Influence of macrophage products on the release of plasminogen activator, collagenase, β -glucuronidase and prostaglandin E_2 by articular chondrocytes

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We describe the effects of products of mononuclear phagocytes on the secretory activity of chondrocytes. The primary confluent cultures of rabbit articular chondrocytes were exposed to standard medium alone or enriched with conditioned medium obtained from cultures of rabbit peritoneal macrophages, the mouse macrophage cell line P388D₁ or human blood mononuclear cells. Four markers of release were assessed, the neutral proteinases plasminogen activator and collagenase, the acid hydrolase β -glucuronidase and prostaglandin E_2 , and the kinetics of their changes were monitored. Chondrocytes that were cultured in standard medium secreted large amounts of plasminogen activator, some β -glucuronidase, but no collagenase, and released only minor amounts of prostaglandin E_2 . The addition of conditioned medium from rabbit macrophages induced a rapid release of large quantities of prostaglandin E_2 and an abundant secretion of collagenase, while abolishing or strongly decreasing plasminogen activator secretion. In addition, β -glucuronidase secretion was markedly enhanced. The decrease in secretion of plasminogen activator appeared to reflect a diminished production, since no evidence was found for the generation of inhibitors or for an accelerated extracellular breakdown of the enzyme. Conditioned media of the mouse and human mononuclear cells influenced the secretory activities of rabbit articular chondrocytes in a similar way, suggesting that the factor (or factors) acting on chondrocytes is produced by a variety of macrophages, and that its action is not species-restricted. The time course and concentration-dependence of the effects observed indicate that the secretion of plasminogen activator and collagenase are influenced in a strictly reciprocal fashion by the macrophage products. The release of prostaglandin E_2 paralleled that of collagenase.

It is well established that connective-tissue cells produce and release proteinases that are capable of degrading a wide variety of proteins, including the major structural macromolecules of connective tissue such as collagens, proteoglycans, laminin and fibronectin (Murphy *et al.*, 1981). Catabolic activities that are important in inflammation and that may also be involved in the normal turnover of the extracellular matrix appear to be modulated by inflammatory cells. Mononuclear phagocytes, for instance, are known to produce a factor that

greatly enhances collagenase and prostaglandin E_2 secretion by synovial cells (Dayer *et al.*, 1979). Deshmukh-Phadke *et al.* (1978, 1980) described a similar factor produced by rabbit peritoneal macrophages, which enhances the secretion of collagenase and neutral proteinases by chondrocytes. Meats *et al.* (1980) found that human synovium releases a factor that stimulates chondrocytes to release prostaglandin E_2 and plasminogen activator. Huybrechts-Godin *et al.* (1979) and Laub *et al.* (1982) showed that co-culturing of macrophages and fibroblasts results in the rapid degradation of collagen.

In order to characterize further the effects of macrophage products on the secretory behaviour of chondrocytes, we have studied the release of

Abbreviation used: DMEM, Dulbecco's modified Eagle's medium.

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plasminogen activator, collagenase, β -glucuronidase and prostaglandin E₂ by untreated and treated chondrocyte cultures.

Our results indicate that macrophage products induce selective changes of the secretory pattern of chondrocytes. Under the influence of the conditioned media from macrophage cultures, the chondrocytes cease to secrete plasminogen activator, which they do under control conditions, and switch to the secretion of large quantities of collagenase and produce in addition considerable amounts of E-type prostaglandins.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, rabbit serum, foetal-calf serum and antibiotics (penicillin/streptomycin solution, 10000 i.u./ml and 10 mg/ml respectively) were from Seromed, Munich, Germany. Hanks balanced salt solution (Gibco Europe, Glasgow, Scotland, U.K.) was made up in the laboratory. Enzymes used for isolating the chondrocytes were hyaluronidase (type I, 415 units/mg of protein; Sigma Chemical Co., St. Louis, MO, U.S.A.), trypsin (type III, 10000 units/mg of protein; Sigma Chemical Co.) and collagenase (136 units/mg of protein; Worthington Biochemical Corp., Freehold, NJ, U.S.A.). Ficoll-Ronpacon 440 for blood mononuclear-cell separation was from Pharmacia Fine Chemicals, Uppsala, Sweden. Acid-soluble starch for macrophage elicitation *in vivo* and digitonin for cell lysis were from Merck, Darmstadt, Germany. Indomethacin, lipopolysaccharide from *Escherichia coli*, trypsin inhibitor from soya bean and 4-aminophenylmercuric acetate were from Sigma Chemical Co. Heparin (178 i.u./mg) was a gift from F. Hoffmann-La Roche, Basel, Switzerland. The chromogenic substrate D-Val-Leu-Lys-4-nitroanilide for plasmin (a gift from T. G. Payne, Sandoz, Basel, Switzerland) was synthesized by conventional peptide chemistry and used as the trifluoroacetate salt. It is also available commercially from Kabi Diagnostica, Stockholm, Sweden. ¹²⁵I-fibrinogen was from Radium-Chemie, Teufen, Switzerland. CR-ITS Premix, a serum replacement for serum-free cell cultures, containing 5 mg of insulin, 5 mg of transferrin and 5 μ g of selenium per litre of medium, was from Collaborative Research, Waltham, MA, U.S.A. Phytohaemagglutinin P was from Difco, Detroit, MI, U.S.A.

