Type-specific collagen degradation by eosinophils

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Despite the association of eosinophils with wound-healing and fibrotic processes, their collagenolytic ability has been poorly defined. By using highly purified eosinophil preparations (>95% eosinophils) obtained from guinea-pigs by peritoneal lavage, we have examined type-specific collagen degradation by eosinophils using sensitive detection methods. The results show that eosinophils contain a metalloprotein that degrades types I and III collagens. This activity was apparent only after the addition of 4-aminophenylmercuric acetate to the reaction mixture, a finding similar to that for latent collagenases described from other sources. No collagenolytic activity against types IV and V collagens could be detected. Thus eosinophils may play a role in the alterations in connective-tissue matrices seen in physiological and pathological states.

The frequent association of inflammatory cells with alteration in the connective-tissue matrix suggests a role for these cells in the observed changes in matrix composition. The frequency with which qualitative rather than quantitative changes in collagen content are encountered in fibrotic states suggests that proteinase secretion by inflammatory cells may be an important factor in these processes. Studies of the inflammatory cells in idiopathic pulmonary fibrosis (Rudd *et al.*, 1981) suggest that those patients with a predominance of eosinophils in the bronchial lavage fluid have a poorer prognosis than those with a predominance of mononuclear cells. Such clinical findings suggest that the composition of the inflammatory infiltrate may be important in determining the pathological changes seen in fibrotic diseases.

Since degradation of collagen, in general, requires the action of specific proteinases, the characterization of the collagenolytic potential of inflammatory cells is essential to the understanding of the role of these cells in matrix alterations. The neutrophil has been relatively well-characterized in terms of its collagenolytic ability. Little, however, is known about the ability of eosinophils to degrade collagen, even though they have been noted during wound-healing (Baker *et al.*, 1976) and are associated with fibrotic diseases such as Loeffler's endocarditis and

eosinophilic fasciitis. The association of eosinophils with these conditions, in which alterations in collagen composition occur, prompted us to investigate type-specific collagen degradation by eosinophils. Other studies (Bassett *et al.*, 1976; Davis *et al.*, 1981) have suggested that eosinophils are capable of degrading type I and III collagens; however, the cell preparations used by these investigators contained a large number of contaminating cells (10% or greater) and the type-specificity of this collagenolytic activity was not well-characterized.

Materials and methods

Materials

HFL-1 human foetal lung fibroblasts (CLL 153) and HT-1080 human fibrosarcoma cells (CLL 121) were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Phosphate-buffered saline (0.01 M-phosphate/0.15 M-NaCl, pH 7.2), HBSS, DMEM and all tissue-culture supplements were purchased from Grand Island Biological Co., Grand Island, NY, U.S.A. Radiochemicals and En³Hance were obtained from New England Nuclear Corp., Boston, MA, U.S.A. All electrophoresis equipment and reagents were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. (NH₄)₂SO₄ was obtained from Schwartz-Mann, Spring Valley, NY, U.S.A. Trizma base, Na₃, NEM, PMSF, EDTA, pepsin, APMA, Ficoll and metrizoate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Heparin-Sepharose was obtained from Pharmacia Fine Chemical Co., Piscataway, NJ, U.S.A., and DEAE-

Abbreviations used: HBSS, Hanks balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; APMA, 4-aminophenylmercuric acetate; β -APN, β -aminopropionitrile; 2-ME, 2-mercaptoethanol; Me₂SO, dimethyl sulphoxide.

cellulose DE-52 was from Whatman, Clifton, NJ, U.S.A. All other chemicals were reagent-grade.

Methods

Cell isolation and purification. Eosinophils were obtained from guinea-pigs by a modification of the method of Gleich & Loegering (1973). Hartley guinea-pigs (500–1000 g) were given an intraperitoneal injection of 20 ml of sterile 0.15 M-NaCl and then lavaged 4 h later with 120 ml of phosphate-buffered saline on a weekly basis. Increased numbers of eosinophils were noted in the peritoneal fluids after 8–12 weeks of lavage.

In order to obtain consistently highly purified eosinophil populations, a two-step isolation procedure was utilized. First, mononuclear leucocytes were separated from the granulocytes by centrifugation of the cells on a Ficoll/metrizoate cushion ($d = 1.077$) at 400 g for 40 min (Boyum, 1968). The pellet from the Ficoll/metrizoate cushion, which contained granulocytes, was washed three times in HBSS and then resuspended in HBSS containing HCl-treated 10% (v/v) foetal-calf serum. Neutrophils were then separated from eosinophils by filtration of the cells through columns of nylon in the presence of serum, as described by Parrillo & Fauci (1978). This purification scheme allowed consistent isolation of cell populations containing more than 95% eosinophils (often more than 99%) as determined by differential counts of 1000 cells of Wright's-stained smears of the final preparation. The remaining 1–5% of the cell population was composed predominantly of mononuclear leucocytes with occasional mesenchymal cells present. Cell recovery was 30–40% of the eosinophils present in the peritoneal fluid.

