COLLAGENASE FROM RABBIT PULMONARY ALVEOLAR MACROPHAGES

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SUMMARY: Rabbit pulmonary alveolar macrophages produce a collagenase which lyses labeled collagen gels, specifically cleaves collagen types I, II and III, is inhibited by ethylenediaminetetraacetate, cysteine, dithiothreitol and serum but is not inhibited by a serine protease inhibitor. Alveolar macrophage collagenase activity can be enhanced by in vivo BCG activation, in vitro latex, silica or mycobacterium activation and by in vitro uncovering of latent enzymatic activity with trypsin treatment. The production of collagenase by unactivated alveolar macrophages and the presence of "latent" collagenase in culture media of alveolar macrophages are examples of significant differences between alveolar and peritoneal macrophages.

Throughout adult life, rabbit lung collagen content remains constant representing approximately 15% of lung dry weight (1). For collagen content to remain stable, the continuous synthesis of collagen by the adult lung (1) must be balanced by continuous degradation. One possible mechanism for lung collagen degradation is by collagenase, an enzyme specific for collagen, believed to be a major cause of extracellular collagen degradation in other organs (2). Cells that may be responsible for the production of collagenase in lung include blood borne polymorphonuclear leukocytes (3), lung parenchymal cells and pulmonary alveolar macrophages. The latter are a likely source since it has been found that the activated peritoneal macrophage will secrete collagenase (4-6) and a preliminary report has suggested that collagenase can be found in the media of cultured PAM (7). The present study demonstrates that rabbit PAM in culture produce a collagenase capable of degrading three types of collagen in a specific fashion. Collagenase production by these macrophages may be stimulated both in vivo and in vitro. The PAM differs from the peritoneal macrophage in that the unstimulated PAM produces a significant amount of collagenase

Abbreviations: PAM, pulmonary alveolar macrophages; BCG, Bacillis Calmette-Guerin; EDTA, ethylenediaminetetraacetate.

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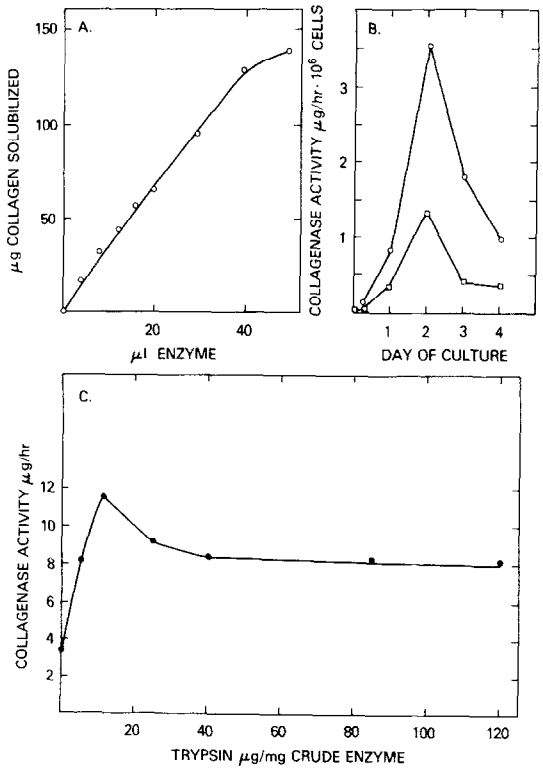

Figure 1. (A) Collagenase activity as a function of enzyme concentration. Increasing amounts of media concentrated from cultures of in vivo BCG activated PAM were added to 200 μg of [^{14}C] collagen in the fibril assay described in Methods. (B) Collagenase found in the media on sequential days of PAM culture. Macrophages were plated as described in Methods. After 2 hrs the cultures were washed free of non-adherent cells and fresh medium was added. At 6, 24, 48, 72 and 96 hrs medium was collected and replenished with fresh medium. Enzyme activity was determined on dialyzed concentrated medium. Data from normal PAM (□——□) and in vivo BCG activated PAM (o——o) are shown. (C) In vitro uncovering of latent collagenase activity with trypsin treatment of PAM media. Thirty μg of dialyzed concentrated medium from 24 to 48 hr of incubation of in vivo BCG activated PAM was incubated with increasing trypsin as described in Methods. Following the trypsin incubation, a 5-fold excess of soybean trypsin inhibitor was added and the resulting collagenase activity quantitated by [^{14}C] collagen fibril lysis.

and that a large part of the collagenase activity found in PAM media is in a latent form which may be activated with trypsin.

