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Pig catabolin is a form of interleukin 1

Cartilage and bone resorb, fibroblasts make prostaglandin and collagenase, and thymocyte proliferation is augmented in response to one protein

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Homogeneous catabolin from pig leucocytes induced proteoglycan breakdown, but not collagen breakdown, in explants of articular cartilage. It augmented lectin-induced proliferation of mouse thymocytes, stimulated production of prostaglandin E_2 and collagenase by fibroblasts and chondrocytes, and increased Ca^{2+} release from mouse calvarial explants, all at concentrations down to 50pM. In view of these effects it was concluded that pig catabolin is a form of interleukin 1.

Catabolin is a protein, produced by pig mononuclear leucocytes when they are cultured with concanavalin A, that induces resorption of proteoglycan in cartilage. It has M_r 21000 and pI4.9 and has been purified to homogeneity (Saklatvala *et al.*, 1983). Proteins highly similar to it are also produced by explants of pig synovial tissue (Saklatvala & Dingle, 1980; Saklatvala, 1981) and by pig synovial fibroblasts (Pilsworth & Saklatvala, 1983).

Interleukin 1 is a protein made by macrophages that activates lymphocytes and is generally assayed by its ability to enhance the proliferation of mouse thymocytes (Mizel, 1982). A form of interleukin 1 has been purified to apparent homogeneity from the mouse cell line P388D$_1$: it has M_r 14000 and pI5.0 (Mizel & Mizel, 1981). Interleukin 1-like molecules have also been found to arise from keratinocytes (Luger *et al.*, 1981), astrocytes (Fontana *et al.*, 1982), dermal fibroblasts (Iribe *et al.*, 1983), 3T3 fibroblasts (Okai *et al.*, 1982) and natural killer lymphocytes (Scala *et al.*, 1984).

Since Mizel *et al.* (1981) have found that highly purified interleukin 1 stimulated production of collagenase and PGE$_2$ by synovial fibroblasts, and other workers have also not been able to separate interleukin 1 from material active on connective tissue cells (Postlewaite *et al.*, 1983; Gowen *et al.*, 1983, 1984), it seemed likely that catabolin and interleukin 1 were identical or closely related.

Here we report some effects of homogeneous catabolin on connective tissue cells and mouse thymocytes and conclude that it is a form of interleukin 1.

Abbreviations used: DMEM, Dulbecco's modification of Eagle's medium; f.p.l.c., fast protein liquid chromatography; h.p.l.c. high-pressure liquid chromatography; PG, prostaglandin; SDS, sodium dodecyl sulphate.

Methods

Purification of catabolin

Catabolin was isolated from culture medium in which pig blood leucocytes had been cultured for 48h with concanavalin A (100μg/ml). The protein was purified by the following steps: (*a*) gel filtration, (*b*) chromatofocusing, (*c*) hydroxyapatite chromatography, (*d*) anion-exchange chromatography (Pharmacia f.p.l.c. MonoQ) and (*e*) reverse-phase h.p.l.c. on Zorbax ODS. This whole procedure has been described in detail, and criteria of homogeneity presented, elsewhere (Saklatvala *et al.*, 1983). The purified catabolin ran as a single band of M_r 21000 on SDS/polyacrylamide-gel electrophoresis (Fig. 1*a*). Catabolin concentration was estimated assuming an $A_{1cm}^{1\%}$ at 280nm = 10.

Synovial catabolin was absorbed batchwise on DEAE-cellulose from 24 litres of medium conditioned by pig synovium and was eluted with 0.13M-NaCl (see Saklatvala, 1981) and purified through steps (*a*) and (*b*) above. The synovial catabolin eluted from the chromatofocusing column at pH4.9.

Cartilage cultures

Discs (2mm) of bovine nasal cartilage were maintained in 96-well microtitre plates in 150μl of

DMEM containing 5% (v/v) heat-inactivated normal sheep serum and 0.1 μg of cortisol/ml for 76h at 37°C in CO_2/air (1:19). Chondroitin sulphate released into the culture medium was then measured by use of the metachromatic dye Dimethyl Methylene Blue (Saklatvala *et al.*, 1983).

