92-kD Type IV Collagenase Mediates Invasion of Human Cytotrophoblasts

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Abstract. The specialized interaction between embryonic and maternal tissues is unique to mammalian development. This interaction begins with invasion of the uterus by the first differentiated embryonic cells, the trophoblasts, and culminates in formation of the placenta. The transient tumor-like behavior of cytotrophoblasts, which peaks early in pregnancy, is developmentally regulated. Likewise, in culture only early-gestation human cytotrophoblasts invade a basement membrane-like substrate. These invasive cells synthesize both metalloproteinases and urokinase-type plasminogen activator. Metalloproteinase inhibitors and a function-perturbing antibody specific for the 92-kD type IV collagen-degrading metalloproteinase completely inhibited cytotrophoblast invasion, whereas inhibitors of the plasminogen activator system had only a partial (20–40%) inhibitory effect. We conclude that the 92-kD type IV collagenase is critical for cytotrophoblast invasion.

All animal embryos face critical developmental decisions when cells are allocated to specific tissue layers in a process called gastrulation. Mammalian embryos have an additional and earlier developmental decision, in which embryonic cells are also allocated to the trophoblast lineage or to embryonic and extra-embryonic membrane fates. The trophoblast differentiation, which results in formation of the placenta, is one of the first critical hurdles the mammalian embryo faces. By the end of the first week of development, human trophoblast cells must be capable of adhering to and rapidly invading the maternal uterine epithelium. These invasive cells form the specialized anchoring chorionic villi that physically connect the fetal and maternal tissues. (For a detailed description of human placental anatomy, including anchoring and floating villi, see Boyd and Hamilton, 1970; Enders, 1976; Fisher et al., 1989; Pijnenborg, 1990.)

Many elements of trophoblast invasion are similar to events that occur during tumor cell invasion. The phenotypic change from carcinoma in situ to invasive carcinoma occurs when tumor cells acquire the ability to penetrate an epithelial basement membrane and invade the underlying stroma (Liotta et al., 1986). Likewise, after a brief adherent stage, cytotrophoblast cells penetrate the basement membrane of the uterine epithelial cells and invade the stroma and its associated arterioles. These similarities suggest that the two invasive processes may share certain common mechanisms. However, unlike tumor invasion, trophoblast invasion is precisely regulated, confined spatially to the uterus and temporally to early pregnancy.

Breaching of the extracellular matrix (ECM)[1] barrier by cells is likely to involve proteolytic enzymes capable of degrading the ECM components. The most important of these enzymes is the collagenase family of metalloproteinases (recently reviewed by Alexander and Werb, 1989; Matrisian, 1990). Interstitial collagenases can degrade collagens of types I, II, and III (Miller et al., 1976), VII and X (Schmid et al., 1986), but the degradation of basement membrane collagens requires specialized enzymes that degrade type IV collagen. The latter proteinases can also degrade collagens of types V, VII, and X, as well as fibronectin, denatured collagens, and casein (Liotta et al., 1979; Collier et al., 1988; Wilhelm et al., 1989; Seltzer et al., 1989; Welgus et al., 1990). The wide specificities that metalloproteinases exhibit may be important in invasion. For example, stromelysin degrades fibronectin, elastin, proteoglycan core proteins, and laminin, as well as the nonhelical regions of collagen types IV, V, VIII, and IX and the amino terminus of type I collagen (Chin et al., 1985; Okada et al., 1986; Herron et al., 1986; Werb, 1989). These metalloproteinases are members of a multi-gene family that includes PUMP-1, two types of stromelysin, interstitial collagenase, and the 72- and 92-kD type IV collagenases (Wilhelm et al., 1989). All members possess sequences necessary for maintenance of the zymogen forms, a zinc-binding catalytic domain, and varying numbers of

1. *Abbreviations used in this paper*: ECM, extracellular matrix; HPL, human placental lactogen; PA, plasminogen activator; PAI, plasminogen activator inhibitor; t-PA, tissue-type PA; u-PA, urokinase-type PA; TIMP, tissue inhibitor of metalloproteinases.

structural regions resembling those found in ECM components.

Tissue-type and urokinase-type plasminogen activators (t-PA and u-PA, respectively), members of the serine proteinase family, also play an important role in ECM degradation (Dano et al., 1985; Ossowski, 1988). Cells transfected with a cDNA encoding u-PA acquired invasive characteristics (Cajot et al., 1989). PAs may play a direct role in ECM degradation, cleaving fibronectin (Quigley et al., 1987) and other components (Quigley, 1979; Fairbairn et al., 1985). Alternatively, PAs may act as part of a proteinase cascade in which plasmin activates procollagenase (Werb et al., 1977). In support of the cascade model, Reich et al. (1988) reported the activation of procollagenase by both t-PA and u-PA, and Mignatti and co-workers (1986) found that invasion of amniotic membranes by B16-BL6 melanoma cells could be blocked by using inhibitors of either PAs, plasmin, or metalloproteinases.

Previous work from this laboratory showed that invasive first trimester human cytotrophoblasts produce a number of metalloproteinases, some of which are absent or greatly reduced in later gestation, noninvasive cells (Fisher et al., 1989). Other investigators showed that term human cytotrophoblasts produce u-PA, but not t-PA (Queenan et al., 1987). Thus, both families of proteinases may play a role in human cytotrophoblast invasion. In the present study we used a model system to test the hypothesis that these proteinases are involved in trophoblast invasion.

Materials and Methods

Cell Isolation and Culture

Cytotrophoblasts were isolated by published methods from first, second (Fisher et al., 1989, 1990), and third trimester (Kliman et al., 1986) human placentas. Enzyme incubation times differed for different lots of collagenase, hyaluronidase, and trypsin. Yields per gram of first, second, and third trimester placentas were 1, 0.5, and 0.25×10^6 cells, respectively. As determined by fluorescent-activated cell sorting, >95% of the cells are cytotrophoblasts. Remaining leukocytes were removed using an antibody to CD-45, a protein tyrosine phosphatase found on bone marrow–derived cells (Charbonneau et al., 1989), but not on cytotrophoblasts (Librach, C. L., and S. J. Fisher, unpublished observations). Briefly, the antibody (HLe; Becton Dickinson and Co., Mountain View, CA, or IgG affinity purified from the GAP 8.3 hybridoma; American Type Culture Collection, Rockville, MD) was coupled to magnetic beads (Advanced Magnetics Inc., Cambridge, MA) and mixed with the cytotrophoblast-enriched Percoll gradient fraction at a density of 25 particles/cell. After incubation for 20 min at 4°C with occasional gentle mixing, the CD-45-positive cells were removed by means of a Bio-Mag Separator (Advanced Magnetics Inc.). Fibroblasts were isolated from first trimester placentas as previously described (Fisher et al., 1989) and were used after the fourth and before the fifteenth passage.

Cells were plated on the laminin-rich extracellular matrix substrate Matrigel (Collaborative Research Inc., Bedford, MA). Only individual lots that contained at least 10 mg/ml protein were used. The Matrigel substrate was not diluted unless otherwise indicated.

Time-Lapse Cinemicrography

To observe aggregate formation, cytotrophoblasts were plated on 35 × 10 mm petri dishes. The central portion of each dish was coated with 100 μl Matrigel, which was gelled for 30 min at 37°C. Cells (1.5×10^6) were plated on the substrate and allowed to attach by incubating for 30 min at 37°C, after which nonadherent cells were removed. A small volume (~400 μl) of fresh medium sufficient to cover the cells was added to the dish, and then the cells and medium were covered with a layer of medium-equilibrated heavy mineral oil to prevent evaporation and to maintain constant CO_2 levels. Additional medium (1.5 ml) was added beneath the mineral oil through a small bore pipette. The petri dishes were transferred to a stage heater (Steier, 1975) connected to a proportional temperature controller (Model 72; Yellow Springs Instrument Co., Yellow Springs, OH) and the central temperature was maintained at 37°C. The dish and heater were placed on the stage of a Nikon Diaphot inverted microscope fitted with Hoffman Modulation Contrast Optics, covered with a plexiglass chamber, and exposed to continuous passage of 5% CO_2 in air over the surface of the oil. Exposures were made at 30 s or 1 min intervals for either 36 or 72 h, using a Bolex H-16 camera as previously described (Sutherland et al., 1990).

