Adhesive and Degradative Properties of Human Placental Cytotrophoblast Cells In Vitro

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Abstract. Human fetal development depends on the embryo rapidly gaining access to the maternal circulation. The trophoblast cells that form the fetal portion of the human placenta have solved this problem by transiently exhibiting certain tumor-like properties. Thus, during early pregnancy fetal cytotrophoblast cells invade the uterus and its arterial network. This process peaks during the twelfth week of pregnancy and declines rapidly thereafter, suggesting that the highly specialized, invasive behavior of the cytotrophoblast cells is closely regulated. Since little is known about the actual mechanisms involved, we developed an isolation procedure for cytotrophoblasts from placentas of different gestational ages to study their adhesive and invasive properties in vitro. Cytotrophoblasts isolated from first, second, and third trimester human placentas were plated on the basement membrane–like extracellular matrix produced by the PF HR9 teratocarcinoma cell line. Cells from all trimesters expressed the calcium-dependent cell adhesion molecule cell-CAM 120/80 (E-cadherin) which, in the placenta, is specific for cytotrophoblasts. However, only the first trimester cytotrophoblast cells degraded the matrices on which they were cultured, leaving large gaps in the basement membrane substrates and releasing low molecular mass ^{3}H-labeled matrix components into the medium. No similar degradative activity was observed when second or third trimester cytotrophoblast cells, first trimester human placental fibroblasts, or the human choriocarcinoma cell lines BeWo and JAR were cultured on radiolabeled matrices. To begin to understand the biochemical basis of this degradative behavior, the substrate gel technique was used to analyze the cell-associated and secreted proteinase activities expressed by early, mid, and late gestation cytotrophoblasts. Several gelatin-degrading proteinases were uniquely expressed by early gestation, invasive cytotrophoblasts, and all these activities could be abolished by inhibitors of metalloproteinases. By early second trimester, the time when cytotrophoblast invasion rapidly diminishes in vivo, the proteinase pattern of the cytotrophoblasts was identical to that of term, noninvasive cells. These results are the first evidence suggesting that specialized, temporally regulated metalloproteinases are involved in trophoblast invasion of the uterus. Since the cytotrophoblasts from first trimester and later gestation placentas maintain for several days the temporally regulated degradative behavior displayed in vivo, the short-term cytotrophoblast outgrowth culture system described here should be useful in studying some of the early events in human placentation.

FORMATION of the human placenta requires a remarkable series of events involving highly specialized fetal and maternal cells. Once the blastocyst adheres to the uterus, the fetal cytotrophoblast cells rapidly penetrate the endometrium. Soon thereafter, mononuclear cytotrophoblasts and multinuclear syncytiotrophoblasts are found mingled with maternal decidual cells throughout much of the placental bed, a condition that persists during the remainder of the pregnancy (see Fig. 1). In addition, groups of cytotrophoblasts migrate through the decidua, invade the walls of the spiral arterioles, and replace the endothelial lining as far as the myometrial segments of these vessels (Brosens and Dixon, 1966; Boyd and Hamilton, 1970; Ramsey et al., 1976; Tuttle et al., 1985). This invasive activity peaks during the twelfth week of pregnancy and declines rapidly thereafter. The result is formation of the human hemochorial placenta, in which blood from the maternal circulation constantly bathes the fetal chorionic villi.

Although several species have hemochorial placentas, there are important features of human placentation that are unique. First, there is generalized mixing of maternal and fetal cells in the placental bed such that there is no definitive boundary between these components. This is in contrast to the mouse where a well-defined boundary exists between the fetal trophoblast cells and the decidua. Second, human trophoblast invasion is extensive, reaching the first third of

the myometrium. Invasion in even closely related primates is limited to the endometrium (Ramsey et al., 1976). These differences suggest that certain of the mechanisms used by human trophoblast cells to penetrate the uterus are unique. Currently, most of what we know about these processes in vivo has been learned from morphological observations of early human implantation sites recovered surgically (Hertig et al., 1956; O'Rahilly, 1973; Enders, 1976). More recently, additional information has been obtained using in vitro models.

Trophoblast outgrowth models have been used to study certain aspects of the dynamic processes thought to occur during implantation in the rodent. Blastocysts have been cultured on plastic, glass, or collagen (Mintz, 1964; Cole and Paul, 1965; Gwatkin, 1966*a,b*; Sherman and Barlow, 1972; Spindle and Pedersen, 1973; Nilsson, 1974; Sherman, 1975*a,b*). Trophoblast outgrowth on more complex substrates such as extracellular matrices (ECMs)[1] (Glass et al., 1983) and on cell monolayers (Cole and Paul, 1965; Salomon and Sherman, 1975; Sherman and Salomon, 1975; Sherman, 1975*a,b*; Glass et al., 1979) has also been studied. These systems offer the advantage of providing a more biologically relevant, three-dimensional substrate for trophoblast migration. Investigators using such systems have consistently found that the outgrowing cells can invade and migrate through ECMs (Glass et al., 1983), uterine stromal cells (Salomon and Sherman, 1975; Sherman and Salomon, 1975; Sherman, 1975*a,b*; Glass et al., 1979), or various other cell types (Cole and Paul, 1965; Salomon and Sherman, 1975; Sherman and Salomon, 1975; Glass et al., 1979). It has been suggested that the clear halo that sometimes forms slightly ahead of the outgrowth may be the result of cytolytic enzymes (Salomon and Sherman, 1975; Sherman and Salomon, 1975).

Consistent with this interpretation are experiments suggesting that exogenous proteinase inhibitors can prevent blastocyst attachment in vitro (Blackwood et al., 1968). Kubo et al. (1981) used the proteinase inhibitor approach to demonstrate that in the mouse, the process of blastocyst attachment requires a trypsin-like activity and the process of trophoblast outgrowth requires both plasminogen activator and trypsin-like activities. Plasminogen activators have been implicated in developmental and pathological processes that require tissue remodeling and cell migration (Strickland and Beers, 1976; Unkeless et al., 1974; Vassalli et al., 1977; Ossowski et al., 1979). Strickland et al. (1976) found that the trophoblastic cells of cultured mouse blastocysts produced plasminogen activators during the time period corresponding to uterine invasion in vivo. However, it was found (Denker, 1977; Denker and Fritz, 1979) that epsilon-aminocaproic acid, an inhibitor of plasminogen activation, did not inhibit rabbit blastocyst attachment, and Glass et al. (1983) found that the same inhibitor did not prevent matrix degradation by cells of mouse trophoblast outgrowths in vitro. The role of plasminogen activators and other proteinases in human placentation has been much less extensively investigated. Cytotrophoblasts isolated from term human placentas were shown to synthesize a urokinase-type plasminogen activator (Queenan et al., 1987). However, there has been no systematic investigation of the developmental regulation of plasminogen activators or other trophoblast proteinases that might contribute to the observed limitation of trophoblast invasion to early gestation.

In vitro models have also been used to study human trophoblast outgrowth. Hatched, human blastocysts have been cultured on monolayers of human endometrial epithelium. This morphological study suggested that, in contrast to many other species including the mouse, cytotrophoblast cells mediate the initial stages of human implantation (Lindenberg et al., 1986). Work from our laboratory has shown that isolated first trimester human chorionic villi can adhere to and rapidly degrade the ECMs on which they are grown (Fisher et al., 1985). The timetable of invasive behavior observed in vitro paralleled that which occurs in vivo in that second trimester villi could adhere to, but not invade, the same matrices. Morphological evidence suggested that matrix degradation was mediated by cytotrophoblast cells that migrated from the villi.

To demonstrate conclusively that cytotrophoblasts can account for the developmentally regulated degradative behavior of human chorionic villi, we developed a method for isolating cytotrophoblast cells that could be applied to placentas from both first and second trimester. The method of Kliman et al. (1986) was used to isolate cytotrophoblasts from term placentas. Two molecules that, within placental villi, are specific for cytotrophoblasts were used as markers to verify the identity of the isolated cells from all three trimesters. These were cytokeratin and cell-CAM 120/80 (hereafter referred to as E-cadherin [Damsky et al., 1983; Yoshido-Noro et al., 1984; Takeichi, 1988]), a calcium-dependent cell adhesion molecule expressed on differentiated epithelia (also known as uvomorulin [Peyreiras et al., 1983] and in avian cells as L-CAM [Gallin et al., 1983]). Culturing the cells on ECM showed that cytotrophoblasts from first, but not second and third, trimester placentas could degrade a complex ECM substrate. The stage-specific behavior of these cells suggests that this short-term culture system will be useful in the study of some of the adhesive and invasive mechanisms required for successful human placentation. As an example of the use of this system, we compared the repertoire of gelatin-degrading proteinases produced by invasive, early gestation cytotrophoblasts with those produced by noninvasive, later gestation cytotrophoblasts and by first trimester human placental fibroblasts. We found that several gelatin-degrading metalloproteinases were uniquely expressed by first trimester cytotrophoblasts.