MATERIAL AND METHODS

Adult rabbits were all from a New Zealand White inbred strain. Lung macrophages were lavaged from normal rabbits ("normal PAM") or rabbits 3 weeks after intravenous injection of 0.5 mg of heat killed BCG in 0.5 ml oil ("in vivo BCG

activated PAM") (8). The macrophages were washed twice with culture medium (Dulbecco's Modified Eagle's Medium), plated in plastic tissue culture dishes at a density of approximately $0.5 \times 10^6/cm^2$ (10^6/ml), allowed to attach for 2 hrs at 37° and re-washed to remove non-adherent cells. Media was collected at 24 hr intervals and replaced with fresh media. Each 24 hr sample of medium was dialyzed (10 mM Tris-HCl, 7.4) and concentrated 10 to 20-fold by lyophilization and reconstitution with water. References to media collection are by days (e.g., media from day 2 represents the media from 24 to 48 hrs of macrophage culture.

Collagenase assays were performed by a modification of the method of Nagi et al (9). Each incubation (200 µl) contained 200 µg of heat reconstituted [^{14}C] guinea pig collagen fibrils (80,000 cpm/mg) and enzyme in final buffer concentrations of 50 mM Tris-HCl (7.4), 5 mM $CaCl_2$ and 100 mM NaCl. Trypsin (5 µg/tube) released approximately 8% of radioactivity from the fibrils and enzymatic activity below this level was not considered significant. Assays were performed under conditions such that: (1) the amount of collagen solubilized was linear with increasing amounts of enzyme (Figure 1A); (2) less than 70% of the total [^{14}C] counts in the fibrils were released; and (3) the digestion of collagen was linear with time. Where indicated, trypsin treatment of enzymes was carried out in 60 µl of 50 mM Tris-HCl (7.4), 5 mM $CaCl_2$ at 22° for 20 min with 1 µg trypsin for each 20 µg of macrophage medium protein. The trypsin was then inactivated by the addition of soybean trypsin inhibitor (5 µg/µg tryspin) and incubation at 22° for 10 min.

Collagen types I and III were purified from rabbit skin; type II was from rabbit sternal cartilage (10,11). To demonstrate collagenase digestion products, 20 µg of concentrated macrophage medium were incubated at 22° with 100 µg of collagen in 50 mM Tris-HCl (7.4), 100 mM NaCl, 5 mM $CaCl_2$ and 0.05% sodium azide for 20 to 72 hrs and electrophoresed on sodium dodecyl sulfate acrylamide gels (1). Specific viscosity measurements were performed as described by Nagi et al (9).

RESULTS

Macrophages were viable for over 4 days in culture as judged by Trypan blue exclusion. Approximately 10% per day detached and were lost during media change; such losses were accounted for in determination of enzyme activity per hr per cell. Over 99.5% of the cells phagocytosed latex particles and were mononuclear and thus were macrophages. A normal adult rabbit yielded $5\text{-}8 \times 10^7$ macrophages; a BCG treated rabbit yielded $6\text{-}9 \times 10^8$ macrophages. Polymorphonuclear leukocytes were never seen.

The presence of collagenase in media of rabbit PAM was proven by several criteria; (1) lysis of [^{14}C] labeled collagen fibrils (Tables I, II); (2) inhibition of fibril lysis by EDTA, rabbit or human serum, cysteine or dithiothreitol (Table I); (3) specific cleavage (at 22°) of types I, II and III collagen leaving two fragments; based on relative migration in sodium dodecyl sulfate acrylamide gels the two fragments represent approximately 70% and 30%

