Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths

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Summary

The maintenance and developmental remodeling of extracellular matrix is crucial to such processes as uterine implantation and the cell migratory events of morphogenesis. When mouse blastocysts are placed in culture they adhere to extracellular matrix, and trophoblast giant cells migrate out onto the matrix and degrade it. The secretion of functional proteinases by developing mouse embryos increases dramatically at the time of implantation. By zymography we identified the major secreted gelatin-degrading proteinase, also known as type IV collagenase, as one migrating at 92×10^3 M_r. Several casein-degrading proteinases were also secreted. The tissue inhibitor of metalloproteinases (TIMP) inhibited all of the embryo-derived proteinases detected by gelatin gel zymography, indicating that they are metalloproteinases, whereas TIMP did not inhibit all of the caseinases. Urokinase was also secreted. Addition of TIMP at 5-500 nM effectively inhibited the degradation of matrix by the trophoblast outgrowths. Blocking antibodies directed against 92×10^3 M_r gelatinase abolished matrix degradation by the trophoblast cells. These observations suggest that several metalloproteinases are regulated in early development and that 92×10^3 M_r gelatinase, in particular, has a rate-limiting function in degradation of the maternal extracellular matrix by trophoblast cells.

Key words: blastocyst, degradation, extracellular matrix, gelatinase, metalloproteinase, tissue inhibitor of metalloproteinases, trophoblast, urokinase.

Introduction

The synthesis and deposition of extracellular matrix (ECM) first takes place during peri-implantation mouse development as the migrating parietal endoderm cells of the blastocyst secrete a basement membrane between themselves and the mural trophectoderm (Mazariegos et al., 1987). This membrane is composed mainly of laminin, fibronectin, and type IV collagen and is potentially a substratum for stromelysin, the 72 and 92×10^3 M_r gelatinases, and plasmin (Alexander and Werb, 1989, 1991). The implantation of the mammalian embryo is an invasive process, beginning in mice at about 4.5 days post coitum. The trophectodermal cells of the blastocyst penetrate the uterine epithelium and its basement membrane and then invade the uterine decidual stroma.

Attachment and outgrowth of blastocysts in culture has been used as a model for implantation (Glass et al., 1983). Preimplantation blastocyst-stage embryos placed in culture attach to and spread on various substrata, including collagen, laminin, heparin, fibronectin, and plastic or glass in the presence of serum (Armant et al.,

1986; Carson et al., 1988; Farach et al., 1987; Sutherland et al., 1988). Embryos attach via the trophoblast cell layer, and trophoblast giant cells migrate out and form an extensive network surrounding the inner cell mass (ICM) (Glass et al., 1983). Preimplantation embryos cultured on a complex ECM composed of elastin, collagens, and glycoproteins degrade the ECM under the spreading trophoblast cells. Trophoblast is a highly invasive tissue (Fisher et al., 1989; Glass et al., 1983) and has long been known to make urokinase-type plasminogen activator (uPA) (Strickland et al., 1976). Although uPA is localized to the invasive margin of the trophoblast (Sappino et al., 1989), its limited substrate range rules it out as the enzyme directly catabolizing ECM (Quigley et al., 1987). Plasmin, the product of activation of plasminogen by uPA, is directly responsible for catabolism of a wider range of substrates. Plasmin (or plasminogen) has not been localized in the early mouse embryo, although each is likely to be present. However, in an earlier study, trophoblast-mediated degradation of ECM was not affected by depletion of plasminogen from the medium or by addition of other inhibitors of serine

proteinases (Glass et al., 1983). Inhibitors of the lysosomal cysteine and aspartic proteinases also did not decrease ECM degradation by embryo outgrowths.

The metalloproteinases are a family of zinc-dependent proteinases that in combination can degrade all of the major components of the ECM, including collagens, fibronectin, elastin, proteoglycans, and laminin (Alexander and Werb, 1989, 1991; Werb, 1989). The expression of the metalloproteinases and their inhibitors is tightly regulated by a range of physiologic factors and responds to many growth conditions. Metalloproteinases are expressed by a variety of cell types, including fibroblasts (Chin et al., 1985), capillary endothelial cells (Herron et al., 1986a,b), and epithelial cells (Werb and Clark, 1989; Collier et al., 1988), and in actively remodeling tissues including bone (Thomson et al., 1989; Rifas et al., 1989), invading tumor cells (Mignatti et al., 1986), involuting rat uterus (Woessner and Taplin, 1988), mammary gland (Talhouk et al., 1991), unfertilized eggs and preimplantation embryos in culture (Brenner et al., 1989), and embryonal carcinoma cells (Adler et al., 1990). In this study we tested the hypothesis that metalloproteinases regulate ECM remodeling during the process of implantation in the mouse.

Materials and methods

Materials

Gonadotropin from pregnant mares' serum, human chorionic gonadotropin from urine of pregnant women, soybean trypsin inhibitor, ovalbumin, and human plasmin were obtained from Sigma. Female mice of the CF1 or CD1 strain, 7-8 weeks old, were purchased from either Harlan or Charles River and housed for one week before use. B6JL F1/J males purchased from Jackson Laboratories were housed individually. Human recombinant tissue inhibitor of metalloproteinases (TIMP) was a gift of Synergen Corp., Boulder, CO. Human α_2-macroglobulin and human α_2-antiplasmin were purchased from Athens Research and Technology, Athens, GA. Human plasminogen was obtained from American Diagnostica, Greenwich, CT. Trasylol (aprotinin) was obtained from Calbiochem. Dulbecco's modified Eagle's medium (DME) was prepared at the Cell Culture Facility, University of California, San Francisco. Lactalbumin hydrolysate (LH) was obtained from GIBCO. Gelatin-Sepharose was purchased from Pharmacia. Fetal bovine serum (FBS) was purchased from Hyclone, Logan, UT.

Polyclonal antibody to mouse uPA (Ossowski, 1988), enriched for immunoglobulin as an $(NH_4)_2SO_4$ precipitate, was a gift of Liliana Ossowski, Rockefeller University, New York, NY.

