Lymphocytes induce resorption of cartilage by producing catabolin

Jeremy SAKLATVALA and Simon J. SARSFIELD
Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, U.K.

(Received 20 October 1981/Accepted 4 November 1981)

Mononuclear cells from pig blood, when cultured in the presence of lectins (concanavalin A or phytohaemagglutinin), release a factor that induces resorption of proteoglycan in explants of live bovine nasal cartilage. The factor had mol.wt. 20 000 and pI 4.6–5.0, and was indistinguishable from the cartilage-catabolic protein catabolin isolated from culture medium of explants of pig synovial tissue. Since much of the catabolin from the mononuclear cells arose from the non-adherent population (98% lymphocytes) it was concluded that lymphocytes (and possibly monocytes) make catabolin.

Resorption of the structural macromolecules of connective tissues occurs in many situations, including inflammation, wound healing, fracture repair, tumour invasion and tissue remodelling during growth. The cellular and molecular mechanisms involved are obscure. Cartilage can be stimulated to degrade its matrix in organ culture and this provides a simple system *in vitro* for the study of connective-tissue resorption. Synovial tissue and other soft connective tissues in culture produce a protein, catabolin, which induces resorption of cultured cartilage by stimulating chondrocytes to degrade their matrix. Catabolin is assayed by its ability to cause release of proteoglycan (measured as glycosaminoglycan) from explants of living cartilage (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980). It has been partially purified from medium in which pig synovial tissue has been cultured. It has a mol.wt. of 17 000–18 000 and pI 4.6–5.0 (Saklatvala, 1981). Cortisol inhibits its production by soft connective tissue, but does not affect its action on cartilage (Saklatvala, 1981; Tyler *et al.*, 1981).

Mononuclear cells from human blood have been shown to produce cartilage-catabolic activity (Jasin & Dingle, 1981). We have therefore examined cells from pig blood to compare their catabolic factors with the pig synovial-tissue catabolin.

Materials and methods

Concanavalin A, whale chondroitin sulphate (grade III), papain, cortisol and protein standards for gel filtration were from Sigma. DME medium and foetal-calf serum were from Gibco. 1,9-

Dimethylmethylene Blue was from Serva Feinbiochemica, PHA from Wellcome, Ficoll 400 (dextran) from Pharmacia, Hypaque sodium (sodium diatrizoate) from Winthrop, DEAE-cellulose from Whatman, Carbowax [poly(ethylene glycol)] from Raymond Lamb. Ultragel AcA 54 and Ampholines were from LKB.

Preparation and culture of pig mononuclear cells

Fresh heparinized pig blood was centrifuged (400 g for 10 min), the serum was discarded and an equal volume of PBS, containing 1 mM-EDTA was mixed with the cells. The cell suspension (10 ml) was layered on to a solution (6 ml) of 6.35% (w/v) Ficoll 400 and 10% (w/v) Hypaque sodium in a sterile plastic centrifuge tube. After centrifugation (400 g for 30 min) the mononuclear cell layer was removed from the PBS-Ficoll/Hypaque interface, the cells were washed three times in PBS and counted in a haemocytometer; examination of a smear showed 98% mononuclear cells, 2% neutrophil leucocytes. These mononuclear cells were separated into adherent and non-adherent populations as follows. The cells were suspended in DME medium, containing 20% heat-inactivated (56°C for 30 min) foetal-calf serum and incubated in a plastic Petri dish for 1.5 h at 37°C in CO₂/air (1:19). The non-adherent cells were poured off and the dish was washed three times with medium. Cells were assessed for their ability to phagocytose particles of carmine: they were incubated with particles for 18 h in DME medium with 10% foetal-calf serum at 37°C in CO₂/air (1:19), and any cell that had two or more particles in its cytoplasm was counted as positive. The mononuclear cell populations (whole, adherent and non-adherent) were then cultured in DME medium with 10% foetal-calf serum (heat-inactivated) for 76 h at 37°C in CO₂/air (1:19) with and without

Abbreviations used: PHA, phytohaemagglutinin; DME medium, Dulbecco's modification of Eagle's medium; PBS, phosphate-buffered saline (1 mM-NaH₂PO₄/18.0 mM-Na₂HPO₄/145 mM-NaCl, pH 7.4).

lectins (concanavalin A or PHA) at $100\mu\text{g/ml}$. After culture the medium was cleared of cells by centrifugation and stored at -20°C until assay.