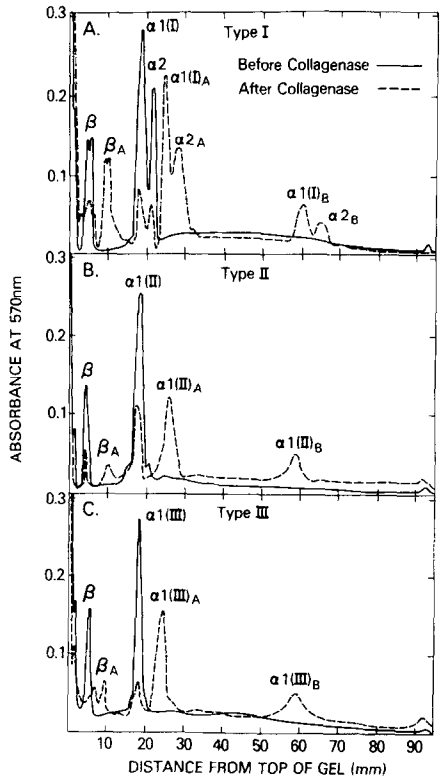

Figure 2. Sodium dodecyl sulfate acrylamide gel electrophoresis of three types of collagen before and after exposure to collagenase produced by in vivo BCG activated PAM. Incubations with (A) rabbit type I collagen; (B) rabbit type II collagen; and (C) rabbit type III collagen were performed for 20, 72, and 20 hrs respectively. Electrophoresis was for 7 hr at 8 ma/gel. Densitometric scans at 570 nm are shown. The nomenclature for collagenase digestion products was adopted from Sakai and Gross (20).

of the size of the original α chains (Figure 2); and (4) reduction of the specific viscosity of type I collagen by 62% (22°, 24 hr), type II collagen by 30% (22°, 72 hr) and type III collagen by 54% (22°, 24 hr) (data not shown). The collagenase produced by PAM was also characterized by the lack of significant inhibition of fibril lysis by phenylmethylsulfonyl fluoride, N-ethylmaleamide or soybean trypsin inhibitor and by lack of activation by parachloromercuribenzoate (Table I). Collagenase activity was maximal after two days following which daily production of enzyme fell. This was true for normal PAM and for in vivo activated PAM (Figure 1B). Medium from the first day of cul-

TABLE I

COLLAGENASE FROM PULMONARY ALVEOLAR MACROPHAGES*

Conditions	Activity Units per 10^6 cells	Percent Activity
PAM culture medium	6.2	100
+ 10 mM EDTA	0.3	5
+ 5% Rabbit serum	0.2	3
+ 5% Human serum	0.3	5
+ 1 mM Phenylmethylsulfonyl fluoride	6.2	100
+ 10 mM Cysteine	1.8	29
+ 10 mM Dithiothreitol	0.6	10
+ 10 mM N-ethylmaleamide	5.4	87
+ 5 mM Parachloromercuribenzoate	5.0	81
+ 50 µg/ml Soybean trypsin inhibitor	6.0	97
+ 0.2 mg PAM homogenate	6.1	98
+ 50 µg PAM culture medium (day 1)	6.6	106

* Collagenase secreted into medium during 24 to 48 hrs of culture of in vivo BCG activated PAM. Activity (1 unit = 1 µg collagen degraded per hr at 36°) represents [^{14}C] collagen released above buffer blanks. Medium was not trypsin treated. Homogenized PAM had no collagenase activity (0.2 mg = $4x10^6$ cells). 50 µg of PAM culture medium (from day 1) had an intrinsic activity of 0.7 units.

ture had low activity but this low level is probably not due to the presence of inhibitors since mixing concentrated day 1 medium with concentrated day 2 medium gave an additive effect (Table I).

Collagenase activity in concentrated, dialyzed culture media can be increased 3 to 9-fold by treatment of the media with trypsin (Table II). Maximal trypsin activation occurred by using 10 µg trypsin/mg protein in the concentrated, dialyzed medium. Excess trypsin caused a mild (usually 20%) decrease in activity from the maximum but large excesses of trypsin did not further diminish activity (Figure 1C). Although there was variation from rabbit to rab

TABLE II

STIMULATION OF THE COLLAGENASE SECRETED BY PULMONARY ALVEOLAR MACROPHAGES*

Conditions	Activity units per 10^6 cells	Activity units per 10^6 cells after trypsin treatment of concentrated enzyme	Percent Stimulation
Culture medium from normal PAM	0.5	1.6	0
+ Latex	1.5	4.4	+175
+ Silica	1.4	7.5	+369
+ M. butyricum	1.6	15.8	+888
Culture medium from *in vivo* BCG activated PAM	3.0	11.2	0
+ Latex	3.2	11.3	+1
+ Silica	2.2	7.8	-30
+ M. butyricum	13.6	35.2	+214