Embryo isolation

Female mice were induced to superovulate by injections in the afternoon with 10 units of gonadotropin from pregnant mares' serum and 48 h later with 5 units of human chorionic gonadotropin. They were caged with the males overnight, and fertilization was assumed to occur at midnight. Two protocols were used to obtain blastocysts, with identical results. Embryos were harvested from the uterus at 3.5 days (blastocyst stage), washed through three drops of Hanks' balanced salt solution with 4 mg ml^{-1} bovine serum albumin,

transferred to 50 μl drops of DME with 10% FBS and 10 mM HEPES under light mineral oil (Sigma), and incubated at 37°C in a 5% CO_2 atmosphere. Alternatively, embryos were harvested at 0.5 day (1-cell stage), treated with hyaluronidase from bovine testis to remove cumulus cells, washed in seven changes of flushing medium, transferred to TE medium (Spindle, 1990) under mineral oil, and incubated at 37°C in a 5% CO_2 atmosphere. In both protocols, the embryos were cultured to the expanded/hatching blastocyst stage before use.

Production of smooth-muscle-cell-derived extracellular matrix

ECM from rat smooth muscle cells (R22) was prepared as described previously (Jones and Werb, 1981). Briefly, R22 cells were plated at a density of 3 × 10^4 cells cm^{-2} in 4-well plates (Nunc). After 24 h, ascorbic acid was added daily to a final concentration of 50 μg ml^{-1}. Four days after plating, L-2,3,4,5-^{3}H-proline (New England Nuclear) was added at 2.0 μCi ml^{-1} and replenished every two days with each medium change. At 14-16 days after plating, the cells were washed twice with phosphate-buffered saline (PBS) and lysed with 0.25 M NH_4OH. The ECM left attached to the well surface was then washed six times with PBS and three times with 70% ethanol, air dried, and stored at 4°C until use.

Collection of proteinases secreted by blastocyst outgrowths

To collect outgrowth-conditioned medium, 40 expanded blastocysts were allowed to hatch and attach to handmade 5 mm round coverslips in DME with 10% FBS. By day 2 the embryos were strongly adherent and flattened. Because FBS contains metalloproteinases, it was necessary to change to a serum-free medium. This was accomplished under oil by removing as much of the medium that contained serum as possible, replacing it 5 times with DME supplemented with 0.2% LH (DME-LH), and culturing finally in 40 μl DME-LH. Medium was collected and replaced on days 6 and 10, or on days 5 and 8.

Degradation of extracellular matrix by blastocyst outgrowths

Expanded blastocysts (40-50) were placed in wells coated with R22 matrix in DME with 10% FBS. For some experiments the medium was removed on day 2 and the embryos were washed three times with serum-free medium and cultured in 0.5 ml DME-LH per well, with or without additional reagents. In other experiments the embryos were plated directly into DME-LH; the embryos adhered just as well as in serum. The reagents used for treatments were added on day 2 as before. Starting on day 4, a 50 μl aliquot was taken from each well, and soluble radioactive peptides were quantified by liquid scintillation spectrometry. The medium removed was replaced daily. On day 7 the embryos were photographed before and after lysis with NH_4OH to visualize the extent of matrix clearing by the outgrowths. All experiments were performed at least three times with 2-4 replicates for each sample.

Purification of 92 × 10^3 M_r gelatinase and preparation of a polyclonal antibody

The mouse macrophage cell line P388D1, clone 2G1, derived in this laboratory, was grown in Nunc 245 cm^2 plates to confluence in DME with 10% FBS. Cells were then washed three times in 0.9% NaCl, and the medium was changed to 100 ml of DME-LH per plate. The medium was collected and replaced every 2-3 days for as long as the cells remained at

least 50% confluent (6-7 collections). The medium was stored at 4°C until use. The cells were centrifuged at 10,000 g and the medium was brought to 60% saturation with $(NH_4)_2SO_4$ overnight at 4°C. The slurry was centrifuged at 10,000 g for 30 min at 4°C, and the precipitate was resuspended in 20 mM HEPES buffer, 1 mM $CaCl_2$, 0.5 M NaCl, pH 7.5, containing 0.05% Brij-35 and 0.02% NaN_3, and dialyzed against two changes of the same buffer with 10% glycerol. Remaining solids were centrifuged at 10,000 g and the supernatant liquid was applied to a gelatin-Sepharose affinity column in the same buffer. The gelatinase was eluted by application of HEPES buffer with 10% dimethyl sulfoxide. The purified enzyme migrated as a single band on silver-stained sodium dodecyl sulfate-polyacrylamide gels. For preparation of a polyclonal antibody, 100 μg of purified 92 × 10^3 M_r gelatinase in PBS was emulsified with Freund's complete adjuvant and injected subcutaneously into a female New Zealand white rabbit (Nita Bell). An additional 100 μg of gelatinase, emulsified with incomplete adjuvant, was injected 2 weeks and 6 weeks later. The gelatinase purified on gelatin-agarose was separated by sodium dodecyl sulfate-gel electrophoresis under unreduced conditions, electroblotted onto nitrocellulose, and stained with India ink. The stained band was cut out of the nitrocellulose and ground into a powder with a mortar and pestle cooled by liquid nitrogen. For the fourth injection, four weeks later, the powder was suspended in 0.5 ml water, emulsified with 0.5 ml Freund's incomplete adjuvant, and injected subcutaneously. In all experiments described we used anti-92 × 10^3 M_r gelatinase IgG from the fifth bleed prepared by affinity chromatography on protein A-Sepharose.