Materials and Methods

Isolation and Culture of Human Cytotrophoblast Cells

We investigated various methods for isolating cytotrophoblasts, including published procedures used for either first (Cotte et al., 1980) or third trimester (Kliman et al., 1986; Nelson et al., 1986) placentas. We found that no single method could be applied successfully to placentas from all stages because of the changes in tissue organization and increases in connective tissue as gestation proceeds. However, we devised an isolation procedure, described below, that could be applied with minimal changes to both first and second trimester placentas.

First and second trimester human placentas were obtained immediately after vacuum aspiration, and the chorionic villi were prepared as previously described (Fisher et al., 1985). The isolation of first and second trimester cytotrophoblast cells from villi has been described in detail elsewhere

1. *Abbreviation used in this paper*: ECM, extracellular matrix.

(Fisher et al., 1989). Briefly, the washed villus pellet was incubated for either 20 (first trimester) or 30 min (second trimester) (5:1, vol/wt/wt) in enzymatic dissociation solution I (PBS containing 500 U/ml collagenase [type IV; Sigma Chemical Co., St. Louis, MO], 200 U/ml hyaluronidase [type I-S; Sigma Chemical Co.], 0.2 mg/ml DNase [type IV; Sigma Chemical Co.], and 1 mg/ml BSA). The villi were separated from the supernatant, which contained the syncytium, by centrifugation and incubated for 10 min in dissociation solution II (PBS containing 0.25% trypsin [type XIII; Sigma Chemical Co.], 2 mM EDTA, and 0.2 mg/ml DNase). The dissociated cells were isolated by centrifugation, resuspended in 4 ml of medium containing 10% FCS, and layered over a preformed Percoll gradient made up in Hanks' balanced salt solution according to the method of Kliman et al. (1986). The gradient was centrifuged (1,000 *g*) for 25 min at room temperature, after which a broad band in the middle of the tube containing the cytotrophoblast cells was removed. The cells were washed several times and resuspended in MEM D-valine medium (Gilbert and Migeon, 1975) containing either 20% dialyzed FCS or 2% Nutridoma, 1% glutamine, and 50 μg/ml gentamicin. 1 ml, containing 5×10^5 cytotrophoblasts, was added per 15-mm tissue culture well. In most cases either the wells or coverslips (12 mm diameter) placed within the wells were coated with an ECM produced by PF HR9 cells as previously described (Fisher et al., 1985). Under these conditions, the cells adhered to produce a confluent monolayer within 4 h. When desired, labeled matrix was produced by incubating the PF HR9 cells with 10 μCi/ml [^{3}H]proline, [^{3}H]glucosamine, or [^{3}H]leucine (30, 30, and 140 Ci/mM, respectively). After 48 h, the ^{3}H-medium was removed and the cells were incubated in unlabeled medium for an additional 48 h before the ECM was isolated.

When this method was applied to term placentas, the yield of cells was very low. Therefore, to isolate term cytotrophoblasts, the washed villus pellet was subjected to three cycles of trypsinization (dissociation solution II) and the resulting cells were purified on a Percoll gradient as described above (Kliman et al., 1986). Yields per gram of first, second, and third trimester placentas were 1, 0.5, and 0.25×10^6 cells, respectively.

We used the high-speed cell sorter at the Lawrence Livermore National Laboratory (Livermore, CA) (Peters et al., 1985) to analyze the purity of the cytotrophoblasts prepared as described above from both first and second trimester placentas. The large size of the cytotrophoblast cells (20–40 μm) and the relatively large volume of the cell occupied by the nucleus made the cytotrophoblast cells readily distinguishable from other possible contaminants such as lymphocytes (10–12 μm). Sorting experiments using light scatter as the method of detection, showed that <1% of the cells in either the first or second trimester preparations displayed the morphological characteristics of lymphocytes. Sorting experiments using fluorescence as the method of detection and the PKK1 anticytokeratin antibody, which within the placental villi stains only trophoblasts, showed that >95% of the cells in preparations of first and second trimester cytotrophoblasts were cytokeratin positive.

Culture of Human Placental Fibroblasts and Choriocarcinoma Cells

Human fibroblasts were isolated from first trimester placentas by further trypsin treatment (dissociation solution II, 30 min at 37°C) of the chorionic villus connective tissue after the removal of cytotrophoblasts. The resulting cells were resuspended in DME H16 containing 10% FCS, 1% glutamine, and 50 μg/ml gentamicin and plated onto 75-mm^2 tissue culture flasks (Costar, Cambridge, MA). The cells were passaged at least six times before plating on ^{3}H-labeled PF HR9 matrices. The JAR human choriocarcinoma cell line (Patillo et al., 1971) was grown in DME H16 containing 10% FCS, 1% glutamine, and 50 μg/ml gentamicin. The BeWo human choriocarcinoma cell line (Patillo and Gey, 1968) was grown in Ham's F-12 medium containing 10% FCS, 1% glutamine, and 50 μg/ml gentamicin. These cell lines were plated on 15-mm tissue culture wells containing ^{3}H-labeled PF HR9 matrix-coated coverslips. The densities required for the cells to reach confluence within 12 h were 5, 4, and 2.5×10^5/ml for human placental fibroblasts, JAR cells, and BeWo cells, respectively.

Preparation of Cultured Cells and Placental Tissue for Immunocytochemistry

Matrix-covered coverslips with cytotrophoblasts were washed once in serum-free medium and fixed either for 5 min in methanol (−20°C) or 10 min in 3% paraformaldehyde in calcium-containing PBS as described by Damsky et al. (1985).

Frozen sections were prepared from first trimester human chorionic villi and from samples of the first trimester human placental bed obtained immediately after vacuum aspiration and curettage, respectively. The tissues were fixed for 30 min with 3% paraformaldehyde in calcium-containing PBS, pH 7.2. After incubation in 0.01 M glycine to quench unreacted aldehyde groups and infiltration with 15% sucrose, the tissues were embedded in OCT (Miles Scientific Div., Naperville, IL) and frozen in liquid nitrogen. Sections (5 μm) were cut using an HR cryostat (Slee International Inc., Tiverton, RI) and collected on 22-mm^2 coverslips. Before staining, the sections were washed for 10 min each in PBS and in PBS containing 0.2% BSA.

Immunocytochemistry

Coverslips with either cultured cytotrophoblasts or sections of placental tissue were exposed to affinity-purified E9 rat monoclonal IgG against the 80-kD fragment of the calcium-dependent cell–cell adhesion molecule, E-cadherin (Damsky et al., 1983; Wheelock et al., 1987). This antigen is present on differentiated epithelia but not on connective tissue, endothelia, muscle, or blood cells. In the fetal chorionic villi it was, therefore, specific for cytotrophoblasts. With regard to the maternal components of the placental bed, it was expressed by uterine epithelium but not by the stroma, decidua, or cellular elements of the spiral arterioles. Coverslips were also stained with a mouse monoclonal antibody, PKK1 (Lab Systems, Chicago, IL), against a broadly distributed epitope of cytokeratin. Cytokeratins are expressed by the epithelial, but not the stromal or vascular, elements of tissues. Decidual cells also do not express cytokeratins. Thus, the anticytokeratin could be used as an additional marker for cytotrophoblasts in the placental bed.

Coverslips were incubated for 60 min with E9 and PKK1 either singly or in combination and then washed. Both fluorescence and peroxidase detection procedures were used to localize the primary antibodies. In the former case, rhodamine-conjugated goat anti-rat IgG (for E9) and fluorescein-conjugated goat anti-mouse IgG (for PKK1; both antibodies were from Cooper Biomedical, Inc., West Chester, PA) were used at a dilution of 1:100. In the latter case, samples were exposed to 1:100 dilutions of biotinylated goat anti-rat or goat anti-mouse antibodies (Sigma Chemical Co.) followed by a 1:100 dilution of streptavidin-peroxidase (Amersham Corp., Arlington Heights, IL). Fluorescently labeled samples were examined with an epifluorescence-phase microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 63× planapo phase lens (1.5 NA) and the appropriate filters to detect both rhodamine and fluorescein. Fluorescently labeled samples were photographed with Tri X film and peroxidase-labeled samples with bright field optics using Panatomic X film (Eastman Kodak Co., Rochester, NY).

Hormone Analyses

A radioimmunoassay was used to measure human chorionic gonadotropin secretion by the trophoblast cells. The culture medium was assayed directly, without prior fractionation (Hussa, 1987). Progesterone was also measured directly, using a radioimmunoassay (DeVilla et al., 1972). Both assays were performed by the Reproductive Endocrinology Laboratory, Department of Obstetrics, Gynecology and Reproductive Biology, University of California at San Francisco (San Francisco, CA).

