ABSTRACT

The ability of cellular vascular components including endothelial cells, smooth muscle cells, and fibroblasts to interact with the collagenolytic activity of invasive human tumor cell lines has been investigated. The human HT1080 fibrosarcoma and Bowe melanoma cells, which rapidly digest collagenous proteins in vitro, failed to dissolve them when co cultured with bovine endothelial cells. This inhibition was not dependent on the ability of endothelial cells to form a monolayer separating the tumor cells from the collagenous substrate. In contrast, little collagenolysis inhibitory activity was detected in bovine vascular smooth muscle cells and human fibroblasts when compared to endothelial cells. Serum-free medium conditioned by endothelial cells inhibited tumor cell-mediated collagenolysis. Our data further suggest that this inhibition was mediated by secreted collagenase inhibitors, since endothelial cell-conditioned medium did not suppress the production of metalloproteinases by the tumor cells but inhibited the activities of collagenases derived from tadpole, rabbit, and human fibroblasts. Treatment of the endothelial cells with cycloheximide suppressed the collagenase inhibitory activity, demonstrating active production of collagenase inhibitors by the cells.

Gel filtration chromatography of endothelial cell-conditioned medium showed the separation of two distinct peaks with inhibitory activities for vertebrate collagenase in the molecular weight range of 70,000 to 75,000 and 30,000 to 35,000, respectively. While the inhibitor with an approximate molecular weight of 30,000 to 35,000 shared many properties with the tissue inhibitor of metalloproteinases, the high-molecular-weight inhibitor demonstrated characteristics not yet described for any collagenase inhibitor.

The production and secretion of inhibitors of vertebrate collagenase by bovine endothelial cells may be of importance in the local control of collagen turnover under physiological as well as pathological conditions.

INTRODUCTION

Malignant cells are characterized by their ability to invade surrounding normal tissues which allows them to have access to blood vessels and lymphatics finally resulting in metastasis (1). Although this process is complex and multifactorial, there is ample evidence that proteases elaborated by tumor cells facilitate malignant spread (2-5). The recent observation of a direct correlation between the metastatic potential of murine tumor cells and their ability to degrade basement membrane collagen has emphasized the importance of collagenases in metastasis (6).

Protease inhibitors may therefore be of importance in protecting host tissues from tumoral invasion. Their role in the natural resistance to malignant invasion of certain tissues, such as cartilage and large arterial blood vessels, has been shown (7-9). We have previously reported that metastatic tumor cell lines degrade highly insoluble extracellular matrices produced in vitro through the production of several proteases including plasminogen activators, elastase, and collagenase (3, 10). This degradative capability was subsequently found to be inhibited by endothelial cells (11). Furthermore, we demonstrated in medium conditioned by bovine endothelial cells the presence of a factor suppressing the synthesis of plasminogen activators by tumor cells (12).

In the present study bovine endothelial cells were found to inhibit the collagenolytic activities elaborated by two human tumor cell lines. Our data suggest that this inhibition was due to the secretion of collagenase inhibitors by endothelial cells rather than to the suppression of metalloproteinase synthesis in the tumor cells.

MATERIALS AND METHODS

Cell Culture Techniques. The human tumor cell lines used were the HT-1080 fibrosarcoma (13) and the Bowe melanoma (14). The endothelial cells were derived from various bovine blood vessels, including the aorta (NCAC1) and the pulmonary artery (APC1) of a newborn calf and the vena cava of a fetal calf (VcC1). The cells were cloned and characterized as previously described (15). Human fibroblasts (Ti) were obtained from foreskin of a healthy individual. Vascular smooth muscle cells were obtained from the aorta of a newborn calf (BANCC1) (16) and the hearts of newborn rats (3). Hurler’s syndrome GM 1391 human fibroblasts were purchased from the National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ. All cell types were grown in Eagle’s minimal essential medium (Grand Island Biological, Santa Clara, CA), containing 10% fetal bovine serum (Irvine Scientific, Irvine, CA), penicillin (100 units/ml), and streptomycin (100 µg/ml).

