Characterization of proteins from human synovium and mononuclear leucocytes that induce resorption of cartilage proteoglycan *in vitro*

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Both human synovial tissue in culture and lectin-stimulated mononuclear leucocytes produced a protein that induced proteoglycan resorption in explants of bovine nasal cartilage and human articular cartilage. On gel filtration the protein had M_r 16 000–20 000 and on isoelectric focusing its pI was 5.2–5.3. The protein corresponded to catabolin, which has previously been identified as a product of cultured porcine synovial tissue and mononuclear leucocytes. The action of partially purified human catabolin was not inhibited by cortisol, although the activity of the leucocyte supernatants from which it had been isolated was inhibited. For this reason it is not possible to be sure that the active factor detected in the bioassay of the crude leucocyte culture supernatants is in fact catabolin.

Although a great deal has been learned about the structure and properties of the biological polymers of connective tissues, little is known about how cells regulate the composition of their surrounding matrix. Breakdown of connective tissue is an important feature of several physiological and pathological processes. For example, chronic inflammatory diseases such as rheumatoid arthritis commonly cause severe tissue destruction. In order to study factors regulating tissue resorption we have used explants of cartilage as a bioassay for substances that cause cartilage resorption. Cartilage maintains its matrix in culture, and the chondrocytes will resorb the proteoglycan of the matrix if they are appropriately stimulated. The fragments of proteoglycan are released into the culture medium, where they are easily measured as chondroitin sulphate. With this bioassay it was found that porcine synovial tissue *in vitro* produced a protein that caused proteoglycan resorption in living cartilage (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980; Saklatvala, 1981), the protein was named 'catabolin'. Further work (Saklatvala & Sarsfield, 1982) revealed that porcine mononuclear leucocytes (lymphocytes and monocytes from blood) when stimulated by the lectins concanavalin A or phytohaemagglutinin produced a

protein that was indistinguishable from the synovial catabolin. Much of the leucocyte catabolin was thought to have arisen from lymphocytes. Catabolin is defined as a small (M_r 15 000–20 000), acidic (pI 4.5–5.5) protein that induces cartilage resorption *in vitro* and whose action is not inhibited by cortisol.

Culture supernatants of phytohaemagglutinin-stimulated human mononuclear leucocytes cause proteoglycan resorption in cartilage, but the activity (in contrast with that of the porcine catabolin) is substantially inhibited by cortisol (Jasin & Dingle, 1981). Because of this inhibition, it seemed that the human mononuclear leucocytes might be making a cartilage-catabolic protein different from porcine catabolin. The experiments described below show that both human synovial tissue and human mononuclear leucocytes make a protein corresponding to porcine catabolin.

Experimental

Materials

DME medium and foetal-calf serum were from Gibco. Concanavalin A, cortisol, retinol, papain, whale chondroitin sulphate (grade III) and protein standards for gel chromatography were from Sigma. Dimethyl-Methylene Blue was from Serva Feinbiochemica, phytohaemagglutinin from Wellcome, Ficoll 400 from Pharmacia, Hypaque sodium (sodium diatrizoate) from Sterling Research Laboratories, DEAE-cellulose DE-52 from Whatman, Ultrogel AcA 54 and Ampholines from LKB,

Abbreviations used: DME medium, Dulbecco's modification of Eagle's medium; phosphate-buffered saline, 1 mM- NaH_2PO_4 /18.0 mM- Na_2HPO_4 /145 mM- NaCl , pH 7.4; MCF, mononuclear cell factor.

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Diaflo ultrafiltration apparatus and PM10 membranes from Amicon, and Carbowax [poly(ethylene glycol)] was from Raymond Lamb. All other chemicals were of analytical-reagent or best available grade.