The specificity of the antibody was verified by immuno-staining of samples blotted onto nitrocellulose. Briefly, the reconstituted $(NH_4)_2SO_4$ precipitate of P388D1 cell-conditioned medium was electrophoresed, unreduced, into 10% polyacrylamide gels, and blotted onto nitrocellulose, which was then blocked with 1% nonfat dry milk in PBS. Strips of the nitrocellulose were then exposed for 2 h to a 1:100 dilution of either preimmune or immune serum in PBS at ambient temperature. After 4 washes (10 min each) of PBS containing 0.2% milk, the strips were incubated in a 1:1000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Miles-Yeda, Israel) for 45 min. After 4 more washes the color was developed by exposure to 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 100 mM Tris-HCl, pH 9.5, with 100 mM NaCl and 5 mM $MgCl_2 \cdot 6H_2O$.

The ability of the antibody to inhibit the gelatin-degrading activity of the 92 × 10^3 M_r gelatinase was tested by coincubation in a soluble gelatinase assay. The purified, autoactivated gelatinase (250 ng) was mixed with 0 - 37.5 μg preimmune IgG or anti-gelatinase IgG. The gelatinase/antibody mixture (100 μl) was added to 50 μl 100 mM Tris-HCl, 30 mM $CaCl_2$, pH 7.6, and allowed to incubate overnight at 4°C. The mixture was then added to 50 μl of 2 mg ml^{-1} heat-denatured (50°C, 30 min) ^{3}H-labeled collagen (gelatin) (gift of Eric Howard, University of California, San Francisco). The mixture was incubated at 37°C for 1 h, and the reaction was stopped by the addition of 100 μl of 45% (w/v) trichloroacetic acid. The radioactivity in the supernatant liquid was measured by liquid scintillation spectrometry.

Zymography

Conditioned medium and lysates of blastocyst outgrowths were analyzed by gelatin, casein and casein/plasminogen zymography (Herron et al., 1986a,b). Samples of conditioned medium were treated with 1 mM 4-aminophenylmercuric acetate (APMA) to activate metalloproteinases as described previously (Brenner et al., 1989). Samples were electrophor-

esed on 10% polyacrylamide gels copolymerized with substrate (1 mg ml^{-1} of either gelatin or casein, or 1 mg ml^{-1} of casein plus 10 μg ml^{-1} of human plasminogen). After electrophoresis, gels were washed twice (15 min) in 2.5% Triton X-100, incubated for 16-48 h at 37°C in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.8, stained in 0.5% Coomassie Blue R250, and destained in 50% methanol. Negative staining indicated the location of active proteinase bands. To demonstrate metalloproteinase activity, the gels were incubated in substrate buffer with 500 nM TIMP, as described previously (Adler et al., 1990).

Results

Blastocyst outgrowths in culture secrete metalloproteinases

The expression of proteinases by early embryos in culture was first investigated by substrate gel electrophoresis of medium conditioned by blastocyst outgrowths and of lysates of outgrowths. Lysates of blastocyst outgrowths contained cell-associated gelatinolytic metalloproteinases migrating at 92 and 85 × 10^3 M_r (Fig. 1A, lanes 1 and 2), mostly as the proenzyme 92 × 10^3 M_r form. In secretions of the outgrowths (Fig. 1A, lanes 3-8) the same proteinases were seen, together with additional proteinases of 60, 65, and >200 × 10^3 M_r. Activities >100 × 10^3 M_r are probably aggregates of the 92 × 10^3 M_r gelatinase (Hibbs et al. 1985). The proenzyme forms of the metalloproteinases can be converted to their active forms by treatment with organomercurial agents such as APMA (Springman et al., 1990). To determine which metalloproteinases were in proenzyme form and which were active, samples were treated with APMA before loading onto the substrate gel. A shift in relative molecular mass of any of the activities on the zymogram was an indicator of latency, as described previously (Adler et al., 1990). Activation by APMA increased the amount of the active enzyme (85 × 10^3 M_r) derived from the 92 × 10^3 M_r proenzyme (Fig. 1A, lane 6). The relationship of the 85 and 92 × 10^3 M_r forms was also shown with the polyclonal antibody (see below). All the gelatinases were inhibited by TIMP, identifying them as metalloproteinases.

Of four caseinolytic proteinases detected in medium conditioned by blastocyst outgrowths (Fig. 1B, lane 9), one with an apparent 92 × 10^3 M_r and a doublet at 85 × 10^3 M_r were inhibited by TIMP (Fig. 1B, lane 10). Another two caseinases of about 120 and 160 × 10^3 M_r were not inhibited by TIMP. Because purified 92 × 10^3 M_r gelatinase is caseinolytic (data not shown), it is likely that the TIMP-inhibitable 92 and 85 × 10^3 M_r embryo caseinases are identical to the 92 and 85 × 10^3 M_r gelatinases. Thus, there were a number of possible target metalloproteinases for TIMP in these experiments.