Production of Radiolabeled Collagen Matrices. Radiolabeled collagen matrices were obtained using a method previously described (3). In brief, rat smooth muscle cells were cultured in 35-mm gelatin-coated tissue culture dishes in the presence of ascorbic acid (25 µg/ml/day) and L-[2,3-3H]proline (0.5 µCi/ml) (45 Ci/mmmole; New England Nuclear, Boston, MA). After 14 days, cells were lysed with 0.025 M NH4OH, and the remaining extracellular matrices were washed 3 times with phosphate-buffered saline (9.3 mM NaH2PO4·H2O, 4.2 mM KH2PO4, 0.132 M NaCl, pH 7.2) and treated with trypsin (type III; Sigma, St. Louis, MO) (20 µg/ml, 37°C, 24 h), followed by exposure to porcine pancreatic elastase (type III; Sigma) (10 µg/ml, 37°C, 24 h) to remove glycoproteins and elastin. Depending on the cell passage, the residual matrices contained from 90,000 cpm to 180,000 cpm of incorporated [3H]proline labeled material with a hydroxyproline:proline ratio of 0.71 ± 0.04. Analysis of these radiolabeled matrices by SDS-PAGE revealed the presence of types I and III collagen isotypes (data not shown).

Digestion Experiments. The cells were plated at the indicated concentrations in 35-mm dishes containing radiolabeled collagen. The fetal bovine serum in the culture medium of these experiments was acid treated (pH 4.2 for 3 h) prior to use to neutralize the serum protease inhibitors (3). The medium was changed every second day, and the radioactivity released was determined by counting aliquots of the supernatant mixed with 5 ml of Biofluor (New England Nuclear) in a scintillation counter. Background counts obtained with matrices incubated with medium alone were subtracted from these values.

Endothelial Cell-conditioned Medium. Endothelial cells (NCAC1) were grown to confluence in 100-mm culture dishes and incubated for 24 h in the absence of serum. The medium was collected, centrifuged (2000 × g, 10 min, 4°C) to remove cells and debris, then 20-fold concentrated by ultrafiltration on a YM10 membrane (Amicon Corp., Danvers, MA), and stored in 1-ml aliquots at -80°C.

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1 This work was supported by Grants CA 29397 and HL 23500 from the NIH, the American Heart Association Greater Los Angeles Affiliate 781-G1-2, and the Concern Foundation.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; APMA, 4-aminophenyl mercuric acetate; TIMP, tissue inhibitor of metalloproteinases; PAGE, polyacrylamide gel electrophoresis.
Visualization of Tumor Cell Metalloproteinase Activities on SDS-Substrate Gels. Tumor cell metalloproteinases were extracted by incubation of the cultured cells in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5% Triton X-100, at 4°C for 30 min, followed by centrifugation (10,000 x g, 5 min). The supernatant was incubated for 3 h at 37°C in the presence of 1 mM APMA to activate latent enzymes which were then separated by electrophoresis on SDS-gelatin substrate acrylamide gels (17). Gelatin (type I; Sigma) was incorporated to the acrylamide polymerization mixture of the separation gel at a final concentration of 0.1%. A 10% separation gel with a 4% stacking gel was used. Lymphoid samples to be analyzed were dissolved in 10 µl of Laemmli buffer (50 mM Tris-HCl buffer, pH 6.8, containing 10% sucrose, 10% SDS, and 1% b-mercaptoethanol) and heated to 100°C for 3 min. After boiling, or reduction on a minislab gel apparatus (Idea Scientific, Coralville, OR). Gels were run at 20 mA/gel at 4°C. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min at room temperature, then overnight at 37°C in 50 mM Tris-HCl buffer, pH 8, containing 10 mM CaCl2 and 0.02% NaN3. After staining in 0.5% Coomassie blue and destaining in a mixture of acetic acid:methanol:water (1:5:4), the protein bands with gelatinolytic activity were identified by the presence of clearing zones. The metalloproteinase nature of these proteinases was confirmed by demonstrating absence of gelatinolysis when gels were incubated overnight in the presence of 20 mM EDTA.

Collagenase Preparation. Purified bacterial collagenase was purchased from Advance Biofacture Corporation (Lynbrook, NY), and purified tadpole collagenase was from New England Nuclear. Crude rabbit fibroblast collagenase was a generous gift of Dr. Z. Werb (University of California-San Francisco, San Francisco, CA). Crude human fibroblast collagenase was obtained from serum-free medium of Hunter's GM1391 fibroblasts treated with cytochalasin D (5 µg/ml; Sigma Chemical Co.). (18, 19). Rabbit and human fibroblast collagenases were activated with trypsin (10 µg/ml) at 22°C for 30 min followed by trypsin inactivation with soybean trypsin inhibitor (50 µg/ml) prior to assay. For each preparation of crude enzyme, a titration curve was obtained, and the specificity of the collagenase was verified by demonstrating the absence of 14C-labeled collagen degradation when 20 mM EDTA was added. One unit of collagenase activity was defined to hydrolyze 1 µg of collagen per min at 37°C.

