen, sectioned and stained with toluidine blue after immunostaining. *Bars* 30 μm. **a** Full thickness section of left (control) supracondylar ridge showing normal metachromasia of cartilage matrix. **b** Section of left (control) intercondylar notch showing normal metachromasia. **c** Section of right (IL-1-treated) supracondylar ridge showing almost complete loss of metachromasia. **d** Section of right (IL-1-treated) intercondylar notch with patchy loss of metachromasia

From these data we conclude that whereas IL-1 (either crude MCM, porcine IL-1α or rHuIL-1α) stimulated collagenase synthesis by chondrocytes in young articular cartilage explants, the synthesis of stromelysin, gelatinase and TIMP-1 was not stimulated by this cytokine. FGFs alone or in combination with IL-1 also had no effect on the production of MMPs by chondrocytes in articular cartilage explants.

Effect of IL-1 on chondrocytes of adult articular cartilage

The response of chondrocytes in adult (5-month-old) articular cartilage to IL-1 was investigated to determine whether the pattern of MMP synthesis was the same as in young cartilage. Full-thickness cartilage was excised from the supracondylar ridge, intercondylar notch and condyle and cultured in paired cultures (each pair containing adjacent condyles etc.) with either medium alone (DMEM with 10% NIRS) vs medium with 1% MCM or medium alone (DMEM with 10% NIRS) vs medium with porcine IL-1α (2 ng/ml). Monensin (5 μM) was added for the last 6 h of the 48 h culture period, then the explants were frozen for immunolocalization and the media assayed for collagenase activity.

The collagenase activity (units/ml) in the media were control, 3.12; MCM, 5.18; IL-1, 3.45. Immunolocalization on the explants revealed no distinct pattern of MMP or TIMP-1 immunofluorescence and no clear effect of either MCM or IL-1. The chondrocytes of all explants (both control and treated) were predominantly negative, although occasionally a few cells in the mid-zone stained for either collagenase or stromelysin (data not shown). Where connective tissue was present alongside cartilage this had occasional small areas of immunofluorescence of either collagenase or stromelysin (cf. Fig. 1d): again this was independent of treatment. Thus collagenase synthesis by chondrocytes was not stimulated in adult articular cartilage when the explants were treated with IL-1, unlike chondrocytes in young cartilage.

Table 4 Metalloproteinase activities in media from young rabbit articular cartilage maintained in Iscove's medium: effect of rHuIL-1α and fibroblast growth factors (FGFs). Full-thickness cartilage explants were taken from defined sites in the joint as described in Table 2. Five rabbits were used and explants were cultured in Iscove's medium, with additions, for 48 h. The media were assayed (duplicate samples) for collagenase, stromelysin and gelatinase and the average values are shown for the number of samples given in parentheses

Treatment	Collagenase U/ml	Stromelysin U/ml	Gelatinase U/ml
Iscove's (3)	0.03	0	0
Iscove's+rHuIL-1α 2 ng/ml (4)	0.21	0.24	0.17
Iscove's+aFGF 100 ng/ml	0	0	0.11
+Heparin 20 μg/ml (2)	0	0	0.11
Iscove's+bFGF 100 ng/ml (2)	0	0	0.06
Iscove's+rHuIL-1α+aFGF +Heparin (3)	0.34	0.38	0.37
Iscove's+rHuIL-1α+gFGF (3)	0.35	0.41	0.24
Iscove's+rHuIL-1α+aFGF +Heparin+bFGF (3)	0.51	0.77	0.48

Table 5 Immunolocalization of matrix metalloproteinases (MMPs) and TIMP-1 and toluidine blue metachromasia of cartilage sections from rabbits after the injection of rHuIL-1 by method 2. Full-thickness cartilage samples were excized from the supracondylar ridge, intercondylar notch and condyle of two rabbits, 24 h after injection of either 100 μg rHuIL-1α or saline into the knee joint spaces. The cartilage explants were cultured with monensin for 24 h, then frozen, sectioned and MMPs and TIMP-1 were immunolocalized by indirect immunofluorescence. The number of chondrocytes with intracellular immunofluorescence were counted and expressed as a percentage of the total number of chondrocytes in the section. The percentages shown are the averages for three sections (see Materials and methods). Adjacent sections were stained with toluidine blue *(Tol. blue)* for assessment of cartilage matrix metachromasia; this is indicated by: + dark staining of all zones; – extensive or complete loss of metachromasia; *ND* not determined