Blastocyst outgrowths also secreted plasminogen activators, predominantly uPA, which was found in the 48 × 10^3 high relative molecular mass form and as 35 × 10^3 low relative molecular mass uPA (Fig. 1C, lane 12). Tissue-type PA was also secreted.
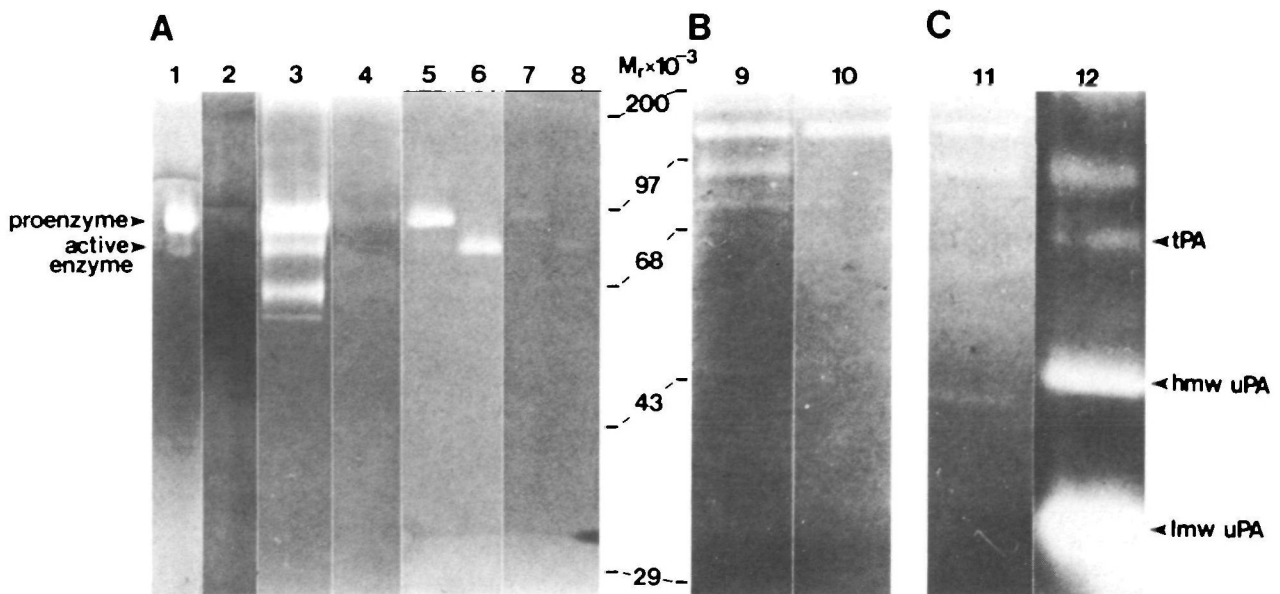
Fig. 1. Zymography of proteinases expressed by blastocyst outgrowths. (A) Gelatin (lanes 1-8) was incorporated into 10% polyacrylamide gels. After electrophoresis of the sample, gels were incubated for 16 h at 37°C in 50 mM Tris-HCl, 10 mM CaCl$_2$, pH 7.8. Segments of the zymograms shown in lanes 2,4,7, and 8 were incubated with 500 nM TIMP after electrophoresis. Samples in lanes 6 and 8 were treated with 1 mM APMA for 1 h before separation on the substrate gels. Lanes 1 and 2 each contain the sodium dodecyl sulfate lysates of 8 outgrowths collected on day 7. Lanes 3 and 4 each contain 2 μl of medium conditioned by 40 outgrowths cultured in 40 μl DME-LH for 120 h and collected on day 7. Lanes 5-8 each contain 2 μl of medium conditioned by 40 outgrowths cultured in 40 μl DME-LH for 72 h and collected on day 6. Gels were stained with Coomassie Blue R250; negative-staining bands indicate the location of proteinases. The migration of proenzyme and active forms of the 92×10^3 M_r gelatinase is shown by arrows. Proenzymes have activity in this system because sodium dodecyl sulfate changes the configuration of the molecule to expose the active site, while not changing its size. (B) Casein (lanes 9,10) was incorporated into 10% polyacrylamide gels. Each lane contains 15 μl of medium conditioned by 40 outgrowths cultured in 40 μl DME-LH for 72 h and collected on day 8. After electrophoresis, gels were incubated for 44 h at 37°C in 50 mM Tris-HCl, 10 mM CaCl$_2$, pH 7.8. Lane 10 was incubated with 500 nM TIMP. Bracket to right of lane 10 shows position of TIMP-insensitive caseinases of about 120 and 160×10^3 M_r. (C) Casein was incorporated into 10% polyacrylamide gels in the absence (lane 11) or presence (lane 12) of 10 μg ml^{-1} human plasminogen. Each lane contains 15 μl of medium conditioned by 40 outgrowths cultured in 40 μl DME-LH for 72 h and collected on day 8. After electrophoresis, gels were incubated for 27 h at 37°C in 50 mM Tris-HCl, 10 mM CaCl$_2$, pH 7.8, and stained with Coomassie Blue R250. Bands in lane 12 that are not present in lane 11 are plasminogen activators. lmw uPA, low relative molecular mass urokinase-type plasminogen activator; hmw uPA, high relative molecular mass urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator. Relative molecular mass markers are shown between panels A and B.

Plasmin is incapable of activating the 92×10^3 M$_r$ gelatinase

It has been suggested that the metalloproteinases collagenase and stromelysin may be activated by an enzyme cascade involving uPA and plasmin (Alexander and Werb, 1989; Werb et al., 1977). However, there is considerable evidence accumulating that suggests that these serine proteinases are not capable of activating the gelatinases. Others have found that avian 70×10^3 M_r (Chen et al., 1991) and human 92×10^3 M_r (Moll et al., 1990) progelatinases cannot be activated by plasmin. A previous study (Glass et al., 1983) and the current work do not point to a role for either plasmin or uPA in ECM degradation by trophoblast. To address the question of how the 92×10^3 M_r gelatinase is activated in vivo, we incubated 92×10^3 M_r gelatinase with plasmin (Fig. 2). Even in the presence of a 2:1 molar ratio of plasmin to metalloproteinases, the proteinases from blastocyst outgrowths were not con-

verted to active forms, whereas APMA fully converted the 92×10^3 M_r gelatinase to its 85×10^3 M_r active form. The same preparation of plasmin activated other metalloproteinases (data not shown). Furthermore, plasmin rapidly inactivated and digested the 85×10^3 M_r active form of the 92×10^3 M_r gelatinase (data not shown).