Methods

Culture of human synovium. Samples of synovial tissue were obtained from normal knee joints after amputation, from the hips and knees of patients undergoing arthroplasty for osteoarthritis, and synovectomy for rheumatoid arthritis. Some samples were obtained at meniscectomy operations. The soft synovial connective tissue was dissected from more dense capsular tissue, and cut into small blocks 2–4 mm³. These were cultured at 37°C in DME medium at 8 g/100 ml in CO₂/air (1:19). Medium was replaced every 48 h and the used medium was pooled. Cultures were terminated after 1 week.

Preparation of mononuclear leucocytes from human blood. Freshly drawn blood from normal subjects was treated with heparin to prevent coagulation. Portions (10 ml) were layered on to 6 ml of 6.35% (w/v) Ficoll 400 and 10% (w/v) Hypaque sodium in sterile centrifuge tubes. After centrifugation (400 g, 30 min) the mononuclear cell layer was removed, washed three times in phosphate-buffered saline and cultured at 30 × 10⁶ cells/ml in DME medium containing 10% (w/v) heat-inactivated (56°C for 30 min) foetal-calf serum and concanavalin A or phytohaemagglutinin (100 µg/ml) at 37°C in CO₂/air (1:19) for 48 h. Culture supernatants were stored at –20°C until used. The mononuclear cell preparations generally contained less than 2% neutrophil leucocytes.

Assay of cartilage resorption with bovine nasal cartilage. This procedure has been fully described elsewhere (Saklatvala, 1981). Discs (4 mm × 1 mm) of cartilage from bovine nasal septum were cultured for 8 days in 1.2 ml of DME medium containing 5% heat-inactivated normal sheep serum at 37°C in CO₂/air (1:19). Medium was changed at 4 days, and samples to be assayed for activity were included in the culture medium. After culture the discs were digested with papain and the amounts of chondroitin sulphate in the digest and medium were measured by using the metachromatic dye dimethyl-Methylene Blue. Each sample was tested on five separate discs and the results were expressed as percentage of total glycosaminoglycan released into the culture medium, ± S.E.M.

Assay of proteoglycan resorption in human articular cartilage. Macroscopically normal articular cartilage was obtained from the femoral condyles of cadavers within 48 h of death. Most samples were obtained within 24 h, and the subjects ranged from 20 to 45 years of age and had no known joint pathology. The cartilage was cut into

pieces about 8 mm³ and cultured exactly as for the bovine cartilage, except that the serum concentration was 10%. Since the cartilage was from cadavers, its viability was checked by culturing it with retinol (10 i.u./ml), a substance known to cause living cartilage to resorb *in vitro*. Glycosaminoglycan release from the cartilage was measured as described for bovine cartilage.

Isoelectric focusing. This was carried out exactly as described previously (Saklatvala & Sarsfield, 1982). Samples (up to 1 ml) were incorporated into 7.5% polyacrylamide-gel cylinders containing 1% Ampholine. After focusing the gels were cut transversely into slices which were homogenized and eluted with phosphate-buffered saline. The eluates were dialysed before they were assayed on cartilage.

Chromatofocusing. This was carried out on a pre-packed MonoP HR5/20 column eluted with Polybuffer 74 according to the manufacturer's directions (Pharmacia Fine Chemicals AB) in a Pharmacia FPLC system equipped with a P-500 pump.

Other methods. Details of chromatography on Ultrogel AcA 54 and DEAE-cellulose are given in the appropriate Figure legends. Ultrafiltration was carried out in a Diaflo (Amicon Ltd.) apparatus with a PM10 membrane. Visking tubing (18/32-in) was acetylated before use by immersion overnight in 25% acetic anhydride in pyridine. Protein concentrations were measured with Folin and Ciocalteu's phenol reagent.

Results

Human synovial culture medium induces resorption of proteoglycan in human articular and bovine nasal cartilage

Table 1 summarizes the results of experiments made with articular cartilage from six different subjects. Unstimulated explants released about 20% of their glycosaminoglycan, but those cultured in the presence of synovial medium released about 50%. The medium had been conditioned with normal synovial tissues from an amputation specimen. The dead (frozen and thawed) cartilage maintained in synovial medium released no more glycosaminoglycan than did the unstimulated controls, demonstrating that living chondrocytes are needed for the resorption and that it is not a direct enzymic degradation caused by the synovial medium. All the cartilage samples were viable as shown by the release induced by retinol. Production of active material from the synovial explants was maximal during the first 48 h and gradually declined to nothing over 7–10 days (results not shown).