Assessment of Matrix Degradation

Cell cultures were examined daily and photographed using a microscope (inverted phase IM; Carl Zeiss, Inc.). The formation of circular areas cleared of matrix was monitored and the experiments were stopped when the retraction of first trimester cytotrophoblast cells from holes in the matrix substrate left approximately half the surface of the culture dish as bare plastic (usually day 4).

We also monitored the ability of first, second, and third trimester cytotrophoblast cells to release into the medium ^{3}H-labeled components from the matrices on which they were grown. Cytotrophoblast cells were plated on the labeled matrices at a density that resulted in formation of a confluent monolayer overnight. The entire volume (1 ml) of culture medium was removed and replaced with fresh medium at either 24- or 48-h intervals. An aliquot (200 μl) of each sample of medium was counted in a scintillation counter (LS 1801; Beckman Instruments, Inc., Fullerton, CA). To determine whether other placental cells degraded matrix substrates, choriocarcinoma cells (BeWo and JAR) and placental fibroblasts were also plated on ^{3}H-labeled matrices. To estimate the rate of spontaneous release of ^{3}H-matrix components, some wells contained ^{3}H-labeled matrices but no cells.

The size of the ^{3}H-labeled matrix components released into the medium by the first trimester cytotrophoblast cells was estimated by gel filtration

chromatography. The remaining 800 μl of medium from these cultures was boiled, acidified, and chromatographed on a 10 × 1-cm column of Sephadex G-50 (fine) eluted with 0.1 M pyridine acetate buffer, pH 5.3. Fractions (0.5 ml) were collected and assayed for radioactivity as described above.

Substrate Gels

We used SDS-polyacrylamide gels containing 1 mg/ml gelatin (type A from porcine skin; Sigma Chemical Co.; Heussen and Dowdle, 1980; McKerrow et al., 1985; Chin et al., 1985) to analyze the changes in trophoblast-associated and secreted gelatin-degrading proteinases that occurred during gestation. For this purpose, cytotrophoblasts and placental fibroblasts were cultured for either 24, 48, or 72 h on plastic tissue culture wells (15 mm) coated with either fibronectin (Collaborative Research, Lexington, MA), laminin (the gift of Dr. Deborah Hall, University of California at San Francisco, San Francisco, CA), or type IV collagen (Collaborative Research). For these experiments, the medium contained 2% Nutridoma-HU (Boehringer Mannheim Biochemicals, Indianapolis, IN) but no FCS. To control for possible exogenous proteinase contamination in the medium or in the protein substrates, some wells contained medium but no cells. Cell-associated and secreted proteinases from ten different preparations of cytotrophoblast cells from all three trimesters were analyzed. The cultures were rinsed three times in PBS and then scraped into Laemmli sample buffer which contained neither reducing agents nor bromphenol blue. The cell extracts were homogenized and centrifuged (1,000 *g*) to remove insoluble material. An aliquot of conditioned medium from the same culture, containing the material released during the previous 24 h, was also solubilized in sample buffer. None of the samples were heated and all were stored on ice until they were applied to the substrate gels (always <1 h). An aliquot of the cell and the medium extracts was removed for protein analysis (Lowry et al., 1951), after which the samples were adjusted to a final concentration of 1 μg protein/μl sample buffer. Routinely, 10 μl was applied to each lane. After electrophoresis the SDS was removed by incubating the gel in 2.5% Triton X-100 (30 min). The gels were incubated 16 h at 23°C in calcium-containing PBS, stained with Coomassie blue, and then destained in 10% acetic acid until the cleared bands were visible and the stacking gel was totally destained.

The class(es) to which the gelatin-degrading enzymes belonged was determined by assaying the effects of inhibitors on proteinase activity. To do so, inhibitors (obtained from Sigma Chemical Co. unless otherwise indicated) of the following classes were used in the concentrations shown: serine proteinase inhibitors—PMSF (2 and 10 mM), epsilon aminocaproic acid (10 and 100 mM), and *p*-nitrophenyl-*p'*-guanidobenzoate (0.2 mM; ICN Pharmaceuticals, Cleveland, OH); metalloproteinase inhibitors—EDTA (1 mM) and 1,10-phenanthroline (0.3 mM); aspartic proteinase inhibitor—pepstatin A (1 mM); and cysteine proteinase inhibitors—trans-epoxy-L-leucyl-amido (4-guanidino) butane (10 and 50 μM), and leupeptin (1 mM; Vega Biotechnologies, Tucson, AZ). In all cases, the inhibitors were added to the buffer in which the gels were incubated. The inhibitors *p*-nitrophenyl-*p'*-guanidobenzoate and PMSF were also included in the sample buffer for solubilizing the cells and medium, and the samples were incubated for 30 min (4°C) before electrophoresis. If it was necessary to dissolve the inhibitor in either dimethyl sulfoxide (e.g., *p*-nitrophenyl-*p'*-guanidobenzoate) or ethanol (e.g., PMSF), control samples and gels were incubated with the solvent alone in the final concentration that had been introduced into either the sample or the incubation buffer.

Results

E-Cadherin and Cytokeratin Are Markers for Human Cytotrophoblast Cells In Situ

The two types of chorionic villi found at the maternal-fetal interface, floating and anchoring villi, are illustrated in Fig. 1. The freely floating villi (Fig. 1 *A*) are constantly bathed by maternal blood and are not directly connected to the uterus. Anchoring villi (Fig. 1 *B*) are embedded in the uterus and attach the fetus to the mother. In floating villi the cytotrophoblast cells lie beneath the syncytiotrophoblastic covering and are separated from the underlying connective tissue stroma of the villus core by a basement membrane. These cytotrophoblast cells fuse to form the overlying syncytium and divide to maintain the complete cytotrophoblast layer present in first trimester chorionic villi. As pregnancy continues, fusing of these cells to form the trophoblast syncytium gradually depletes this population of cytotrophoblast cells, possibly because of a decrease in their rate of division. Thus, this layer is incomplete by the second trimester of pregnancy and composed of isolated, single cells at term. The anchoring villi contain an additional population of cytotrophoblasts that adhere to the uterine epithelium, migrate into the endometrium, and invade the spiral arterioles. For the fetal portion of the placenta to be expelled from the uterus, these cellular anchoring connections must be severed.

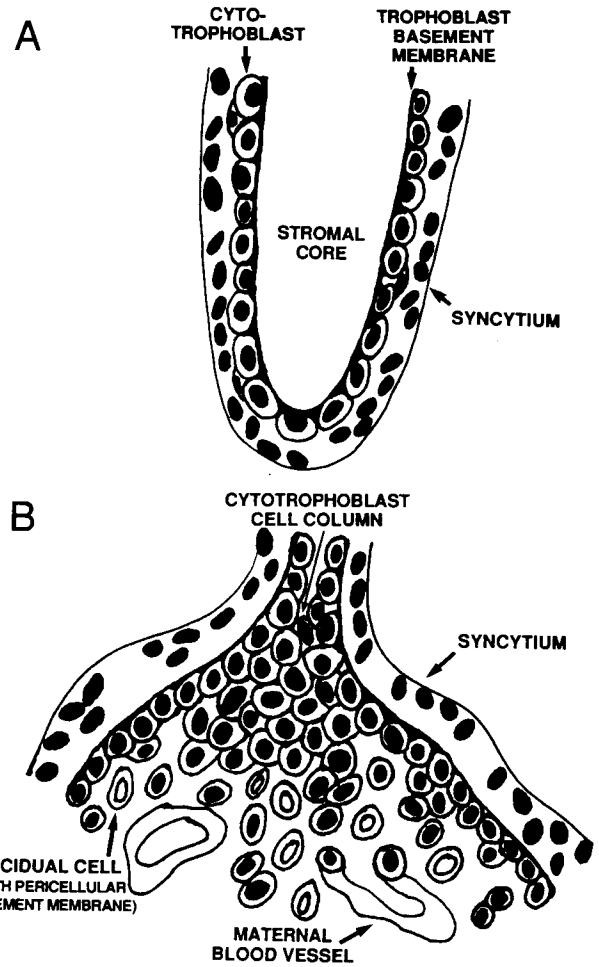

Figure 1. Anatomy of the maternal-fetal interface depicting the floating and anchoring chorionic villi of the human placenta. Cytotrophoblasts located in floating villi (*A*) lie beneath the trophoblast syncytium and are not directly exposed to maternal blood. Cytotrophoblasts of the anchoring villi (*B*) lie beneath the syncytium and extend through the endometrium to line the spiral arterioles, thus attaching the fetus to the uterus.