Assay for Collagenase and Collagenase-inhibitory Activities. Collagenase activity was determined with a fibril assay using succinimidyl propionate 3H-labeled type I collagen extracted from rat tail tendon (New England Nuclear) (20) or with a 14C-labeled collagen film assay (21). 14C-labeled collagen was obtained by acetylation of type I rat tail tendon collagen with [1-14C]acetic anhydride (250 µCi; Irvine Scientific, Irvine, CA) (22). The final preparation had a specific activity of 250 cpm/µg of protein, and its purity was verified by SDS-PAGE (data not shown). Concentrations of collagenase in the linear part of the titration curve were selected for the inhibitory assays. Samples (50 µl) tested for inhibitory activity were preincubated at 4°C for 15 min with indicated amounts of collagenase mixed in 150 µl of 50 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM NaCl and 10 mM CaCl2. The mixtures were tested for residual collagenase activity in the presence of radiolabeled collagen. With bacterial and tadpole collagenases, assays were performed using fibrilar succinimidyl propionate 3H-labeled collagen at 22°C for 24 h. For rabbit and human fibroblast collagenases, assays were done on a 14C-labeled collagen film (50 µg/microtiter well) at 37°C for 3 h. Controls included collagenase mixed with serum-free regular medium, test sample without enzyme, and buffer without collagenase but with trypsin and soybean trypsin inhibitor. One unit of inhibitor activity was defined as the amount giving 50% inhibition of 2 units of collagenase (23).

SDS-Polyacrylamide Gel Electrophoresis. Electrophoretic analysis of the 3H-labeled collagen degradation products by bacterial and tadpole collagenases was performed on SDS slab gels with a 3% stacking gel and a 5–15% gradient separation gel. Electrophoresis were done at room temperature with 30 mA per plate. Protein containing bands were visualized by autoradiography after fluorography in Enhance (New England Nuclear) or by silver stain (Biorad Laboratories, Richmond, CA).

Gel Filtration Chromatography. Serum-free medium conditioned by endothelial cells (100 ml) was concentrated by precipitation in 80% saturation of ammonium sulfate. The precipitate was dissolved in 2 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM NaCl, 10 mM CaCl2, and 0.05% (w/v) Brij 35, exhaustively dialyzed against the same buffer and chromatographed at 4°C on a Sephadex G-100 column (2.6 cm x 90 cm). Fractions (4 ml) were 4-fold concentrated by ultrafiltration and tested for collagenase activity, collagenase inhibition, and protein content.

Inactivation of Inhibitors of Collagenase. Trypsin resistance of inhibitors was determined by incubation of the samples with indicated concentrations of trypsin for 5 min at 37°C, followed by trypsin inactivation with a 5-fold excess of soybean trypsin inhibitor. Heat sensitivity was determined by incubation at indicated temperatures for 30 min prior to testing. Acid sensitivity was tested by adjusting the pH of the sample to pH 3 by addition of 1 N HCl followed by incubation at room temperature for 30 min and subsequent neutralization with 1 N NaOH. Reduction-alkylation was performed by the addition of 2-mercaptoethanol (10 mM) at 4°C for 16 h, followed by carboxymethylation with 20 mM iodoacetic acid at 22°C for 1 h. After reduction-alkylation, samples were exhaustively dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM NaCl and 10 mM CaCl2 before assay. Reversibility of collagenase inhibition was tested by treatment of the inhibitor-collagenase mixture with APMA (1 mM) for 30 min before assay for collagenase activity. Results were compared with the activity of untreated samples.

Protein Determination. Protein determination was done according to Bradford (24) using bovine serum albumin as standard.

RESULTS

Co-culture of Human Tumor Cells with Bovine Aortic Endothelial Cells. Radiolabeled collagenous matrices were used to study the suppression of the tumor cell-mediated collagenolytic activity by cloned bovine aortic endothelial cells. While the endothelial cells degraded only trace amounts of the radiolabeled collagen, the HT1080 fibrosarcoma (Fig. 1A) and Bowe melanoma (Fig. 1B) cells released 60,000 and 63,000 cpm, respectively, of radioactivity during the 10-day experiment. In contrast, when the tumor cells were grown on collagen matrices previously seeded with confluent endothelial cells, little collagen degradation was observed. Two other clones of bovine endothelial cells derived from the pulmonary artery of a newborn calf (APC11) and the vena cava of a fetal calf (VFC12) also inhibited degradation of radiolabeled collagen matrices by bovine endothelial cells and HT1080 (A) or Bowe melanoma cells (B). Bovine endothelial cells (NCAC1) were plated at 5 x 105 cells/35-mm dish on radiolabeled collagen matrices. 30 mM EDTA were added at 10° cell dish. Degradation of the matrix was followed by measuring the amount of radioactivity released every second day. Background counts from dishes incubated with medium alone were subtracted. Points, mean cumulative counts from duplicate dishes. Similar results were obtained in 2 separate experiments. V, NCAC1 alone; L, tumor cells alone; A, tumor cells and endothelial cells.