Light Microscopy

To observe invasion by light microscopy, cells were plated on Matrigel plugs formed in size 00 BEEM capsules (Polysciences, Inc., Warrington, PA). The top portion of the capsule was cut off, and the remainder was sterilized by UV irradiation. Matrigel (325 μl) was then poured into the capsule and gelled for 30 min at 37°C. First, second, and third trimester cytotrophoblasts ($2–4 \times 10^5$) were plated on the plugs. After 24, 48, or 72 h the Matrigel plugs were fixed with 3% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min, and then pushed out from the capsules with a glass rod and fixed for an additional 30 min. Next, the plugs were washed in several changes of cacodylate buffer, dehydrated in acetone, and embedded in JB4 according to the method of Beckstead (1985). Sections (2 μm) were cut on a JB4 microtome, transferred to coverslips, and air dried at room temperature. Alternate sections were stained with hematoxylin and eosin or with antibody against human placental lactogen (HPL), a hormone produced exclusively by trophoblasts. The latter sections were incubated with primary antiserum (anti-HPL; Dako, Glostrup, Denmark, 1:1,000) overnight at 4°C and then with secondary antiserum (peroxidase-conjugated goat anti–rabbit IgG; Jackson Immunoresearch Laboratory Inc., West Grove, PA, 1:200) for 45 min at room temperature. The peroxidase reaction was developed using 3,3′-DAB and the sample was counterstained with Gill's hematoxylin as previously described by Beckstead (1985). As a control, some sections were incubated with primary or secondary antibody alone. Photographs were taken using an Olympus (BH series) microscope.

Invasion Assay

To quantify invasion, cytotrophoblasts were plated on Transwell inserts (6.5 mm; Costar, Cambridge, MA) containing polycarbonate filters with 3-, 5-, and 8-μm pores. The upper surface of the filters was coated with 10 μl Matrigel. In experiments testing the effects of proteinase inhibitors on invasion, three times the desired concentration of the inhibitor or control substance was mixed with the Matrigel (1:2, vol/vol) and coated on the filter. Coated filters were incubated at 37°C for 30 min to allow the matrix to gel. Cytotrophoblasts (2×10^5) or human placental fibroblasts (2×10^5) were plated on the filter in 200 μl of Dulbecco's Modified Eagle's H21 MEM containing 2% Nutridoma (Boehringer Mannheim, Indianapolis, IN), 50 μg/ml gentamicin, and the inhibitory compound or antibody (described below) to be tested. Medium added to both the top and bottom of the culture contained the same concentration of the inhibitory or control substance as the Matrigel. The cultures were incubated for 24 h; because of its instability, an additional amount of one inhibitor, PAI-1, was added after 12 h.

After 24 h, the Transwell inserts were washed twice with medium, and the cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min. The samples were then rinsed in cacodylate buffer and processed for scanning EM. The filters were passed through increasing ethanol concentrations (5 min each time) up to 100%. While still attached to the inserts, the samples were critical point–dried, then the filters were cut off the inserts with a scalpel blade and mounted with either the upper or lower sides exposed for coating. The filters were sputter-coated with a 20–25-nm layer of gold palladium, and the specimens were examined in a JEOL (JSM-840) scanning electron microscope. Alternatively, samples were fixed as above, washed in cacodylate buffer, and examined without drying in a charge-free anticontamination system mounted on an SX-40A scanning electron microscope (International Scientific Instruments, Pleasanton, CA).

For quantification, ten representative photographs (150×) of each filter underside were taken in a systemic fashion from all sectors. The resulting photographs were analyzed to estimate the surface area of the filter covered by invasive cells. Photographs were overlaid with a grid consisting of points forming equilateral triangles, and the number of cells that overlapped with grid points was counted by an independent observer. The data were expressed as the percentage of grid points that overlapped with invading cells (Weibel and Bolender, 1973). For each condition tested, at least two filters

were examined per experiment and the experiments were repeated on three different occasions.

Immunofluorescence Microscopy

The identity of cells found on the underside of the filters was confirmed with antibodies that within the placenta are specific for cytotrophoblasts. These were rabbit anti-HPL and a mouse anticytokeratin mAb (PKK1; Lab Systems, Chicago, IL). The cytotrophoblasts that remained on the upper side of filters were removed with a cotton swab. Cells that had invaded the matrix and emerged on the underside of the filter were triple stained with Hoechst nuclear stain (Sigma Chemical Co., St Louis, MO), anti-HPL (1:1,000), and PKK1 (1:100). The primary antibodies were demonstrated by the appropriate secondary antibody, either rhodamine-conjugated goat anti-rabbit IgG (1:200) (Cooper Biomedical Inc., West Chester, PA) or fluorescein-conjugated goat anti-mouse IgG (1:200) (Cooper Biomedical Inc.).

Substances Tested for Inhibitory Activity in the Cytotrophoblast Invasion Assay

Metalloproteinase Inhibitors. Human recombinant tissue inhibitor of metalloproteinases (TIMP) (5, 50, and 500 nM; gift of Dr. D. Carmichael, Synergen Corp., Boulder, CO); TIMP (5, 50, and 500 nM) and TIMP-2 (5, 50, and 500 nM) purified from human transformed fibroblasts (gifts of Dr. Eric Howard, Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, CA); 1,10-phenanthroline (50 and 500 μM, Sigma Chemical Co.); HONHCO-CH_2CH(i-Bu)CO-Tyr(OMe)-NHMe (20 nM, 200 nM, and 2 μM [K_i = 2 nM], Reich et al., 1988); and an analogue of this compound (t-butoxy Leu-Tyr (OMe)NHMe; 20 nM, 200 nM and 2 μM), which had no collagenolytic inhibitory activity as determined using the thiopeptolide Ac-Pro-Leu-Gly-SCH[CH_2CH(CH_3)$_2$]-CO-Leu-Gly-OC_2H_5 (Bachem, Philadelphia, PA) (Weingarten et al., 1985).

Serine Proteinase Inhibitors. Plasminogen activator inhibitor-1 (PAI-1) (10 nM, 100 nM, 1 μM, and 10 μM; American Diagnostica, New York, NY); PAI-2 (10 nM, 100 nM, 1 μM, and 10 μM; gift of Dr. Robert Cohen, University of California, San Francisco); aprotinin (0.1, 1, and 10 U/ml; Sigma Chemical Co.) and α_2-antiplasmin (10 nM, 100 nM, and 1 μM; American Diagnostica).

Antibodies. An affinity-purified IgG from rabbit polyclonal antibody 647 that blocks interstitial collagenase function (2.5, 5, 10, and 20 μg/ml; Birkedal-Hansen et al., 1987); sheep antiporcine 92 kD gelatinase (Murphy et al., 1989) affinity purified using mouse 92 kD type IV collagenase conjugated to Sepharose 4B (2.5, 5, 10 and 20 μg/ml; Dr. Eric Howard, Laboratory of Radiobiology and Environmental Health, University of California, San Francisco); mouse mAb 7-18, which blocks u-PA function (2.5, 5, 10, and 20 μg/ml; Birkedal-Hansen et al., 1987); purified sheep IgG, and purified rabbit IgG (Jackson ImmunoResearch Laboratories Inc., Avondale, PA).

Immunoblotting

The identity of metalloproteinases secreted by cytotrophoblasts was confirmed by immunostaining nitrocellulose transfers. Conditioned medium from equal numbers (1.5×10^6) of first, second, and third trimester cytotrophoblasts, and from placental fibroblasts, was collected after 24 h in culture. The protein concentration was determined (Lowry et al., 1951) and 10 μg was mixed with an equal volume of sample buffer. The samples were subjected to SDS-PAGE (10% gels) and transferred by blotting to nitrocellulose (Towbin et al., 1979). Nonspecific staining was blocked by incubating the transfers for 1 h in TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4) containing 5% nonfat dried milk (Carnation). The transfers were then incubated for 48 h with anti-interstitial collagenase or anti-92-kD type IV collagenase diluted 1:100 in the blocking buffer. The blots were washed (5 min each time) once in TBS, twice in TBS containing 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS, and twice in TBS, and then were incubated for 3 h with HRP-conjugated, affinity-purified donkey anti-sheep IgG diluted 1:100. The blots were washed again as described above and the color was developed by adding a solution consisting of 6 mg diaminobenzidine/10 ml TBS and 0.03% H_2O_2. Control blots were exposed to secondary antibody alone.