TIMP inhibits extracellular matrix degradation by blastocyst outgrowths

Degradation of the ECM secreted by R22 cells (a rat aortic smooth muscle cell line) is a useful assay for cellular invasive capacity (Glass et al., 1983). Blastocysts attach to and grow on R22 matrix as well as on plastic or glass. Initially, trophoblast cells grow out as a sheet, but after 4 days they begin to separate from one another, and small areas of matrix clearing become microscopically visible. This clearing of matrix under trophoblast cells correlates with the release of soluble
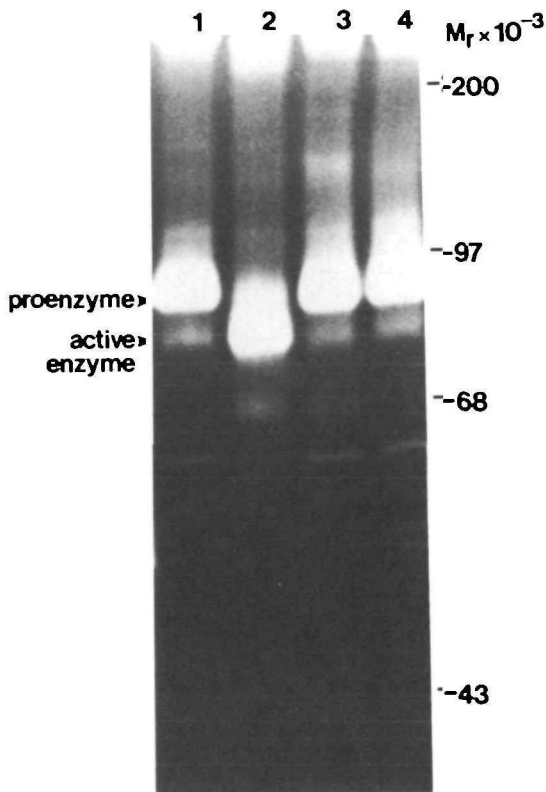
Fig. 2. Effect of plasmin on activation of the 92×10^3 M_r gelatinase. Conditioned medium (9 μl) from 30 blastocyst outgrowths cultured for 96 h in 35 μl of DME-LH was electrophoresed in 10% polyacrylamide gels with incorporated gelatin (1 mg ml^{-1}). The gel was incubated for 17 h in 50 mM Tris-HCl, 10 mM CaCl$_2$, pH 7.8, before staining. Lane 1, unactivated sample; lane 2, sample treated with 1 mM APMA (1 h, 37°C); lane 3, sample treated with 10.7 nM human plasmin; lane 4, sample treated with 0.7 nM human plasmin. Molecular weight markers are shown on the right.

Fig. 3. Effect of TIMP on solubilization of peptides from R22 matrix. ^{3}H-labeled peptides released into the medium of blastocyst outgrowths (40 per well) cultured on R22 smooth muscle cell matrix in DME-LH (□) or DME-LH with 500 nM TIMP (O) were measured from a 50 μl sample taken each day of the experiment from a total volume of 0.5 ml. The 50 μl volume was replaced each day with fresh DME-LH with or without TIMP. Each datum point is the mean of 3 wells. Bars indicate s.d.

fragments from the matrix; however, various inhibitors of serine, cysteine, and aspartic proteinases are unsuccessful at suppressing biochemical and morphologic clearing of the R22 matrix (Glass et al., 1983). Because metalloproteinases are the major secreted proteinases of blastocyst outgrowths, we added TIMP to cultures grown on R22 matrix to measure the contribution of metalloproteinases to the process of implantation in culture. At a concentration of 500 nM, TIMP dramatically inhibited the release of soluble radiolabeled peptides, continuing throughout the culture period of 7 days (Fig. 3). The effect of TIMP was clearly visualized after lysis of the embryonic cells and removal of outgrowths with NH$_4$OH (Fig. 4). In outgrowths not treated with TIMP, a zone of clearing of ECM was associated with trophoblast giant cells under and around the ICM; the zone reached the full extent of the outgrowth by 7 days (Fig. 4C). In the presence of TIMP, ECM clearing was diminished or absent (Fig. 4D).
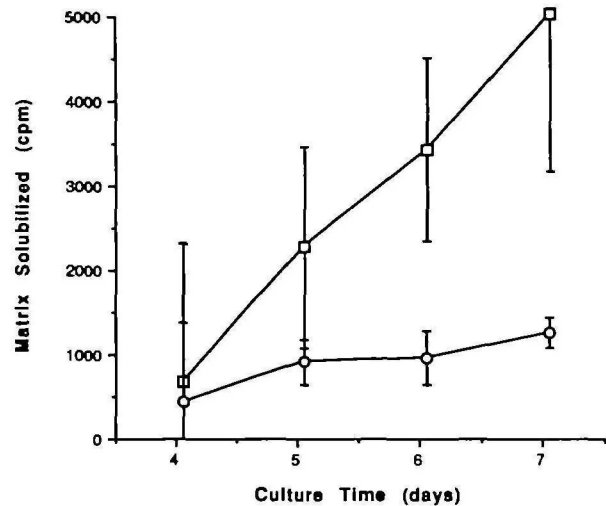
92×10^3 M$_r$ gelatinase is responsible for most of the lytic capacity of blastocyst outgrowths

The results with TIMP clearly suggest a role for metalloproteinases in the degradative capacity of blastocyst outgrowths; we further characterized this degradation with blocking antibodies to the major proteinases. We first observed that purified 92×10^3 M_r gelatinase degraded the R22 matrix (data not shown). We then prepared an antibody to the 92×10^3 M_r gelatinase that recognized only this enzyme in macrophage-conditioned medium (Fig. 5A) and found that the degradation of soluble radiolabeled gelatin by 92×10^3 M_r gelatinase could be abolished by coincubation with this antibody (Fig. 5B). Blastocyst outgrowths cultured on matrix in the presence of anti-92×10^3 M_r gelatinase IgG released fewer radiolabeled peptides than outgrowths cultured with preimmune IgG (Figs 5C, 6). This reduction was not affected by the addition of 500 nM TIMP to the cultures (Fig. 5C), suggesting that the 92×10^3 M_r gelatinase was rate-limiting for all the TIMP-inhibitable degradation by the embryos.