Treatment of serum

For the acid treatment, serum was acidified to pH 2 with 3 M-HCl, stirred for 2 h at 20°C, and then

titrated back to pH 6.8 with 3 M-NaOH (Unkeless *et al.*, 1974).

Heat-inactivation consisted in incubation at 56°C for 30 min.

Chondrocyte culture

Chondrocytes were released from slices of distal femur articular cartilage taken from 900–1000 g New Zealand White rabbits by sequential treatment at room temperature with 0.05% hyaluronidase for 10 min, 0.2% trypsin for 15 min and 0.2% collagenase for 30 min. The cells were then incubated overnight at 37°C in DMEM enriched with 10% (v/v) heat-inactivated rabbit serum, 3.5 mg of glucose/ml and 0.2% collagenase (Benya *et al.*, 1977). The isolated chondrocytes were suspended in DMEM (100000 cells/ml) and were seeded in 15 ml portions into 75 cm² Corning tissue-culture flasks. The medium was supplemented with 5% (v/v) heat-inactivated rabbit serum and 1% (v/v) antibiotics solution. Culturing was performed at 37°C in a humidified CO₂ incubator. The cultures were optically confluent after 6 days.

Conditioned medium from rabbit peritoneal macrophages

New Zealand White rabbits (900–1000 g) were injected intraperitoneally with 60 ml of a 1% (w/v) starch suspension in iso-osmotic saline (0.9% NaCl). Then 3 days later the peritoneal cavity was washed with 250 ml of DMEM containing 1% (v/v) antibiotics solution and 500 i.u. of heparin. The cell suspension was centrifuged for 10 min at 360 g, the pellet was washed with DMEM, the cells were re-suspended at the density of 10⁶/ml in DMEM containing 5% (v/v) heat-inactivated rabbit serum and 1% (v/v) antibiotics solution, and 35 ml portions of this suspension were seeded into 150 cm² Corning tissue-culture flasks. After 5 h at 37°C, non-adherent cells were eliminated by decanting the medium and washing twice with serum-free DMEM. Culturing was continued for 24 h in serum-free DMEM containing antibiotics as described by Deshmukh-Phadke *et al.* (1978). Results from the literature (Simon & Willoughby, 1982) and our experiments (J. Schnyder, unpublished work) have shown that the cells must be in culture for hours in order to produce chondrocyte-stimulating activity. A brief exposure of minutes was insufficient (Mizel *et al.*, 1978a).

Some cultures were supplemented with 30 μ g of *E. coli* lipopolysaccharide/ml. The medium was then collected, cleared of cells and debris by centrifugation at 1300 g and stored at -20°C. At the end of the culture period, viability of the macrophages was at least 95%, as assessed by Trypan Blue exclusion.

Conditioned media from other sources

Conditioned medium from human peripheral-blood mononuclear cells was prepared as described by Bettens *et al.* (1982). Briefly, the blood of clinically healthy donors (22–32 years old) was centrifuged, the plasma diluted with 3 vol. of Hanks balanced salt solution, layered on Ficoll-Ronpacon 440 and centrifuged at 600g for 25 min. The cell-containing interphase was aspirated, washed twice with Hanks balanced salt solution and resuspended at a density of 2×10^6 cells/ml in RPMI 1640 medium, supplemented with 5% (v/v) heat-inactivated foetal-calf serum. The cultures were incubated for 4h before being harvested. Some cultures were supplemented with phytohaemagglutinin P at final dilution 1:1000.

Conditioned medium from the mouse macrophage cell line P388D₁ was produced by the method of Mizel *et al.* (1978a), with minor modifications. P388D₁ cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated foetal-calf serum in 25cm² Corning tissue-culture flasks. When the cells formed a confluent monolayer, they were washed twice with medium and incubated in RPMI 1640 medium or DMEM enriched with 1% heat-inactivated foetal-calf serum for 72–144h. The media were then cleared by centrifugation and stored at –20°C. Some of them were heat-inactivated for 30min at 56°C (Oppenheim *et al.*, 1982). A concentrate was prepared from P388D₁-cell supernatant. Confluent P388D₁-cell cultures were incubated in DMEM containing CR-ITS Premix for 10 days. The conditioned medium was cleared by centrifugation and concentrated about 20-fold with a YM-5 ultrafilter (Amicon Corp., Lexington, MA, U.S.A.). The concentrate was then dialysed overnight at 4°C against distilled water and freeze-dried. The residue was resuspended in distilled water, and a clear solution obtained by centrifugation. This final solution was about 50-fold concentrated with respect to the initial conditioned medium.