The depletion of proteoglycan from human articular cartilage cultured in the presence of synovial medium is shown in Fig. 1; there is marked

Table 1. Release of glycosaminoglycan from cultured human articular cartilage

Samples of femoral condylar cartilage were cultured for 8 days as described under 'Methods'. The synovial culture medium was made with tissue from the knee of a 55-year-old man after amputation. The results are means $\pm$ S.E.M. The dead cartilage was prepared by freezing and thawing five times.

Agent added to culture	Dose	State of cartilage	Human articular cartilage specimen no. ...	Glycosaminoglycan released during culture (% of total)				
				1	2	3	4	5
None	—	Live		19 $\pm$ 1	25 $\pm$ 2	21 $\pm$ 2	25 $\pm$ 3	16 $\pm$ 2
Retinol	10 i.u./ml	Live		38 $\pm$ 3	79 $\pm$ 4	33 $\pm$ 4	55 $\pm$ 3	59 $\pm$ 4
Human synovial medium	0.5 ml/ml	Live		42 $\pm$ 3	49 $\pm$ 5	45 $\pm$ 3	51 $\pm$ 4	35 $\pm$ 2
	0.5 ml/ml	Dead		—	18 $\pm$ 2	—	20 $\pm$ 2	—

Fig. 1. Vertical section of explants of cartilage from a femoral condyle of a 27-year-old man after culture for 8 days in (a) control medium and (b) medium containing 50% synovial medium made with a sample of synovium obtained at a meniscectomy operation on a 25-year-old man

There was no cortisol added to the cartilage culture. The tissue was fixed in Bouin's solution for 24 h and 6 μ m sections were stained with Toluidine Blue [0.5% (w/v) in 5% (v/v) ethanol]. The sulphated glycosaminoglycans are stained metachromatically a deep purple colour. The articular surface is marked by the arrows. The surface zone in (a) is characteristically unstained.

reduction in the metachromatic staining of the matrix with Toluidine Blue.

Human synovial medium also induced release of glycosaminoglycan from bovine nasal cartilage; the dose-response curve (Fig. 2) was approximately

Fig. 2. Release of glycosaminoglycan from bovine nasal cartilage induced by human synovial culture medium Culture medium made with synovial tissue from an osteoarthritic hip joint was assayed on bovine nasal cartilage at increasing concentrations in the presence (O) and absence (●) of cortisol (0.1 μ g/ml). Vertical bars indicate the S.E.M.

linear and unaffected by culturing the cartilage in the presence of cortisol (0.1 μ g/ml).

Fractionation of human synovial medium

Previously it has been found that when cortisol (0.1 μ g/ml) was included in the cartilage assay the release of glycosaminoglycan from unstimulated cartilage was lowered, but the action of pig catabolin was not inhibited. This improved the sensitivity and overall performance of the assay (Saklatvala, 1981; Saklatvala & Sarsfield, 1982).

Fig. 3. Gel filtration on Ultrogel AcA 54 of (a) concentrated human synovial medium and (b) material prepared from the synovial medium by chromatography on DEAE-cellulose

