Activation of human leucocyte procollagenase by rheumatoid synovial tissue culture medium

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Wize, J., Abgarowicz, T., Wojtecka-Łukasik, E., Książny, S., and Dancewicz, A. M. (1975). *Annals of the Rheumatic Diseases*, 34, 520–523. Activation of human leucocyte procollagenase by rheumatoid synovial tissue culture medium. Production of a factor activating leucocyte procollagenase by tissue culture of rheumatoid synovium was shown. The activator was isolated, partly purified, and shown to be thermolabile, nondialysable, and had no activity toward casein, haemoglobin, histones, and PZ-peptide. The activity of the activator was partly decreased by trypsin.

A conspicuous migration of leucocytes to inflamed joints is observed in chronic rheumatoid arthritis. This disease is associated with progressive destruction of collagenous articular and juxta-articular structures such as cartilage, bone, and tendon. It is likely that synovial collagenase(s) (Evanston, Jeffrey, and Krane, 1968; Harris, DiBona, and Krane, 1969, 1971) and collagenase originating from polymorphonuclear leucocytes (Lazarus, Daniels, Brown, Bladen, and Fullmer, 1968) play a key role in this process.

Furthermore, there are indications that the collagenolytic activity of rheumatoid synovial fluid may be enhanced due to the conversion of leucocyte procollagenase to the active form (Kruze and Wojtecka, 1972). Thus it can be assumed that rheumatoid synovial fluid contains an endogenous factor able to activate leucocyte procollagenase. However, the origin of the activator present in rheumatoid synovial fluid is unknown.

Procollagenase activator has been shown in culture medium of tadpole explants (Harper and Gross, 1972) and mouse long bone (Vaes, 1972). It is reasonable then to assume that the activator present in rheumatoid but not in osteoarthrotic synovial fluid (Oronsky, Perper, and Schroder, 1973) may originate from rheumatoid synovial tissue. The results presented in this paper show that rheumatoid synovial tissue culture produces a factor activating human leucocyte procollagenase.

Materials and methods

CULTURE TECHNIQUE

Synovial tissue from patients with rheumatoid arthritis, obtained at operation, was trimmed of fat and cut into 3 mm² pieces. Explants were cultured up to 5 days in disposable flasks in Eagle's medium as described by Evanston and others (1968). Culture media were harvested daily and replaced with fresh ones. Each day's medium was centrifuged and dialysed against 0.05 mol/l TRIS-HCl buffer pH 7.5, containing 0.005 mol/l CaCl₂.

HUMAN LEUCOCYTE PROCOLLAGENASE

White blood cells were obtained from normal adult donors. Isolation of leucocytes and preparation of leucocyte homogenate was performed as described by Kruze and Wojtecka (1972). Leucocyte homogenate was chromatographed on DEAE Sephadex A-50 column to separate the collagenase-procollagenase mixture from the bulk of protein. Eluting buffer was 0.02 mol/l TRIS-HCl pH 8.6, containing 0.002 mol/l CaCl₂ on which a linear gradient from 0 to 0.4 mol/l NaCl was superimposed. The peak eluted between 0.06 and 0.12 mol/l NaCl was taken as a partially purified procollagenase.

ISOLATION OF ACTIVATOR FROM CULTURE MEDIUM

Pooled, dialysed culture media were freed from collagenase by its adsorption on collagen fibrils according to Harper, Bloch and Gross (1971). The resulting medium devoid of synovial collagenase was used as crude preparation of the activator in assays of its action on leucocyte procollagenase. In order to isolate the activator to identify it, pooled

dialysed culture media were concentrated using Aquacide (Calbiochem, Los Angeles, Calif.), then dialysed against 0.1 mol/l TRIS-HCl buffer pH 7.5, containing 1 mol/l NaCl and 0.005 mol/l CaCl_2 , and chromatographed on a 2.4 cm $\times$ 75 cm column of Sephadex G-150 equilibrated with the same buffer. The fractions containing the activator were pooled, dialysed against 0.01 mol/l TRIS-HCl buffer pH 7.5, containing 0.005 mol/l CaCl_2 and 0.2 mol/l NaCl, and further purified by separation on Sephadex G-100 column (1.6 cm $\times$ 75 cm). The effluent fractions containing the activator were pooled and stored at -20°C .