Cartilage slices cut from pig metacarpal heads were cultured on Millipore SC filter membrane (8 μm) supported by a stainless steel grid in DMEM containing 15% (v/v) heat-inactivated normal rabbit serum (Wellcome Research Laboratories) for 16 days at 37°C in CO_2/air (1:19). Medium was changed every 4 days.

Cell culture

Chondrocytes were isolated by sequential enzyme digestion (Schwartz, 1979) of pig metacarpal head cartilage and used in primary culture. Fibroblasts were obtained by enzymic digestion (Dayer *et al.*, 1979) of synovium or lung, and cultured through five to eight passages in DMEM with 10% (v/v) foetal calf serum before use. Human embryonic foreskin fibroblasts (Flow 7000) were used in passages 16–30.

Assay of prostaglandin

PGE_2 was assayed by specific radioimmunoassay (Jaffe & Behrman, 1979) using rabbit anti-PGE_2– bovine serum albumin (Steranti Research; batch no. RB1112/1; specificity: PGE_2, 100%; PGE_1, 2%; PGF, 0.2%; PGB_2, 25%). Standard [5,6,8,11,12,14,15(n)-^{3}H]PGE_2 (5.92 TBq/mmol— came from Amersham International. The standard curve was constructed for the range 10–1000 pg/assay tube.

Assay of collagenase

Collagenase was measured by digestion of [^{14}C]-acetylated rat skin collagen fibrils (Sellers & Reynolds, 1977). Before assay, samples of serum-containing medium were treated for 10 min with trypsin (100 μg/ml) at 37°C, followed by soya bean trypsin inhibitor (500 μg/ml) for 20 min. This procedure saturates the serum proteinase inhibitor α_2-macroglobulin and activates latent collagenase (Dayer *et al.*, 1979). A unit is defined as the amount of enzyme degrading 1 μg of collagen/min at 35°C.

Mouse calvarial bone resorption assay

Detailed description of the assay can be found elsewhere (Reynolds, 1976). Mice aged 1–2 days were injected with $^{45}CaCl_2$ (1 μCi/mouse) 4 days before the experiment. Explants of half-calvaria were cultured on stainless steel grids in 1.5 ml of P_4 medium (a modification of BGJ medium) containing 5% (v/v) rabbit serum for 24h at 37°C in CO_2/air (1:19). The paired bones were then transferred to either control or test medium and incubated for 48 h. $^{45}Ca^{2+}$ in explants and medium was then measured in a liquid-scintillation counter. To be sure that the changes in isotope release were cell-mediated, catabolin was also tested on dead bones (killed by three cycles of refreezing and thawing) and found to be without effect.

Interleukin 1 assay on mouse thymocytes

Thymocytes from C3H/HeOla mice aged 6–12 weeks (Olac 1976) were cultured in flat-bottomed microtitre plates (1.5×10^6 cells/0.2 ml per well) in RPMI 1640 (Gibco) containing phytohaemagglutinin (1 μg/ml), 10^{-5}M-2-mercaptoethanol, 10% (v/v) foetal calf serum and the material under test. After 48 h cultures were pulsed for 16 h (0.5 μCi/well) with [^{3}H]thymidine (Amersham International). Subsequently cells were harvested and washed and their uptake of ^{3}H was measured in a scintillation counter.

Results

Action on pig articular cartilage

Bovine nasal cartilage, which is used in the defining bioassay of catabolin, is permeated by blood vessels and their supporting connective tissue. To prove that the pure protein acts directly on chondrocytes it was tested at 200 pM on pig articular cartilage which is avascular (Fig. 1*b*). Compared with the control (Fig. 1*c*), all the proteoglycan was lost after 16 days culture, but, even at this relatively high concentration, the collagen matrix was intact as judged by van Gieson staining (Fig. 1*d*). Explants cultured in 1 nM-catabolin with either 15% (v/v) rabbit serum or 10% (v/v) foetal-calf serum also showed no evident collagen loss. Spent medium from stimulated cultures contained increased amounts of trypsin-activatable collagenase compared with controls (Fig. 2). No activity was found without trypsin activation.