The specificity of the affinity-purified anti-92 kD antibody, diluted 1:2 in blocking buffer, was determined by immunoblotting essentially as described above except that bands were identified by means of the Enhanced Chemiluminescence Detection System (Amersham Corp., Arlington Heights, IL) and demonstrated using Hyperfilm (Amersham Corp.).

Zymography on Gelatin Substrate Gels

The effects of inhibitors on cytotrophoblast-associated and -secreted gelatin-degrading proteinases were analyzed in SDS-polyacrylamide gels copolymerized with 1 mg/ml of gelatin (type A from porcine skin; Sigma Chemical Co.; Heussen and Dowdle, 1980; McKerrow et al., 1985; Chin et al., 1985). Culture of the cells and the methods used for running the gels have been described previously (Fisher et al., 1989). The inhibitors were added in the following concentrations to the buffer in which the gels were incubated: TIMP, 350 nM; HONHCO-CH_2CH(i-Bu)CO-Leu-Tyr(OMe)NHMe, 20 nM; and the noncollagenolytic analogue of this compound in which leucine was substituted with a t-butyloxycarbonyl group, 20 nM. The gels were incubated for 16 h at room temperature in calcium-containing PBS either with or without inhibitors, stained with Coomassie blue, and then destained with 10% acetic acid in 50% methanol until the cleared bands were visible and the stacking gel was totally destained.

Results

Cytotrophoblasts Aggregate, then Invade Basement Membrane-like Matrices

When first trimester cytotrophoblasts were plated as single cells on Matrigel-coated plastic tissue culture wells they exhibited striking social behavior. By the first 24 h of culture they formed large, multicellular aggregates; single cells were only rarely seen (Fig. 1 *A*), suggesting that aggregation was a property of a majority of the cells. In addition, these aggregates were often connected by long (>100 μm) cellular projections. In contrast, cytotrophoblasts isolated from placentas of >20 wk gestation and cultured under the same conditions for 24 h formed smaller aggregates that contained fewer cells and no cellular projections. These were interspersed among numerous single cells (Fig. 1 *B*). By cinemicrography, we observed aggregation of first trimester cytotrophoblasts. Within the first hour of plating the cells rapidly adhered to the matrix as a dispersed monolayer (Fig. 1 *C*). After 8 h the cells had migrated toward one another, forming aggregates (Fig. 1 *D*). During the next 16 h these multicellular structures increased in size as the smaller aggregates coalesced with the larger ones (Fig. 1, *E* and *F*). In addition, throughout the course of the experiment numerous cells undergoing rapid cycles of expansion and contraction were visible, suggesting that some of the cytotrophoblasts were pumping fluid, an important property of trophoblast cells.

To examine the nature of the interaction of these aggregates with the underlying matrix, we sectioned Matrigel plugs on which multicellular cytotrophoblast aggregates had formed (Fig. 2). Between 24 and 72 h in culture, cells from the aggregates progressively invaded the underlying matrix (Fig. 2 *A*). Cells found within the Matrigel showed interesting morphological differences when compared with those that had not penetrated the matrix. The invading cells had relatively larger nuclei with prominent nucleoli. In addition, many had large vacuoles. Individual cells did not invade in isolation. Instead, the invasion front was often organized into finger-like cell columns (Fig. 2 *B*). The morphological changes that occurred during Matrigel invasion were accompanied by increased staining with anti-HPL as compared with cells that remained above the matrix surface (Fig. 2 *C*) and with freshly isolated cytotrophoblasts (data not shown). This is in agreement with the finding that in tissue sections, extravillous placental bed cytotrophoblasts that have invaded the uterus are strongly positive for HPL (Kurman et al.,

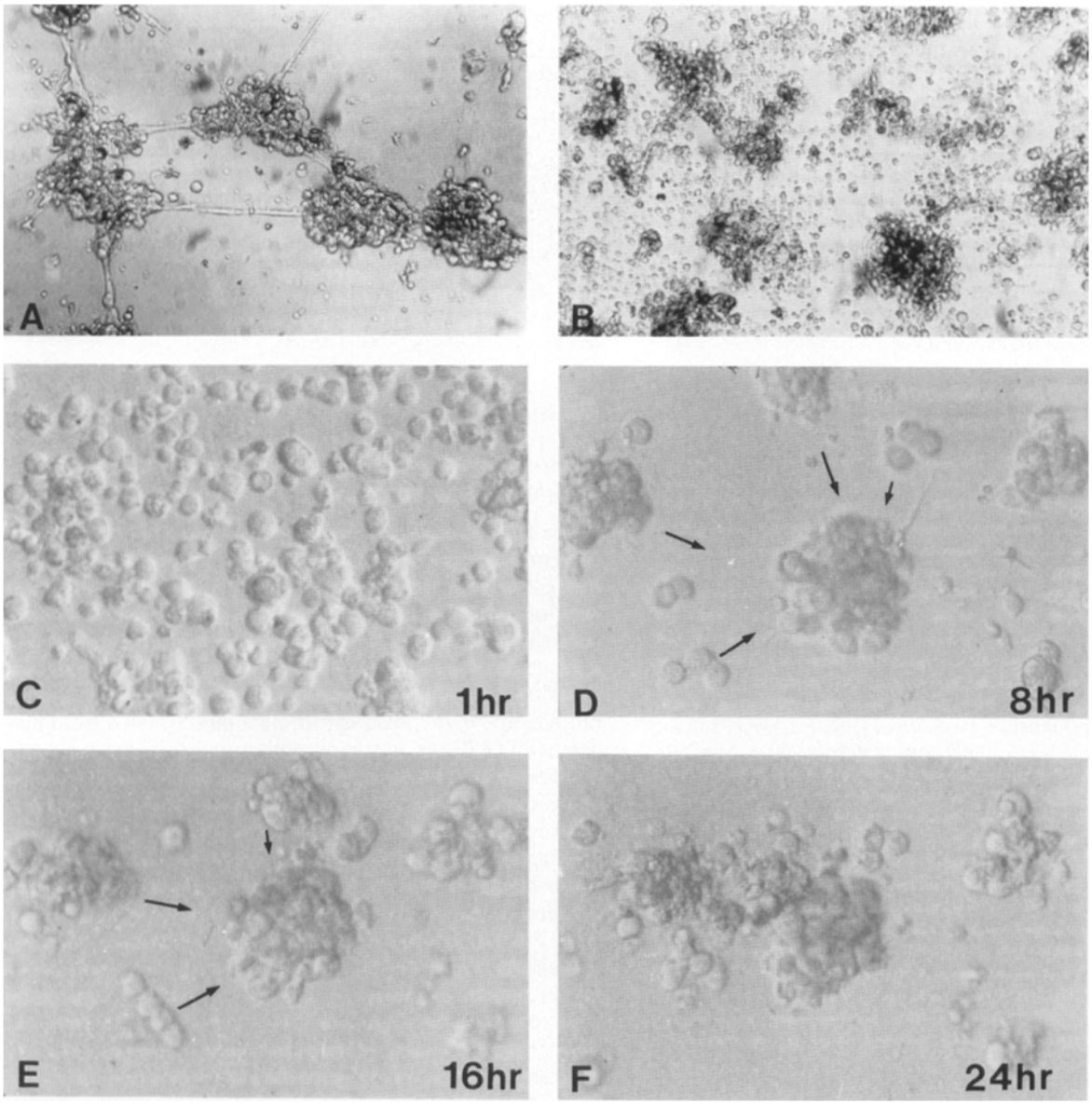

Figure 1. First trimester human cytotrophoblasts aggregate on Matrigel. Human cytotrophoblasts were isolated from first or third trimester human placentas and plated as a dispersed monolayer in plastic tissue culture wells coated with undiluted Matrigel. (*A*) After 24 h first trimester cells formed discrete, multicellular aggregates that were often connected by cellular processes. (*B*) In contrast, third trimester cells formed smaller aggregates, and numerous single cells were visible covering the surface of the Matrigel. (*C–F*) Time-lapse cinemicrography was used to study the time course of first trimester cytotrophoblast aggregation. After 1 h in culture the cytotrophoblasts appeared as a dispersed monolayer (*C*) During the next several hours the cells rapidly aggregated. A cellular process is seen projecting from an aggregate at 8 h (*D*). Smaller aggregates then coalesced with larger ones at 16 and 24 h (*E* and *F*).

