Expression of a Metalloproteinase That Degrades Native Type V Collagen and Denatured Collagens by Cultured Human Alveolar Macrophages

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Abstract

Human pulmonary alveolar macrophages obtained by bronchoalveolar lavage from both normal controls and smokers secreted in vitro a neutral proteinase that degraded denatured collagens. Optimal expression of the proteinase was detected after 3–5 d of culture. The proteinase could not be detected in the media of cultures that had been treated with 0.5 μg/ml of cycloheximide. The gelatinase had an $M_r$ of 90,000 and was immunologically cross-reactive with human neutrophil gelatinase. When newly synthesized $^{35}$S-methionine–labeled proteins were analyzed, the proteinase appeared to be a major secretion product of alveolar macrophages. Chromatography on gelatin–Sepharose gave a single peak of activity that was predominantly composed of the 90,000-mol-wt proteinase. The proteolytic activity in the gelatin-Sepharose-purified material was inhibited by EDTA and 1,10-phenanthroline, but not by N-ethylmaleimide or phenylmethylsulfonyl fluoride, indicating that the proteinase was a metalloproteinase. The partially purified material was also capable of degrading native type V collagen and this degradation was inhibited in the presence of an antibody to neutrophil gelatinase. The data suggest that human alveolar macrophages in culture elaborate a metalloproteinase that degrades both native type V collagen and denatured collagens.

Introduction

The major human phagocytes, the monocyte–macrophage and the neutrophil, are derived from a common precursor in the bone marrow. However, many of their characteristics during maturation in the bone marrow and after release into the circulation are divergent. Neutrophils differentiate within the bone marrow during which time they synthesize most, if not all, their proteinases destined for later intracellular digestion or extracellular release (1). Upon release from the bone marrow, the neutrophil has a short life span during which its proteinases are either released in response to specific stimuli (2, 3) or nonspecifically released upon cell death. In contrast, cells of the monocyte lineage are long-lived, versatile cells capable of adopting multiple phenotypes in response to developmental and environmental regulators (4).

Although resident macrophages may be relatively quiescent in normal tissue, they show a striking increase in their secretory repertoire during inflammatory processes. Among their secretory products, in such a circumstance, are neutral proteinases, including metalloproteinases such as interstitial collagenase and elastase (5, 6). Thus, in an inflammatory state, the expression of neutral proteinases involved in connective tissue destruction appears to be enhanced. Such proteinases may directly contribute to the tissue destruction seen in diseases such as rheumatoid arthritis and pulmonary emphysema.

Over the past decade, our knowledge of the collagenous composition of the extracellular matrix has greatly expanded. At present the collagen family is comprised of >25 distinct gene products that encode at least 11 collagen types (7, 8). Corresponding studies of the proteinases related to collagenous matrix turnover were given impetus by the observation that traditional interstitial collagenases were incapable of degrading types IV and V collagens (9, 10). Subsequently, a number of proteinases have been described that degrade the various minor collagenous components of the extracellular matrix.

Among these proteinases is a metalloproteinase that has been termed by various investigators as gelatinase (11–13), type V collagenase (14), or metalloproteinase II (15). This proteinase degrades native type V collagen and its cartilage counterpart, type XI collagen. Whereas it also degrades denatured collagens of all types, it shows no activity against native types I, II, III, and IV collagens. The proteinase derived from neutrophils appears to be relatively specific for collagenous components of the extracellular matrix as it does not catalyze the degradation of the glycoproteins present in the matrix (12). Similar proteinases have been described in rabbit bone cultures (16) and “activated” rabbit alveolar macrophage cultures (14). As types V and XI collagens are pericellular collagens that are important in cell matrix communication (17), their depletion may lead to abnormal repair processes after inflammatory events.

In this study, we have examined the ability of human alveolar macrophages to secrete a metalloproteinase that degrades native type V collagen as well as denatured collagens. The identification of a gelatinase with functional and immunological similarities to neutrophil gelatinase suggests that human macrophages may play a direct role in the observed connective tissue remodeling that accompanies inflammation.