Treatment of chondrocytes with conditioned medium

The conditioned medium of rabbit peritoneal macrophages or the control medium (incubated in a culture flask without cells) was added to the chondrocyte cultures to a final concentration of 20%. Acid-treated serum (5%, v/v), which is free of proteinase inhibitors, was used for the chondrocyte cultures. Portions (0.5ml) were removed from the culture flask after 1, 2, 4, 8, 12, 24, 30 and 36h. After 48h the whole medium was changed and the sampling was repeated according to the same scheme. The samples of medium were centrifuged at 1300g to remove dead cells and stored at –20°C. These media were assayed for collagenase,

plasminogen activator, β -glucuronidase and prostaglandin E₂.

Collagenase assay

Thermally reconstituted trypsin-resistant fibrils of ¹⁴C-labelled rat skin collagen were used, as described by Cawston & Barrett (1979). One unit of collagenase hydrolyses 1 μ g of collagen fibrils/min at 35°C. Since all collagenase was found to be latent, assays were performed after activation with 650 μ M-4-aminophenylmercuric acetate.

Plasminogen activator assay

Two methods were used, a photometric assay of the plasmin-dependent amidolysis of D-Val-Leu-Lys-4-nitroanilide and the radiometric measurement of fibrinolysis on plates. The latter was the technique of choice in the past, and was employed in the present work for comparative purposes only. In the photometric assay, 25 μ l samples were incubated at 37°C for 30–180min with 500 μ l of 0.1M-Tris/HCl buffer, pH8.0, containing 1mM substrate and either 2.5 μ g of human plasminogen purified by affinity chromatography (Deutsch & Mertz, 1970) or no plasminogen. After the reaction had been stopped with 4 μ g of soya-bean trypsin inhibitor in 400 μ l of the above buffer, the absorbance was measured at 405nm. The plasmin-independent amidolysis of the substrate never exceeded 5% of the plasmin-dependent amidolysis and was usually undetectable. The overall reaction rate of this assay is a function of time squared (Christensen & Müllertz, 1977; Drapier *et al.*, 1979). A straight line with a slope corresponding to half the acceleration rate is obtained when the absorbance is plotted against the square of time (Drapier *et al.*, 1979). In order to express enzyme activity in units of μ mol of substrate hydrolysed/min, which depend on the rate of hydrolysis rather than on the acceleration rate, the velocity curve was calculated as the first differential of the quadratic function (Fig. 1).

Enzyme activity was calculated from the formula below, where ϵ ($\epsilon = 10054 \text{ M}^{-1} \cdot \text{cm}^{-1}$) is the molar absorption coefficient of 4-nitroaniline, t is the time in minutes and ΔA the absorbance at 405nm of the sample minus the absorbance of the blank:

$$1 \text{ munit/ml} = \frac{2\Delta A \times 0.925 \times 10^6}{\epsilon \times t^2 \times 0.025}$$

Fibrinolysis was assayed on 24-well tissue-culture plates (Linbro; Flow Laboratories, McLean, VA, U.S.A.) coated with fibrin. ¹²⁵I-fibrinogen was sterilized by filtration and dispensed into the 24-well plates (20 μ g per well; 5 nCi/ μ g), as described by Unkeless *et al.* (1974). At 1 day before the

Fig. 1. Plot of the mathematical function $y = 0.1x^2$. The parabolic quadratic curve (●—●), the curve after linearization (■--■) and the first differential of time dy/dx (velocity curve) (—) are given.

chondrocytes were plated, fibrinogen was converted into fibrin by incubation for 12 h with phosphate-buffered saline (composition: 8.00 g of NaCl, 0.20 g of KCl, 1.44 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 0.20 g of KH_2PO_4 per litre, pH 7.4) containing 10% (v/v) foetal-calf serum. After being washed four times with phosphate-buffered saline, the plates were sterilized overnight with u.v. light. Rabbit articular chondrocytes were suspended in DMEM (100 000 cells/ml), and 0.5 ml of the suspension was added to each well. The medium was supplemented with 5% (v/v) heat-inactivated rabbit serum, 1% (v/v) antibiotics solution and 100 μg of soya-bean trypsin inhibitor/ml. Cultures that reached confluence were washed four times with phosphate-buffered saline, supplied with DMEM (plus 1% antibiotics solution) containing 20% (v/v) of either conditioned medium of rabbit peritoneal macrophages or control medium and then incubated for 48 h with or without plasminogen (10 ng/well). The control medium and conditioned medium were dispensed on plates without cells and were treated in the same way as plates containing cells. After 1, 2, 5, 8, 12, 24, 34 and 48 h, 25 μl portions were removed for radioactivity measurements. The same culture plates coated with ^{125}I -fibrin were also used to assay plasminogen activator in samples of culture media as described by Snyder & Baggiolini (1978).