Fig. 1. Degradation of radiolabeled collagen matrices by bovine endothelial cells and HT1080 (A) or Bowe melanoma cells (B). Bovine endothelial cells (NCAC1) were plated at 5 x 105 cells/35-mm dish on radiolabeled collagen matrices. EDTA were added at 10° cell dish. Degradation of the matrix was followed by measuring the amount of radioactivity released every second day. Background counts from dishes incubated with medium alone were subtracted. Points, mean cumulative counts from duplicate dishes. Similar results were obtained in 2 separate experiments. V, NCAC1 alone; L, tumor cells alone; A, tumor cells and endothelial cells.
tumor cell-mediated collagenolysis (Table 1). Similar inhibitions of collagenolysis were observed when endothelial cells were either plated at low density (Fig. 2A) or 4 days after seeding of the tumor cells (Fig. 2B). Thus, the inhibition of collagen degradation was not due to the formation of an endothelial cell monolayer separating the tumor cells from the substrate.

Inhibition by Other Mesenchymal Cells. Bovine vascular smooth muscle cells (BANCCl) and human fibroblasts (T1) were found to display as much and more collagenolytic activity than the tumor cells when plated on the collagen matrices (Fig. 3, A and B). When smooth muscle cells were cocultivated with HT1080 cells, the amount of collagen digested was less than additive, supporting some inhibitory effect. Cocultivation of tumor cells with human fibroblasts demonstrated an additive collagen degradation, suggesting absence of significant inhibition. The ability to suppress the collagenolytic activity of human tumor cells was most prominent in endothelial cells and in particular those derived from the aorta (NCACl). Therefore these cells were used for further studies.

Inhibition of Tumor Cell-mediated Collagenolysis by Medium Conditioned by Endothelial Cells. The effect of various concentrations of endothelial cell-conditioned medium on the collagenolytic activity of HT1080 cells is shown in Fig. 4. Endothelial cell-conditioned medium, 10% (O) or 50% (A), was added to the culture medium of HT1080 cells plated on radiolabeled collagen matrices 3 days prior to seeding HT1080 cells at 10^6 cells/dish. The degradation of the matrix was determined as described in Figs. 1 and 2. Points, mean of triplicate dishes. The lower degree of digestion observed with HT1080 in B compared with A is due to the use of labeled collagen matrices with different specific activities. C, HT1080 fibrosarcoma alone; M, smooth muscle cells or fibroblasts alone; A, tumor and mesenchymal cells.

### Table 1 Collagen degradation by HT1080 fibrosarcoma cells and bovine endothelial cells

<table>
<thead>
<tr>
<th>Cells in culture</th>
<th>[H]Proline released (cpm)</th>
<th>% of inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial</td>
<td>Tumor</td>
<td></td>
</tr>
<tr>
<td>V.Cf</td>
<td>HT1080</td>
<td>386 ± 390</td>
</tr>
<tr>
<td>APCI</td>
<td>25,550 ± 1,180</td>
<td>0</td>
</tr>
<tr>
<td>V.Cf</td>
<td>HT1080</td>
<td>1,560 ± 380</td>
</tr>
<tr>
<td>APCI</td>
<td>5,467 ± 850</td>
<td>79</td>
</tr>
</tbody>
</table>

* Percentage of inhibition = (1 - cpm released by cocultures / cpm released by endothelial cells + cpm released by tumor cells) × 100.

** Mean ± SD.

Fig. 2. Degradation of radiolabeled collagen matrices by HT1080 fibrosarcoma cells and bovine endothelial cells added at various concentrations (A) or different time (B). Bovine endothelial cells (NCACl) were plated 3 days prior to seeding (A) or 4 days after plating of HT1080 fibrosarcoma cells (B). The degradation of the matrix was followed by measuring the amount of radioactivity released. Points, mean cumulative counts from duplicate dishes. C, HT1080 alone; A, HT1080 and NCACl seeded at 2 x 10^6 cells/35-mm dish (confluent culture); A, HT1080 and NCACl seeded at 0.5 x 10^6 cells/35-mm dish (nonconfluent culture). Arrow (B) indicates the time endothelial cells were seeded.