Rabbit no.	Source of cartilage	Saline-treated joint					IL-1-treated joint				
		Tol. blue	CL	SL	GL	TIMP-1	Tol. Blue	CL	SL	GL	TIMP-1
1	Supracondylar ridge	+	1.5	0.9	1.4	0.4	–	17.2	11.6	2.3	0
	Intercondylar notch	+	0.9	0.5	0	0	–	2.8	6.2	0	0
	Condyle	ND	ND	ND	ND	ND	–	8.2	7.4	0	0
2	Supracondylar ridge	+	0	0	0	0	–	0	0	0	0
	Intercondylar notch	+	0	0	0	0	–	0	0	0	0
	Condyle	+	0	8.3	0	0	–	0	0	0	0

The effect of intra-articular IL-1α in vivo

To study the effect of IL-1 in vivo three different protocols were used because methodologies of previous workers had varied. Firstly, the effect of 3 injections of 20 ng porcine IL-1α into the knee joint of a rabbit was investigated, assessing the distribution of MMPs and TIMP-1 within the articular cartilage by immunolocalization at 3 days after the last injection, the time of maximal glycosaminoglycan loss ([33], method 1). Sections of cartilage from all positions in the left knee injected with vehicle alone were completely negative when stained with antibodies to MMPs and TIMP-1, and subsequent staining of these sections with toluidine blue confirmed normal metachromasia of the cartilage matrix (Fig. 4a, b). Cartilage sections from the right knee injected with IL-1α when stained with antibodies to MMPs and TIMP-1 were also negative, although all explants showed marked loss of metachromasia of the cartilage matrix (Fig. 4c, d).

To exclude the possibility that either MMP synthesis could have occurred earlier or that the source of IL-1 was critical, a higher dose of rHuIL-1α (100 ng) was injected into the knee joints of two New Zealand White rabbits (method 2). Cartilage and synovium were removed 24 h post-injection and samples either cultured in monensin for 6 or 24 h or frozen directly. Cartilage samples either frozen directly or cultured for 6 h were negative for all antisera. The results of immunolocalization of MMPs on cartilage cultured for 24 h are shown in Table 5. The low incidence of positive cells in these cartilages probably represents the normal turnover rate in these growing animals (aged approximately 10–12 weeks; rabbits 1 and 2 gained 137 g and 82 g respectively in weight in the 6 days prior to death). There was no consistent increase in MMP synthesis in the joints treated with rHuIL-1. There was marked loss, however, of toluidine blue metachromasia from the cartilage matrix of these joints but no loss in the saline treated joints (Table 5).

As previous workers had reported a transient increase in stromelysin mRNA and protein following injection of IL-1β [21], a further rabbit was injected with 200 ng IL-1β (method 3). Monensin was injected into the joint spaces 9 h post-IL-1 injection and the animal was killed 3 h later. Cartilage was removed as before, frozen, sectioned and stained for MMPs and TIMP-1. Cartilage sections from both saline and IL-1-treated joints had normal toluidine blue metachromasia. No cells positive for MMP or TIMP-1 were present, apart from sections taken from the intercondylar notch of the IL-1 treated joint

which contained a small number of mid-zone chondrocytes (3%) positive for stromelysin only.

Taking these data together, we found no immunohistochemical evidence for a significant increase in MMP synthesis and secretion by chondrocytes following intra-articular injection of IL-1, although cartilages excised from methods 1 and 2 showed histological depletion of proteoglycan. With all three methods, synovia from both control and IL-1-treated joints were predominantly normal but contained small foci of inflammatory cell infiltration in which a few cells contained intracellular MMPs and TIMP-1.

Discussion

Firstly, we demonstrate that 1- to 2-week-old rabbit cartilage explants synthesize collagenase in culture in response to MCM and to a lesser extent with IL-1. Collagenase was assayed in the culture medium and immunolocalized within chondrocytes with a distinct distribution pattern: the subarticular cells had both intracellular immunofluorescence and fluorescence of surrounding matrix whereas hypertrophic chondrocytes had intracellular fluorescence without matrix staining, and fluorescence was usually absent in articular cells. The addition of dexamethasone caused a reduction in both MCM-stimulated and IL-1-stimulated collagenase synthesis, but not complete inhibition. Secondly, stromelysin, gelatinase and TIMP-1 could not be demonstrated by immunofluorescence within chondrocytes of any zone, indicating that synthesis of these was not induced by MCM or IL-1. Thirdly, FGFs alone or in combination with IL-1 did not modify the responses of chondrocytes to IL-1. Fourthly, collagenase synthesis was not stimulated in adult articular chondrocytes when explants were treated with either MCM or IL-1. Fifthly, intra-articular injection of IL-1 into rabbit knee joint spaces caused marked loss of cartilage metachromasia but there was no increase in MMP or TIMP-1 synthesis by chondrocytes.