The inhibition of ECM clearing and degradation by the blastocyst outgrowths was restricted to the inhibition of metalloproteinases. α_2-Macroglobulin, a wide-spectrum proteinase inhibitor from serum that inhibits metalloproteinases, was also effective at suppressing ECM degradation by outgrowths. Inhibitors of serine proteinases, particularly uPA and plasmin (soybean trypsin inhibitor, Trasylol, and α_2-antiplasmin) had no effect. Even at very high concentrations, a blocking antibody to uPA (Ossowski, 1988) inhibited ECM degradation by a maximum of 25%. This could be due
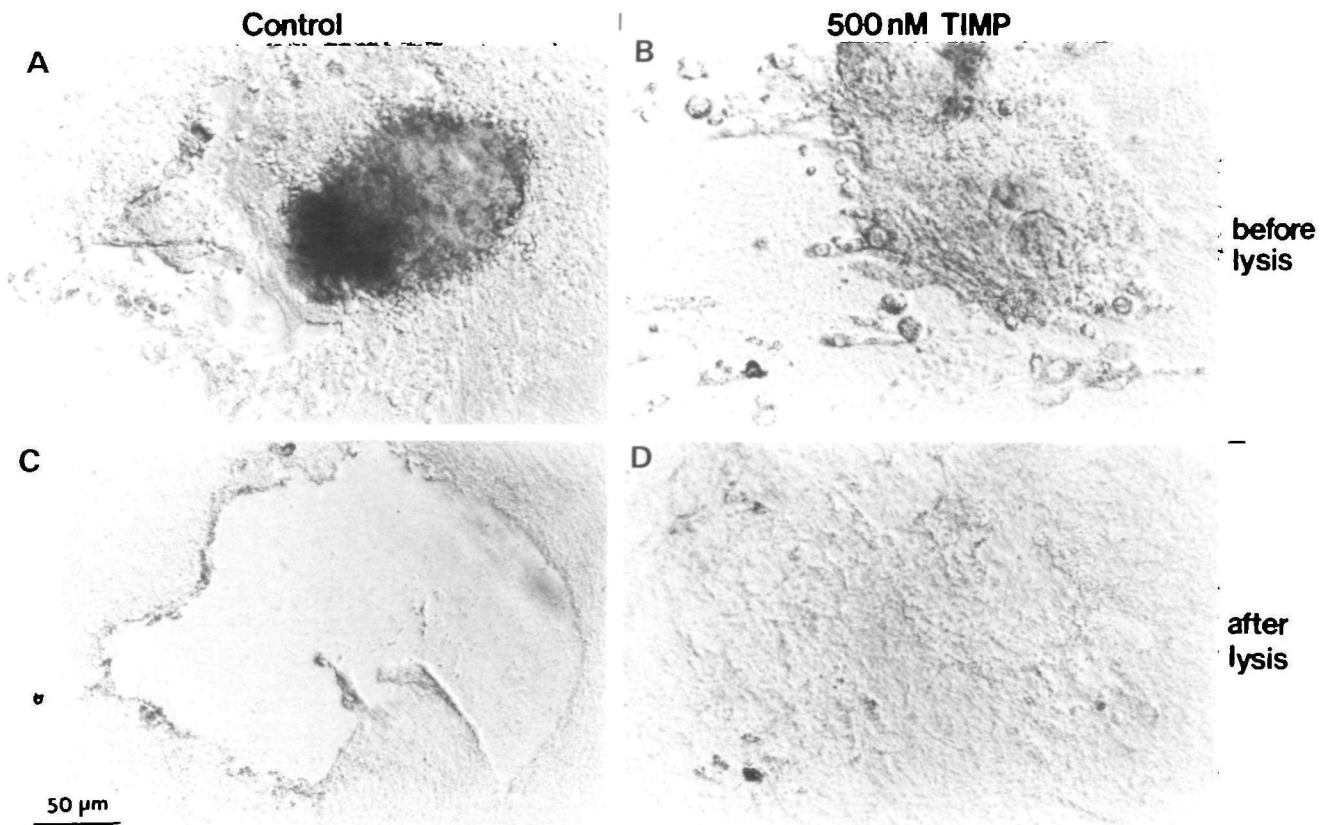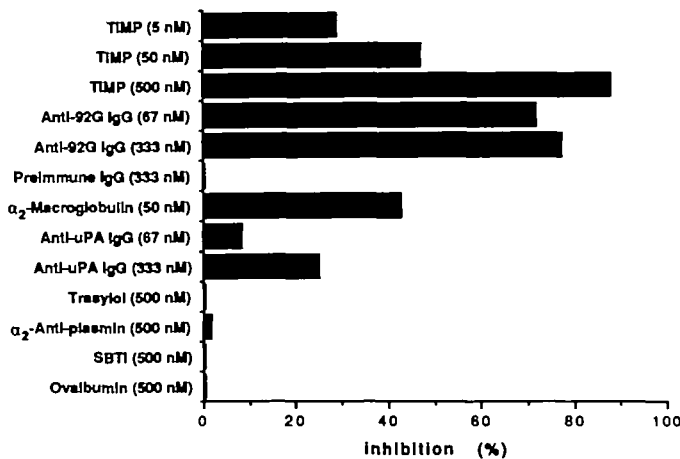
Fig. 4. Effect of TIMP on morphologic clearing of ECM by trophoblast. Blastocyst outgrowths (40 per well) cultured on R22 matrix were photographed on day 7 before (A,B) and after (C,D) lysis by NH₄OH. In outgrowth cultures not treated with TIMP (C), ECM clearing was widespread beneath the trophoblast cells. In outgrowth cultures treated with 500 nM TIMP (D), ECM clearing did not occur or was greatly diminished.
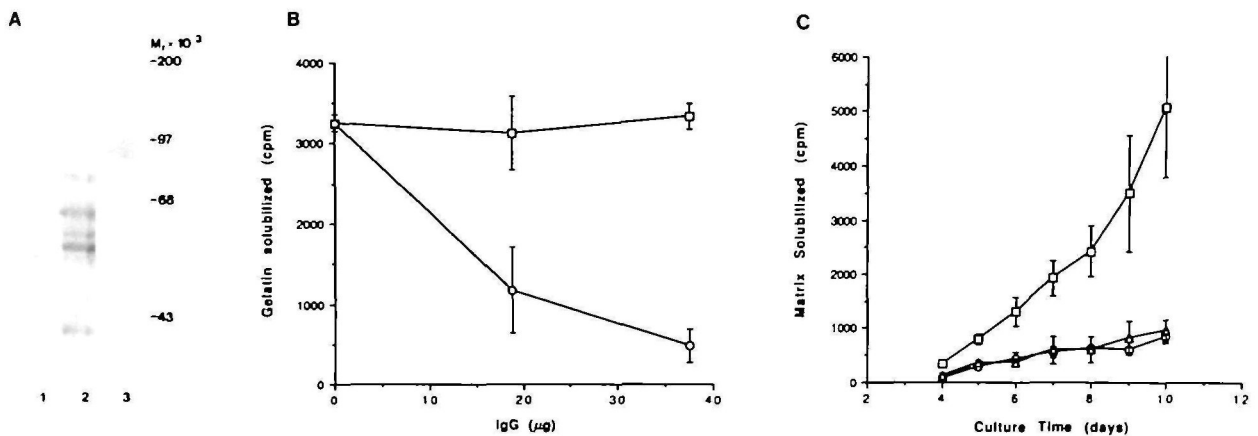
Fig. 5. Contribution of the 92×10^3 M_r gelatinase to the ECM-degrading metalloproteinase activity of trophoblast outgrowths. (A) Specificity of the polyclonal antibody to gelatinase by immunostaining of samples of 50× concentrated, unreduced conditioned medium from P388D1 cells blotted onto nitrocellulose. Lane 1, 1:100 dilution of preimmune IgG; lane 2, India ink stain of total protein; lane 3, 1:100 dilution of anti-92×10^3 M_r gelatinase IgG. Molecular weight markers are shown on the right. (B) Incubation of 250 ng of pure activated 92×10^3 M_r gelatinase from P388D1 cells with 18.75 or 37.5 μg preimmune IgG (□) or anti-92×10^3 M_r gelatinase IgG (○) in a volume of 150 μl, followed by addition of 50 μl of ³H-labeled gelatin (1 h, 37°C). After centrifugation, the ³H solubilized was quantified by liquid scintillation spectrometry. (C) Blastocyst outgrowths (40 per well) cultured on R22 matrix in the presence of 50 μg ml⁻¹ preimmune IgG (□), 50 μg ml⁻¹ anti-92×10^3 M_r gelatinase IgG (○), or 50 μg ml⁻¹ anti-92×10^3 M_r gelatinase IgG with 500 nM TIMP (△). Each data point is the mean of two wells; bars indicate range.