In cross sections of floating chorionic villi from first trimester human placentas (Fig. 2) the cytotrophoblast cell layer is located between the stromal core of the villus and the syncytiotrophoblast covering. The monoclonal antibody E9, against the 80-kD fragment of E-cadherin, stains only the cells of this cytotrophoblast layer and is confined to areas of cell-cell contact (Fig. 2 *A*). When a monoclonal antibody (PKK1) specific for cytokeratins was used to stain sections of

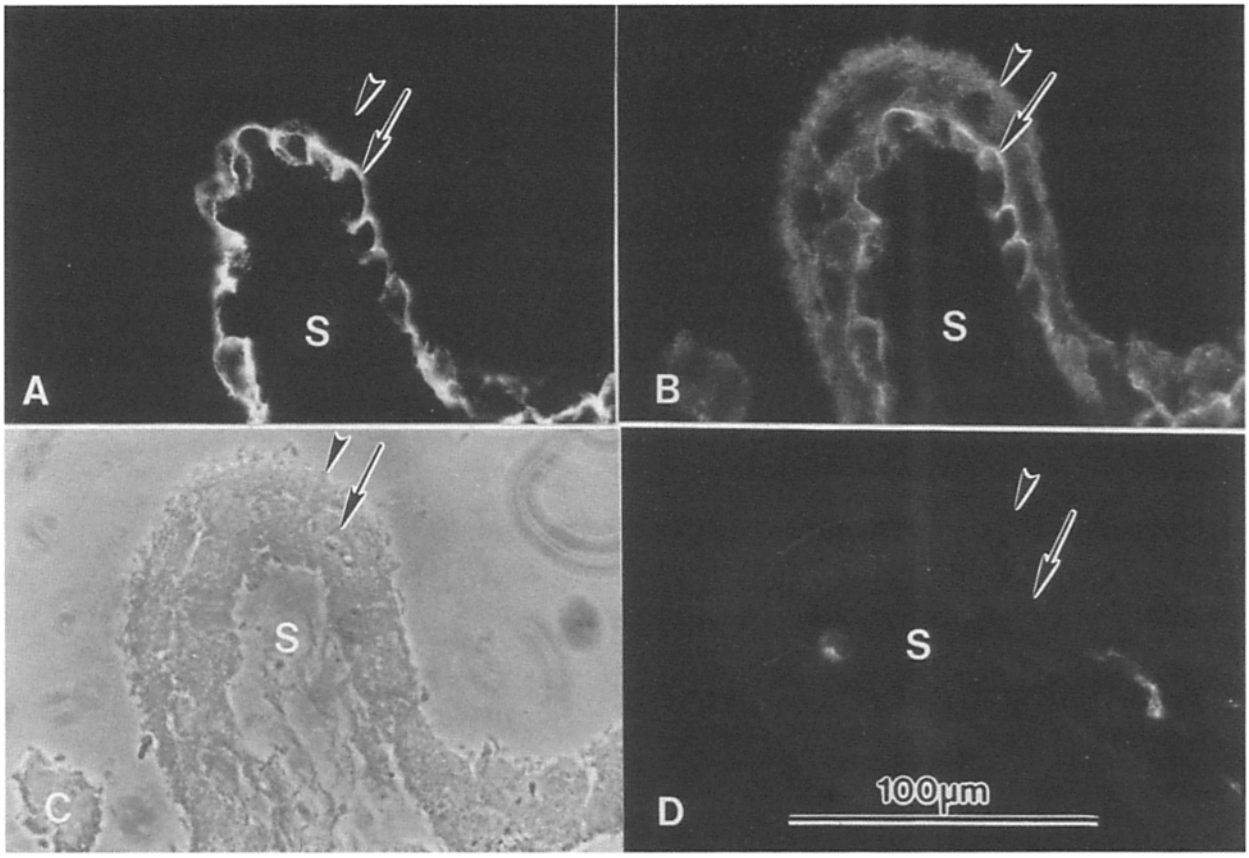

Figure 2. Immunofluorescence staining of cytotrophoblast cells in placental villus using the E9 rat monoclonal antibody against E-cadherin (cell-CAM 120/80) and cytokeratins (PKK1). The same section was stained with E9 (*A*) and with PKK1 (*B*). (*C*) A phase micrograph of the same field. (*D*) A similar section stained with nonimmune rat IgG and goat anti-rat rhodamine. Arrowheads point to the syncytiotrophoblast layer. Arrows point to the cytotrophoblast layer. *S*, stromal core of the placental villus.

floating villi (Fig. 2 *B*), a similar pattern of staining was obtained except that this antibody also reacted with the trophoblast syncytium.

Tissue containing the invasive population of cytotrophoblast cells that had migrated into the uterus was obtained by biopsy of the placental bed (Figs. 3 and 4). Clusters of cytotrophoblast cells are important components of the anchoring villi, which are embedded in the endometrium and anchor fetal tissues to the uterus. These cytotrophoblasts are found mixed with maternal decidual cells throughout the placental bed and extend into the myometrial layer of the uterus. The cytotrophoblast cells of the placental bed stained with the anti-E-cadherin monoclonal antibody E9 (Fig. 3 *A*). A similar pattern of staining was obtained when placental bed biopsies were incubated with a monoclonal antibody (PKK1) specific for cytokeratins (Fig. 3 *B*). The large cells with pale nuclei, probably decidual cells, are not stained with either antibody. When a section of the placental bed was stained with both antibodies the same subset of cells was identified (Figs. 4, *A* and *B*). Thus, both E-cadherin and cytokeratin are useful antigens to identify cytotrophoblasts in complex cell mixtures.

Characterization of Isolated Cytotrophoblasts

When first trimester cytotrophoblasts were plated onto 15-mm tissue culture wells containing 12-mm glass coverslips coated with the ECM synthesized by the PF HR9 teratocarcinoma cell line, the cells preferentially attached to the matrix-coated coverslips. Few, if any, adhered to the uncoated tissue culture plastic, which was exposed at the perimeter of the well (Fig. 5 *A*). Since the E9 anti-E-cadherin antibody stained cytotrophoblast cells in both the chorionic villi and in the placental bed, this antibody was used to confirm that the cells isolated and cultured from human placentas were cytotrophoblasts. Fig. 5 *B* shows a region of a cytotrophoblast monolayer that was stained with E9 after 24 h in culture. Fig. 5 *C* shows a phase-contrast micrograph of the monolayer shown in Fig. 5 *B*. As was observed when chorionic villi were incubated with the same antibody (Fig. 2 *A*), the antigen was localized to areas of cell-cell contact. Identical results were obtained when cells isolated from second and third trimester human placentas were stained with E9 (data not shown). These experiments confirmed that the cells obtained from all trimesters and cultured on ECMs were cytotrophoblasts.

Hormone synthesis by the cytotrophoblast cells was also characterized. Between 48 and 72 h in culture, a confluent monolayer of first trimester cytotrophoblast cells plated on a 12-mm coverslip secreted a total of 4,000 mIU of human chorionic gonadotropin and 30 ng of progesterone/ml of medium. During the same time period second trimester