For certain studies, mononuclear leucocytes were isolated from the interface of the Ficoll/metrizoate cushion. This cell population contained 99% mononuclear leucocytes as monitored by differential counts of Wright's-stained smears of the preparation.

Preparation of cell extracts. Purified cells (1×10^6 cells/ml) were suspended in 0.01 M-Tris/HCl/1 M-NaCl/0.1% Triton X-100/0.02% NaN_3 , pH 7.6, and subjected to two cycles of freeze-thawing, followed by sonication for 60 s at 50 W. The suspension was clarified by centrifugation (27000 g, 30 min). Further extraction of the pellet failed to result in additional solubilization of collagenolytic activity. The supernatant was dialysed against 0.01 M-Tris/HCl/0.15 M-NaCl/0.02% NaN_3 , pH 7.6, and concentrated 50-fold by using an Amicon ultrafiltration chamber equipped with a YM-5 membrane.

Preparation of substrates. Biosynthetically labelled types I and III collagens were obtained by incubating confluent HFL-1 lung fibroblasts with

[^{14}C]proline (0.5 $\mu\text{Ci/ml}$) and [^{14}C]glycine (0.5 $\mu\text{Ci/ml}$) in DMEM containing ascorbic acid (50 $\mu\text{g/ml}$), β -APN (50 $\mu\text{g/ml}$) and 20% dialysed foetal-calf serum for 24 h. The culture medium was harvested in the presence of 10 mM-NEM/1 mM-PMSF/5 mM-EDTA. The procollagens were precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 25% (w/v). After dialysis to remove the $(\text{NH}_4)_2\text{SO}_4$, types I and III procollagens were separated on DEAE-cellulose as described by Smith *et al.* (1972). The isolated procollagens were dialysed against 0.5 M-acetic acid and then treated with pepsin (100 $\mu\text{g/ml}$) for 18 h at 4°C to convert the procollagens into native collagens. After termination of pepsin digestion by adjusting the pH to 8.0 with saturated Tris, the samples were dialysed against 0.05 M-Tris/HCl/0.15 M-NaCl/0.02% NaN_3 , pH 7.6.

Native type IV collagen was biosynthetically labelled by using cultures of HT-1080 fibrosarcoma cells, a tumour line that has been shown to synthesize type IV collagen (Alitalo *et al.*, 1980), in a manner similar to that described for types I and III collagens. Confluent cultures were incubated with [^{14}C]proline (10 $\mu\text{Ci/ml}$) and [^{14}C]glycine (10 $\mu\text{Ci/ml}$) in DMEM containing ascorbic acid (50 $\mu\text{g/ml}$), β -APN (50 $\mu\text{g/ml}$), 1% foetal-calf serum and antibiotics for 48 h. The medium was harvested in the presence of proteinase inhibitors and precipitated with $(\text{NH}_4)_2\text{SO}_4$ as described above. After resolubilization of the $(\text{NH}_4)_2\text{SO}_4$ precipitate in 0.1 M-acetic acid, collagen was dialysed against phosphate-buffered saline and applied to a column of heparin-Sepharose as described by Sakishita *et al.* (1980). The unbound fractions were pooled and dialysed against 0.05 M-Tris/HCl/0.15 M-NaCl/0.02% NaN_3 , pH 7.6.

Type V collagen was isolated from a pepsin extract of human amnion by the procedure of Rhodes & Miller (1978) and was a gift from Dr. Jerome Seyer. Type V was acetylated with [^{14}C]acetic anhydride by the method of Cawston & Barrett (1979).

Determination of collagenolytic activity. Collagenolytic activity was assessed by incubation of 50 μl of cell extract with 5000 c.p.m. of labelled collagen in a reaction mixture containing 50 mM-Tris/HCl/5 mM- CaCl_2 /0.02% NaN_3 , pH 7.6, for 18 h. Types I and III collagens were incubated at 27°C in the presence of arginine (50 mM). The assay for type V collagen degradation was modified by the inclusion of 0.5 M-NaCl in the reaction mixture to maintain the collagen in solutions. Assays for the degradation of types IV and V collagen were performed at 32.5°C. The higher temperature of incubation was used with types IV and V collagens to maximize the detection of degradation of these collagens. Latent collagenolytic activity was activated with 1 mM-4-amino-

phenylmercuric acetate (APMA) as described by Sellers *et al.* (1977). Other methods of activation were not investigated. Stock solutions of proteinase inhibitors used were: 0.5 M-1,10-phenanthroline in Me_2SO ; 0.1 M-PMSF in Me_2SO ; 0.1 M-NEM in 0.05 M-Tris/HCl, pH 7.6; and 0.1 M-EDTA in 0.1 M-Tris/HCl, pH 7.6. All assays were terminated by the addition of EDTA to a final concentration of 10 mM.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed by the method of Laemmli (1970) with a slab-gel apparatus. Gels of radiolabelled substrates were processed for fluorography by a modification of the method of Bonner & Laskey (1974). After fixation in 50% (v/v) methanol containing 10% (w/v) trichloroacetic acid, the gels were impregnated with En^3Hance , dried and then fluorographed with pre-fogged Kodak XAR-5 X-ray film. Fluorographs were exposed for 4–10 days.