* Collagenase secreted into medium during 24 to 48 hrs of culture of either normal or *in vivo* BCG activated PAM. Activity (1 unit = μg collagen degraded per hr at 36°) represents [^{14}C] collagen released above buffer blanks. Trypsin treatment was as described in text: an amount of trypsin necessary to give maximal enzyme activity (Figure 1C) was used. Latex (mean diameter 1.01μ; 100 μg/10^6 cells), silica (mean diameter 5μ; 100 μg/10^6 cells) or heat-killed Mycobacterium butyricum (100 μg/10^6 cells) were added only on day 1 of culture. At the end of day 1, greater than 99.5% of PAM contained five or more latex particles or two or more silica particles respectively. Percent stimulation was calculated using post-trypsin treatment activity values.

bit in production of collagenase (Figure 1B vs. Table II), there was excellent reproducibility within a given set of cultures from the same rabbit.

Collagenase activity was found only in PAM media; homogenates of macrophages obtained directly from rabbits did not have detectable activity even after trypsin treatment. No collagenase inhibitor could be found on mixing concentrated medium from 10^6 PAM with homogenate from $4x10^6$ PAM (Table I).

PAM can be stimulated *in vivo* or *in vitro* to produce more collagenase. Latex, silica or M. butyricum all stimulate collagenase secretion by cultured normal PAM with the latter being the most active (Table II). The *in vivo* BCG activated PAM produces up to 7-fold more collagenase than the unstimulated

normal PAM (Table II, Figure 1B). In vivo BCG activated PAM can be further stimulated in vitro by heat killed micobacteria but not by latex or silica (Table II).

DISCUSSION

An enzymatic activity can be found in the media of cultured rabbit pulmonary alveolar macrophages which specifically cleaves native collagen under physiologic conditions and is inhibited by EDTA, cysteine and serum. By the general criteria established in studies with similar enzymatic activity produced by tadpole tails, synovium, bone, skin, ascites tumor cells and polymorphonuclear leukocytes, this is due to a vertebrate collagenase (2,12).

In vitro uncovering of latent collagenase activity with trypsin treatment of the media has been seen in collagenase secreted by other cells in culture (13,14). It is not clear whether this in vitro trypsin "activation" is due to reversal of collagenase inhibition, proenzyme activation or other as yet undefined mechanisms.

A normal PAM can be activated in vitro to produce more collagenase by a variety of means (Table II). Although collagenase production appears to be a general phenomenon accompanying stimulation of macrophage metabolic activity, some stimuli appear more potent than others with mycobacterial products yielding maximal collagenase activity. Interestingly, the macrophages activated in vivo by BCG cannot be activated in vitro by a nonspecific stimulant such as latex particle phagocytosis but they can be additionally activated in vitro by exposure to mycobacterial products.

Collagenase production by the PAM differs from the collagenase production by the peritoneal macrophage in two respects. First, the PAM from the normal animal will produce collagenase without any activation, while the peritoneal macrophage does not produce collagenase unless activated (4-6). This is not surprising since several studies have shown that the PAM is generally in a more active state (15). Secondly, PAM media has collagenase in a latent form that can be uncovered by trypsin while all of the collagenase found in the media of the peritoneal macrophage is already in an active form. In contrast to these differences, collagenase

production by both macrophage types is maximally stimulated by mycobacterial products and collagenase activity cannot be found in homogenate of either cell type

Three classes of isolated cell types have been shown to produce a collagenase under culture conditions: (1) mesenchymal cells (13,16); (2) blood cells (3); and (3) tissue cells originally derived from blood (4-6,17). In the normal lung, blood cells are confined to the vascular space and are probably not important in the normal destruction of collagen. The PAM is derived from blood, yet traverses the lung parenchymal interstitium before becoming an "alveolar" macrophage (15,18). Thus, it is possible that its collagenase may take part in the remodeling of lung collagen (19).

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