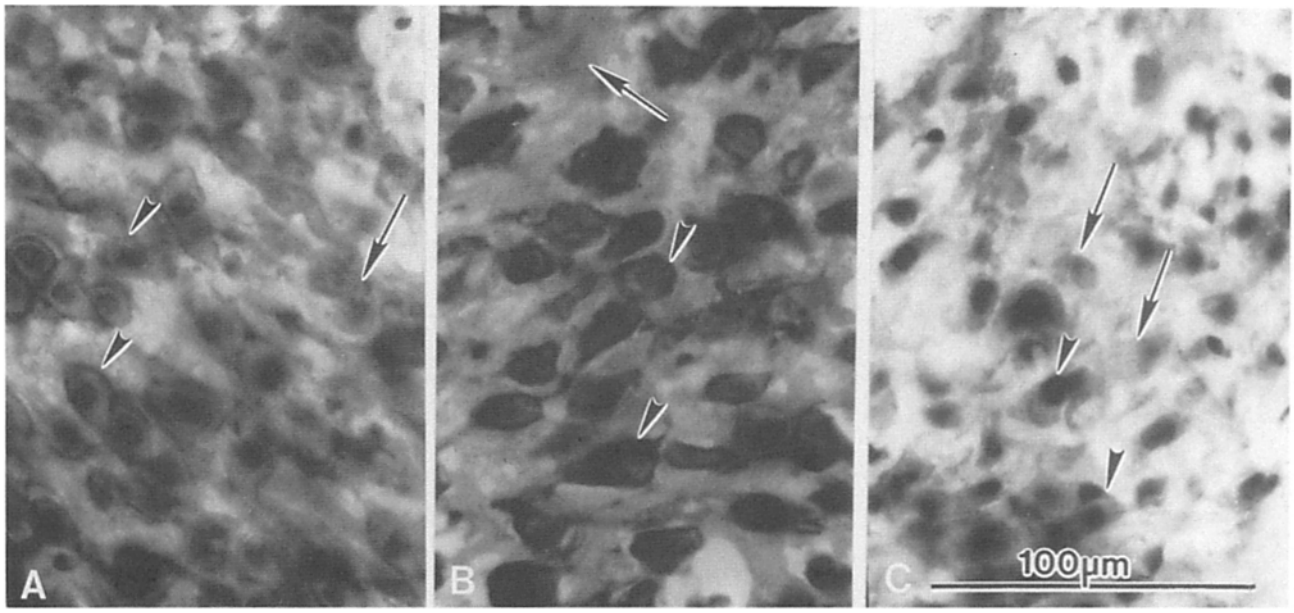

Figure 3. Immunoperoxidase staining of cytotrophoblast cells in placental bed biopsies using antibodies against E-cadherin (E9) and cytokeratins (PKK1). (*A*) A large number of E9-positive cells are present in the placental bed. Unstained cells are likely to be components of the decidua. (*B*) A staining pattern similar to that shown in *A* was obtained when PKK1 was used to stain the placental bed. (*C*) As a control, nonimmune rat IgG was used in place of the primary antibody. Arrowheads point to cytotrophoblasts. Arrows point to decidual cells.

cytotrophoblast cells cultured under the same conditions synthesized 300 mIU of human chorionic gonadotropin and 80 ng of progesterone/ml of medium.

First Trimester Cytotrophoblasts Degrade ECMs In Vitro

The behavior of cytotrophoblasts cultured from first trimester human placentas was markedly different from that of cytotrophoblasts cultured from placentas of the second trimester onward. When first trimester cytotrophoblast cells were plated as a confluent monolayer in either serum- or Nutridoma-containing medium, small, usually circular, holes in both the monolayer and the matrix substrate appeared after the first 24 h in culture (Fig. 6 *A*). The absence of matrix in these holes was verified by scanning electron microscopy. Such gaps formed only when the cells were plated as a monolayer and were never observed in more sparse cultures. In ad-

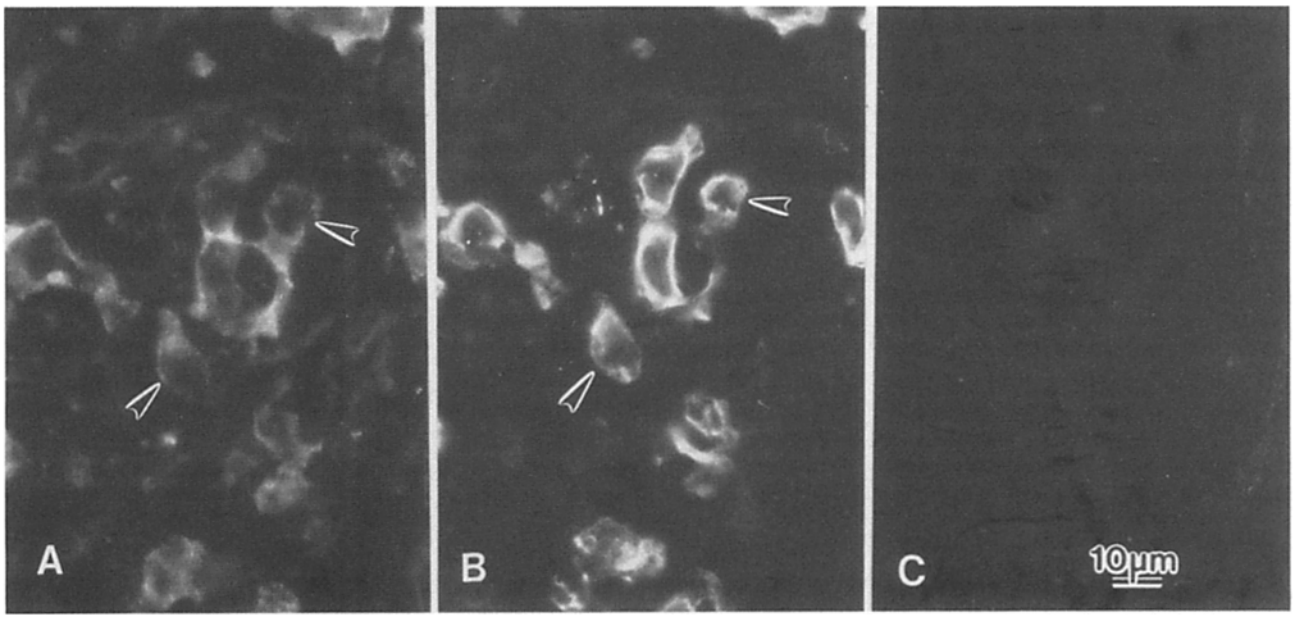

Figure 4. Anti-E-cadherin and anticytokeratin antibodies identify the same cells in placental bed biopsies. The same frozen section was stained with E9 (*A*) and PKK1, an anticytokeratin antibody (*B*). (*C*) An adjacent section stained with nonimmune rat IgG and goat anti-rat rhodamine.

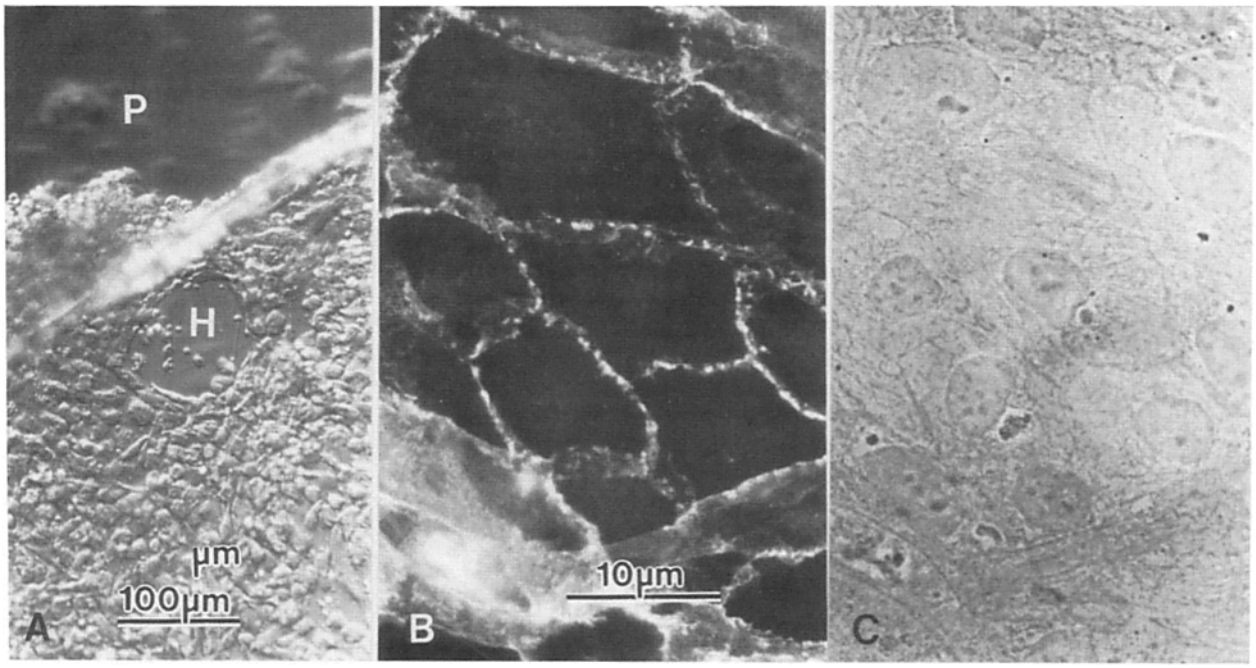

Figure 5. Isolated first trimester cytotrophoblasts cultured for 24 h on PF HR9 ECM. (*A*) Cells attached and spread on the matrix-coated coverslip, but failed to adhere to the uncoated tissue culture plastic (*P*). A small hole (*H*), cleared of matrix, is visible at the left edge of the coverslip. (*B*) A portion of the monolayer stained with E9 after 24 h in culture. (*C*) A phase-contrast micrograph of the monolayer taken at higher magnification.