Fig. 3. Degradation of radiolabeled collagen matrices by HT1080 cells and bovine smooth muscle cells (BANCCl) or human fibroblasts (T1). Bovine smooth muscle cells (A) and human fibroblasts (B) were plated at 5 x 10^5 cells/35-mm dish on radiolabeled collagen matrices 3 days prior to seeding HT1080 cells at 10^6 cells/dish. The degradation of the matrix was determined as described in Figs. 1 and 2. Points, mean of triplicate dishes. The lower degree of digestion observed with HT1080 in B compared with A is due to the use of labeled collagen matrices with different specific activities. C, HT1080 fibrosarcoma alone; M, smooth muscle cells or fibroblasts alone; A, tumor and mesenchymal cells.

Fig. 4. Degradation of radiolabeled collagen matrices by HT1080 cells in the presence of endothelial cell-conditioned medium. HT1080 cells (2 x 10^6 cells/35-mm dish) were grown on radiolabeled collagen matrices in growth medium containing concentrated serum-free medium conditioned by NCACl. Final concentration of conditioned medium was 1x (O) or 5x (A). Controls were grown in regular medium (C). The degradation of the matrix was determined as described in previous figures.

Fig. 5. Effect of Endothelial Cell-conditioned Medium on Metalloproteinase Activities of Tumor Cells. The effect of endothelial cell-conditioned medium on the production of metalloproteinases by tumor cells was analyzed by SDS-gelatin substrate acrylamide gel electrophoresis. Prior to extraction, tumor cells were incubated for 16 h with various concentrations of serum-free medium conditioned by bovine aortic endothelial cells. Extracts containing similar amount of proteins (5.4 ± 0.8 µg) were then loaded on SDS-gelatin acrylamide gels (Fig. 5). Tumor cell conditioned medium was 20-fold concentrated by ultrafiltration and added at final concentrations of 1x and 5x to the culture medium of HT1080 cells plated on radiolabeled collagen matrices. This resulted in a decrease of radioactivity released of 17 and 24%, respectively. Since similar cell counts were found under these conditions, these data suggested the presence of a soluble factor inhibiting collagenolysis by the tumor cells.

Effect of Endothelial Cell-conditioned Medium on Metalloproteinase Activities of Tumor Cells. The effect of endothelial cell-conditioned medium on the production of metalloproteinases by tumor cells was analyzed by SDS-gelatin substrate acrylamide gel electrophoresis. Prior to extraction, tumor cells were incubated for 16 h with various concentrations of serum-free medium conditioned by bovine aortic endothelial cells. Extracts containing similar amount of proteins (5.4 ± 0.8 µg) were then loaded on SDS-gelatin acrylamide gels (Fig. 5). Tumor cell...
extracts demonstrated the presence of several zones of gelatinolytic activity. Cell-associated metalloproteinases were extracted in 0.5% Triton X-100, activated with APMA, and analyzed on gelatin-substrate acrylamide gels. Proteins with gelatinolytic activity were identified after staining with Coomassie blue and are shown by the presence of clearing zones. Lanes 1 to 4, HT1080 cells in the presence of regular medium (Lane 1), or endothelial cell-conditioned medium at final concentrations of 1x (Lane 2), 5x (Lane 3), or 10x (Lane 4). Lanes 5 to 8, human melanoma cells in the presence of regular medium (Lane I), or endothelial cell-conditioned medium at final concentrations of 1x (Lane 6), 5x (Lane 7), or 10x (Lane 8). Molecular weight standards are indicated by arrows (x10^3).

Fig. 5. Gelatin substrate acrylamide gel electrophoresis of tumor cell extracts. HT1080 and human melanoma cells were grown in the presence of the indicated concentrations of endothelial cell-conditioned medium. Cell-associated metalloproteinases were extracted in 0.5% Triton X-100, activated with APMA, and analyzed on gelatin-substrate acrylamide gels. Proteins with gelatinolytic activity were identified after staining with Coomassie blue and are shown by the presence of clearing zones. Lanes 1 to 4, HT1080 cells in the presence of regular medium (Lane 1), or endothelial cell-conditioned medium at final concentrations of 1x (Lane 2), 5x (Lane 3), or 10x (Lane 4). Lanes 5 to 8, human melanoma cells in the presence of regular medium (Lane 5) or endothelial cell-conditioned medium at final concentrations of 1x (Lane 6), 5x (Lane 7), or 10x (Lane 8). Molecular weight standards are indicated by arrows (x10^3).