1984), whereas noninvasive villous cytotrophoblasts stain weakly for HPL. In contrast to first trimester cells, cytotrophoblasts isolated from term placentas formed smaller aggregates among single cells and did not penetrate the surface (Fig. 2, *D–F*). As previously described, some of these aggregates may be forming a multinucleate syncytium (Kliman and Feinberg, 1990; Kliman et al., 1986).

Cytotrophoblast Invasion of Basement Membrane Matrix Is Developmentally Regulated

To further study and quantify invasion, we plated freshly isolated cytotrophoblasts on Matrigel-coated polycarbonate filters containing 3-, 5-, or 8-μm pores. The ability of cells to penetrate the Matrigel and/or migrate through defined-size pores to the underside of the filter has been positively correlated with invasive potential in several studies (Tullberg and Burger, 1985; Kramer et al., 1986; Albini et al., 1987; Hendrix et al., 1987; Repesh, 1989). Since the assay depends on the integrity of the matrix coating, we first investigated the conditions under which Matrigel forms a complete barrier. By scanning EM, the upper surface of a Matrigel-coated polycarbonate filter containing 8-μm pores had a smooth, amorphous appearance and no visible holes (Fig. 3). Thus, no cracks or other major aberrations were introduced during coating, incubation for 24 h with medium, and

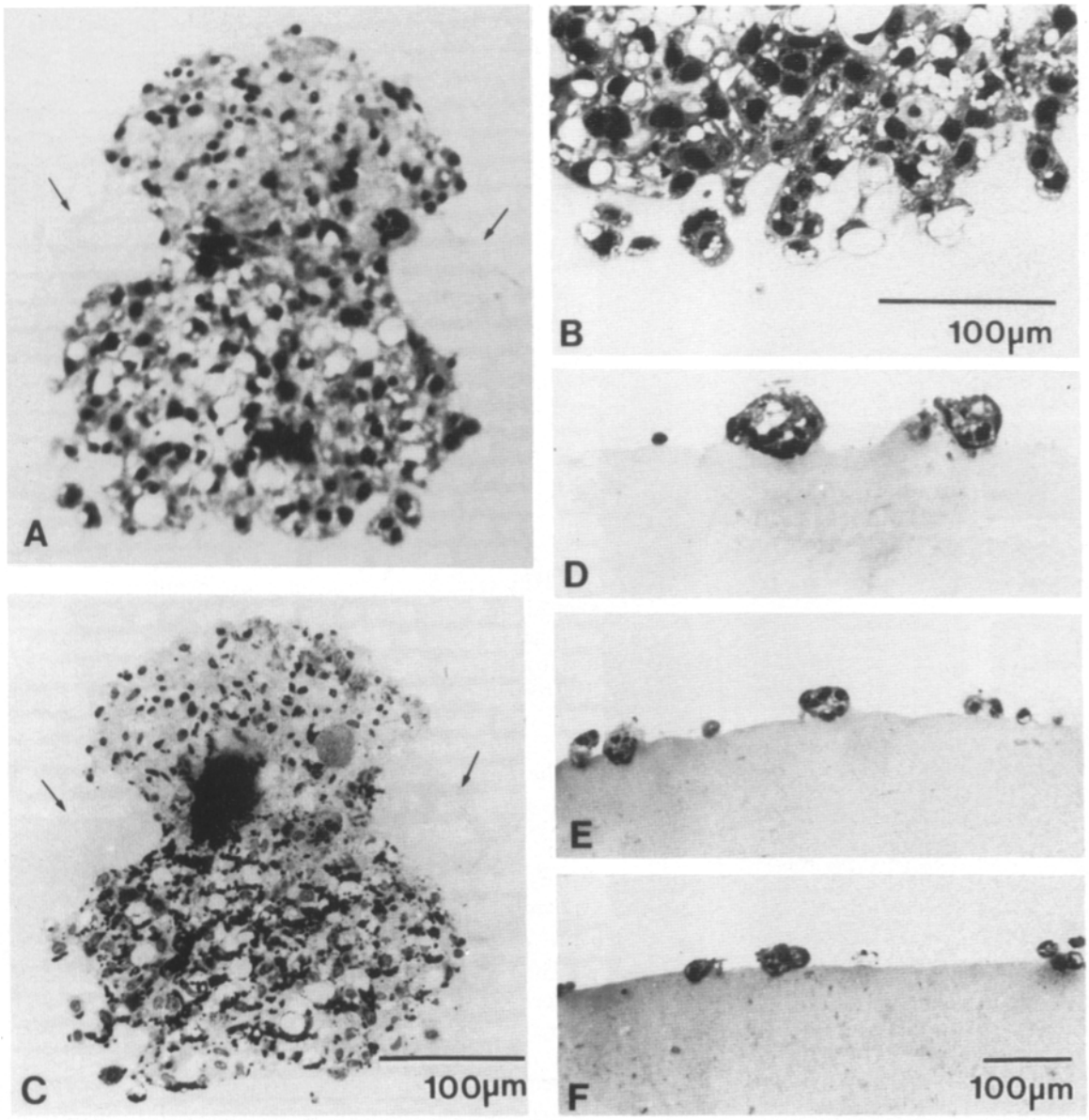

Figure 2. First trimester human cytotrophoblasts invade Matrigel. (*A*) Cross sections of first trimester human cytotrophoblast aggregates stained with hematoxylin and eosin showed that by 72 h they had invaded well below the surface of the Matrigel (*arrows*). (*B*) The invasion front was often organized into cell columns. (*C*) A serial section to *A* shows that cytotrophoblast penetration of the Matrigel was accompanied by an increase in immunoreactive human placental lactogen. A similar increase is also observed in the human placental bed. (*D–F*) In contrast, sections of term cytotrophoblasts plated on Matrigel for 72 h and stained with hematoxylin and eosin exhibited only shallow penetration of the matrix.

preparation of the sample for scanning EM. The undersurface showed the filter pores with no Matrigel coating visible. Because the introduction of inhibitors required dilution of the Matrigel, we examined the effect of this manipulation on the integrity of the matrix coating. At concentrations below 20% (vol/vol), the Matrigel formed an incomplete layer on the top of the filter and flowed through the pores to the underside (not shown). Therefore, a 1:2 vol/vol mixture of inhibitor or antibody to Matrigel was used for each invasion experiment.

Scanning EM of the upper surface of the Matrigel-coated filters on which first trimester cytotrophoblasts were plated for 24 h revealed the remarkable morphology of the multicellular aggregates. The elaborate processes extending from the surfaces of cells at the periphery of the aggregates were particularly striking. These may be related to the invadopodia seen in Rous sarcoma virus–transformed chick embryo fibroblasts and implicated in the local degradation of matrix by these cells (Chen, 1989). At points of contact with these processes, the substrate often showed signs of disruption that were not visible in the Matrigel coating when cells were not present (see Fig. 3) or when noninvasive cells such as term cytotrophoblasts (Fig. 4 *E*) or placental fibroblasts (data not shown) were plated on this substrate. Examination of the underside of filters with 3-μm pores showed cellular processes projecting from the openings (Fig. 4 *B*), suggesting that the