Assay of glycosaminoglycan release from bovine cartilage

Medium from mononuclear cell cultures was assayed for its ability to induce proteoglycan (measured as chondroitin sulphate) release from cultured discs of bovine nasal cartilage. A detailed account of this procedure has been given previously (Saklatvala, 1981). Discs of bovine nasal cartilage ($4\text{ mm} \times 1\text{ mm}$) were cultured in DME medium with 5% heat-inactivated normal sheep serum and cortisol ($0.1\mu\text{g/ml}$) for 8 days at 37°C in CO_2/air (1:19), medium being changed after 4 days. After culture the discs were digested with papain and the amounts of chondroitin sulphate in the digest and in the medium were measured by using the meta-chromatic dye, Dimethylmethylene Blue. Samples under test were included in the medium throughout the culture. Each was tested on five separate discs and the results expressed as percentage glycosaminoglycan released into the culture medium $\pm$ S.E.M.

Preparation of partially purified catabolin

Pig synovial tissue was cultured in DME medium, and 12 litres of used medium was adjusted to 95% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ at 4°C . The precipitated protein was chromatographed on DEAE-cellulose, then on Ultrogel AcA 54 as described previously (Saklatvala, 1981). Fractions were assayed for catabolin activity on bovine nasal cartilage, active material was concentrated, dialysed against 1% glycine and stored at -20°C until used.

Isoelectric focusing

Samples (1ml) were incorporated into 7.5% acrylamide gel cylinders ($5.9\text{ mm} \times 60\text{ mm}$) containing 1% Ampholine for either a pH3–10 or pH4–6 gradient. Gels were focused for 1.25 h, starting at 1 mA/gel constant current, and switching over to 250 V constant voltage. Electrode solutions were 0.1% NaOH (cathode) and 0.2% H_3PO_4 (anode). After focusing the gels were sliced, homogenized and eluted for 48 h with either 1 ml of water (for pH measurement) or 1 ml of PBS (for assay). The PBS eluates were dialysed against a large volume of PBS before assay on cartilage.

Results

Culture supernatants of all three mononuclear cell populations (whole, adherent and non-adherent) induced release of cartilage glycosaminoglycan, provided that the cells had been cultured in the presence of PHA or concanavalin A (Fig. 1). The lectins had no effect on the cartilage at the

Fig. 1. Release of glycosaminoglycan from explants of bovine cartilage induced by culture supernatants of pig mononuclear cells

(a) Culture supernatant of the whole population of mononuclear cells assayed on bovine cartilage at two concentrations. Results for cells at $10^7/\text{ml}$ cultured without lectin (■), with PHA ($100\mu\text{g/ml}$; ▲) and with concanavalin A ($100\mu\text{g/ml}$; ●) are shown. (b) Assay of the supernatants of the adherent cells cultured at 0.3×10^7 cells/ml (no lectin, □; PHA, △; concanavalin A, ○) and non-adherent cells cultured at 0.6×10^7 cells/ml (no lectin, ■; PHA, ▲; concanavalin A, ●).

concentrations used. Allowing for the difference in cell density (non-adherent cells were cultured at twice the density of the adherent) the non-adherent cells produced more activity per cell than the adherent population. Of the non-adherent cells 98% were lymphocytes and 2% were monocytes (as judged by phagocytosis) and since monocytes were 30% of the whole mononuclear population the adherence step removed 90% of these cells. Quantification of activity in the bioassay is only approximate but in other experiments we have generally found that the non-adherent cells produced as much, or more activity, per cell than the adherent ones.

Active medium for biochemical fractionation was prepared by culturing the whole mononuclear cell population at 10^7 cells/ml in the presence of concanavalin A ($100\mu\text{g/ml}$) and 10% foetal-calf serum. Gel filtration of this on Ultrogel AcA54 (Fig. 2) revealed a single peak of active material (60% recovery), which was eluted at a position corresponding to mol.wt. 20000 ($\pm 10\%$). The arrow indicates the elution position of the pig synovial tissue catabolin (mol.wt. $18000 \pm 10\%$); the difference was not significant.