COLLAGEN

Acid soluble calf skin collagen was isolated and purified by the method of Kang, Nagai, Piez, and Gross (1966). For assays, approximately 0.2% collagen solution in cold 0.05% acetic acid was used. Before use, collagen solution was dialysed against 0.05 mol/l TRIS-HCl pH 7.5 containing 0.2 mol/l NaCl and 0.005 mol/l CaCl_2 , and centrifuged to remove small fragments and particles not dissolved. The origin of collagen was checked by its susceptibility to the action of trypsin added in a final concentration of 1% to collagen. Only 3% of collagen was digested in this test.

ASSAY OF THE ACTIVATOR

The presence of the activator and its relative activity was estimated from the difference in the collagenolytic activity of the systems containing procollagenase with or without the activator.

ASSAY OF COLLAGENOLYTIC ACTIVITY

Collagenolytic activity was measured by determination of hydroxyproline content in soluble peptides released from reconstituted collagen fibrils as described elsewhere (Kruze and Wojtecka, 1972). In addition, collagenolytic activity was tested by measuring changes in viscosity of collagen solution incubated in the presence of the systems tested. Viscosity measurements were performed at 25°C using Ostwald type of viscometer.

OTHER METHODS

Proteolytic activity of the activator was assayed using casein (Kunitz, 1947), urea denatured haemoglobin (Anson, 1939), and histones (Davies, Rita, Krakauer, and Weissmann, 1971) as substrates. Assay for collagen-peptidase activity using 4-phenylazobenzyloxy-carbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine as a substrate was performed as described by Wunsch and Heidrich (1963). Hydroxyproline was determined by the method of Stegemann and Stalder (1967) and protein by the method of Lowry, Rosebrough, Farr, and Randall (1951). Polyacrylamide gel disc electrophoresis of collagen degradation products was carried out according to the procedure of Nagai, Gross, and Piez (1964).

Results and discussion

During the first 2 days of culturing rheumatoid synovium no significant collagenase activity was detected in the medium. During the following days the collagenase was produced in the excreted medium with the yield similar to that reported by Evanson and

others (1968). When leucocyte procollagenase was supplemented with crude preparation of the activator, the collagenolytic activity of the system was increased significantly (Table I).

When attempting to isolate the activator, the media which were pooled from the 2nd to 5th day of culture were subjected to molecular sieve chromatography on Sephadex G-150 and then on Sephadex G-100. The elution profile obtained from the second chromatography had a single, well separated peak of the activator (Fig. 1). The partially purified activator had very low collagenolytic activity but a pronounced ability to activate the leucocyte procollagenase (Table II), but this activity was destroyed by heating for 10 minutes at 100°C ; heating for 10 minutes at 60°C or incubation with trypsin caused only partial decrease

Table I Effect of rheumatoid synovium culture media devoid of collagenase activity on human leucocyte procollagenase

Incubation system (Day of culture)	Collagenolytic activity* (μg hydroxyproline released)
1st	27.2
2nd	95.6
3rd	100.8
4th	105.6
Control without medium	10.0

* The buffer blank of 15 μg hydroxyproline and hydroxyproline contained in culture medium were subtracted from the samples. The incubation system consisted of 0.02 ml leucocyte procollagenase (300 μg protein), culture medium harvested at a day indicated (100 μg of protein), 1.5 ml collagen gel (300 μg hydroxyproline), and 0.05 mol/l TRIS-HCl buffer pH 7.5, containing 0.005 mol/l CaCl_2 added to final volume of 3 ml.