β -Glucuronidase, lactate dehydrogenase, alkaline phosphodiesterase I and DNA assays

Established methods were used for the assay of β -glucuronidase (Snyder & Baggiolini, 1978) and

DNA (Kapuscinski & Skoczylas, 1977). Lactate dehydrogenase was measured kinetically by using a Titertek system (Flow Laboratories). Samples (50 μl) were mixed with 50 μl of substrate solution made up to ensure the final concentration of reactants described by Bergmeyer & Bernt (1970), and the changes in absorbance were recorded at 492 nm. Since enzyme activity is lost on freezing and thawing, assays were always performed on fresh samples. Alkaline phosphodiesterase I was measured as described by Edelson & Erbs (1978).

Prostaglandin E_2 assay

A radioimmunoassay for E-type prostaglandins (Miles Laboratories, Elkhart, IN, U.S.A.) was used in accordance with the supplier's prescription.

Results

Enzyme secretion and prostaglandin E_2 release

The time course for the release of four different markers of secretory activity, namely the two neutral proteinases plasminogen activator and collagenase, the lysosomal hydrolase β -glucuronidase and prostaglandin E_2 , by confluent rabbit articular chondrocytes is shown in Fig. 2. The cells were cultured for 96 h with standard medium either without additions (○ symbols) or supplied with medium conditioned by rabbit peritoneal macrophages (● symbols). In both cases the medium was changed after 48 h.

Under control conditions (standard medium alone) large amounts of plasminogen activator and moderate amounts of β -glucuronidase were secreted (Figs. 2a and 2c), but no collagenase and only small amounts of prostaglandin E_2 (Figs. 2b and 2d). Secretion of plasminogen activator was observed only after a lag period of about 24 h. A similar lag period was observed again on change of medium. In sharp contrast with the controls, the secretory pattern of chondrocytes that were cultured in the presence of macrophage-conditioned medium was essentially reversed. In this case, large amounts of collagenase and prostaglandin E_2 (Figs. 2b and 2d) were released, but little or no plasminogen activator (Fig. 2a). The secretion of β -glucuronidase was significantly enhanced (Fig. 2c). Furthermore, no apparent lag phases were observed in the release curves.

In a few experiments (results not shown) 1 μM -indomethacin was used to test the effect of a blockade of cyclo-oxygenase. Under these conditions prostaglandin E_2 production was suppressed or decreased to a minimum, in both control and conditioned medium-treated chondrocyte cultures. The effect on the other secretory parameters, however, was minimal. A slight increase in the rate of secretion of plasminogen activator was

Fig. 2. Effect of rabbit-peritoneal-macrophage-conditioned medium on enzyme secretion and prostaglandin E₂ production by rabbit articular chondrocytes

Confluent cultures of chondrocytes were treated with 20% macrophage-conditioned medium (●) or control medium (○). At indicated times samples were removed for the detection of plasminogen activator (a), collagenase (b), β-glucuronidase (c) or prostaglandin E₂ (d). After 48 h the culture medium was aspirated and the culturing was continued for a second period with fresh medium containing 20% macrophage-conditioned or control medium. Means from quadruplicate cultures are given; S.E.M. is given as a vertical bar when larger than the symbol's radius.

Table 1. Cell-associated enzyme activities of chondrocytes cultured for 48 h with or without macrophage-conditioned medium
Chondrocytes were cultured for 48 h with 20% of the different conditioned media or 20% of corresponding control described in the Materials and methods section. After the incubation, the medium was removed, and the cells were washed three times with phosphate-buffered saline and lysed with aq. 0.05% digitonin solution. Results are given as means ± S.E.M. for two to four experiments.

Enzyme	No. of expts.	Specific activity (munits/10 μg of DNA)	
		No additions	Conditioned medium added
Lactate dehydrogenase	2	316 ± 24	319 ± 124
β-Glucuronidase	4	1.8 ± 0.1	1.9 ± 0.2
Alkaline phosphodiesterase I	3	49.5 ± 1.9	35.9 ± 6.7
Plasminogen activator	4	0.7 ± 0.1	1.5 ± 0.3*

* $P < 0.05$.

observed in control cultures, and collagenase and β-glucuronidase secretion were slightly inhibited in the cultures supplied with macrophage-conditioned medium.