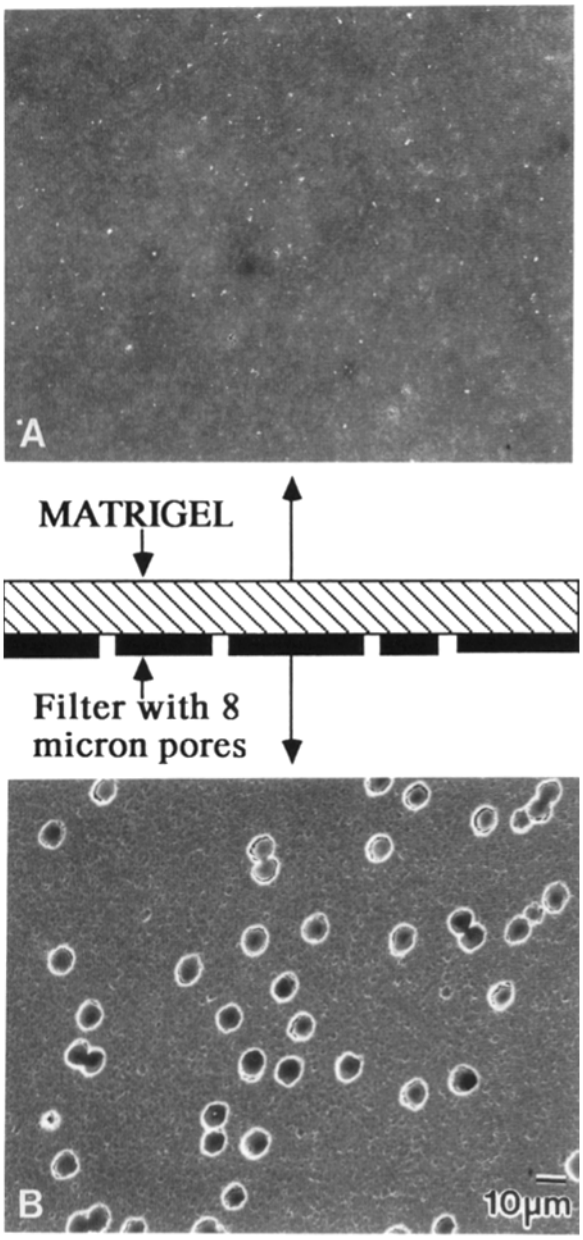

Figure 3. A system for quantifying cytotrophoblast invasion. A polycarbonate filter containing 8-μm pores was coated with Matrigel. Scanning EM of the top of the filter showed a smooth amorphous coating through which no pores were visible. Thus, the Matrigel coating formed a continuous barrier through which the invading cells must pass to reach the underside of the filter. In contrast, scanning EM of the lower surface of the filter showed the pores, but no Matrigel coating.

processes play an important role in invasion. However, entire cells were unable to pass through these pores to the undersurface.

When filters containing 5- or 8-μm pores were used, both processes and cell bodies of first trimester cytotrophoblasts were readily visible on the undersurface (Fig. 4 *D*). Filters containing 8-μm pores were used in subsequent experiments. We consistently noted interesting morphological details that were related to the emergence of the cytotrophoblasts on the underside of the filters. In many areas we observed pseudopodia, some of which were connected to elongated cell bodies (Fig. 4, *C* and *D*). Elsewhere entire, flattened cells were visible. These observations suggest a sequence of events in which cytotrophoblasts first extend cell processes through the pores, and then form attachments on the underside of the filter that assist the migration of the remainder of the cell body. Once the cell emerges on the underside of the filter, it assumes a more flattened shape. Thus, our data suggest that the formation of complex cellular processes by these epithelial cells is an important aspect of the invasion process.

Third trimester cells plated on Matrigel-coated filters formed smaller aggregates interspersed among single cells (Fig. 4 *E*). These cells did not form the elaborate processes that were characteristic of the first trimester cytotrophoblasts. In general, the surface of the Matrigel, including areas adjacent to the cells, appeared smooth and showed little evidence of the disruption observed with the first trimester cytotrophoblasts. Examination of the underside of the filter showed that neither cells nor processes were visible (Fig. 4 *E*).

To identify first trimester cells that emerged on the underside of the filters as trophoblasts, we used triple staining with Hoechst nuclear stain, PKK1 (anticytokeratin), and anti-HPL (Fig. 5). All invading cells, identified as Hoechst-stained nucleated cells in the field (Fig. 5 *A*), also stained with an antibody to HPL, a hormone made exclusively by cytotrophoblasts (Fig. 5 *B*). Reactivity with PKK1, an mAb that recognizes some cytokeratins and within the placenta stains only cytotrophoblasts (Fisher et al., 1989), was more variable in both intensity and pattern (Fig. 5 *C*). It is possible that the trophoblasts, like certain tumor cells (Lichtner et al., 1979; Auersperg et al., 1989), modulate their repertoire of cytokeratins during invasion.

Synthesis of the 92-kD Type IV Collagenase by Cytotrophoblasts Is Developmentally Regulated

Previously, using substrate gel zymography, we showed that the pattern of metalloproteinases produced by cytotrophoblast cells is developmentally regulated (Fisher et al., 1989). In the present study, to identify some of these cytotrophoblast metalloproteinases in conditioned medium, we blotted with antibodies that recognize two members of this family, interstitial collagenase and the 92-kD type IV collagenase (Fig. 6). Antibodies to the 92-kD enzyme also cross-reacted with the 72-kD type IV collagenase which was present throughout gestation. Both placental cytotrophoblasts and fibroblasts (data not shown) secreted interstitial collagenase, but the amount detected did not change during gestation. In contrast, the synthesis of the 92-kD type IV collagenase was highest during early gestation and was not detected at term. This observation was in keeping with our previous observation that a 92-kD metalloproteinase activity was greatly reduced in term cells as compared with first trimester cells (Fisher et al., 1989). Taken together, these two results, obtained by detecting immunoreactive protein and enzyme activity respectively, suggest that synthesis of the 92-kD proteinase decreases dramatically during gestation.

Metalloproteinase Inhibitors and Antiproteinase Antibodies Block Cytotrophoblast Invasion

We first used substrate gel zymography to assess the effects of various inhibitors on cytotrophoblast metalloproteinase activity. All the gelatin-degrading metalloproteinases ob-