FIG. 1 Molecular sieve chromatography of the activator on Sephadex G-100. 2 mg of the activator separated by chromatography on Sephadex G-150 column were applied to Sephadex G-100 column (1.6 cm $\times$ 75 cm) and eluted with 0.01 mol/l TRIS-HCl buffer pH 7.5, containing 0.005 mol/l CaCl_2 and 0.2 mol/l NaCl. 3.2 ml fractions were collected at a rate of 18 ml/h. $\times$ = activator activity; $\bullet$ = collagenase activity; $\circ$ = absorbance at 280 nm

Table II Effect of partially purified activator from culture medium of rheumatoid synovial tissue on human leucocyte procollagenase

Incubation system	Collagenolytic activity* (μ g hydroxyproline released)
Full system	79.0
Without procollagenase	2.5
Without activator	10.0
Activator heated 10 min at 100°C	15.0
Activator heated 10 min at 60°C	65.0
Activator trypsinized†	46.2

* The buffer blank of 15 μ g hydroxyproline was subtracted from all samples.

† The activator (5 μ g protein) was preincubated with trypsin (10 μ g/ml) at room temperature for 30 min. After preincubation, 400 μ g of soyabean trypsin inhibitor was added and collagenolytic activity was assayed as described in 'Materials and methods'. The incubation system consisted of 0.02 ml leucocyte procollagenase (300 μ g protein), 0.3 ml activator (5 μ g protein), 1.5 ml collagen gel (300 μ g hydroxyproline), and 0.05 mol/l TRIS-HCl buffer pH 7.5, containing 0.005 mol/l CaCl_2 added to final volume of 3 ml.

in activity. There was no activity toward casein, haemoglobin, and histones, the common substrates of proteolytic enzymes. It had also no activity toward PZ-peptide.

Viscosity measurements showed that partially purified activator had no effect on collagen solution but added together with procollagenase increased its collagenolytic activity (Fig. 2). The composition of

FIG. 2 Viscometric assay of collagenolytic activity generated by treatment of leucocyte procollagenase preparation with partially purified activator from rheumatoid synovium culture medium. 0.05 ml (750 μ g protein) of leucocyte procollagenase and 1 ml of activator (20 μ g protein) were mixed and incubated for 2 h at 37°C. This solution was mixed with 1 ml of 0.2% collagen solution and 0.05 mol/l TRIS-HCl buffer pH 7.5, containing 0.005 mol/l CaCl_2 and 0.5 mol/l NaCl in final volume of 3 ml and transferred to Ostwald's viscometer. Measurements were performed at 25°C. x = activator; o = leucocyte procollagenase; ● = leucocyte procollagenase preincubated with activator

the collagen solution treated with procollagenase and activator was analysed by disc electrophoresis on polyacrylamide gels. The separation pattern obtained was essentially the same (Fig. 3) as that described for products of collagen degradation by animal collagenases (Lazarus and others, 1968; Harris and others, 1969).

The results show that the activator of leucocyte procollagenase is produced by rheumatoid synovium in tissue culture. The activator probably belongs to the class of procollagenase activators produced in tadpole explant and bone explant tissue cultures and regarded as specific proteinase (Harper and others, 1971; Vaes, 1972). These activators act as specific proteinase(s) converting procollagenase into collagenase through limited proteolysis. However, there is as yet no proof to confirm this mechanism of procollagenase activation by the activator from rheumatoid synovial tissue culture medium.

Production of the activator by the rheumatoid synovium in tissue culture might account for the presence of the activator in the synovial fluids of patients with rheumatoid arthritis (Kruze and Wojtecka, 1972).

Collagen degradation process seen in rheumatoid arthritis depends on a number of factors. Production of the activator of leucocyte procollagenase in conjunction with leucocyte immigration into synovial fluid and with procollagenase and collagenase released from leucocytes seems to contribute significantly to the destructive process in rheumatoid arthritis.

FIG. 3 Acrylamide gel electrophoresis of thermally denatured reaction products of incubation mixtures described in Fig. 2. A = collagen; B = collagen treated with activator; C = collagen treated with procollagenase; D = collagen treated with procollagenase and activator. α refers to the single polypeptide chain, β to the cross-linked dimers of the α chains

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