Possible changes in the activities of some cell-associated enzymes were assessed at the end of a

48 h culture period. As shown in Table 1, exposure to the conditioned medium did not alter the activities of lactate dehydrogenase, alkaline phosphodiesterase I and β-glucuronidase. The activity of cell-associated plasminogen activator rose to about twice the control value. The activities recovered in

the cells, however, were considerably lower than those released by control chondrocytes during the same culture period.

Addition of the conditioned medium did not induce chondrocyte proliferation and was not cytotoxic, as indicated by the fact that the DNA content of the cultures remained constant over the whole experimental period.

Experiments with chondrocytes on fibrin-coated culture dishes

A common technique for the detection of cellular plasminogen activator assesses the release of radioactivity on culture of the cells directly on a layer of ^{125}I -fibrin. In order to compare our results with those reported by others, we also adopted this method in some experiments. Data are shown in Fig. 3. When chondrocytes were cultured with standard medium alone (controls), plasminogen-dependent release of radioactivity occurred during the whole culture period of 48 h. The rate of release

Fig. 3. Effect of rabbit-peritoneal-macrophage-conditioned medium on plasminogen-dependent degradation of ^{125}I -fibrin by rabbit articular chondrocytes

Confluent rabbit articular chondrocytes that had been cultured on ^{125}I -fibrin-coated 24-well tissue-culture plates were treated with 20% macrophage-conditioned medium (●) or control medium (○). Cumulative values for plasminogen-dependent fibrinolysis are expressed in c.p.m./well. The radioactivity measured in the media incubated without cells was in the range 700–1000 c.p.m./well during the whole experiment. The total lysis of ^{125}I -fibrin determined by addition of trypsin was about 50000 c.p.m./well. Mean values from triplicate cultures are given; S.E.M. is indicated as a vertical bar when larger than the symbol's radius.

was highest during the first 12 h and then decreased progressively. By contrast, in the presence of macrophage-conditioned medium plasminogen-dependent hydrolysis of the fibrin coat, which initially was virtually identical with that of control chondrocytes, ceased after the first 12 h. Plasminogen-independent fibrinolysis was tested in parallel, but was not affected by the treatment with conditioned medium. For both control and treated chondrocytes, it accounted for about 25% of total fibrinolysis of control chondrocytes in 48 h (results not shown).

Effects of conditioned media from other sources

Conditioned media from different sources, the mouse macrophage cell line P388D₁ and mononuclear cells from human peripheral blood, were compared with that of rabbit macrophages in seven separate experiments, of which three were chosen for Table 2. All three types of conditioned medium were active on rabbit chondrocytes: they induced the secretion of collagenase, which was always undetectable under control conditions, strongly enhanced prostaglandin E₂ release and markedly lowered the secretion of plasminogen activator. The results of selected cases with large variations in the chondrocyte response are shown in Table 2 to underline the opposite behaviour of the two proteinases. When chondrocytes responded to the conditioned medium with a high rate of collagenase secretion, only minimal amounts of plasminogen activator were detectable. However, when collagenase secretion was low, the rates of release of plasminogen activator were intermediate to high, similar to the amount found in the media of untreated chondrocytes.

In additional experiments we determined the response of chondrocytes to partially purified human mononuclear-cell factor (generously given by Dr. J.-M. Dayer and Dr. C. Zavadil, Geneva, Switzerland; see Dayer *et al.*, 1981). As shown in Fig. 4, increasing concentrations of the factor progressively diminished the secretion of plasminogen activator. The curve showing this effect is nearly reciprocal to those representing the release of collagenase and prostaglandin E₂, which increase in parallel with the concentration of the factor supplied to the chondrocyte culture medium. A similar pattern of response was obtained by exposing chondrocytes to increasing amounts of conditioned medium from cultures of mouse macrophage cell line P388D₁. The P388D₁-cell medium was concentrated as described in the Materials and methods section, and increasing portions of the concentrate (100, 250, 500 and 750 μl) were added to 100 ml of DMEM supplemented with 5% acid-treated rabbit serum and 1% antibiotics, which was used as the medium for the

Table 2. *Effect of the addition of different mononuclear-cell-conditioned media on enzyme secretion and prostaglandin E₂ production by chondrocytes*

Chondrocytes were cultured for 48 h with 20% of the different conditioned media or 20% of corresponding control medium. Results are given as mean values $\pm$ S.E.M. for triplicate cultures. Abbreviations used: LPS, *E. coli* lipopolysaccharide; PHA, phytohaemagglutinin P. Human mononuclear cells and P388D₁-cell-conditioned media were generously given by Dr. F. Kristensen and Dr. F. Bettens, Institute of Clinical Immunology, Inselspital, Bern, Switzerland.