Action on fibroblasts and chondrocytes

Table 1 shows the effect of catabolin on the synthesis of PGE_2 and trypsin-activatable collagenase by various connective tissue cells. Pig synovial fibroblasts produced up to four times more collagenase and nine times more PGE_2 in 100 pM-catabolin; smaller but significant increases were seen with 50 pM-catabolin. Primary cultures of pig articular chondrocytes responded to the protein at similar concentrations with increases in production of PGE_2 and collagenase. In all these cultures collagenase was only detectable after trypsin activation. Mouse, human and rabbit fibroblasts also responded to catabolin with increased production of PGE_2 and showed similar sensitivity to the protein as the pig cells. None of

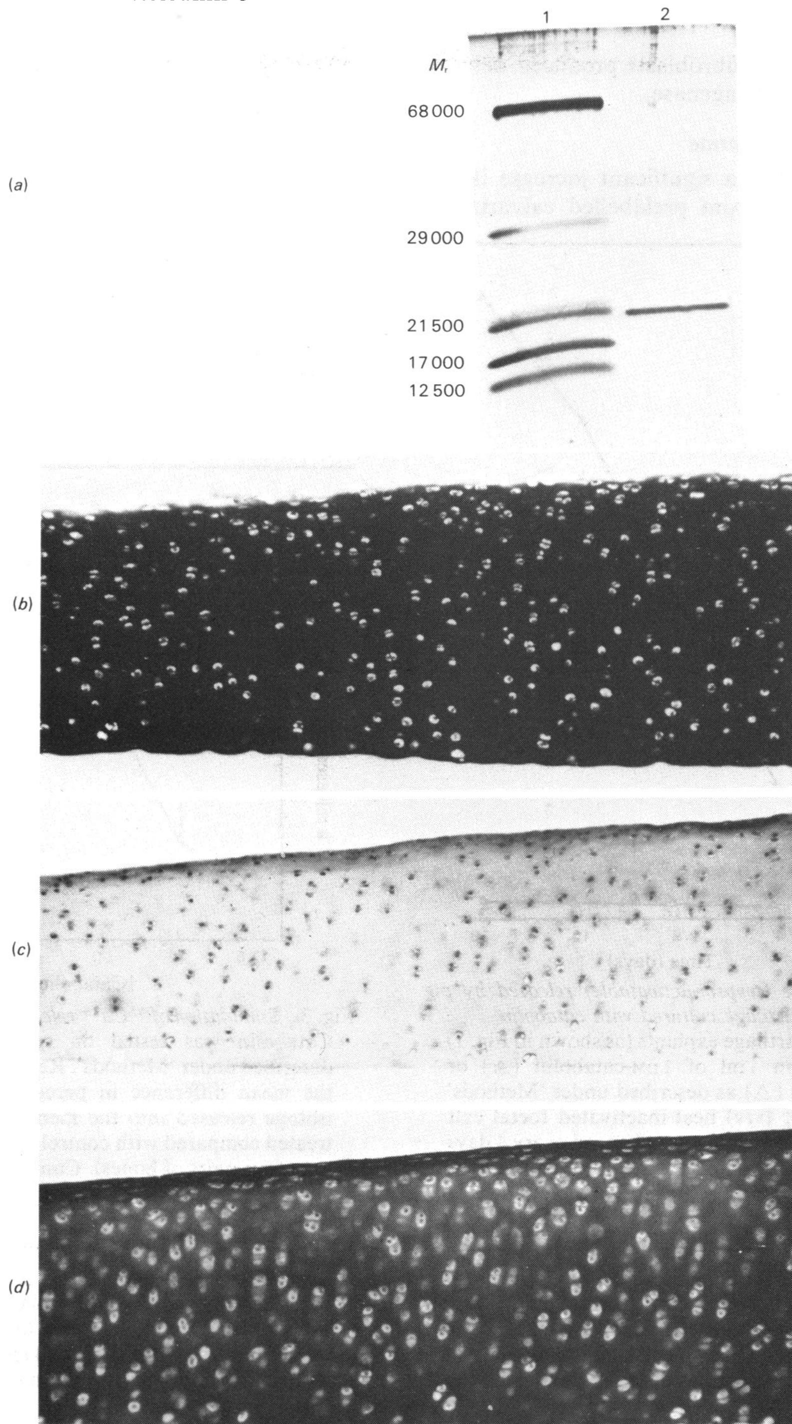

Fig. 1. *Purity of catabolin and effect on articular cartilage*
(*a*) SDS/polyacrylamide-gel electrophoresis of catabolin. A 12.5%-polyacrylamide slab in which samples were run with reduction in an ammediol buffer system (Wyckoff *et al.*, 1977) and stained with silver is shown. Lane 1, M_r markers are serum albumin (68000) carbonic anhydrase (29000), soya bean trypsin inhibitor (21500), myoglobin (17000) and cytochrome *c* (12500); lane 2, 0.1 μg of catabolin. (*b*)–(*d*) The effect of catabolin on pig articular cartilage. (*b*) Cartilage cultured for 16 days and stained with Toluidine Blue after fixation in Bouin's fluid; 8 μm section, ×25. (*c*) As (*b*) but the culture medium contained 200 pM-catabolin. (*d*) As (*c*) but stained for collagen with van Gieson's stain.