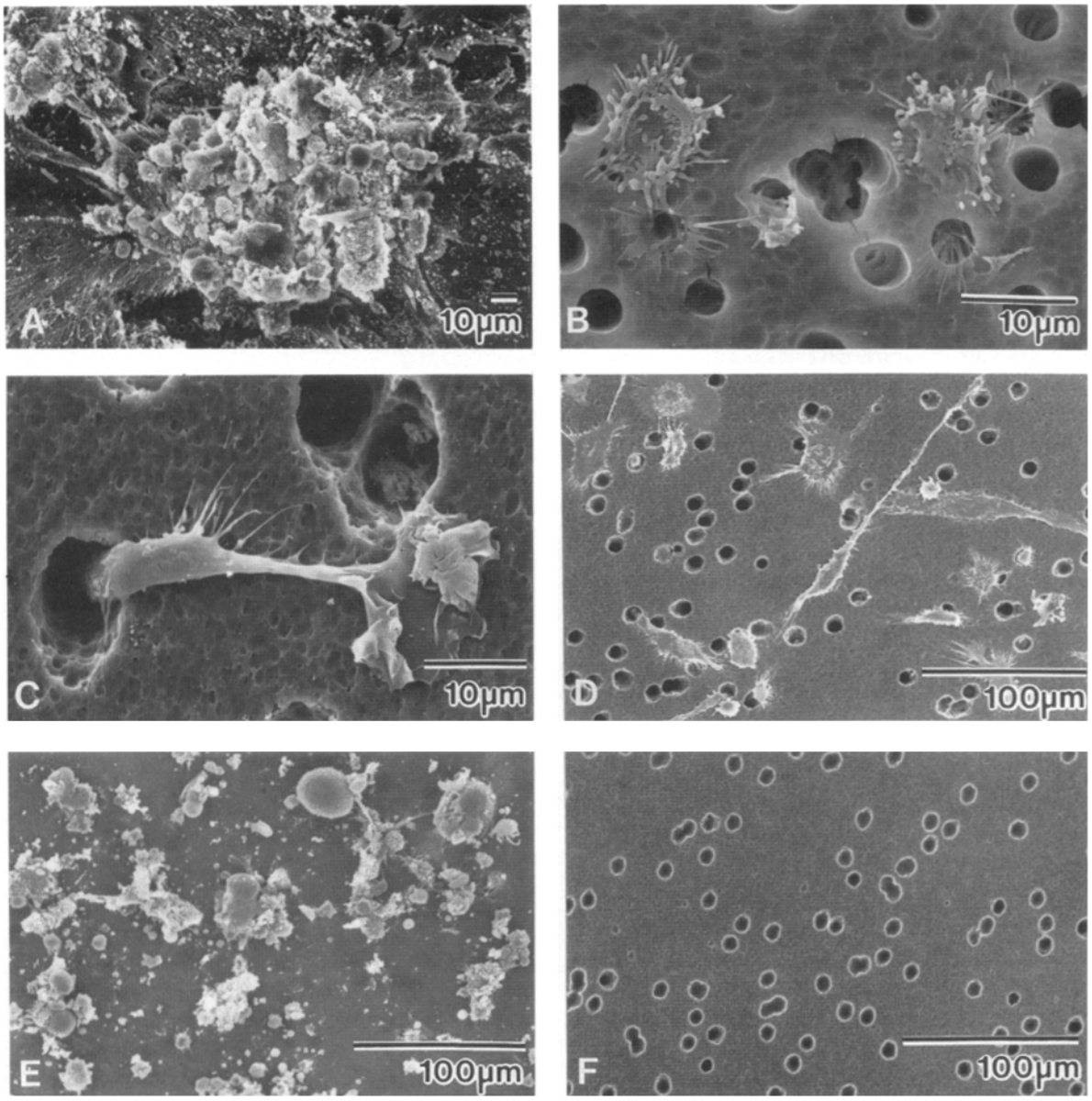

Figure 4. Scanning EM of cytotrophoblast invasion of Matrigel-coated polycarbonate filters. (*A*) Scanning EM of the upper surface of Matrigel-coated filters showed that the first trimester cytotrophoblast aggregates interacted with the matrix via complex cellular processes. (*B*) When the cells were plated on filters containing 3-μm pores, numerous processes protruded through the openings and were visible on the underside of the filter, but the passage of cell bodies was restricted. (*C*) In contrast, when the cells were plated on filters with 8-μm pores, entire cells emerged on the filter underside. (*D*) After 24 h numerous cells had undergone this process. (*E*) Compared with the early-gestation cytotrophoblasts, term cells cultured for 24 h formed smaller, less compact aggregates on the surface of the Matrigel; none penetrated to the underside of the filter (*F*).

served in our earlier study (Fisher et al., 1989) were inhibited by recombinant TIMP and a synthetic collagenase inhibitor that has been used to prevent tumor cell metastasis in vivo (Reich et al., 1988). A related peptide in which the free amino group of leucine was substituted with a t-butyloxycarbonyl group had no inhibitory activity (data not shown).

We next determined whether proteinase inhibitors and function-perturbing antibodies reactive with matrix metalloproteinases and u-PA were able to inhibit cytotrophoblast invasion. In control cultures of cytotrophoblasts, 30–35% of the surface area on the underside of the filter was covered by cells, as determined by morphometric analysis (Fig. 7 *A*). Two natural inhibitors, TIMP and TIMP-2, effectively blocked cytotrophoblast invasion. Recombinant TIMP (Fig. 7, *B* and *C*) and TIMP purified from transformed human fibroblasts (Fig. 8 *A*) inhibited cytotrophoblast invasion in a dose-dependent manner, producing virtually complete inhibition at 500 nM. TIMP-2, also purified from transformed human fibroblasts, completely blocked cytotrophoblast invasion at 500 nM (Fig. 8 *A*), but the IC_{50} of TIMP (5 nM) was substantially less than that of TIMP-2 (60 nM). A dose-dependent inhibitory effect was also obtained with a synthetic transition state analogue of metalloproteinases (Fig. 8 *B*). The IC_{50} was ~5 nM and complete inhibition was achieved at

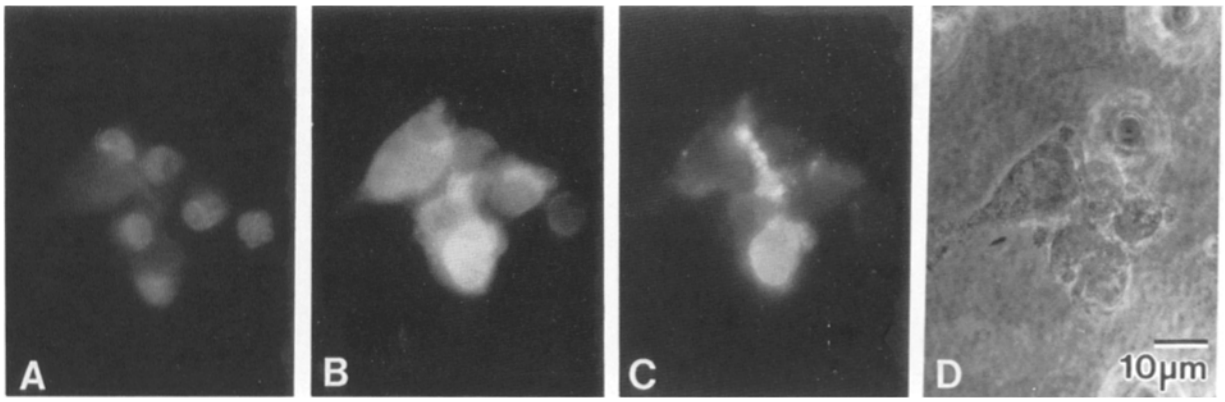

Figure 5. Invasive first trimester placental cells express cytotrophoblast-specific antigens. Cells from the underside of the filter were triple-stained with (*A*) Hoechst's nuclear stain, (*B*) anti-HPL, and (*C*) an anticytokeratin antibody (PKK1). (*D*) A phase micrograph of the same field.

2 μM. As a control for possible toxicity of this compound, we used a related peptide with no inhibitory activity and observed no effect on cytotrophoblast invasion (Fig. 8 *B*).

To determine which metalloproteinase was most active during the invasion process, we used proteinase-specific inhibitory antibodies in the assay. To do so, we affinity purified the anti-92-kD sheep polyclonal antibody. By immunoblotting, the resulting purified IgG recognized only a 92-kD band in first trimester human cytotrophoblast–conditioned medium (compare Fig. 6 with Fig. 9 *A*). This affinity-purified antibody also completely inhibited cytotrophoblast invasion at the highest concentrations tested (Fig. 9 *B*). As a control, purified sheep IgG had no effect on invasion (data not shown). In contrast, a polyclonal antibody that inhibits interstitial collagenase function (Birkedal-Hansen et al., 1989) had no effect on cytotrophoblast invasion even when used at 20 μg/ml, four times the IC_{50} (Fig. 9 *B*). As a control, purified rabbit IgG also had no effect on invasion (data not shown). These results provide strong evidence that the 92-kD type IV collagenase is an important mediator of human cytotrophoblast invasion.

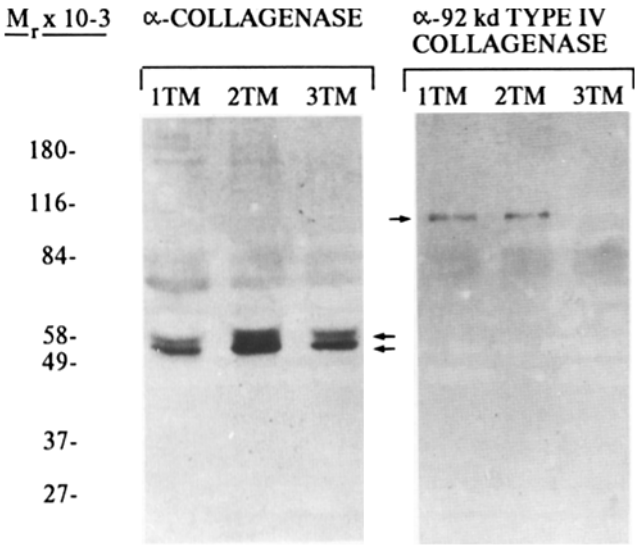

Figure 6. Cytotrophoblast expression of the 92-kD Type IV collagenase is developmentally regulated. Conditioned medium from equal numbers of first, second, and third trimester (*TM*) cytotrophoblasts was electrophoretically separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were probed with metalloproteinase-specific antibodies. The synthesis of interstitial collagenase remained constant throughout gestation but production of the 92-kD type IV collagenase was not detectable at term. Weak staining at 72 kD represents cross reactivity with the 72-kD type IV collagenase.

The morphology of the cells cultured in the presence of TIMP, TIMP-2, the synthetic inhibitor, and the function-perturbing antibodies remained unchanged. Interestingly, TIMP had no effect on the aggregation of cytotrophoblasts on the top of the Matrigel, and when cells managed to penetrate, elongated pseudopodia were still visible. However, the extent of matrix disruption around the aggregates was diminished significantly. These observations suggest that the proteolytic component of invasion was inhibited, but not the cell behavior parameters.

