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Degradation of Extracellular Matrix by the Trophoblastic Cells of First-Trimester Human Placentas

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First-trimester human placental villi were cultured on ^{3}H-leucine-labeled extracellular matrices isolated from the PF HR9 and PYS-2 cell lines. Both cell lines produced an extracellular matrix that contained basement membrane-specific macromolecules, including type IV collagen, laminin and proteoglycan. Both matrices promoted outgrowth of cells from the villi which, according to morphological criteria, were identified as cytotrophoblastic cells. As the cells migrated from the attachment site, they caused a marked focal dissolution of the matrix which was accompanied by a concomitant release of ^{3}H-labeled material into the media. Approximately half of this material chromatographed near the inclusion volume of Sephadex G-50, indicating that the labeled matrix components had been degraded. This phenomenon was dependent on the age of the placenta. Second-trimester placental villi also adhered to the matrix, but no areas of dissolution were formed and no significant amounts of radioactivity were released into the medium. These results suggest that culture of first-trimester human placental villi on extracellular matrices may be useful for the study of some of the early embryonic events leading to human implantation, during which the trophoblastic cells erode the uterine epithelium.

Key words: human placenta, cytotrophoblastic cells, extracellular matrix, degradation

Implantation in the human is an active process that occurs in two phases [1]. First, the blastocyst is apposed to the uterine luminal epithelium. Second, the trophoblastic cells that comprise the fetal portion of the placenta erode the endometrium and associated basement membrane, penetrate the uterine stroma with its connective tissue matrix, and ultimately line the uterine blood vessels. This process results in the formation of the human hemochorial placenta in which fetal tissues are bathed constantly by maternal blood.

Currently, little is known concerning the mechanisms that mediate human implantation. Certain aspects of the events involved in this process in other species have been studied in vitro. The trophoblastic cells of isolated mouse blastocysts are

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capable of mediating the penetration of cell monolayers [2–5] and the digestion of isolated extracellular matrix [6]. The results reported in the present study indicate that the trophoblastic cells of isolated terminal villi from first-trimester human placentas are also capable of solubilizing basement membrane components, thus mimicking in vitro certain of the embryonic processes involved in implantation.

MATERIALS AND METHODS

The PF HR9 cell line was obtained through the courtesy of Dr. Erkki Ruoslahti at the La Jolla Cancer Research Foundation. The PYS-2 cell line was the kind gift of Dr. John M. Lehman, Department of Pathology, University of Colorado, Denver. Placental fibroblasts were isolated from first-trimester human placentas as previously described [7] and were used between the fifth and tenth passages. All cells were cultured routinely in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Sterile Systems, Logan, UT) and 10–50 μg/ml of gentamicin. The cells were passaged at regular intervals using either trypsin alone or trypsin - EDTA.

Isolation of Extracellular Matrices

The PF HR9 and PYS-2 cells, which were used for matrix production, were seeded onto plastic wells, 22 mm in diameter (Costar, Cambridge, MA) at 5×10^5 cells per well. The wells were precoated with bovine plasma fibronectin (50 μg/ml in PBS, 30 min at 25°C), isolated as previously described [8–10]. The culture medium was replaced every second day with DMEM containing 10% fetal bovine serum, 50 μg/ml ascorbic acid, and 10 μg/ml gentamicin. After 7–10 days, the cells were incubated for 48 hr in medium containing 5 or 10 μCi/ml L-3,5-^{3}H-leucine. The labeling media was supplemented with 10% fetal bovine serum and 10-50 μg/ml gentamicin. Fresh ascorbic acid (50 μg/ml) was added after the first 24 hr of the labeling period.

After 48 hr the labeled matrices were isolated according to a modification of the procedures previously described [11]. The culture dishes were washed twice at 37°C with hypotonic buffer (10 mM Tris-HCl, 0.5 mg/ml bovine serum albumin, 0.1 mM $CaCl_2$, pH 7.5) then incubated with the same buffer for 10 min at 37°C, during which the cells became swollen. Lysis was accomplished by a brief extraction (2 min, 37°C) with 0.5% NP-40 nonionic detergent diluted in hypotonic buffer [11]. The dishes were washed four times with distilled water and then incubated for 15 min at room temperature with 0.1 N ammonium hydroxide [12, 13]. They were rinsed three times with PBS containing 10% fetal calf serum and incubated for 1 hr in DMEM plus 10% fetal bovine serum to extract any residual detergent. In all cases, the matrices were used immediately after isolation.

To determine the electrophoretic profile of labeled matrix components, the matrices were solubilized immediately in loading buffer and separated on 5% SDS-polyacrylamide slab gels according to the method of Laemmli [14]. Labeled components were visualized after fluorography according to the method of Laskey and Bonner [15]. Laminin and type IV collagen were isolated from the EHS tumor [16] and used as reference protein standards.