Methods

Cell preparation and culture. Human alveolar macrophages were obtained by bronchoalveolar lavage from ten cigarette smokers undergoing routine diagnostic procedures or two nonsmoking, drug-free normal volunteers. All cell populations utilized contained >85% alveolar macrophages. Contamination by polymorphonuclear leukocytes was always <5%. The remaining cells identified were lymphocytes. The cell composition was determined by differential counts of Wright–Giemsa stained material. The cells were cultured at a density of 10⁵ alveolar macrophages/well in 24-well plates (surface area 2.1 cm²). Linbro, Flow Laboratories, Inc., McLean, VA) in Dulbecco’s modified Essential
medium (Gibco, Grand Island, NY) containing 0.2% lactalbumin hydrolysate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25
μg/ml amphotericin B. 2 h after plating, the nonadherent cells were
removed by washing three times with culture media. The adherent
population was > 95% macrophages, as determined by nonspecific
esterase staining (18). The culture media was again changed 24 h
after plating and then at 2-3 d intervals. The aspirated media were treated
with Tris–HCl, pH 7.6 (final concentration, 20 mM), phenylmethylene-
sulfonyl fluoride (PMSF; final concentration, 2 mM), and NaN3 (final
concentration, 0.02%) and then frozen at −20°C until analysis.

Collagen preparation. Type I collagen was prepared from an acid
extract of fetal bovine skin by the method of Glimcher et al. (19) and
radiolabeled as described by Cawston and Barrett (20). Type V colla-
gen was isolated from human amnion by the procedure of Rhodes and
Miller (21) and was a gift from Dr. Jerome M. Seyer.

Assay methods. Gelatinolytic activity was determined by the
method of Harris and Krane (22) using heat-denatured (15 min at
60°C) bovine type I collagen as the substrate, as previously described
(13). Units of enzyme activity are expressed in micrograms of
gelatin degraded per min. Type V collagenolytic activity was deter-
mined by incubating 100 μg of native type V collagen with the enzyme
preparations in a reaction mixture containing 60 mM Tris–HCl, 5 mM
CaCl2, 0.1% Triton X-100, 0.02% NaN3, pH 7.6, for 18 h at 32.5°C.
After termination of the assay by the addition of EDTA (final concen-
tration, 10 mM), the reaction products were analyzed on 7.5% poly-
crylamide gels. Previous viscometric studies have indicated that the
incubation temperature was below the melting temperature of type V
collagen (14). All assays utilizing unpurified material were done in the
presence of 2 mM PMSF. To activate any latent enzyme present,
aminocephylmecuric acetate was added to the incubation mixture at a
final concentration of 1 mM (23).

Demonstration of enzyme activity in SDS–polyacrylamide gels. When
gelatinase activity was to be determined, conventional SDS-gels (24)
were run in the usual manner except that gelatin (2 mg/ml) was
included in the running gel, the samples were not boiled or reduced before electrophoresis and the gel was cooled to 4°C during electropho-
resis. After electrophoresis the gel was washed in 2.5% Triton X-100
(wt/vol) to reenat the proteinase, rinsed in water, and then incubated
in 50 mM Tris–HCl, 5 mM CaCl2, 0.01 mN ZnCl2, 0.02% NaN3, pH
7.6, for 16 h at 37°C (25), as previously described (13). The zones of
enzyme activity were indicated by negative staining.

Immunoblotting. Samples for immunoblotting were concentrated
tenfold by pressure dialysis using a YM-10 membrane (Amicon Corp.,
Danvers, MA). The samples were electrophoresed on SDS–polyacry-
lamide gels and then transferred electrophoretically to nitrocellulose
by the method of Towbin et al. (26) and developed as previously de-
scribed (13).

Gelatin-Sepharose chromatography. Gelatin–Sepharose was pre-
pared by linking heat-denatured bovine type I collagen to CNBr-acti-
vated Sepharose (reference 13; Pharmacia Fine Chemicals, Piscataway,
NJ). Pooled supernatants from alveolar macrophage cultures were
concentrated tenfold by pressure dialysis, dialyzed against the starting
buffer, and applied to a 1 × 10 cm column that had been previously equilibrated with 20 mM Tris–HCl, 5 mM CaCl2, 0.05% Brij-35 (vol/
vol), 0.02% NaN3 (wt/vol), pH 7.6, containing 150 mM NaCl (starting buffer). Flow rate was 40 ml/h and 5 ml fractions were collected. The
column was washed with the starting buffer until the absorbance had
returned to baseline and then the column was washed with the starting buffer containing 1 M NaCl. The proteolytic activity was eluted with 1
M NaCl and 5% dimethyl sulfoxide (Me2SO) in the starting buffer.

Antibody preparation. Antibodies were prepared, as previously de-
scribed (13), either by immunizing with the 92-kD form of neutrophil
gelatinase obtained by preparative gel electrophoresis or by immuniz-
ing with the purified native form of the proteinase. Both antibodies
reacted with all three forms of neutrophil gelatinase and were shown to
be monospecific by immunoblotting techniques. However, the anti-
body against the native form of the proteinase was more effective in
immunoprecipitation studies. Purified IgG was obtained by affinity
chromatography on protein A Sepharose (27).