these other species' fibroblasts produced detectable quantities of collagenase.

Action on mouse calvariae

Catabolin caused a significant increase in the release of $^{45}Ca^{2+}$ from prelabelled calvariae at 50 pM and the response was maximal at 100 pM (Fig. 3). The protein was at least as active in this assay as other known bone resorbing agents such as parathormone and 1,25-dihydroxyvitamin D_3 (active at 50 nM and 2 nM respectively; Reynolds, 1976).

Action on mouse thymocytes

Catabolin augmented thymocyte proliferation (Fig. 4). For the batch of thymocytes used, the dose–response curve lay in the range 20–200 pM. The same batch of protein assayed on bovine nasal cartilage was active in the 2–20 pM range (Fig. 4). Partially purified pig synovial catabolin was

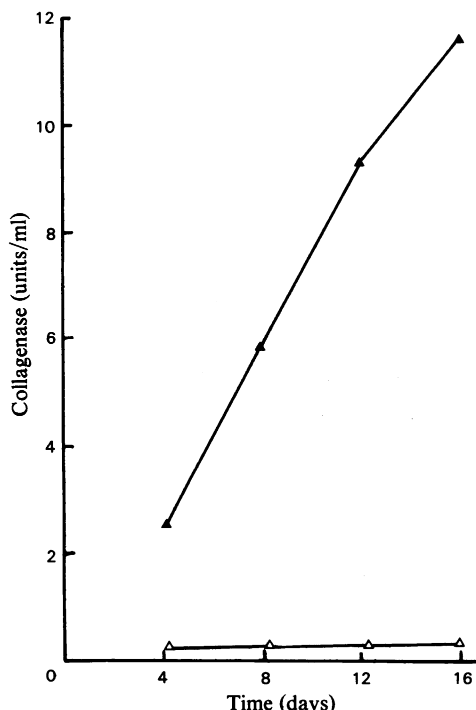

Fig. 2. *Collagenase (trypsin-activatable) released by pig articular cartilage cultured with catabolin*

Four articular cartilage explants (as shown in Fig. 1) were cultured in 1 ml of 1 nM-catabolin (▲) or control medium (△) as described under 'Methods' except that 10% (v/v) heat-inactivated foetal calf serum was used. Medium was changed every 4 days and the spent medium was assayed for collagenase after trypsin activation. Results are plotted as a cumulative total.