(a) Human synovial culture medium (100 ml) made with synovial tissue from an osteoarthritic hip joint was concentrated to 2.5 ml in dialysis tubing with Carbowax and chromatographed on a column (1.5 cm $\times$ 90 cm) of Ultrogel AcA 54 eluted at 10 ml/h with phosphate-buffered saline. Fractions (2.3 ml) were collected and assayed on bovine nasal cartilage at 8% concentration in culture medium containing cortisol (0.1 μ g/ml). ●, Glycosaminoglycan release (bars show S.E.M.); —, A_{280} . (b) Synovial culture medium (2 litres), obtained by pooling culture medium made with samples of synovium from various rheumatoid and osteoarthritic joints, was diluted 10-fold with water and adjusted to pH 8.5 with NaOH. A slurry of DEAE-cellulose DE52 (50 ml, about 15 g of dry resin) equilibrated with 20 mM-glycine/HCl buffer, pH 8.5, was added and stirred for 15 min at room temperature. The slurry was allowed to settle, and after the supernatant had been discarded, was packed into a column, washed with 10 column-vol. of equilibrating buffer and active material was eluted with the buffer containing 0.13 M-NaCl. This active eluate was concentrated to 3 ml over an Amicon PM10 ultrafiltration membrane and chromato-

graphed on the same column as for (a). Fractions (2.7 ml) were assayed at 2% on bovine nasal cartilage in the presence of cortisol (0.1 μ g/ml). The column was calibrated with Blue-Dextran, serum albumin, carbonic anhydrase, soya-bean trypsin inhibitor, cytochrome *c* and aprotinin.

Since cortisol did not inhibit the action of the human synovial medium, it was included in the nasal cartilage bioassays on fractions from chromatography and isoelectric focusing. In preliminary experiments it was found that active material from human synovium did not pass through acetylated dialysis membrane or through PM10 ultrafiltration membranes.

Synovial medium concentrated 50-fold in dialysis tubing by Carbowax [poly(ethylene glycol)] was chromatographed on Ultrogel AcA 54 (Fig. 3a). A peak of active material eluted at a position corresponding to M_r 20 000–25 000; it represented about 60% of the activity applied to the column. In order to purify partially a larger amount of active material, 2 litres of pooled medium made with synovium from various osteoarthritic and rheumatoid patients were subjected to preliminary chromatography on DEAE-cellulose, followed by chromatography on Ultrogel AcA 54 (Fig. 3b). The main peak of active material eluted at a position corresponding to M_r 16 000–21 000. The fractions at 40–45% elution volume showed significant activity associated with material of higher molecular weight. This has not been found repeatedly, and it may be caused by the protein of the main active peak aggregating or binding to other proteins. The fractions of the main active peak were pooled and subjected to isoelectric focusing; active material focused in the region pH 5.0–5.5 in a pH 3–10 gradient (Fig. 4a) and at pH 5.2 in a pH 4–6 gradient (Fig. 4b). The eluates from the pH 4–6 gradients were also assayed on human articular cartilage, without cortisol in the assay (Fig. 4b), and the distribution of activity was the same as with the bovine cartilage.

From these experiments, it was concluded that human synovium produced a protein corresponding to the 'catabolin' made by porcine synovium (Saklatvala, 1981).

Supernatants of lectin-stimulated human mononuclear leucocytes induce resorption of proteoglycan in bovine nasal cartilage

Mononuclear leucocytes separated from human blood by Ficoll/Hypaque centrifugation were cultured in the presence of concanavalin A or phytohaemagglutinin for 48 h. The culture supernatants were then assayed at increasing concentrations on

graphed on the same column as for (a). Fractions (2.7 ml) were assayed at 2% on bovine nasal cartilage in the presence of cortisol (0.1 μ g/ml). The column was calibrated with Blue-Dextran, serum albumin, carbonic anhydrase, soya-bean trypsin inhibitor, cytochrome *c* and aprotinin.

Fig. 4. Isoelectric focusing of material from cultured human synovium

Active material after chromatography on DEAE-cellulose and Ultrogel AcA 54 (shown in Fig. 3b) was concentrated by ultrafiltration, dialysed against 1% glycine and focused on polyacrylamide gels. Gels were sliced along the pH gradients, and the slices were homogenized and eluted. The eluates were assayed. (a) Eluates from a pH 3–10 gradient assayed on bovine nasal cartilage. ●, Glycosaminoglycan release ($\pm$ S.E.M.); ----, pH gradient. (b) Eluates from a single gel (pH 4–6 gradient) were assayed on bovine nasal cartilage (●) as in (a) and on human articular cartilage (○). The results for the human cartilage are expressed as a ratio, test sample/control.