dition, only a subpopulation of the cells appeared to be involved in the initial process of rapid clearing since islands of cells that retained the morphologic characteristics of an intact monolayer were always present in these cultures. During the next 24 h the holes rapidly expanded and coalesced until these cleared areas occupied a major portion of the tissue culture well (Fig. 6 *B*). After 72 h, significant expansion of the existing holes usually stopped and no new holes formed in the discrete islands that remained of the original cell monolayer. Since cytotrophoblasts adhered poorly in the absence of matrix, they accumulated at the periphery of the cleared areas. In contrast, when cytotrophoblasts isolated from second trimester (Fig. 6 *C*) onward were plated on matrix, no holes in the monolayer were seen, even after 1 wk

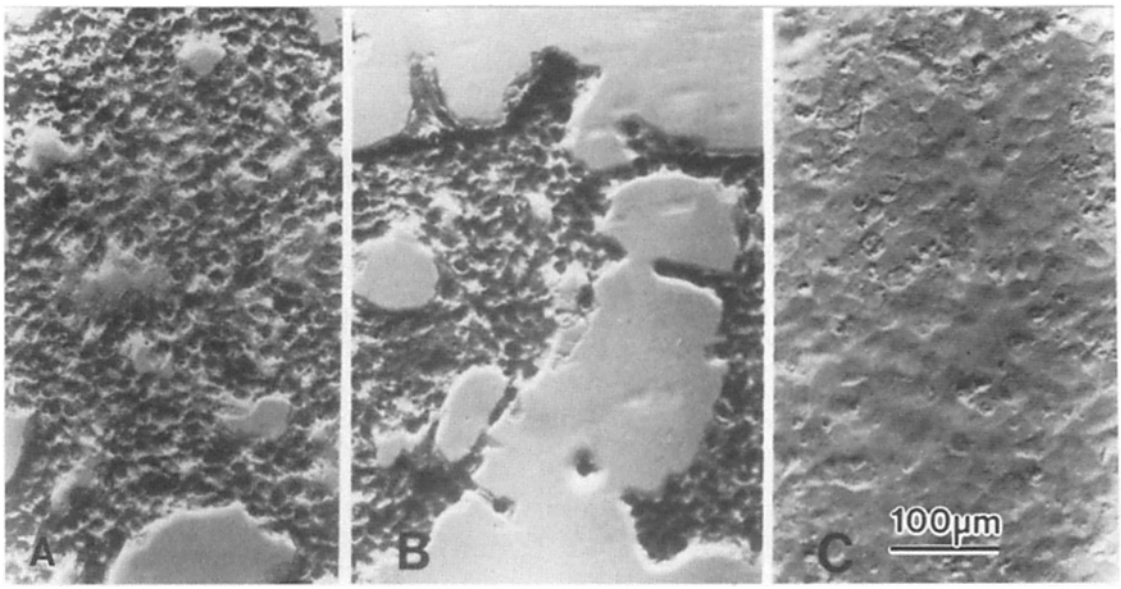

Figure 6. Cytotrophoblast degradation of the matrix substrate. First trimester human cytotrophoblast cells were plated under the conditions required to produce a confluent monolayer overnight. After 24 h, small circular areas devoid of both matrix and cells appeared (*A*) and were rapidly expanded during the next 48 h in culture (*B*). Second trimester human cytotrophoblast cells (*C*) did not invade the matrix substrates on which they were cultured and remained as a confluent monolayer even after 72 h in culture.

in culture. Together, these results suggest that human cytotrophoblast cells retain in vitro the stage-specific degradative behavior they exhibit in vivo.

To verify that the cells at the sites of actual matrix clearing were cytotrophoblasts, first trimester cultures in the initial phases of degrading the matrices on which they were plated were stained with the anti-E-cadherin antibody E9. As the gaps enlarged (Fig. 7) the free edges of the cells bordering the hole no longer expressed E-cadherin. In addition, the morphology of the plasma membrane surface lacking the antigen became highly convoluted and bright points of fluorescence were often visible intracellularly. E9 staining was still retained at the points where these cells contacted other cytotrophoblasts. At the same time cells located near, but not directly adjacent to, the developing holes retained their connections with other cytotrophoblasts, and the distribution of E9 staining in these areas was identical to that seen in the original, intact monolayer. These results indicated that the cells at the sites of matrix degradation were cytotrophoblasts, and that matrix degradation was accompanied by a loss of E-cadherin at the convoluted cell borders facing the matrix-free holes.

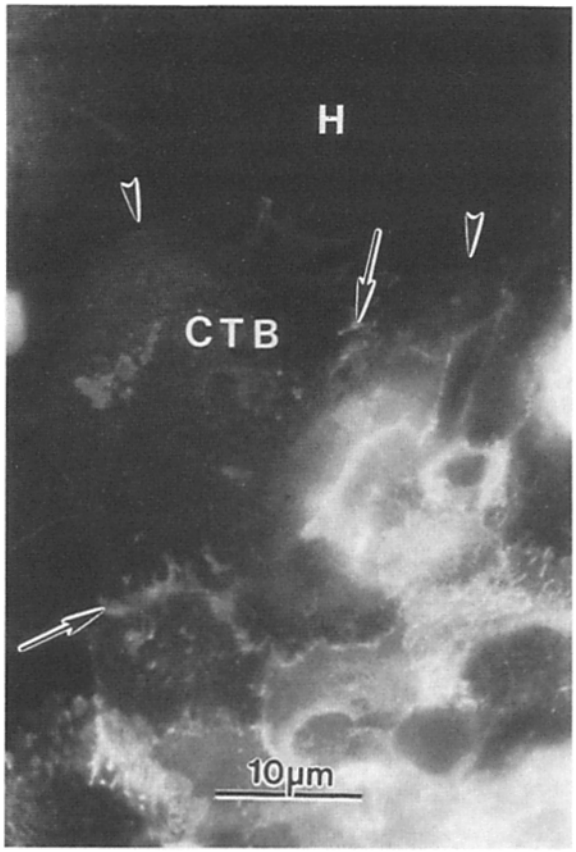

Figure 7. Distribution of E-cadherin in cells associated with matrix dissolution. During the initial stages of matrix clearing, the cells immediately adjacent to the hole retain E-cadherin at their points of contact with other cells. However, the regions of the cell surface facing the hole are frequently devoid of E-cadherin staining and are highly convoluted. Arrows point to areas of cell–cell contact. Arrowheads mark edge of cell bordering the hole. *H*, hole; *CTB*, cytotrophoblast.

Degradation of ^{3}H-Labeled PF HR9 Matrix by Cytotrophoblasts, Placental Fibroblasts, and Human Choriocarcinoma Cells

Cytotrophoblasts were plated on ^{3}H-labeled matrices to confirm that formation of holes in confluent monolayer cultures of first trimester cells was indicative of active matrix degradation and not solely the result of mechanical penetration. The experiment was repeated six times using matrices labeled with [^{3}H]glucosamine, [^{3}H]proline, and [^{3}H]leucine. In each case at least half the total radioactivity incorporated into the matrix was solubilized by the first trimester cytotrophoblast cells during the first 3 d in culture. However, the absolute counts released varied among experiments due to differences in ^{3}H-label incorporation into the matrix. Figs. 8 and 9 summarize the results from one such experiment. Fig. 8, *A–C*, respectively, shows the pattern of release into the medium of [^{3}H]glucosamine-, [^{3}H]proline-, and [^{3}H]leucine-labeled matrix components as a function of time in culture. During the first 3 d, first trimester cytotrophoblast cells rapidly solubilized both protein- and carbohydrate-containing matrix components. Chromatography on Sephadex G-50 of the ^{3}H-labeled matrix components released into the medium (Fig. 9) showed that the solubilized components had been extensively degraded. At least half of the protein ([^{3}H]proline and [^{3}H]leucine) fragments that were released by first trimester cytotrophoblast cells were in a low molecular mass form (<2,500 D). In comparison, the [^{3}H]glucosamine components found in the medium were not as extensively degraded. Only a third of these ^{3}H-labeled fragments entered the column, and their average molecular mass was greater than that of the labeled protein fragments. These results suggest that first trimester cytotrophoblast cells are able to substantially degrade the ECM substrates on which they are plated. In addition, the time course of this activity paralleled the appearance of holes in the first trimester cytotrophoblast monolayers.

Second and third trimester cytotrophoblast cells showed a greatly reduced ability to liberate glucosamine-containing matrix material. The cumulative release of [^{3}H]glucosamine components by cells isolated from a 15-wk human placenta was ~30% of that released by first trimester cells (Fig. 8 *A*). Similar results were obtained with [^{3}H]proline (Fig. 8 *B*) and [^{3}H]leucine (Fig. 8 *C*) matrix components. Cells isolated from later second trimester (data not shown) and third trimester placentas showed degradation levels similar to that of the early second trimester cytotrophoblasts. Thus, cytotrophoblast-associated degradative activity was reduced substantially early in the second trimester.