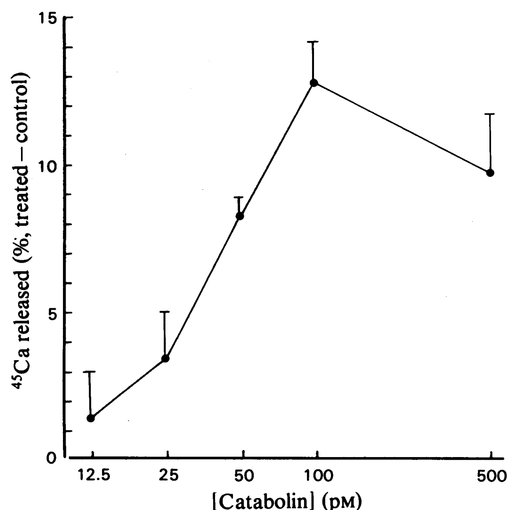

Fig. 3. *Stimulation of $^{45}Ca^{2+}$ release from mouse calvariae*

Catabolin was tested on calvarial explants as described under 'Methods'. Results are expressed as the mean difference in percentage of total bone isotope released into the medium from catabolin-treated compared with control bones ±S.E.M. (for at least four pairs of bones). Control release of $^{45}Ca^{2+}$ lay in the range 11–16% for live bones and 6–8% for dead bones.

Table 1. *Effect of catabolin on production of prostaglandin and collagenase by fibroblasts and chondrocytes*

Freshly-isolated chondrocytes (plated at 2.5×10^5 cells/ml), and serially-passaged fibroblasts [plated at $(0.6–1)\times10^5$/ml] were grown to confluence in 30 mm Petri dishes. Catabolin was added to groups of five dishes in DMEM containing 10% (v/v) foetal calf serum (2 ml/dish) and left on the cells for 4 days. PGE_2 and collagenase in the culture medium were measured as described under 'Methods'. PGE_2 is shown as ng/ml and collagenase as units/ml, both ±S.E.M.

		Response				
Cell type	[Catabolin] (pM) ...	0	20	50	100	250
Pig articular chondrocytes	PGE_2	9.5±0.4	48±7	149±10	259±25	675±43
	Collagenase	0.01	–	–	–	7.5±0.9
Pig synovial fibroblasts	PGE_2	39±19	75±9	186±28	303±43	–
	Collagenase	7.3±2.2	4.4±0.8	16±1.3	24.3±4.5	–
Human foreskin fibroblasts	PGE_2	28±7	–	118±8	176±17	–
Mouse lung fibroblasts	PGE_2	1.3±0.2	–	8.6±0.8	15.8±1.5	20.5±0.2
Rabbit synovial fibroblasts	PGE_2	136±14	–	300±26	420±56	–