35S-methionine labeling and immunoprecipitation studies. Alveolar
macrophages that had been cultured for 48 h were washed three times with
methionine-free DME and then incubated with methionine-free
DME supplemented with 1 mM methionine and 50 μCi 35S-methio-
nine (New England Nuclear, Boston, MA) for 16 h. The labeled media
proteins were precipitated by quinique sulfate (28). Immunoprecipita-
tion was performed, as described by Mischel et al. (29), using 10 μg of
purified IgG of either preimmune or immune serum except that Em-
pigen was substituted for NP-40 as the detergent. Precipitated proteins
were analyzed on 10% polyacrylamide gels. After fixation and treat-
ment with Enhance (New England Nuclear) the gels were dried and
fluorographed as previously described (30). Densitometry was per-
formed using a laser densitometer (LKB Instruments, Inc., Gaithers-
burg, MD) equipped with an integrator (Hewlett-Packard, Co., Palo
Alto, CA).

Results

Identification of gelatinolytic activity in the media of alveolar
macrophage cultures. Initially, the culture supernatants were
assayed for functional gelatinase activity as described in the
Methods. Alveolar macrophages from both normal volunteers and
smokers secreted gelatinolytic activity into the culture media (Fig. 1).
While the maximal enzyme activity was noted in the 1st 3–5 d of culture,
continued production of enzyme activity was noted for up to 12 d of culture. The enzymatic activity was present in a latent form (> 99%). The functional activity from the various cell preparations differed substantially with the total cumulative activity ranging from 8 to 26 U/107 cells. The basis for the variability in functional activity in these preparations is not clear; however, it is possible that the concomitant secretion of collagenase inhibitor in some cell preparations (31) may be responsible for the variability in functional gelatinase expression. Collagenase inhibitor is also an effective gelatinase inhibitor (Hibbs M. S., and G. P.
Stricklin, unpublished observations). As similar results were
obtained with cells from smokers and nonsmokers, the re-
mainder of the studies were performed with cells from smokers
since the number of cells obtained were higher.

The spectrum of gelatinolytic proteinases present in the
macrophage culture media was similar in all preparations as
determined by a substrate gel technique. The majority of the
gelatinolytic activity was detected at an Mr of 90,000 (Fig. 2).
Some higher molecular weight material (250,000 D) was also
noted. It is unclear at present whether the higher molecular

1. Abbreviations used in this paper: Me2O, dimethyl sulfoxide; PMSF, phenylmethylene sulfonyl fluoride.

Figure 1. Cumulative gelatinase activity in alveolar macrophage culture media. Alveolar macrophages from normal individuals or uninfected
smokers were obtained by bronchoalveolar lavage and cultured. Functional
gelatinase activity in the media was determined using denatured 14C-la-
teled type 1 collagen. Results are expressed as U/107 macrophages. A rep-
resentative experiment is shown.

---,-, Smoker; —, nonsmoker.
weight form represents the native secreted species of the enzyme or an aggregate. Such large molecular weight species have also been observed in our studies of neutrophil gelatinase (13). Further biosynthetic studies will be necessary to resolve these questions.

Immunoreactivity of the macrophage proteinase with neutrophil gelatinase. As the major molecular weight species seen on gelatin-substrate gels was similar to the molecular weight of one of the forms of neutrophil gelatinase (13), the reactivity of this protein with a polyclonal antibody prepared against the 92-kD species of neutrophil gelatinase was examined by immunoblotting. Strong reactivity at M, of 90,000 was noted in the media from the 3–5-d cultures (Fig. 3) that corresponded to the peak activity seen on gelatin-substrate gels. Less reactivity was noted at other time periods, which may be related to the smaller amounts of antigen present. Although the immunoreactivity generally paralleled the activity as demonstrated by substrate gel analysis, it is possible that the neutrophil and macrophage proteinases share common antigenic determinants but are not completely immunologically identical.

Biosynthesis of gelatinase by alveolar macrophages. As several reports have indicated that alveolar macrophages secrete stored neutrophil proteinases (32, 33), studies were undertaken to establish that the detected gelatinolytic proteinase was a biosynthetic product of the macrophage. Initially, we examined the distribution of the proteinase between the culture media and the cell layer 48 h after the initial plating of the macrophages. This time point was chosen since any contami-

![Figure 2](image1.png)

**Figure 2.** Identification of gelatinase by substrate gel analysis. 60 μl of concentrated (tenfold) conditioned media were electrophoresed on an SDS-polyacrylamide gel (6%) containing 2 mg/ml gelatin. After renaturation of the enzyme by washing the gel in 2.5% Triton, the gels were incubated for 16 h at 37°C. Zones of negative staining represent areas of enzyme activity.