To determine whether the ability to degrade matrix substrates was a unique characteristic of first trimester human cytotrophoblast cells, first trimester placental fibroblasts and human choriocarcinoma cell lines BeWo and JAR were also plated on radiolabeled PF HR9 ECMs (Fig. 8). For all the radiolabeled matrices tested, the release of radioactivity by all three cell types was either comparable with or less than that of the second trimester cytotrophoblast cells. In addition, daily observation of the cultures using phase-contrast microscopy showed that no gaps formed in any of the fibroblast or choriocarcinoma monolayer cultures.

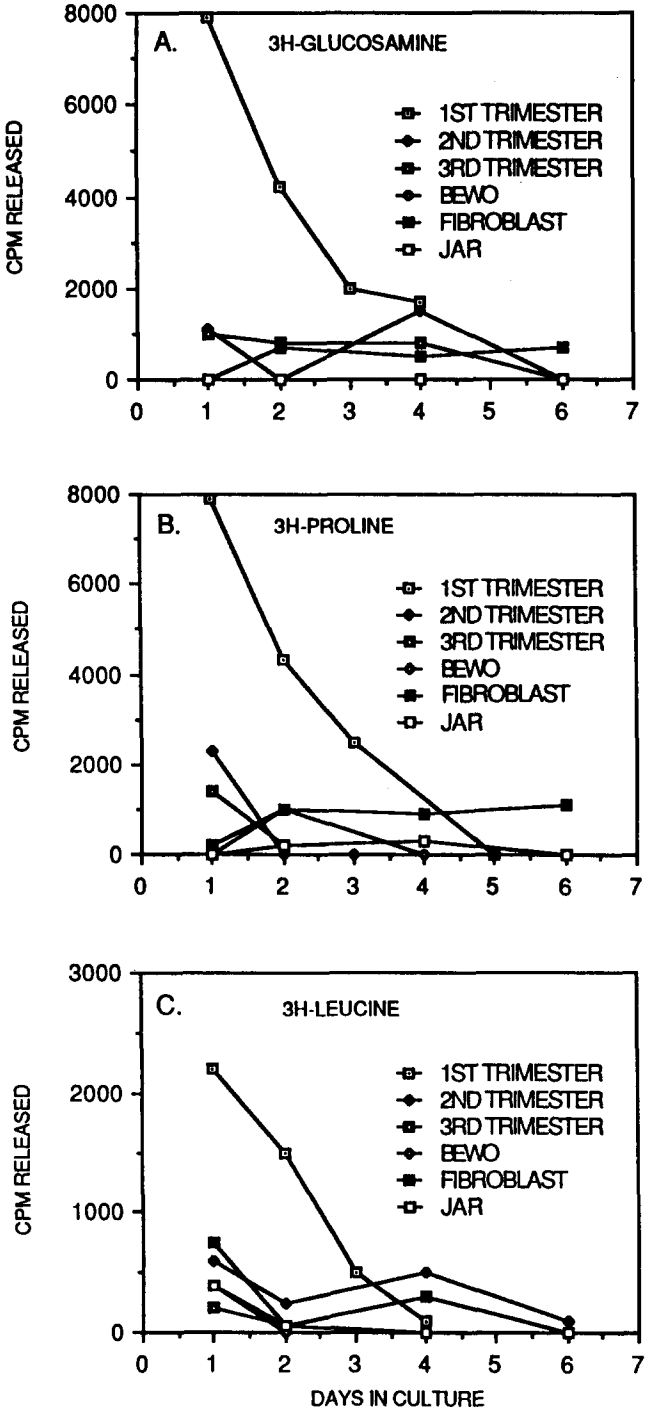

Figure 8. Degradation of ^{3}H-labeled PF HR9 matrix by cytotrophoblasts, placental fibroblasts, and the human choriocarcinoma cell lines BeWo and JAR. Counts released daily from (*A*) [^{3}H]glucosamine-, (*B*) [^{3}H]proline-, and (*C*) [^{3}H]leucine-labeled matrices are shown. Each data point is the mean of the ^{3}H-matrix components released into the medium from three wells. The bars represent the standard error of the mean. Each data point was corrected for the spontaneous release of labeled matrix components by subtracting the mean of the counts released into the medium from three wells that contained ^{3}H-labeled matrices but no cells.

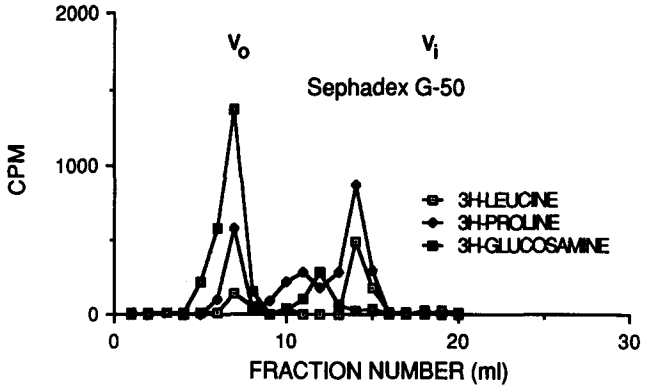

Figure 9. Degradation of ECM by first trimester human cytotrophoblast cells. The culture medium from first trimester cells grown for 3 d on ^{3}H-labeled PF HR9 matrices was pooled and chromatographed on Sephadex G-50. Medium from wells that contained ^{3}H-matrices but no cells was also chromatographed on Sephadex G-50. The counts per minute for each control fraction was subtracted from the experimental fraction to correct for the spontaneous release of labeled matrix components. V_o, void volume; V_i, inclusion volume.

Analysis of Cytotrophoblast-associated and Released Proteinases

We used substrate gels copolymerized with gelatin to determine whether the stage-specific degradative activity of first trimester human cytotrophoblasts coincided with the expression of a unique set of proteinase activities by these cells (Fig. 10). For this purpose the cytotrophoblasts were cultured in serum-free medium containing 2% Nutridoma on either fibronectin, laminin, or type IV collagen—all components of the PF HR9 matrix (Fisher et al., 1985)—rather than on the matrix itself. This was necessary since preliminary analysis on gelatin-containing substrate gels of PF HR9 matrices cultured in Nutridoma-containing medium in the absence of cells showed the presence of proteinase activities in the 60–100-kD region of the gel. No proteinase activities were associated with defined substrates or Nutridoma-containing medium used in the experiments described below.

Cell extracts prepared from first trimester cytotrophoblasts plated on fibronectin had a complex pattern of gelatin-degrading proteinases ranging from 68 to >200 kD (Fig. 10 *A*). The proteinase patterns were identical when the cells were plated on laminin or type IV collagen (data not shown). Cytotrophoblasts isolated from second and third trimester placentas displayed much simpler patterns of proteinase expression. A single major gelatin-degrading proteinase, of relative molecular mass 92 kD, and minor, higher molecular mass activities were detected in second trimester cells. By term, the activity of the 92-kD proteinase was significantly reduced. We consistently found that the molecular mass of the proteinase in the 90-kD region of the first trimester cytotrophoblast extracts was slightly lower than that detected in the second trimester and term cells. Thus, several proteinases were present in first trimester cells and absent in later stages. This marked change occurred rapidly between the first and second trimesters. The only exception was a high molecular mass (>200-kD) activity that was present in extracts of cytotrophoblast cells from all three trimesters. Extracts of first trimester human placental fibroblasts had a sim-