Isoelectric focusing on polyacrylamide gels was carried out with the active material from gel filtration. Eluates from homogenized slices of the gels were assayed on bovine nasal cartilage. On a pH3–10 gradient (Fig. 3a) active material focused in

Fig. 2. Gel filtration of culture medium of pig mononuclear cells on Ultrogel AcA 54

Mononuclear cell medium (2 ml) was chromatographed on a column (1.5 cm $\times$ 90 cm) of Ultrogel AcA 54, which was eluted with PBS at 6 ml/h. Fractions (3 ml) were assayed at 6.5% for activity on bovine nasal cartilage. Results are means of quintuplicate assays $\pm$ S.E.M. (●). A_{280} was also recorded (—). The column was calibrated with Blue Dextran, serum albumin, carbonic anhydrase, soya-bean trypsin inhibitor, cytochrome *c* and aprotinin. The arrow shows the elution position (peak) of the partially purified catabolin from pig synovial tissue chromatographed on the same column.

the region pH 4–5. On a narrow-range gradient (pH 4–6) active material focused between pH 4.6 and 5.1 (Fig. 3b). In another gel, run in parallel but loaded with the pig synovial-tissue catabolin, active material was found in exactly the same slices (Fig. 3b). The eluates of the pH 4–6 gel containing mononuclear cell material (shown in Fig. 3b) were assayed on pig articular cartilage and the two active slices (9 and 10) corresponded to the peak of activity on bovine cartilage (Fig. 3c). All the cartilage assays were done in the presence of cortisol (0.1 μ g/ml); this had no inhibitory effect on the degradation induced by the blood-cell supernatants (Fig. 4).

Discussion

The factor from the blood mononuclear cells was indistinguishable from the synovial-tissue catabolin as judged by its charge, molecular weight and activity, as well as its failure to be inhibited by cortisol. Their identical distribution on narrow-range isoelectric-focusing gradients strongly suggests that they are the same protein. A major part of the activity arose from the non-adherent cells, so lymphocytes must make catabolin. As some activity came from the adherent cells, monocytes also may make catabolin but because this population contained substantial numbers of lymphocytes (10–20%) it is not possible to be certain upon this point. Our results with pig cells differ from those reported for human mononuclear cells (Jasin & Dingle, 1981) in that much of our active material arose from the

Fig. 3. Isoelectric focusing of cartilage-catabolic factor from blood mononuclear cells and catabolin from synovial tissue

Mononuclear cell medium (300 ml) was concentrated 30-fold over an Amicon PM10 Ultrafiltration membrane and chromatographed on Ultrogel AcA 54 as shown in Fig. 2 except that a larger column (4.4 cm $\times$ 100 cm) was used. Fractions active on bovine cartilage were pooled, concentrated 30-fold and dialysed against 1% glycine. This material was focused in polyacrylamide gels. (a) Material from mononuclear cells in a pH 3–10 gradient. Eluates from gel slices were assayed on bovine nasal cartilage at 1.3%. ●, Glycosaminoglycan release (means $\pm$ S.E.M.); (—), pH. (b) pH 4–6 gradient, parallel gels which contained mononuclear cell material (●) and partially purified catabolin (○) prepared as described in the Materials and methods section. Eluates were assayed as in (a). (c) The pH 4–6 gel containing mononuclear cell material shown in (b) was assayed on pig articular cartilage (metacarpophalangeal joint). The pig cartilage was cultured exactly as the bovine. Results are means of triplicate assays.

non-adherent cells (i.e. lymphocytes), and it was not inhibited by cortisol. These could be species differences.

Various proteins that stimulate cultured

Fig. 4. Effect of cortisol on the release of proteoglycan from explants of bovine cartilage caused by culture medium of blood mononuclear cells

Medium from the whole mononuclear cell population (cultured at 10^7 cells/ml + concanavalin A at $100 \mu\text{g/ml}$) was assayed on bovine nasal cartilage at increasing concentrations without (●) and with (○) cortisol ($0.1 \mu\text{g/ml}$) in the cartilage culture medium. Bars represent $\pm$ S.E.M.