Results

Degradation of collagens by eosinophils

Collagenolysis of type I collagen by eosinophils was not evident when unlabelled substrates were utilized thus biosynthetically labelled substrates were prepared and incubated with eosinophil extract as described under 'Methods'. The biosynthetically labelled substrates contained nanogram quantities of substrate and required fluorography for detection. After termination of the reaction by the addition of EDTA, the samples were freeze-dried. The freeze-dried samples were solubilized in Laemmli sample buffer with 2-ME and analysed on 7.5% (w/v) polyacrylamide gels. The gels were fluorographed as described under 'Methods'. The results are shown in Fig. 1. No degradation was noted in the absence of APMA (track 8); however, collagenolysis was observed in the presence of this mercurial compound (track 7). Addition of 1,10-phenanthroline (5 mM) totally inhibited the proteolytic activity (track 6), but addition of PMSF (1 mM) (track 5) or NEM (10 mM) (track 4) was not inhibitory. The results indicate that eosinophils contain a latent metalloproteinase which degrades types I and III collagens, a finding similar to that for the classic interstitial collagenases (Welgus *et al.*, 1981). The lack of inhibition of collagenolysis by PMSF suggests that eosinophils do not contain a neutrophil elastase-like serine proteinase capable of degrading type III collagen (Mainardi *et al.*, 1980b; Gadek *et al.*, 1980).

Since types IV and V collagens have been shown to be resistant to the action of interstitial collagenase (Woolley *et al.*, 1978; Welgus *et al.*, 1981), we decided to determine whether the eosinophil was capable of degrading these collagens. To examine this, the eosinophil extract was incubated with

Fig. 1. Degradation of biosynthetically labelled collagens

^{14}C -labelled type I or type III collagen (5000 c.p.m.) obtained from human lung fibroblast cultures was incubated with 50 μl of eosinophil extract for 18 h at 27°C in a reaction mixture containing 50 mM-Tris/HCl/50 mM-arginine/5 mM- CaCl_2 /0.02% NaN_3 , pH 7.6. The reaction was terminated by the addition of EDTA (10 mM). The samples were freeze-dried, resolubilized in Laemmli sample buffer with 2-ME, analysed on 7.5%-polyacrylamide gels and then fluorographed. The APMA concentration was 1 mM. (a) Represents reaction with type I collagen. (b) Shows reaction with type III collagen. Track 9, collagen alone; track 8, collagen + cell extract; track 7, collagen + cell extract + APMA; track 6, collagen + cell extract + APMA + 1,10-phenanthroline (5 mM); track 5, collagen + cell extract + APMA + PMSF (1 mM); track 4, collagen + cell extract + APMA + NEM (10 mM); track 3, collagen + cell extract + APMA + 1% Me_2SO ; track 2, collagen; track 1, collagen + APMA + 1% Me_2SO . Abbreviation used: bf, buffer front. $\alpha 1$ and $\alpha 2$, intact collagen chains; $\alpha 1^A$ and $\alpha 2^A$, N-terminal cleavage products of collagen chains obtained after digestion with collagenase.

^{14}C -labelled type IV collagen or ^{14}C -labelled type V collagen as described under 'Methods'. No degradation of either type IV or type V collagen was noted in the presence or absence of APMA (results not shown). Thus, though eosinophils are capable of degrading the interstitial collagens, we were unable to demonstrate any collagenolytic activity against these types IV or V

collagens. This is in contrast with the neutrophil, which contains elastase, a potent type IV collagenase (Mainardi *et al.*, 1980a).

Collagen degradation by mononuclear cells

Since the eosinophil preparations contained 1–4% mononuclear leucocytes, the ability of mononuclear leucocyte extract to degrade types I and III collagens was examined to exclude the possibility that the proteinase present in the eosinophil extract was derived from mononuclear leucocytes. All procedures were identical with those described for the eosinophil. No degradation of either type I or type III collagen was noted, even when APMA was included in the reaction mixture (results not shown). Thus it appears unlikely that mononuclear cells could be the source of the collagenolytic activity seen in the eosinophil extract.

The presence of eosinophils during the post-inflammatory phase of wound-healing (Baker *et al.*, 1976), a time when remodelling of newly synthesized collagen is occurring, suggests that an interstitial collagenase elaborated by eosinophils could play an important role in this process. While the alterations in connective-tissue composition in the fibrotic processes accompanying hypereosinophilic states remain undefined, it is possible that collagenolysis could participate in the initiation and/or the perpetuation of such fibrosing reactions. The further characterization of the collagenolytic enzymes of eosinophils, the stimuli leading to their secretion and their association with alterations in matrix composition should lead to a better understanding of the role of these cells in both normal and pathological processes.

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