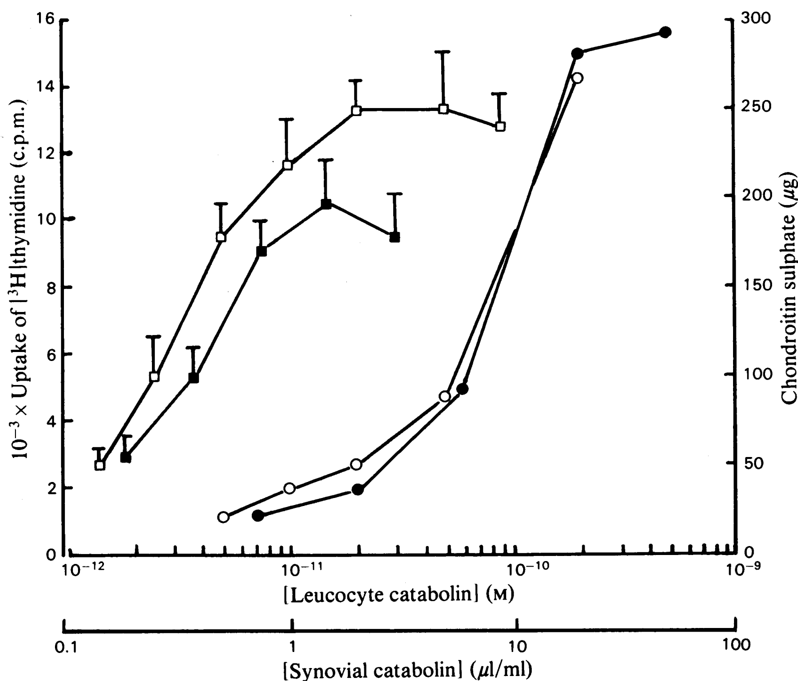

Fig. 4. *Dose–response curves for catabolin in the thymocyte assay and on bovine nasal cartilage*
Pure leucocyte catabolin was assayed on mouse thymocytes (●) and bovine nasal cartilage (■) at the molar concentrations indicated. [³H]Thymidine uptake by thymocytes is shown as mean c.p.m./well of triplicate assays. Cartilage assay results are means of quadruplicate assays (±S.E.M.) and are the amounts (μg) of chondroitin sulphate released/76h per disc. The concentration of partially purified synovial catabolin tested in the thymocyte assay (○) and cartilage assay (□) is expressed as μl/ml. The alignment of the concentration scales is arbitrary.

assayed on the same cartilage and thymocytes (Fig. 4) and its dose–response curves closely paralleled those of leucocyte catabolin.

Discussion

Pig catabolin augments thymocyte proliferation and stimulates fibroblasts to make PGE_2 and collagenase. These are actions it shares with the interleukin 1 purified from the mouse cell line $P388D_1$ (Mizel *et al.*, 1981), although it is is a larger protein (M_r 21000 versus M_r 14000). Human interleukin 1 copurifies through several steps with material that stimulates connective tissue cells (Mizel *et al.*, 1981, Postlethwaite *et al.*, 1983; Gowen *et al.*, 1984) and induces Ca^{2+} release in the mouse calvarial assay (Gowen *et al.*, 1983). Human interleukin 1 shows charge heterogeneity, the two main forms having pI values around 5 and 7 (Gowen *et al.*, 1983, 1984); rabbits (Murphy *et al.*, 1980) and mice (Lachman & Metzgar, 1980) also have more than one isoelectric form. Interleukin 1 may be a single protein whose heterogeneity arises from post-translational modification, or a group of different proteins with similar biological activities. Since interleukin 1 lacks a biochemical definition it is defined by its biological property of lymphocyte activation, and it is therefore reasonable to conclude that pig catabolin is a form of interleukin 1.

Catabolin was first found as a product of explants of synovial tissue (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980) but it has not been possible to purify the synovial protein to homogeneity. The equivalence of their activity on cartilage and thymocytes, together with the similarity in their M_r and isoelectric point (Saklatvala & Sarsfield, 1982) strongly suggests that the synovial and leucocyte catabolins are identical, both being a form of interleukin 1. The reason for the greater potency of catabolin on bovine cartilage than thymocytes is not known, but it could be that there are receptors of different affinity for the protein.

Catabolin failed to cause collagen resorption, but the detectable collagenase that was produced by the stimulated cartilage was all in a latent form. Activation of latent collagenase, which is thought to be a pro-enzyme (Nagase *et al.*, 1981), is likely to be a critical step if collagen resorption is to occur.

Highly purified interleukin 1 is pyrogenic (Rosenwasser *et al.*, 1979; Murphy *et al.*, 1980) and

stimulates production of acute phase proteins (Sztein *et al.*, 1981), and proteolysis in muscle (Baracos *et al.*, 1983). It may therefore not only regulate immune responses and connective tissue cells but also mediate systemic effects of inflammation. Whether pig catabolin can cause these other effects, and whether it causes tissue resorption *in vivo*, require investigation.

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