connective-tissue cells (fibroblasts and chondrocytes) to secrete proteinases and prostaglandins have been suggested to cause resorption of connective-tissue matrix, and their relationship to catabolin must be considered. Generally these factors have come from monocytes or macrophages (Deshmukh-Phadke *et al.*, 1978, 1980; Dayer *et al.*, 1979; Ridge *et al.*, 1980; Hamilton & Slywka, 1981) and synovial explants (Meats *et al.*, 1980; Robinson *et al.*, 1981). The best characterized is the monokine described by Dayer *et al.* (1979), which stimulates synovial fibroblasts to produce collagenase and prostaglandins, and which is now thought to be interleukin 1 (Mizel *et al.*, 1981). Interleukin 1 (previously called lymphocyte-activating factor) is produced by monocytes (or macrophages) and stimulates thymocyte and lymphocyte proliferation (Mizel & Farrar, 1979). Murine interleukin 1 has mol.wt. 14 000 and pI 5.0–5.5 (Mizel, 1980; Mizel & Mizel, 1981). Human interleukin 1 has pI 6.5 (Wood, 1979). Its action on synovial fibroblasts is inhibited by glucocorticoids (Dayer *et al.*, 1976). The relationship between these various factors will only be clarified when they are purified, but catabolin apparently differs from interleukin 1 in the following respects: (a) physicochemically; (b) it

originates from lymphocytes (and possibly from monocytes); and (c) its action is not inhibited by glucocorticoids. It must be emphasized that the relationship of the stimulation of proteinase secretion from dedifferentiated connective-tissue cells to matrix resorption in intact tissues has never been established, but catabolin is assayed by its ability actually to cause such resorption.

Production of catabolin by lectin-stimulated lymphocytes suggests a mechanism by which antigen-triggering of lymphocytes might cause connective-tissue resorption. Accumulations of lymphocytes at sites of inflammation could stimulate resorption of adjacent connective tissues. An obvious example in relationship to the work described here is the degradation of cartilage caused by chronic inflammation in rheumatoid arthritis.

We thank Dr. Lynn Pilsworth for advice on estimating the number of phagocytic cells, Mrs. Valerie Curry for skilled technical work, Mrs. Beryl King and Mrs. Bridget Wright for synovial culture medium and the Arthritis and Rheumatism Council and the Medical Research Council for financial support.

References

- Dayer, J.-M., Krane, S. M., Russell, R. G. G. & Robinson, D. R. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 945–949
- Dayer, J.-M., Breard, J., Chess, L. & Krane, S. M. (1979) *J. Clin. Invest.* **64**, 1386–1392
- Deshmukh-Phadke, K., Lawrence, M. & Nanda, S. (1978) *Biochem. Biophys. Res. Commun.* **85**, 490–496
- Deshmukh-Phadke, K., Nanda, S. & Lee, K. (1980) *Eur. J. Biochem.* **104**, 175–180
- Dingle, J. T., Saklatvala, J., Hembry, R., Tyler, J., Fell, H. B. & Jubb, R. (1979) *Biochem. J.* **184**, 177–180
- Hamilton, J. A. & Slywka, J. (1981) *J. Immunol.* **126**, 851–855
- Jasin, H. E. & Dingle, J. T. (1981) *J. Clin. Invest.* **68**, 571–581
- Meats, J. E., McGuire, M. B. & Russell, R. G. G. (1980) *Nature (London)* **286**, 891–892
- Mizel, S. B. (1980) *Mol. Immunol.* **17**, 571–577
- Mizel, S. B. & Farrar, J. J. (1979) *Cell Immunol.* **48**, 433–436
- Mizel, S. B. & Mizel, D. (1981) *J. Immunol.* **126**, 834–837
- Mizel, S. B., Dayer, J.-M., Krane, S. M. & Mergenhagen, S. E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2474–2477
- Ridge, S. C., Oronsky, A. L. & Kerwar, S. S. (1980) *Arthritis Rheum.* **23**, 448–454
- Robinson, D. R., Bastian, D., Hamer, P. J., Pichiarallo, D. M. & Stephenson, M. L. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5160–5164
- Saklatvala, J. (1981) *Biochem. J.* **199**, 705–714
- Saklatvala, J. & Dingle, J. T. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1225–1231
- Tyler, J. A., Fell, H. B. & Lawrence, C. E. (1981) *J. Pathol.* in the press
- Wood, D. D. (1979) *J. Immunol.* **123**, 2395–2399