TIMP (5 nM)
TIMP (50 nM)
TIMP (500 nM)
Anti-92G IgG (67 nM)
Anti-92G IgG (333 nM)
Preimmune IgG (333 nM)
α_2-Macroglobulin (50 nM)
Anti-uPA IgG (67 nM)
Anti-uPA IgG (333 nM)
Trasylol (500 nM)
α_2-Anti-plasmin (500 nM)
SBTI (500 nM)
Ovalbumin (500 nM)

0   20   40   60   80   100

Inhibition (%)

Fig. 6. Effect of inhibitors of metalloproteinases and serine proteinases on matrix degradation by blastocyst outgrowths (40 per well) cultured on R22 matrix for 7 days. The percent inhibition of matrix degradation with each treatment was computed as the difference in ^3H counts released between days 5 and 7. Results are the mean of three wells. Anti-92G IgG, anti-92 × 10^3 M_r gelatinase IgG; SBTI, soybean trypsin inhibitor.

to small amounts of contaminating α_2-macroglobulin in the preparation (Fig. 6).

Discussion

We have demonstrated that the metalloproteinases are expressed during peri-implantation development and that their inhibitor, TIMP, can alter the degradative and invasive properties of cells even at this early developmental time point. In previous work, mRNA for the metalloproteinases and TIMP was shown to be expressed during preimplantation development, increasing at the blastocyst stage (Brenner et al., 1989). We have shown here that metalloproteinases function during the development of blastocysts to differentiating outgrowths in culture. One process that clearly depends on the metabolism of ECM components takes place during this time of development. This is the invasion of the trophoblast through the uterine epithelium and expansion of the ectoplacental cone in the decidualizing stroma to establish the fetal-maternal blood sinus. The correlation of the invasive ability of cells with their expression of metalloproteinases has been documented for a number of malignant cell types (Mignatti et al., 1986; Bernhard et al., 1990). We show here that TIMP inhibited the lytic capacity of trophoblast outgrowths, implicating the metalloproteinases in the trophoblast invasive process.

The activity of metalloproteinases is regulated by their naturally occurring inhibitor TIMP, a glycoprotein of 28.5 × 10^3 M_r (Alexander and Werb, 1989). The purified natural inhibitor, or an equally active unglycosylated human recombinant form of it, has been used to modulate the invasive and/or proteolytic properties of various cell types, including melanoma cells (Schultz et

al., 1988), chondrocytes (Gavrilovic et al., 1987), choriocarcinoma cells (Yagel et al., 1988), osteoblasts (Thomson et al., 1987), and M5076 tumor cells (Thorgeirsson et al., 1982). When TIMP production is reduced in Swiss 3T3 cells by antisense TIMP mRNA, the cells become more invasive in an amnion invasion assay in vitro and more tumorigenic and metastatic in vivo (Khokha et al., 1989). That metalloproteinases and their inhibitors are important to tissue morphogenesis was demonstrated by Fukuda et al. (1988), who found that TIMP enhanced cleft formation in 12-day mouse salivary gland in culture.

The proteinase inhibitor α_2-macroglobulin, which inhibits all classes of proteinases including metalloproteinases, also significantly inhibited the degradation of ECM by mouse blastocyst outgrowths. This observation reiterates the inhibition observed with TIMP; however, α_2-macroglobulin is unlikely to be a significant player in ECM remodeling. This inhibitor is a large molecule (725 × 10^3 M_r), and there are probably steric restrictions for protein access during early development.