bovine nasal cartilage in the presence or absence of cortisol (Fig. 5). When assayed without steroid, supernatants from both concanavalin A- and phytohaemagglutinin-stimulated cells induced proteoglycan resorption; concanavalin A had generated more active material than phytohaemagglutinin. When the leucocyte supernatants were assayed in the presence of cortisol, there was inhibition of release of glycosaminoglycan. The degree of inhibition was estimated from the slopes of the dose–response curves to be about 70–80%. This was in marked contrast with the lack of inhibition of the activity of the synovial medium (Fig. 2).

Fractionation of human-mononuclear-leucocyte medium

Concentrated medium from blood mononuclear leucocytes stimulated with concanavalin A was chromatographed on Ultrogel AcA 54 and the fractions were assayed on bovine nasal cartilage in the absence of cortisol (Fig. 6). A peak of active

material eluted at a position corresponding to M_r 15 000–20 000. Chromatofocusing was carried out on the active peak from gel filtration (Fig. 7a). The fractions were assayed for activity in the presence or absence of cortisol (Fig. 7b). In either case, a single active component was found which eluted from the column at pH 5.3–5.4. The height of the peak was approximately the same with or without cortisol, which showed that the cortisol was not inhibiting the isolated protein, although it strongly inhibited the original activity of the culture medium (Fig. 5). The pH at which a protein elutes from a chromatofocusing column may differ from its pI, but isoelectric focusing of the material from gel filtration showed that the active protein had an isoelectric point of 5.3 (Fig. 8).

Discussion

Human synovium in culture produced a protein that induced proteoglycan resorption in explants of

Fig. 5. Effect of cortisol on the release of glycosaminoglycan from explants of bovine cartilage induced by culture supernatants of human mononuclear leucocytes. The leucocyte culture supernatants were assayed on bovine cartilage at increasing concentrations without (●) or with (○) cortisol (0.1 µg/ml). The supernatants were prepared by culturing the leucocytes in the presence of (a) phytohaemagglutinin (100 µg/ml) and (b) concanavalin A (100 µg/ml) as described under 'Methods'.

cartilage and which was very similar to the catabolin previously identified as a product of porcine soft connective tissue and mononuclear leucocytes

Fig. 6. Gel filtration on Ultrogel AcA 54 of concentrated supernatants from cultured mononuclear leucocytes. Mononuclear leucocytes were cultured in the presence of concanavalin A (100 µg/ml) as described under 'Methods'. A portion (8 ml) of supernatant was concentrated 4-fold with Carbowax and chromatographed on a column (1.5 cm × 90 cm) of Ultrogel AcA 54 eluted at 6 ml/h with phosphate-buffered saline. Fractions (3 ml) were assayed at 2% on bovine nasal cartilage. No cortisol was present in the incubation mixture. ●, Glycosaminoglycan release (± S.E.M.); ----, A_{280} .

(Saklatvala, 1981; Saklatvala & Sarsfield, 1982). The human synovial catabolin had M_r 16000–20000 on gel filtration and pI 5.2, and its action was not inhibited by cortisol. A protein of similar characteristics was also isolated from the culture supernatants of human mononuclear leucocytes stimulated by concanavalin A. It is difficult to explain why the activity of the original leucocyte culture supernatants was inhibited by cortisol when the active protein isolated from them was not. Two possible explanations are: (a) the activity of the culture supernatants was actually due to some other factor whose action was suppressed by cortisol and the action of the catabolin was masked; or (b) the action of catabolin was suppressed by cortisol in the presence of other substances in the crude mixture. The recovery of activity from chromatofocusing was about 50% and there was no good evidence of any cortisol-suppressible substance on fractionation, so that the second explanation is currently more plausible. Until the disappearance of the inhibition by cortisol upon purification is understood, caution is needed in interpreting the results of cartilage bioassays on the supernatants of cultured human leucocytes, since it is impossible to be sure that it is