PAs have long been implicated in trophoblast invasion because of their early expression during trophoblast differentiation and their presence at the site of implantation (Strickland et al., 1976; Sappino et al., 1989). Because u-PA (Queenen et al., 1987) and its inhibitors, PAI-1 and PAI-2, are expressed by human cytotrophoblasts in vivo (Feinberg et al., 1989), we tested the effect of inhibitors of this serine proteinase enzyme system on cytotrophoblast invasion. Exogenous PAI-1, PAI-2, and a polyclonal inhibitory antibody to u-PA inhibited cytotrophoblast invasion by no more than 40% (Fig. 10, *A* and *B*). Because the action of u-PA is usually mediated by its product, plasmin, we determined the effects of the plasmin inhibitors aprotinin and α_2-antiplasmin (Fig. 10 *C*). Aprotinin, which inhibits plasmin both in solution and bound to the cell membrane (Stephens et al., 1989), had a partial (40%) inhibitory effect, similar to that obtained with the u-PAIs. In contrast, α_2-antiplasmin, which inhibits soluble but not membrane-bound plasmin (Plow et al., 1986), had little effect. These results suggest that u-PA and plasmin play a role in cytotrophoblast invasion, but cannot totally account for this process.

Discussion

The most critical phase of early mammalian development is formation of a specialized maternal–fetal interface. Tro-

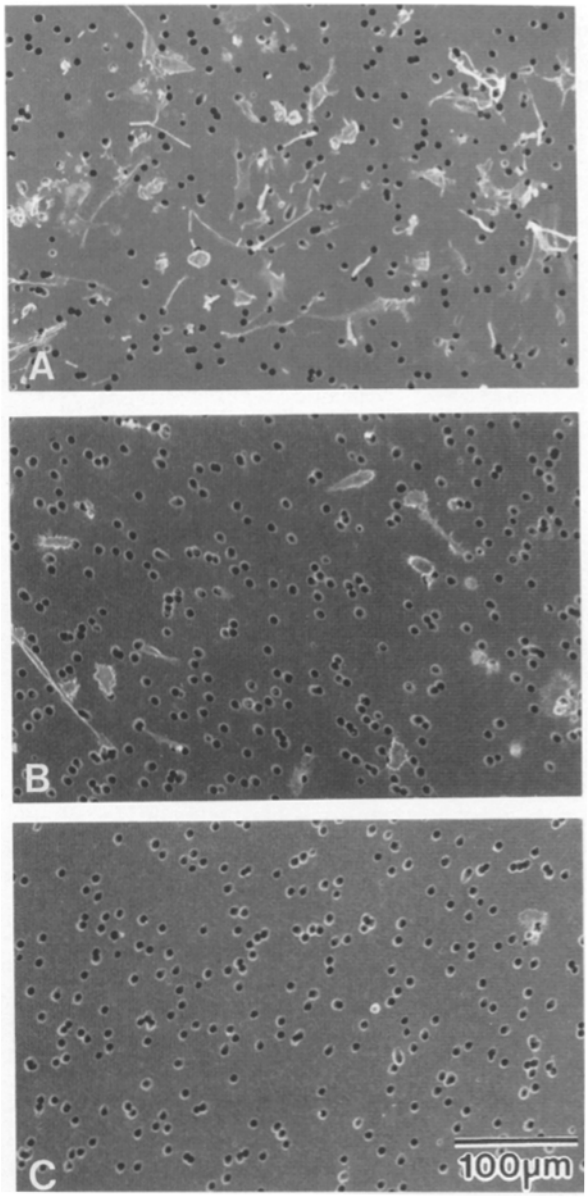

Figure 7. Recombinant TIMP inhibits cytotrophoblast invasion in vitro in a dose-dependent manner. Cytotrophoblast invasion was assessed by morphometric analysis of wet scanning electron micrographs of the undersides of the filters. (*A*) Control; (*B*) 50 nM TIMP; (*C*) 500 nM TIMP.

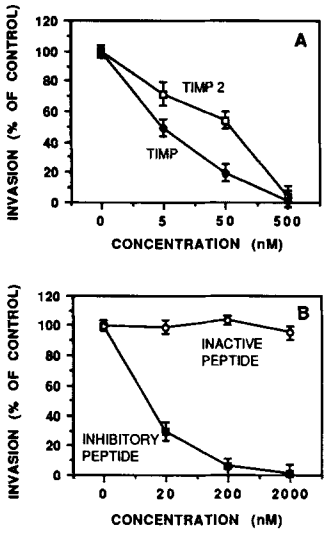

Figure 8. TIMP (*A*), TIMP-2 (*A*), and a synthetic collagenase inhibitor (*B*) inhibit cytotrophoblast invasion in vitro in a dose-dependent manner. Values are means of three determinations; bars indicate standard deviations.

phoblast penetration of the uterine lining, which establishes this interface, is the first invasion process carried out by the embryo. We investigated the molecular mechanisms mediating this process. Our experiments show that invasion of basement membrane matrix by purified first trimester human cytotrophoblasts involves three distinct processes: (*a*) a social organization with large multicellular aggregates or columns; (*b*) extension of elaborate processes; and (*c*) ECM degradation by a cascade of proteolytic enzymes, rate limited by the 92-kD type IV collagenase.

Culture of cytotrophoblasts on a basement membrane–like matrix resulted in an interesting progression of events that was initiated by the formation of large, multicellular aggregates and concluded with extensive invasion of the substrate. The aggregates and their invasion of the matrix morphologically resembled the anchoring villi that invade the uterus during early pregnancy. This was confirmed by the observation that penetration of the matrix was accompanied by a marked increase in production of HPL, paralleling the in vivo synthesis of this hormone by invasive trophoblast cells (Kurman et al., 1984). In addition, the cells in the aggregates often interacted with the matrix via complicated processes that have been postulated to facilitate invasion of tumor cells (Chen, 1989). Aggregation of term cytotrophoblast cells in suspension culture has previously been described. Outgrowths from these aggregates had syncytial coverings and hollow cores resembling floating chorionic villi (Babalola et al., 1990) and could attach to matrices (Kliman and Feinberg, 1990). We found that cytotrophoblasts isolated from term placentas and plated directly on the matrix formed small aggregates, lacked invadopodia, and did not penetrate the surface. Our results correspond to the developmentally regulated invasive properties cytotrophoblasts exhibit in vivo and suggest that the mechanisms which function in this model are similar to those that operate in the placental bed during implantation of the mammalian embryo. Thus, while the formation of aggregates and invadopodia probably facilitate invasion, production of the 92-kD type IV collagenase is absolutely required for this process.

PAs have been implicated by others in trophoblast invasion. Strickland et al. (1976) found that trophoblastic cells of cultured mouse blastocysts produced PAs during the time period corresponding to uterine invasion in vivo, and Sappino et al. (1989) detected u-PA mRNA in invading mouse trophoblasts. However, Denker (1977) found that ϵ-aminocaproic acid, an inhibitor of PAs, did not inhibit rabbit blastocyst attachment, and Glass et al. (1983) found that the same inhibitor did not prevent matrix degradation by mouse trophoblastic cells in vitro.

These results suggest that other proteinases play an important role in trophoblast invasion. Recently, Brenner et al. (1989) showed that mouse embryos secrete functional metalloproteinases (collagenase and stromelysin) during the periimplantation period. Results from this laboratory (Fisher et al., 1989) showed that invasive first trimester human cytotrophoblasts synthesize a complex set of metalloproteinases,

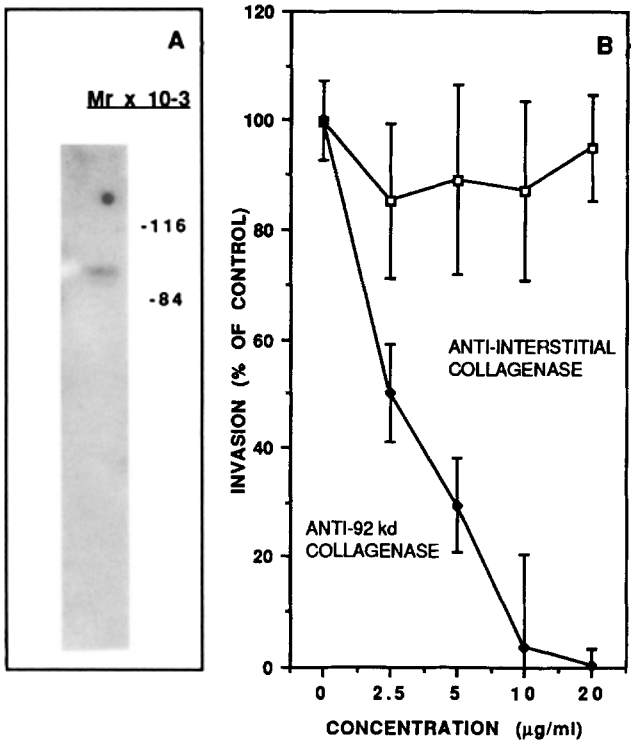

Figure 9. An antibody that inhibits the function of the 92-kD type IV collagenase totally inhibits trophoblast invasion in vitro. A function-inhibiting antibody that reacted only with the 92-kD type IV collagenase was produced by affinity purification (see Materials and Methods) of the polyclonal antibody that reacted with both the 72- and 92-kD enzymes (Fig. 6). By Western blotting with first trimester cytotrophoblast-conditioned medium, the affinity-purified antiserum reacted with the 92-kD but not the 72-kD enzyme (*A*). This antibody totally inhibited invasion at the highest concentrations tested (*B*), whereas an antibody that perturbs the function of interstitial collagenase had no effect on cytotrophoblast invasion (*B*). Values are means of three determinations; bars indicate standard deviations.