![Figure 3](image2.png)

**Figure 3.** Immunoreactivity of alveolar macrophage culture media with an antibody to neutrophil gelatinase. 60 μl of concentrated (tenfold) media were electrophoresed on a 6% polyacrylamide gel, transferred to nitrocellulose, and reacted with an antibody to human neutrophil gelatinase.

![Figure 4](image3.png)

**Figure 4.** Gelatinase expression is dependent on protein synthesis. (A) The gelatinase in the culture media and cell layer extracts from 10⁷ macrophages was identified by substrate gel analysis. Media and cells were harvested after 48 h of culture. Media represents the amount of gelatinase secreted between 24 and 48 h in culture. (B) Alveolar macrophage cultures were treated with the indicated amount (micrograms per milliliter) of cycloheximide for 48 h, and the culture media was analyzed for gelatinase activity by substrate gel analysis and functional gelatinase activity (percent inhibition).
Characterization of the gelatinolytic activity present in the media of alveolar macrophage cultures. To facilitate characterization of the gelatinolytic proteinase secreted by alveolar macrophages, the proteinase was isolated from the culture media by affinity chromatography on gelatin–Sepharose as described in the Methods. Fig. 6A shows that the gelatinolytic activity was sharply eluted with Me2SO. When the fractions were screened on gelatin-substrate gels (Fig. 6B), the gelatinase activity present in the starting material was found to be totally adsorbed to the affinity matrix and was eluted in the Me2SO fraction. No activity was noted in the 1 M NaCl fraction (not shown). Although there were several minor bands of gelatinolytic activity in the concentrated material, the 90,000-mol-wt form is the major proteinase.

As we have observed the phenomenon of autodegradation in concentrated preparations of gelatinases purified from other sources (reference 13, unpublished observations), we examined the possibility that the additional lower molecular weight bands were related to autoactivation of the proteinase. The purified proteinase was incubated with 1 mM aminophenylmercuric acetate at 37°C for 16 h. Then the activated and the autoactivated material was analyzed by substrate gel analysis. As seen in Fig. 7, the molecular weight of the activated proteinase is clearly shifted to those corresponding to the minor bands of activity seen in the fraction obtained by gelatin–Sepharose chromatography. These data suggest that these minor bands of activity are related to autocatalysis.

After isolation of the proteinase, the inhibition of the gelatinase by standard proteinase inhibitors was examined. Metal chelating agents such as 1,10-phenanthroline and EDTA were effective inhibitors. The serine proteinase inhibitor, PMSF, and the thiol proteinase inhibitor, N-ethylmaleimide, failed to inhibit the gelatinolytic activity (Table I). Also, if substrate gels of either the crude or the partially purified material were incubated in the presence of these proteinase inhibitors, only metal chelating agents were found to be effective inhibitors (not shown). These results demonstrated that the gelatinolytic activity was attributable to a metalloproteinase.

Since a subset of gelatinolytic proteinases have been shown to degrade type V collagen (12–14), the ability of the gelatinase isolated by gelatin–Sepharose chromatography to degrade native type V collagen was examined. In these experiments, the proteinase was incubated with type V collagen for 18 h at 37°C and the reaction products were separated on a 6% polyacrylamide gel. As seen in Fig. 8, the material isolated by gelatin–Sepharose also degraded native type V collagen. To assure that this type V degrading activity was related to the 90,000-D proteinase, the ability of the antibody to neutrophil gelatinase to inhibit the type V degradation was also assessed. When antineutrophil gelatinase IgG was included in the reaction mixture, the type V degradation was almost completely inhibi-
Table I. Inhibition of Alveolar Macrophage Gelatinase*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activity µg gelatin degraded/min per ml</th>
<th>Inhibition %</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>3.7</td>
<td>—</td>
</tr>
<tr>
<td>1,10-phenanthroline (5 mM)</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>EDTA (10 mM)</td>
<td>0.16</td>
<td>96.0</td>
</tr>
<tr>
<td>N-ethylmaleimide (10 mM)</td>
<td>3.5</td>
<td>5.0</td>
</tr>
<tr>
<td>PMSF (2 mM)</td>
<td>3.6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* 30 µl of gelatin–Sepharose-isolated gelatinase were preactivated with 1 mM aminophenylmercuric acetate for 1 h at 37°C and then incubated with 100 µg gelatin for 6 h at 37°C in the presence of the indicated inhibitor.