Source of conditioned medium added to chondrocyte culture	Activity released		
	Collagenase (units/ml)	Prostaglandin E ₂ (ng/ml)	Plasminogen activator (munits/ml)
Control medium + LPS	<0.01	234 $\pm$ 7	1.54 $\pm$ 0.69
Rabbit macrophages + LPS	2.06 $\pm$ 0.04	776 $\pm$ 42	0.03 $\pm$ 0.01
Control medium	<0.01	132 $\pm$ 2	1.64 $\pm$ 0.03
Rabbit macrophages	0.86 $\pm$ 0.03	398 $\pm$ 19	0.15 $\pm$ 0.01
Control medium	<0.01	65 $\pm$ 7	2.47 $\pm$ 0.47
Rabbit macrophages	0.13 $\pm$ 0.02	799 $\pm$ 21	0.43 $\pm$ 0.09
Control medium	<0.01	65 $\pm$ 7	2.47 $\pm$ 0.47
Human mononuclear cells	1.94 $\pm$ 0.04	792 $\pm$ 56	0.01 $\pm$ 0.001
Control medium	<0.01	65 $\pm$ 7	2.47 $\pm$ 0.47
Human mononuclear cells	0.52 $\pm$ 0.04	1706 $\pm$ 49	0.02 $\pm$ 0.01
Control medium + PHA	<0.01	91 $\pm$ 2	4.21 $\pm$ 0.08
Human mononuclear cells + PHA	0.12 $\pm$ 0.02	363 $\pm$ 10	0.33 $\pm$ 0.05
Control medium	<0.01	132 $\pm$ 2	1.64 $\pm$ 0.03
P388D ₁ cells*	1.72 $\pm$ 0.03	405 $\pm$ 26	0.14 $\pm$ 0.01
Control medium	<0.01	122 $\pm$ 22	3.45 $\pm$ 0.05
P388D ₁ cells	0.48 $\pm$ 0.02	450 $\pm$ 15	0.10 $\pm$ 0.01
Control medium	<0.01	67 $\pm$ 1	2.70 $\pm$ 0.17
P388D ₁ cells	0.06 $\pm$ 0.001	167 $\pm$ 7	1.54 $\pm$ 0.03

* Conditioned medium was heated at 56°C for 30 min.

chondrocytes. As shown in Fig. 5, the release of collagenase and prostaglandin E₂ increased markedly in dependence of the amounts of conditioned medium added, and that of plasminogen activator fell correspondingly. In this experiment, plasminogen activator was determined in parallel by the chromogenic substrate and the ¹²⁵I-fibrin-coated-plate method. An excellent linear correlation was found between the two sets of data, the correlation coefficient r^2 being 0.97.

Stability of the cellular products

A number of control experiments were made to test the stability of the enzymes and of prostaglandin E₂ under the experimental conditions adopted. Cell-free culture media from rabbit chondrocytes in the presence or in the absence of macrophage-conditioned medium were incubated at 37°C, and collagenase, plasminogen activator and prostaglandin E₂ were assayed after 0, 8, 24 and 48 h. Collagenase activity remained constant, whereas plasminogen activator and prostaglandin E₂ decreased with time (Table 3). Freezing and thawing of the samples did not affect collagenase or plasminogen-activator activity, but lowered prostaglandin E₂ recovery to 82 $\pm$ 2% (mean $\pm$

S.E.M. for 47 determinations). β -Glucuronidase was previously found to be stable under comparable conditions (Schnyder & Baggiolini, 1978).

Search for possible inhibitors of plasminogen activator

To explore the possibility that the marked decrease in plasminogen activator induced by the macrophage-conditioned medium could result from the appearance of plasminogen-activator inhibitors, several mixing experiments were performed. Media from chondrocytes treated between 0 and 48 h with macrophage-conditioned medium containing collagenase but little plasminogen activator were mixed in a ratio of 1:1 with 48 h media from untreated chondrocyte cultures containing high activities of plasminogen activator. These mixtures were incubated for 1 h at 37°C before addition of plasminogen and substrate for activity determination. No change in the activity of plasminogen activator, corrected for dilution, was observed in any of the mixtures tested (results not shown). In further experiments, different dilutions of media containing plasminogen activator were mixed with equal volumes of media that were suspected to contain inhibitors. The results are presented in Fig. 6. The dilution curve of media

Fig. 4. Effect of partially purified human mononuclear-cell factor on the release of plasminogen activator, collagenase and prostaglandin E_2 by rabbit chondrocytes