The inhibition of tadpole collagenase was further investigated by electrophoretic analysis of the 3H-labeled collagen degradation products (Fig. 6). In the absence of endothelial cell-conditioned medium, two fragments from a single peptide bond cleavage site for tadpole collagenase and complete degradation of the radiolabeled collagen by bacterial collagenase were observed. Endothelial cell-conditioned medium did not change the activity of bacterial collagenase but significantly inhibited the degradation of type I collagen by tadpole collagenase. These observations confirmed the presence of specific collagenase inhibitor(s) in endothelial cell-conditioned medium.

Possible absorption and subsequent release by endothelial cells of inhibitors present in serum were ruled out by exposing the endothelial cells to cycloheximide (Table 3). Medium conditioned for 24 h by endothelial cells in the presence of cycloheximide (2 µg/ml) had no collagenase inhibitory activity, demonstrating active biosynthesis of the inhibitors by the cells. This treatment resulted in 82% decrease of the overall protein synthesis with a cell viability of 89%. Therefore the endothelial cells produced collagenase inhibitors.

Identification of Two Types of Collagenase Inhibitors from Medium Conditioned by Endothelial Cells. The inhibitory activity of the endothelial cell-conditioned medium had a specific activity of 0.8 unit/mg after precipitation in 80% ammonium sulfate. Gel filtration chromatographic analysis of ammonium sulfate precipitate resulted in the appearance of two peaks with inhibitory activities against rabbit collagenase (Fig. 7A). The first peak was found in the molecular weight range of 70,000–75,000 and demonstrated a maximal specific activity of 6.4 units/mg of protein. The second peak was observed in the molecular weight range of 30,000–35,000 with a maximal specific activity of 4.2 units/mg of protein. No collagenase activity was detected in any of the individual fractions even after activation with trypsin (data not shown).

The fractions with high inhibitory activities, Peak I (Fractions 20–22) and Peak II (Fractions 26–29), were pooled and analyzed by SDS-polyacrylamide electrophoresis (Fig. 7B). Peak I demonstrated the presence of 2 major bands with molecular weights of 94,000 and 62,000, whereas Peak II showed a major band with a molecular weight of 29,000. Additional tests showed that the inhibitory activity present in Peak I (M, 70,000–75,000) was resistant to trypsin and reduction-carboxymethylation but sensitive to heat (Table 4). In contrast, the inhibitor present in Peak II (M, 30,000–35,000) was resistant to heat up to 80°C, but sensitive to trypsin and reduction-carboxymethylation. Both peaks of inhibitors remained active after acid treatment. Exposure to organomercurials of the inhibitor of Peak I incubated with rabbit collagenase resulted in partial recovery of the collagenase activity, suggesting a partially reversible enzyme-inhibitor complex. In contrast, no collagenase activity was recovered after similar treatment of Peak II.

DISCUSSION

Bovine aortic endothelial cells were found in the present study to inhibit the collagenolytic activities displayed by the human HT1080 fibrosarcoma and Bowe melanoma cells. In contrast to previous observations showing the production by endothelial cells of a factor inhibiting the plasminogen activator synthetic activity by the HT1080 cells (12), the inhibition of collagen degradation documented in the present study was not due to the release of a factor by the endothelial cells which blocks the production of tumor cell metalloproteinases.

The collagenase inhibitory activity was found in the serum-free medium conditioned by the endothelial cells, but only under conditions which allowed for protein synthesis by the cells. This finding suggests that endothelial cells actively produce and secrete collagenase inhibitors. In addition, the molecular weights of the endothelial cell inhibitors described are different from that of α₂-macroglobulin (M, 725,000), a major inhibitor present in serum (25). It is also unlikely that these endothelial cell inhibitors belong to a class of cationic glycoproteins with the activity is reversed by treatment with organomercurials or pro-
Collagenase inhibition by endothelial cells

Table 2 Inhibition of collagenase by medium conditioned by bovine aortic endothelial cells