ECM was degraded under the trophoblast cells, suggesting the presence of a cell-associated enzyme or highly localized activity. Examination of cell lysates confirmed the presence of both the 92 × 10^3 M_r gelatinase proenzyme and its 85 × 10^3 M_r active form. The 92 × 10^3 M_r progelatinase and corresponding active form represented the bulk of secreted matrix-degrading activity. A blocking antibody to the 92 × 10^3 M_r gelatinase inhibited ECM lysis by up to 79%, and a combination of blocking antibody and TIMP did not increase the inhibition. We can assume that other metalloproteinase activities did not contribute significantly to the lytic process. Preliminary data from immunolocalization in post-implantation embryos indicate that the 92 × 10^3 M_r gelatinase is temporally regulated, being expressed by trophoblast giant cells at the invasive front into the uterus at days 5-9 and disappearing at day 10 (C.M. Alexander, O. Behrendtsen, M. Flannery, and Z. Werb, unpublished data).

The fact that the serine proteinase inhibitors Trasylol, α_2-antiplasmin, and soybean trypsin inhibitor had little effect on ECM lysis minimizes the potential role of plasmin and uPA. This was a somewhat unexpected result, considering that uPA is made by, and localized to, the trophoblast (Sappino et al., 1989). Plasmin (70 × 10^3 M_r) was not detected in the conditioned medium or lysates of embryo outgrowths analyzed by zymography, in which proteinases the size of plasmin were absent or inhibited by TIMP; hence, if plasmin were present, it could be present only in catalytic quantities. Metalloproteinases are secreted as inactive proenzymes, and removal of a propeptide may be essential to their activity (Werb, 1989; Alexander and Werb, 1991). Plasmin generated from plasminogen by uPA is one of the proteinases capable of activating metalloproteinases; this has been demonstrated for procollagenase and prostromelysin (Werb et al., 1977; He et al., 1989). Plasmin may be involved in the cascade of ECM lysis upstream of metalloproteinases, acting as a metallopro-

teinase activator. However, in a previous study (Glass et al., 1983) plasminogen did not alter the degradation of ECM by trophoblast outgrowths. Our data indicate that plasmin is incapable of activating the $92 \times 10^3 M_r$ gelatinase. Thus, the involvement of plasmin in an activation cascade for metalloproteinases may be restricted to enzymes such as procollagenase. We do not know the mechanism of activation of $92 \times 10^3 M_r$ gelatinase in vivo; however, the trophoblast outgrowths, like macrophages, another naturally invasive cell type (Werb et al., 1980), were clearly capable of activating the enzyme in both cell-associated and secreted forms, as shown by the presence of the $85 \times 10^3 M_r$ active form of the enzyme.

What then is the role of uPA? High expression and localization to trophoblast in vivo (Sappino et al., 1989) suggest a function in the implantation process. Perhaps, as our data suggest, uPA has little function in ECM degradation, either directly as a proteinase or indirectly as a metalloproteinase activator. An alternative function might be to regulate fibrin formation in the areas where maternal blood vessels are breached. Such a role would be consistent with observed effects on fibrinolysis, but not ECM lysis, in transgenic animals overexpressing uPA (Heckel et al., 1990). This hypothesis remains to be tested.

That the proteinase/inhibitor balance may regulate cell migration has been hypothesized (Werb and Clark, 1989; Alexander and Werb, 1991), and the migration-regulating activity of a proteinase inhibitor is not unprecedented; other investigators have identified a serine proteinase inhibitor (protease nexin-1) that promotes neurite outgrowth (Gloor et al., 1986). Several lines of evidence implicate the $92 \times 10^3 M_r$ gelatinase, also called invadolysin (Alexander and Werb, 1991), as the major rate-limiting metalloproteinase in most physiologic and pathologic invasive processes. The human placenta also exhibits an invasive phase during implantation, although in many respects it is quite different from that of rodents. Human cytotrophoblasts, which are invasive only in the first trimester of gestation, express the $92 \times 10^3 M_r$ gelatinase in active form only during the first trimester and not during the second and third trimesters (Fisher et al., 1989). Librach et al. (1991) have also implicated this enzyme in the invasion of basement membrane matrices by first-trimester human cytotrophoblasts. Several other proteinases, including collagenase, uPA, and the $72 \times 10^3 M_r$ gelatinase, are produced by cytotrophoblasts during both the invasive and the noninvasive phases of development. Puistola et al. (1989) have reported that human cleavage-stage embryos secrete a type IV collagen-degrading activity. Polymorphonuclear leukocytes and macrophages also express the $92 \times 10^3 M_r$ gelatinase and release it in response to chemotactic stimuli (Hibbs and Bainton, 1989). The secretion of $92 \times 10^3 M_r$ gelatinase, but not of the other metalloproteinases or uPA, also correlates with metastasis of viral oncogene-transformed fibroblasts (Bernhard et al., 1990).

We have not yet identified whether there is a role for

TIMP in the development of the early embryo. We have not detected TIMP or any other metalloproteinase inhibitor on inhibitor zymograms of outgrowth-conditioned medium. There is also evidence that TIMP is expressed by the maternal deciduum, presumably to control the outgrowth of the implanting embryo (Nomura et al., 1989; C.M. Alexander, M. Flannery, O. Behrendtsen, and Z. Werb, unpublished observations). An implantation-stage embryo transplanted to an ectopic site can invade uncontrollably during its invasive phase (Enders et al., 1981; Sherman and Wudl, 1976), indicating that the deciduum is a limiting environment. It is difficult to test the effects of exogenous TIMP on post-implantation embryos in utero. However, with the aim of establishing the physiologic importance of the metalloproteinases in various developmental and adult remodeling processes, our effort is directed to the determination of TIMP and metalloproteinase concentrations in vivo (Alexander et al., 1991). Genetic studies, using targeted gene mutation and transgenesis, or organ and explant studies using blocking antibodies should reveal how TIMP and the metalloproteinases are balanced in the animal, so that excess lytic metalloproteinases are scavenged and the processes of invasion and migration are tailored to generate particular cell-cell contacts and tissue architecture.

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