Mononuclear-cell factor (a gift from Dr. Dayer) was material purified as described by Dayer *et al.* (1981) and freeze-dried in the Tris buffer used for elution. The powder was dissolved in DMEM and dialysed against this medium during 24 h. Portions were then added to the chondrocytes in DMEM supplemented with 5% acid-treated rabbit serum and 1% antibiotics, and plasminogen activator (Δ), collagenase ($\square$) and prostaglandin E_2 ($\circ$) were determined in the culture medium after 48 h. Means from triplicate cultures are given; s.e.m. is indicated by a vertical bar when larger than the symbol's radius. The numbers on the abscissa refer to mg of freeze-dried material added to the medium.

containing plasminogen activator was the same when they were mixed with culture media from treated chondrocytes or with media that were incubated in culture dishes without cells. In no instance was the activity of plasminogen activator influenced by the dilution, ruling out the presence of active inhibitors in the media of treated chondrocytes. Finally, the possibility of a proteolytic degradation of plasminogen activator in the media of treated chondrocytes was tested. Media from control and from treated chondrocyte cultures were incubated as such or in 1:1 mixtures for 48 h at 37°C. The activity of plasminogen activator recovered after incubation with the media of treated chondrocytes was somewhat lower than without (0.80 ± 0.18 versus 0.98 ± 0.18 munit/ml in four assays), but the difference was not statistically significant.

Fig. 5. Effect of a concentrate of P388D₁-cell-conditioned medium on the release of plasminogen activator, collagenase and prostaglandin E_2 by rabbit chondrocytes

Increasing portions of the concentrate (indicated on the abscissa) were added to the medium employed to culture the chondrocytes, and the amounts of plasminogen activator (see below), collagenase ($\square$) and prostaglandin E_2 ($\circ$) released in 48 h were determined. Plasminogen activator was determined with the chromogenic substrate of plasmin (Δ) and on 125 I-fibrin-coated plates ($\blacktriangle$) as described in the Materials and methods section. Means from triplicate cultures are given; s.e.m. is indicated by a vertical bar when larger than the symbol's radius.

Table 3. Stability of prostaglandin E_2 and of plasminogen activator

Chondrocytes were cultured for 2 days. The medium was aspirated aseptically, centrifuged to remove cells or cell debris, and then incubated for another 2-day period on fresh culture dishes without cells. Samples were taken at the time points indicated, stored at -20°C and then assayed for prostaglandin E_2 and plasminogen activator. Results are given as mean values $\pm$ s.e.m. for two experiments run in triplicate.

Time of incubation (h)	Prostaglandin E_2		Plasminogen activator	
	(ng/ml)	(%)	(munits/ml)	(%)
0	144.4 ± 13.7	100	3.54 ± 0.21	100
8	122.9 ± 0.6	85	3.32 ± 0.26	94
24	110.8 ± 1.6	76	2.91 ± 0.17	82
48	95.3 ± 1.8	66	2.36 ± 0.15	67

Discussion

Rabbit articular chondrocytes in confluent cultures release large amounts of plasminogen activator, moderate amounts of β -glucuronidase and

Fig. 6. Failure to detect inhibitors of plasminogen activator in media from treated chondrocytes

Samples (15 μ l) of differently diluted 48h culture media from untreated chondrocytes, which contained plasminogen activator, were mixed either with 15 μ l of 48h culture media from treated chondrocytes (●) or with control media that had been incubated in culture wells without cells (○). After 1h at 37°C the chromogenic substrate and plasminogen were added, and after 2h at 37°C the extinctions were measured. Mean values from triplicate cultures are given.

some prostaglandin E₂. Products elaborated in culture by mononuclear phagocytes from different species, i.e. rabbit peritoneal macrophages, macrophages of the mouse cell line P388D₁ and mononuclear cells from human blood, radically modify the chondrocyte secretion pattern. They induce the secretion of collagenase, they increase massively the production of prostaglandin E₂, they diminish or even suppress the secretion of plasminogen activator and they increase β -glucuronidase secretion. Similar effects are observed when the partially purified human mononuclear-cell factor prepared by Dayer *et al.* (1981), instead of the macrophage-conditioned media, is supplied to the chondrocytes. Kinetic analysis of the time course and concentration-dependence of the effect of the macrophage products show that the secretion of the two neutral proteinases is influenced in a strictly reciprocal fashion. The release of prostaglandin E₂ parallels that of collagenase. Proteinase secretion, however, appears to be independent of cyclo-oxygenase activity, since it is influenced only marginally, if at all, by the cyclo-oxygenase-blocker indomethacin.