<table>
<thead>
<tr>
<th>Collagenase source</th>
<th>Amount</th>
<th>Collagenase (cpm)</th>
<th>Collagenase conditioned medium (cpm)</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial</td>
<td>0.5 µg</td>
<td>232,645 ± 7,310×</td>
<td>189,620 ± 5,670</td>
<td>18</td>
</tr>
<tr>
<td>Tadpole</td>
<td>1 µg</td>
<td>80,410 ± 6,300</td>
<td>29,245 ± 5,400</td>
<td>64</td>
</tr>
<tr>
<td>Rabbit fibroblast</td>
<td>0.03 units</td>
<td>1,404 ± 179</td>
<td>0 ± 100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.04 units</td>
<td>1,770 ± 267</td>
<td>310 ± 130</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>0.07 units</td>
<td>3,173 ± 170</td>
<td>2,450 ± 74</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>0.11 units</td>
<td>4,990 ± 70</td>
<td>4,328 ± 642</td>
<td>13</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>0.02 units</td>
<td>1,000 ± 16</td>
<td>141 ± 29</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>0.03 units</td>
<td>1,424 ± 149</td>
<td>462 ± 111</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>0.06 units</td>
<td>2,925 ± 99</td>
<td>2,161 ± 544</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>0.13 units</td>
<td>5,994 ± 224</td>
<td>4,779 ± 585</td>
<td>21</td>
</tr>
<tr>
<td>Endothelial cell-conditioned medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unactivated</td>
<td>50 µl</td>
<td>0 ± 2</td>
<td></td>
<td>0.13 units</td>
</tr>
<tr>
<td>Activated</td>
<td>50 µl</td>
<td>25 ± 25</td>
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</table>

* Mean ± SD of triplicate samples.

Table 3 Effect of treatment with cycloheximide on rabbit collagenase inhibition

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Amount of 14C-labeled collagen released (cpm)</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>4284 ± 171×</td>
<td>100</td>
</tr>
<tr>
<td>Collagenase + medium from NCACl₂</td>
<td>2288 ± 529</td>
<td>47</td>
</tr>
<tr>
<td>Collagenase + medium from NCACl₂ treated with cycloheximide</td>
<td>5420 ± 820</td>
<td>0</td>
</tr>
<tr>
<td>Medium from untreated NCACl₂</td>
<td>139 ± 16</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mean ± SD from quadruplicate samples.

Fig. 6. SDS-polyacrylamide gel electrophoresis of the degradation of 3H-labeled collagen by bacterial and tadpole collagenase. Bacterial and tadpole collagenses were incubated for 24 h at 22°C with serum-free medium or 20x-concentrated serum-free medium conditioned by endothelial cells (50 µl) in the presence of 3H-labeled type I collagen. After incubation, the entire samples containing digested and undigested 3H-labeled collagen were lyophilized and electrophoresed on a SDS-PAGE gradient gel (15–5%). Protein containing bands were visualized by autoradiography after fluorography with Enhance. Lane 1, no enzyme; Lane 2, tadpole collagenase; Lane 3, bacterial collagenase; Lane 4, tadpole collagenase plus endothelial conditioned medium; Lane 5, bacterial collagenase plus endothelial cell-conditioned medium. Collagen (α1, α2) and dimers (β) are indicated. p8, p5, α1/5, α2/5, and α1β are degradation products (75–25%) from a single peptide bond cleavage for tadpole collagenase.

tinases. Gel filtration chromatography demonstrated the presence of 2 distinct inhibitors of classical vertebrate collagenses with apparent molecular weights of 30,000–35,000 and 70,000–75,000, respectively. The low-molecular-weight inhibitor shows many characteristics of the TIMP, a class of recently described inhibitors extracted from various tissues and body fluids (23, 27, 28) and also synthesized by many cells including fibroblasts (19, 29), pig smooth muscle cells (30), rat brain capillary cells (31), human platelets (32), and mouse fibrosarcoma cells (33). These characteristics include resistance to heat and acid treatment, inactivation by trypsin and reduction alkylation, and irreversible complex formation with classical vertebrate collagenase (34). TIMP has a molecular weight of 28,500 by SDS-polyacrylamide gel electrophoresis and 34,000 by gel filtration chromatography, which is consistent with our findings. Definitive proof of identity will be obtained by Western blotting using monospecific antibodies for TIMP (19).

The high-molecular-weight collagenase inhibitor seems to be different from any well-characterized collagenase inhibitor. Its resistance to trypsin and reduction alkylation and its sensitivity to heat are in marked contrast to the low-molecular-weight inhibitor, suggesting that these 2 inhibitors are unrelated. It is unlikely to be a dimer form of the low-molecular-weight inhibitor, since its activity is preserved by reduction with 2-mercaptetoethanol, whereas the activity of the latter was partially abolished by such treatment. A collagenase inhibitor with a molecular weight of 76,000 has recently been isolated from fluid of rheumatoid joints, but it has so far not been further characterized (35). Additional studies are necessary to look for a possible identity.