Discussion

In this report, we have identified a gelatinolytic proteinase that is secreted by cultured human alveolar macrophages. The proteolytic activity is present predominately in the form of a 90-kD proteinase, though a higher molecular weight form is also evident. As has been reported for other neutral metalloproteinases secreted by monocyte–macrophages (5, 6), the proteinase does not appear to be stored in the cell and its secretion is dependent on active protein synthesis. Although cycloheximide could interfere with secretion in an indirect manner, the demonstration of immunoprecipitable, newly synthesized 35S-labeled gelatinase clearly indicates that the proteinase is a biosynthetic product of alveolar macrophages. Also, this proteinase appears to be a major product of the macrophage.

Characterization of the proteolytic activity revealed that it appears to be due to a metalloproteinase. The partially purified material also exhibits the ability to degrade native type V collagen. Although the isolated material is predominantly 90 kD, a number of minor bands of gelatinolytic activity are also present. The higher molecular weight species could represent aggregates that form after concentration by the chromatographic procedure. Such species were also observed in our previous studies with neutrophil gelatinase. Those studies demonstrated that the higher molecular weight species seen in association with the 90-kD proteinase also degraded type V collagen (13). The lower molecular weight bands appear to be related to autodegradation and partial activation of the proteinase during isolation. The spontaneous generation of multiple molecular weight species of metalloproteinases has been previously reported (34, 35). The ability of the neutrophil gelatinase antibody that recognizes the 90-kD proteinase to inhibit the type V degrading activity indicates that this proteinase is responsible for both activities. Interestingly, similar to neutrophil gelatinase, the proteinase shows no detectable activity against native types I–IV collagens, elastin, or glycoproteins such as fibronectin or laminin (data not shown). Thus, the data are consistent with our previous studies (13, 14) and those of other investigators (12, 16) that have demonstrated that gelatin-specific metalloproteinases with a M, in the range of 85,000–95,000 are consistently found to be type V collagenases.

Despite the complexities of the system, it is clear that human alveolar macrophages secrete proteolytic activity that degrades both denatured collagens and native type V collagen.

Figure 8. Degradation of type V collagen by gelatinase isolated by affinity chromatography and the inhibition of degradation in the presence of anitneutrophil gelatinase IgG. 0.5 µg of the gelatinase preparation obtained by gelatin–Sepharose chromatography were incubated with 50 µg of type V collagen for 18 h at 32.5°C and the reaction products separated on a 7.5% polyacrylamide gel. Antibody dilution was 1:500. Lane 1, collagen plus antigelatinase IgG; lane 2, collagen plus enzyme; lane 3, collagen plus enzyme plus antigelatinase IgG; lane 4, collagen plus enzyme plus unrelated immune IgG.

3, collagen plus enzyme plus antigelatinase IgG; lane 4, collagen plus enzyme plus unrelated immune IgG.
tinase more closely resembles the neutrophil form of this protease (13) as the predominant gelatinase in fibroblast culture media is 67 kDa and the native form of fibroblast enzyme is not reactive with our antibodies to neutrophil gelatinase (44). Further studies will be needed to determine whether these differences are due to posttranslational processing or alterations in gene expression.

In addition to the studies on interstitial collagenase, a metal-dependent type IV collagenase has been detected in peripheral blood monocytes and certain types of macrophages in culture (45). In contrast to the interstitial collagenases, the expression of this protease was maximal within the first 6 h of culture and progressively declined after that time. Thus, it appears that human macrophages secrete several metalloproteinases that may participate in degradation of the collagenous components of the extracellular matrix. The detection of these proteinases may be impeded by the presence of collagenase inhibitor (tissue inhibitor of metalloproteinases) as well as by the complexities of their regulation.

In the present study, we have defined a third collag enolytic metalloproteinase, gelatinase-type V collagenase, as another secretory product of human macrophages. Its secretion gener ally paralleled the secretion of interstitial collagenase; however, gelatinase secretion appears to be more sustained. Although the data suggest that the protease is constitutively secreted, additional studies will be needed to clearly define the regulation of this protease. Indeed, the further study of the collag enolytic metalloproteinases secreted by human macrophages in terms of both collagen type specificity and regulation of expression should provide important insights into the role of these cells in collagen degradation in both physiologic and pathologic processes.

Acknowledgments

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References