Our observations that products of rabbit peritoneal macrophages induce collagenase and

prostaglandin E₂ release by rabbit chondrocytes confirm the reports by Deshmukh-Phadke *et al.* (1978), Ridge *et al.* (1980) and Nolan *et al.* (1982). The time course of collagenase secretion shown in Fig. 2(b) closely corresponds to that reported by Ridge *et al.* (1980). Our study shows, in addition, how the other secretory parameters change, in the same culture experiments, when macrophage products of different origins are added. That untreated chondrocytes secrete large amounts of plasminogen activator and that macrophage products suppress this secretory activity have not been reported before.

Results suggesting a reciprocal regulation of the secretion of collagenase and plasminogen activator were obtained in some other experimental systems. Gordon & Werb (1976) found that the addition of colchicine to thioglycollate-elicited macrophages enhanced the release of collagenase and other metallo-proteinases and lowered that of plasminogen activator. Golds *et al.* (1983) have reported that in synovial-cell cultures all-*trans*-retinoic acid enhanced the secretion of plasminogen activator while suppressing that of collagenase. A parallel rise in collagenase and prostaglandin E₂ production was observed by Brinckerhoff *et al.* (1979) in a study on rabbit synovial fibroblasts that were stimulated with phorbol myristate acetate, and by Dayer *et al.* (1979) in a study on human rheumatoid synovial cells stimulated with conditioned media of human blood mononuclear cells. Data by Berger *et al.* (1977) also appear in keeping with our results. These authors found that the secretion of plasminogen activator by human rheumatoid synovial cells was much lower (about one-twentieth) of that observed in the less-activated cells obtained from non-rheumatoid synovium.

In apparent contradiction with the above observations are the reports by Meats *et al.* (1980) and Laub & Vaes (1982), describing an increase in fibrinolytic activity induced by human synovium in human chondrocytes and by macrophage-conditioned media in rabbit fibroblasts. In these studies, however, the target cells were cultured on fibrin-coated plates. Under such conditions, the fibrin substratum used for the detection of plasminogen activator is degradable, not only by enzyme that is secreted, but also by cell-associated enzyme exposed at the outer surface of the plasmalemma. In fact, chondrocytes that were cultured in the presence of macrophage-conditioned medium and that secreted only minor amounts of plasminogen activator contained on average more than twice the amount of cell-associated enzyme as control chondrocytes (Table 1). The possibility that plasminogen activator is, at least in part, an ecto-enzyme in chondrocytes was investigated by the method of DePierre & Kar-

novsky (1974), with the use of the non-penetrating diazonium salt of sulphanilic acid to inactivate surface-exposed enzyme. This procedure resulted in a 90% decrease of cell-associated plasminogen-activator activity, suggesting that in these cells bound plasminogen activator is largely an ecto-enzyme.

The discrepancy between our findings and those of Meats *et al.* (1980) and Laub & Vaes (1982) cannot be explained by the different methodology employed. The chromogenic substrate mainly used in our study was found to be at least as accurate for the detection of plasminogen activator as the traditional labelled fibrin on plates. Furthermore, our experiments with chondrocytes seeded on fibrin-coated plates confirmed the finding that macrophage-conditioned medium decreased the secretion of plasminogen activator by chondrocytes. The actual data, however, underline the main problem of this methodology: the fibrin substrate is accessible to both cell surface-bound and secreted plasminogen activator. The most likely interpretation of the data shown in Fig. 3 is that during the first 12 h fibrinolysis was due largely to cell-associated enzyme and only later (in control cultures) to free, i.e. secreted, enzyme. This view is in keeping with the results obtained by the analysis of plasminogen activator in the culture media (Fig. 2a), which demonstrates secretion after a lag period only.

A further problem connected with the cultures on fibrin-coated dishes is that the fibrinolytic activity depends heavily on the amount of plasminogen supplied. At the concentration of 10 ng/ml, which is well above that supplied with fresh sera, plasminogen still is the limiting factor of fibrinolysis. The activity was 15–50-fold higher in the presence of 1 µg of plasminogen/ml. Under those conditions, however, the chondrocytes rapidly digested the substrate to which they were attached and tended to fall off (results not shown).

The nature of the factor that is present in the macrophage-conditioned media and that influences chondrocyte secretion is still hypothetical. In view of the constant pattern of chondrocyte response obtained, it is reasonable to assume that a similar active principle was present in the different conditioned media used. This factor is likely to be Interleukin 1, a well-recognized product of mononuclear phagocytes, for two main reasons. It is active across species barriers, a property that distinguishes Interleukin 1 from other cytokines (Oppenheim *et al.*, 1982), and it has a profile of activity resembling that of the human mononuclear-cell factor obtained by Dayer *et al.* (1981), which was found to be very similar to Interleukin 1 produced by the mouse macrophage cell line P388D₁ (Mizel *et al.*, 1981).

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