Whether these collagenase inhibitors are responsible for the inhibition of collagen degradation by tumor cells observed in
the presence of endothelial cell or endothelial cell-conditioned medium is only suggested by our data. The ability of these inhibitors to suppress tumor cell-mediated collagenolysis will have to be demonstrated when larger amounts of purified inhibitors will be available.

The physiological role of collagenase inhibitors in the vascular endothelium is presently unknown. We speculate that it may be of importance for local control of collagenolysis. This hypothesis is supported by the observation that most mesenchymal cells produce simultaneously proteases and their specific inhibitors when stimulated by phorbol diesters or mitogens. Fibroblast, e.g., produce collagenase as well as TIMP (19) and prourokinase and its inhibitor protease-nexin (36). Endothelial cells have previously been shown to produce plasminogen activators as well as a very stable inhibitor of these enzymes (37). The collagenase inhibitors isolated in the present study were also tested for antiplasminogen activator activity. No such inhibitory activity was found in the fractions containing the collagenase inhibitors when tested on a fibrin plate assay (data not shown), suggesting that these inhibitors are distinct from plasminogen activator inhibitors. Collagenolytic activity has been found in endothelial cells derived from various sources (38, 39). In contrast to these previous reports no collagenase inhibitory activity was detected in the culture medium of our bovine aortic endothelial cells. This may be due to the fact that endothelial cells were not treated with phorbol diesters or mitogens. In contrast, these cells secreted significant amounts of inhibitors.

Our cocultivation experiments demonstrate that endothelial cells inhibit the degradation of interstitial collagens by tumor cells. In addition, we found that endothelial cells display more anticollagenase activity than smooth muscle cells or fibroblasts, the other cellular components of a vessel wall. Our findings may explain in part the natural resistance of vessel walls to invasion and degradation by tumor cells. This hypothesis is supported by the observation that concentrated medium conditioned by endothelial cells inhibits the matrix degradation by tumor cells. Other investigators have recently demonstrated the production of TIMP by murine fibrosarcoma cells, showing that the production of such inhibitor was not restricted to tumor cells. Other investigators have recently demonstrated the production of type IV specific collagenase, since this type of inhibitor may be of importance in the prevention of tumor cell extravasation from the circulation.

Table 4 Characteristics of the two collagenase inhibitors identified in endothelial cell-conditioned medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conditions</th>
<th>Peak I</th>
<th>Peak II</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>99.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>10 μg/ml, 37°C, 30 min</td>
<td>98.6</td>
<td>93.7</td>
</tr>
<tr>
<td></td>
<td>100 μg/ml, 37°C, 30 min</td>
<td>97.5</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>250 μg/ml, 37°C, 30 min</td>
<td>91.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Heat</td>
<td>37°C, 30 min</td>
<td>98.3</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td>50°C, 30 min</td>
<td>98.7</td>
<td>98.7</td>
</tr>
<tr>
<td></td>
<td>80°C, 30 min</td>
<td>21.8</td>
<td>98.3</td>
</tr>
<tr>
<td>Acid</td>
<td>pH 3, 30 min</td>
<td>96.9</td>
<td>99.2</td>
</tr>
<tr>
<td>2-Mercaptoethanol and</td>
<td>10 mM, 4°C, 16 h</td>
<td>99.0</td>
<td>52.0</td>
</tr>
<tr>
<td>isooxacetic acid</td>
<td>20 mM, 22°C, 1 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Aminophenylmercuric</td>
<td>1 mM</td>
<td>54.6</td>
<td>95.5</td>
</tr>
</tbody>
</table>

Fig. 7. A, gel filtration chromatography of endothelial cell-conditioned medium. Serum-free endothelial cell-conditioned medium (100 ml) was precipitated in 80% saturation ammonium sulfate. The precipitate was collected by centrifugation; resuspended in 2 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl, 10 mM CaCl₂, and 0.05% (w/v) Brij 35; and dialyzed against the same buffer prior to loading. Fractions (4 ml) were collected, concentrated (4x) by ultrafiltration, and tested for protein content and collagenase and collagenase inhibitory activities. 

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3585
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REFERENCES


3586
# Inhibition of Tumor Cell Collagenolytic Activity by Bovine Endothelial Cells

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