The response of chondrocytes in articular cartilage to MCM and IL-1 varies according to their position within the cartilage explant as well as with the age of animal. The greater effect of MCM, compared to IL-1, suggests that it contains other factor(s) that modify MMP synthesis. In terms of collagenase synthesis, subarticular chondrocytes from young rabbit cartilage seem to be most responsive to both MCM and IL-1, with articular chondrocytes significantly less so. Two studies, using antibodies to IL-1-induced epitopes on chondrocytes within cultured explants of 6- to 9-month-old pig articular cartilage [9, 11] concluded that there is a heterogeneity in response of individual chondrocytes to activation by IL-1, a view which concurs with our results. The effect of age on the sensitivity and response of human cartilage chondrocytes to IL-1 in explant culture with regard to proteoglycan synthesis has been reported [14, 19, 30]. These studies showed that immature cartilage responded more rapidly and was more sensitive to IL-1 than mature cartilage, agreeing with our data in the rabbit.

In contrast to the induction of collagenase, the failure to induce the synthesis of stromelysin, gelatinase and TIMP-1 by chondrocytes in young cartilage explants cultured with IL-1 indicates the complex nature of the regulation of MMP synthesis in tissues. These results agree with data obtained from the immunolocalization of MMPs in the rabbit growth plate [2] and in two rabbit models of arthritis [17], showing that each MMP has a unique pattern of synthesis. Collagenase mRNA was found to be differentially expressed in vivo in synovium and cartilage [42] and in chondrocyte monolayers [7], suggesting that regulation of each MMP is independent and that their roles are separate. In vitro studies on human fibroblasts suggest that gelatinase is also modulated differently [12, 32]. All these results contrast markedly with observations on cultured cells, where IL-1 treatment induced co-ordinate or near co-ordinate expression of MMPs [27, 41].

We failed to detect a significant increase in either MMP or TIMP-1 synthesis by chondrocytes following intra-articular injection of IL-1 by three protocols. Although few animals were used in these experiments, we monitored multiple cartilage sites from each joint, which included both weight-bearing and non-weight-bearing areas. These observations contrast with other data [21, 42] showing a transient increase in stromelysin mRNA by Northern hybridization analysis and protein but no collagenase [42]. However, our results agree with unpublished results (Cockett, M and Henderson, B personal communication) in which cartilage samples were analysed by in situ hybridization. Cartilage removed at 8, 12, 24 and 48 h after a single intra-articular injection of IL-1 into rabbit knee joints [18, 34] was negative for both collagenase and stromelysin mRNA. It is possible that these discrepancies could result from the levels of individual chondrocyte synthesis being too low to detect by in situ hybridization, and by immunohistochemical techniques using our antisera, although low levels have been detected previously [2, 17].

Another possible explanation of our negative results is that the loss of proteoglycan following intra-articular injection of IL-1 is not mediated by increased synthesis of known MMPs. Analyses of the products of proteoglycan degradation suggest the involvement of a proteinase that cleaves the aggrecan core protein after certain glutamic acid residues [39] but the identity of this enzyme (aggrecanase) is unknown. Members of the MMP family have been suspected of involvement because application of MMP inhibitors to IL-1 treated cartilage in vitro reduce proteoglycan release [1, 4–6, 31], although release was not inhibited by TIMP-1 [1, 37]. Potent inhibition was obtained by addition of cytochalasins B or D [37], raising the possibility that degradation of cartilage proteoglycan may involve movement of the cell membrane or endocytosis, and receptor-mediated endocytosis followed by extensive intracellular degradation was also suggested by Morales and Hascall [24]. More recently

cysteine endopeptidases were implicated [3], specifically cathepsin B [4]. These authors concluded that at least two pathways of cartilage proteoglycan breakdown exist, which may converge at the activation of an as yet uncharacterized matrix prometalloproteinase.

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