Fig. 7. FPLC chromatofocusing of material from human mononuclear leucocytes

A portion (38 ml) of supernatant from mononuclear leucocytes cultured in the presence of concanavalin A was concentrated to 3 ml over an Amicon PM10 ultrafiltration membrane and chromatographed on Ultrogel AcA 54 as shown in Fig. 6. The active fractions were pooled and ultrafiltration was used to concentrate them 10-fold and change the buffer to 25 mM-Bistris {2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol} adjusted to pH 7.1 with iminodiacetic acid. The sample was applied to a MonoP HR5/20 chromatofocusing column in a Pharmacia FPLC system. The column had been equilibrated with the Bistris buffer. The column was eluted with Polybuffer 74 adjusted to pH 4.0 with iminodiacetic acid. The flow rate was 1 ml/min at a pressure of 2 MPa (20 bar). Fractions (1 ml) were collected and their pH was measured. They were assayed at 0.4% (v/v, of culture medium) on bovine nasal cartilage with and without cortisol at 0.1 μ g/ml. ●, Glycosaminoglycan release ($\pm$ S.E.M.) without cortisol in the assay; ○, glycosaminoglycan release with cortisol present. —, A₂₈₀; ----, pH.

catabolin that is being detected. Furthermore, the dose-response curves for these supernatants have generally been erratic with large standard errors.

The samples of synovium were obtained from a variety of sources (normal subjects and persons with osteo- and rheumatoid arthritis), and there were

differences in the amount of active material that they produced. Samples from patients with arthritis did not make noticeably more than those from normal subjects (judged as activity/wet weight), but because of the difficulties in quantifying the bioassay, only a relatively large difference would have been detected.

Fig. 8. Isoelectric focusing of material from human mononuclear leucocytes

Active fractions from gel filtration of mononuclear leucocyte culture supernatants (Fig. 6) were pooled, concentrated, dialysed against 1% glycine and focused in polyacrylamide gel in a pH 3–10 gradient. The gel was sliced along the gradient. Equal amounts of the eluates of homogenized slices were assayed on bovine cartilage in the presence of cortisol (0.1 µg/ml). ●, Glycosaminoglycan release ($\pm$ S.E.M.); ----, pH gradient.

It is possible that cells resorb the polymers of connective-tissue matrix by releasing proteinases. Monocytes and macrophages produce a factor that stimulates cultured fibroblasts and chondrocytes to produce collagenase, prostaglandins (Dayer *et al.*, 1976, 1979; Korn *et al.*, 1980; Deshmukh-Phadke *et al.*, 1980) and plasminogen activator (Hamilton & Slywka, 1981). The agent responsible has been called 'mononuclear cell factor' (MCF) and a substance of similar activity has been found in supernatants of human synovial explants (Meats *et al.*, 1980; Robinson *et al.*, 1981). Subsequently it has been reported that MCF activity may be attributable partly, if not wholly, to interleukin 1, a protein made by monocytes and macrophages that stimulates thymocyte and lymphocyte proliferation. Murine interleukin 1 has been purified to apparent homogeneity (Mizel & Mizel, 1981). Human interleukin 1 has an isoelectric point in the region pH 6.6–7.0 (Wood, 1979; Lachman *et al.*, 1977). It is reasonable to expect that catabolin might be the same substance as MCF, but its isoelectric point (pH 5.2) is quite different from that of interleukin 1. Perhaps interleukin 1 and catabolin are different

proteins which both contribute to the overall MCF activity of the supernatants of cultured leucocytes. Whether or not catabolin does have MCF-like activity requires investigation.

Another agent that affects connective-tissue cells is the human monocyte product that stimulates synovial fibroblasts to make plasminogen activator (Hamilton & Slywka, 1981). This factor was found to have an M_r about 30000 on gel chromatography and it was separated from interleukin 1 (Hamilton *et al.*, 1982); its relationship to MCF and catabolin needs to be established.

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