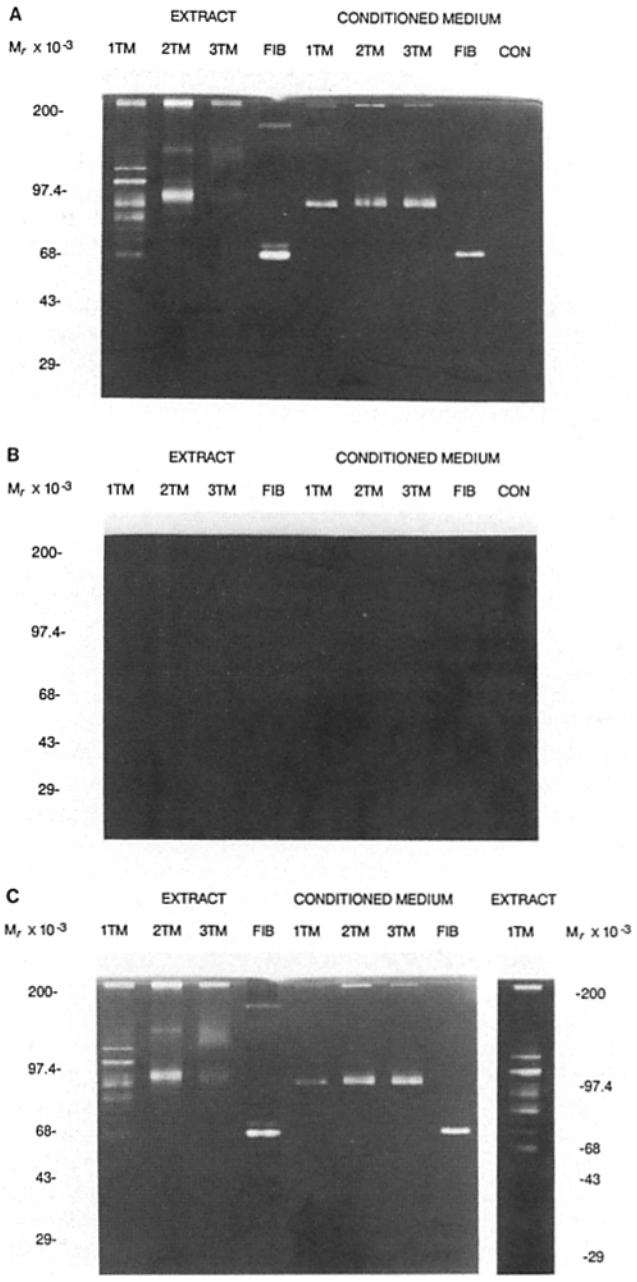

Figure 10. Proteinase profiles of extracts and conditioned medium from first trimester, second trimester, and term cytotrophoblasts and first trimester placental fibroblasts cultured on fibronectin. Samples were analyzed on 10% polyacrylamide gels containing 1 mg/ml gelatin. (*A*) The pattern of cell-associated and released proteinases revealed several activities unique to first trimester cytotrophoblasts. *1TM*, first trimester cytotrophoblasts; *2TM*, second trimester cytotrophoblasts; *3TM*, term cytotrophoblasts; *FIB*, first trimester human placental fibroblasts; *CON*, medium control. (*B*) 1,10-Phenanthroline, an inhibitor of metalloproteinase activity, completely abolished all the proteolytic activity. Inhibitors of serine proteinase activity (*C*) had no effect. The gel to the left was incubated in PMSF; the lane to the right, containing a cell extract prepared from first trimester cytotrophoblast cells, was incubated in epsilon-aminocaproic acid. Other inhibitors of serine, aspartic, and cysteine proteases were tested (see Materials and Methods) and had no effect.

ple pattern of two proteinase activities, of relative molecular mass 68 and 75 kD. Analysis of gelatin-degrading activity in cytotrophoblast-conditioned medium showed that cytotrophoblasts from all three trimesters secreted only the 90-kD proteinase into the medium. The placental fibroblasts secreted only the 68-kD proteinase. The repertoires of cell-associated and released proteinase activities were stable over the first 72 h of culture, which coincided with the period of most active ECM degradation by the first trimester cytotrophoblasts (Fig. 8).

All of the proteinases visualized in this assay, including those unique to first trimester cytotrophoblasts, were inhibited by 1,10-phenanthroline, suggesting they are metalloproteinases (Fig. 10 *B*). Several inhibitors of other classes of proteinases, including those capable of inhibiting plasminogen activators, were also tested (see Materials and Methods). None of these inhibitors had any effect. Typical of the results we obtained are the gels shown in Fig. 10 *C*, which were incubated in the presence of PMSF and epsilon-aminocaproic acid. Taken as a whole, these data suggest that many of the cell-associated metalloproteinases expressed by early cytotrophoblasts are different from those expressed by either later gestation cytotrophoblasts or fibroblasts. Significantly, changes in proteinase expression by cytotrophoblasts correlate temporally with changes in their invasive behavior observed both in vivo and in the in vitro culture system we have used.

Discussion

The results reported show that human cytotrophoblasts of all gestational ages adhered preferentially to basement membrane–like substrates in vitro. In first trimester cultures this initial adherent phase was soon followed by trophoblast degradation of the PF HR9 matrix, resulting in formation of large gaps devoid of both cells and matrix. The appearance of these holes coincided with the release of low molecular mass, radiolabeled matrix components into the culture medium. In contrast, neither holes in the monolayers nor substantial degradative activity were observed in cultures of cytotrophoblast cells isolated from the second trimester onward. Thus, the expression of this degradative activity in vitro paralleled closely the highly regulated timetable of uterine invasion by trophoblasts in vivo. This degradative activity appeared to be unique to first trimester cytotrophoblast cells since cultures of placental fibroblasts and human choriocarcinoma cell lines failed to display invasive characteristics under identical assay conditions.

The striking changes in ECM degradative activity observed in cytotrophoblast cultures of different gestational ages suggested that first trimester cytotrophoblasts would express unique proteinase activities. Using gelatin–substrate SDS-PAGE and subsequent incubation of gels in the presence or absence of proteinase inhibitors, we showed that this was indeed the case for both cell-associated and secreted proteinases. Specifically, early cytotrophoblasts expressed a number of metalloproteinases that were not found in later stage cytotrophoblasts or in first trimester placental fibroblasts, neither of which displayed degradative behavior using the culture conditions and assays described. To our knowledge this is the first report that trophoblast metalloproteinase expression is developmentally regulated during gestation.

The presence of a complex proteolytic enzyme profile is consistent with the fact that first trimester cytotrophoblasts must penetrate a variety of maternal cell layers and their associated basement membranes, as well as components of the interstitial matrix, to access the maternal circulation. At the same time these processes must be carefully controlled temporally so as to physically limit invasion to the superficial segments of the myometrium. Abnormally extensive invasion leads to premalignant or malignant conditions such as placenta accreta and choriocarcinoma, respectively. Abnormally shallow invasion is associated with preeclampsia (Robertson et al., 1975), a serious condition characterized by fetal intrauterine growth retardation, hypertension, and proteinuria (Hughes, 1972). These specialized requirements suggest that a number of complex regulatory processes are involved in the interactions of cytotrophoblasts and ECM during implantation and placentation. Our results are evidence that the temporal regulation of metalloproteinases is one important component. Other proteinases are also likely to play a role. For example, both urokinase-type and tissue-type plasminogen activators have been proposed as activators of procollagenase in tumor cells (Reich et al., 1988). Similar proteinase cascades may also be involved in trophoblast invasion (Yagel et al., 1988). Processes other than the timed synthesis of proteinases are also likely to be involved in regulating the complex behavior of trophoblasts during gestation.

Other features of trophoblast behavior in vivo suggest that specialized adhesive mechanisms are also important during the invasive phase of gestation. Within the fetal portion of the placenta, the cytotrophoblasts of the floating villi sit on an extensive basement membrane located between the stromal core and the syncytiotrophoblast covering of the chorionic villus. As they differentiate, the cytotrophoblast cells of the floating villi that fuse to form the syncytium, detach from their own basement membrane without any evidence of invading this membrane. On the other hand, cytotrophoblasts of the anchoring villi do penetrate basement membranes associated with the uterine epithelium and the endothelium of spiral arterioles (Brosens and Dixon, 1966; Boyd and Hamilton, 1970; Ramsey et al., 1976; Tuttle et al., 1985). In addition, a unique pericellular basement membrane, present from the earliest stages of pregnancy onward, surrounds individual decidual cells (Wewer et al., 1985, 1986). It is tempting to speculate that trophoblast adhesive interactions with these various basement membrane components play an important role in controlling the directed migration of the cells during uterine invasion. Finally, the population of cytotrophoblast cells that invade the superficial segments of the spiral arterioles, replace the endothelial lining of these vessels and adhere to the newly exposed vascular basement membrane. Thus, during the early stages of placentation the trophoblast cells adhere to, detach from, penetrate, and finally adhere again to basement membranes found in a variety of locations. In this regard, first trimester cytotrophoblasts share many of the properties of metastatic tumor cells (Liotta et al., 1986).

The degradative behavior exhibited in vitro by freshly isolated cytotrophoblasts closely parallels that thought to occur in vivo. This suggests that this experimental system may enable investigators to elucidate the mechanisms underlying other important interactions that occur during the early stages of human placentation. We have already shown that the cytotrophoblasts from first trimester placentas express several metalloproteinases that are not found in the cytotrophoblasts of later gestational age or in first trimester placental fibroblasts. Currently we are determining the specificity and localization of these proteinases. We are also investigating whether the coordinate modulation of adhesion receptors contributes to the temporally regulated behavior of trophoblast cells. It is clear that first trimester cells must be able to migrate through, as well as degrade, maternal ECM to reach the circulation. On the other hand, cytotrophoblasts of later gestational age may be less motile. In addition, modulation of adhesion receptors may be required in response to the unique proteolytic environment present during the invasive phase of placentation. Experiments using the culture system described here to address these points should offer interesting insights regarding the mechanisms responsible for the temporally regulated adhesive and degradative behavior of human trophoblast cells during gestation.

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