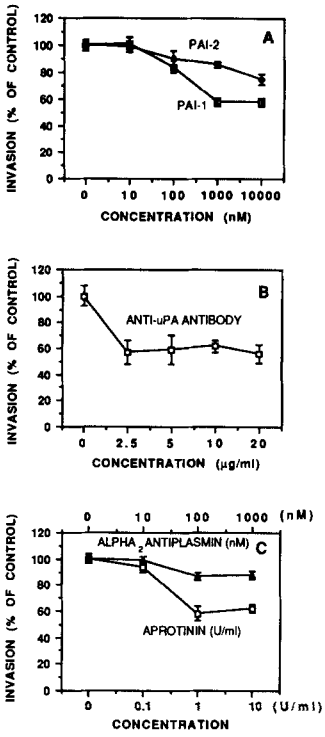

Figure 10. PAIs only partially inhibit cytotrophoblast invasion in vitro. (*A*) PAI-1 and PAI-2 inhibited cytotrophoblast invasion by 40% and 20%, respectively. (*B*) A function-perturbing antibody that inhibited u-PA activity had the same effect as PAI-1. (*C*) Aprotinin, an inhibitor of membrane-bound plasmin, had a greater inhibitory effect (40%) than α_2-antiplasmin, an inhibitor of soluble plasmin. Values are means of three determinations; bars indicate standard deviations.

of which only a subset is secreted by noninvasive term cells. Characterization of these metalloproteinases by substrate gel (gelatin) zymography demonstrated several prominent bands, including activities of 72 and 92 kD. In the present study, immunoblotting with specific antisera confirmed the identity of these enzymes as the recently characterized type IV collagenases (Murphy et al., 1985; Collier et al., 1988; Wilhelm et al., 1989) and showed that early gestation invasive cells secreted the highest levels of these proteinases. The 72-kD enzyme is secreted by a variety of normal human cells, including fibroblasts, as well as by some tumor cells (Collier et al., 1988). The 92-kD enzyme is produced by keratinocytes, granulocytes, macrophages, and some tumor cells, but has not been detected in any normal, unstimulated cells of fibroblast lineage (Wilhelm et al., 1989; Bernhard et al., 1990), and may very well be used for migration and/or metastasis. Consistent with these data, we found that the 72-kD, but not the 92-kD, enzyme was produced by placental fibroblasts (Fisher et al., 1989). These cells, which are noninvasive in our model system, also produced both u-PA and t-PA. Therefore, we hypothesized that the 92-kD type IV collagenase, which was unique to cytotrophoblast cells, is critical for invasion.

The question of how cytotrophoblast invasion is so closely regulated remains to be determined (recently reviewed in Alexander and Werb, 1989). One possible mechanism is regulation by factors that control metalloproteinase synthesis. Interestingly, the 92-kD type IV collagenase can be induced by EGF, interleukin 1, and phorbol esters (Wilhelm et al., 1989), suggesting possible mechanisms that might contribute to the developmentally regulated synthesis we observed. Since CSF-1 (Regenstreif and Rossant, 1989; Arceci et al., 1989) and TGF-α (Han et al., 1987) are produced by the uterus at the time of trophoblast invasion, these molecules might have interesting effects on both the metalloproteinase and PA systems. Finally, metalloproteinase synthesis can be regulated by steroid hormones (Werb, 1978). This suggests that hormone levels at the maternal–fetal interface could directly or indirectly control the production of proteinases required for invasion.

Trophoblast invasion may also be regulated by factors controlling metalloproteinase activation. The mechanism by which the 92-kD type IV procollagenase is activated is currently unknown. Interstitial collagenase can be activated through a u-PA–plasmin cascade; subsequent cleavage by stromelysin produces an increase in the specific activity of the collagenase (He et al., 1989). However, this cascade does not play a significant role in activation of the 92-kD procollagenase (Cui, T.-Y., and S. J. Fisher, unpublished observations; Behrendtsen, O., C. M. Alexander and Z. Werb, manuscript submitted for publication). Interestingly, an inhibitor that blocks the activity of membrane-bound plasmin was as effective as inhibition of u-PA, whereas an inhibitor of only soluble plasmin had virtually no effect, suggesting that this proteinase activity is localized to the cell surface. This is in agreement with our previous observation, using substrate gel zymography, that first trimester cytotrophoblast cell extracts contained a proteinase of slightly lower molecu-

lar mass than the 92-kD enzyme that was also associated with term cells and was secreted into the medium by cytotrophoblasts from all stages of gestation. It is likely that the activated form of the type IV collagenase is associated with invasive cells, whereas the proenzyme form is associated with noninvasive cells and is secreted into the medium (Fisher et al., 1989).

Proteinase inhibitors also play an important role in modulating levels of enzyme activity. TIMP inhibits collagenases that degrade interstitial and basement membrane collagens (Thorgeirsson et al., 1982) by forming complexes with the active forms of the enzymes. In addition, TIMP can complex with the proenzyme form of the 92-kD collagenase (Wilhelm et al., 1989). That TIMP can modulate tumor invasion is suggested by its ability to prevent tumor cells from traversing amnion in vitro (Thorgeirsson et al., 1982; Mignatti et al., 1986) and from forming metastatic colonies in vivo (Schultz et al., 1988). This inhibitor may also regulate trophoblast invasion. In the presence of TIMP, mouse blastocysts decreased their matrix degradation and increased their parietal endoderm migration (Brenner et al., 1989). In vivo the murine decidua expresses high levels of TIMP, which prescribe the limits of trophoblast invasion (Nomura et al., 1989). In the present study we found that exogenous TIMP and TIMP-2 could completely inhibit cytotrophoblast invasion in vitro, suggesting a possible function for these inhibitors in regulating human cytotrophoblast invasion in vivo. Because human cytotrophoblasts synthesize only low levels of TIMP (Cui, T.-Y., L. Zhang, and S. J. Fisher, unpublished observations), any metalloproteinase inhibitors that function in the placental bed are probably of maternal origin. The decidua may be an important source of inhibitors, since a reduction in decidual reaction leads to extensive trophoblast invasion through the uterine wall, a condition termed placenta accreta.

As to PAIs, only three naturally occurring inhibitors, PAI 1, PAI 2, and proteinase nexin 1, can react rapidly enough to be effective in vivo (Saksela and Rifkin, 1988). Although proteinase nexin 1 has been shown to inhibit the degradation of a smooth muscle cell matrix by fibrosarcoma cells in vitro (Bergman et al., 1986), most of the evidence that any of these inhibitors can regulate ECM breakdown is indirect. For example, transforming growth factor β induces PAI 1 (Laiho et al., 1987) while increasing the pericellular deposition of fibronectin (Ignotz and Massague, 1986; Roberts et al., 1986) and the expression of cell-matrix and cell-cell receptors (Heino et al., 1989; Ignotz et al., 1989). Thus, as with the proteinases themselves, the inhibitors can function as integral components of a pathway, suggesting complex mechanisms of regulation.

The literature on the rate-limiting processes in tumor invasion is complex, which may reflect the heterogeneity in mechanisms used by different tumor types, and even by different cells within a tumor. However, these abnormal processes probably share common mechanisms with invasive processes that occur normally. Our data suggest that the 92-kD type IV collagenase plays an integral role in one such normal process, that of human cytotrophoblast invasion.

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