Assessment of Solubilization

First- and second-trimester human placentas were obtained immediately after vacuum aspiration and term placentas after normal vaginal deliveries. The placentas

were washed in several volumes of PBS (10°C). The fetal portion was dissected free of any adherent decidua and again rinsed in PBS to remove any remaining blood. The cotyledons were transferred to petri dishes containing 10 ml PBS, and the branched terminal villi were isolated by stripping the main stem villi with forceps. The large villous cores were removed, and the PBS containing the terminal villi was transferred to a sterile culture tube. The villi were washed once with PBS, isolated by centrifugation (800 × g, 5 min), and washed two additional times in DMEM H21 containing 4.5 mg glucose/ml, 10% fetal bovine serum, and 10 μg/ml gentamicin. The pellet was resuspended in the same medium, and 0.5 ml containing approximately 100 villi was added to some of the culture dishes that contained labeled matrices. Control dishes for testing adherence of villi to matrices contained medium and villi but were not coated with matrix. Control dishes for testing the ability of trophoblastic cells to solubilize matrix contained matrices and medium, but no villi. After 24 hr the medium was changed on both the control dishes and dishes which contained villi and thereafter at regular intervals. The solubilization of labeled matrix components was determined by counting 50 μl of media from each culture dish in a Packard scintillation counter. The amount removed was replaced with an equal volume of fresh medium.

At the end of each experiment (21 days), the medium from all the experimental dishes was pooled, boiled, acidified and chromatographed on a 30 × 1.5 cm column of Sephadex G-50 (fine). The column was eluted with 0.1M pyridine acetate buffer, pH 5.3. Fractions (1 ml) were collected and assayed for radioactivity. Medium from the control dishes also was pooled and analyzed in an identical manner.

In other experiments, placental fibroblasts were adjusted to 9×10^5 cells/ml and added to isolated matrices. Control cultures were as previously described. For the quantitation of macromolecular ^{3}H-radioactivity present, the dishes were extracted in cold trichloroacetic acid. The extracted matrices were hydrolyzed with 1N NaOH, neutralized and assayed for radioactivity.

The cultures were examined daily using a Nikon inverted phase-contrast microscope, and photomicrographs were taken at regular intervals. At the conclusion of each experiment, the culture dishes were stained with either Toluidine blue or Coomassie blue.

Electron Microscopy

Cultured cells were fixed at room temperature for 2 hr using a solution of 2.5% glutaraldehyde in 0.08M sodium cacodylate buffer, then post-fixed at room temperature for 90 min in 1.5% aqueous osmium tetroxide. Cells were dehydrated in a graded series of ethanol solutions (70, 95, 100%) and embedded in Epon 812 resin. Sections having a pale gold interference color were cut on an LKB Nova ultramicrotome, collected on formvar coated slot grids, counter-stained with uranyl acetate and lead citrate, and viewed on a Carl Zeiss 10A transmission electron microscope.

RESULTS

Fluorograms of SDS-polyacrylamide gels of ^{3}H-leucine PF HR-9 matrix components showed three major bands (Fig. 1). By electrophoretic migration with authentic standards, the 200,000 and 400,000 molecular weight component were identified as the two subunits of laminin and the 180,000 molecular weight component as the α2 chain of type IV (pro) collagen. In this gel system the α1 chain of type IV (pro) collagen co-migrated with the 200,000 laminin subunit. The appearance of a minor high molecular weight band was variable and probably represents aldehyde-derived

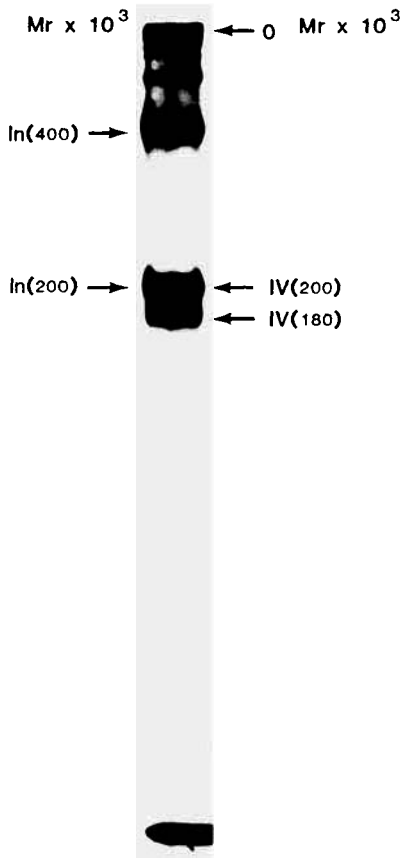

Fig. 1. Fluorogram of 5% SDS-polyacrylamide slab gel of ^{3}H-leucine-labeled macromolecules present in the isolated matrix components (PF HR9). The major bands comigrated with purified laminin (In) and type IV collagen (IV) as indicated. O indicates origin.

cross-linked collagen monomers. A similar electrophoretic pattern was obtained when ^{3}H-leucine PYS-2 matrix components were analyzed.

First trimester human placental villi rapidly attached to the extracellular matrices derived from both cell lines tested (PF HR9, PYS-2). After 24 hr the medium was changed, thus removing any villi that did not attach. The percent of adherent villi was the same for both matrices and was several times greater than was observed using uncoated plastic culture dishes. Villi that did not attach appeared to be those that were prevented by crowding from reaching the bottom of the dish.

Following attachment, the trophoblastic cells began to migrate from the villus surface onto the matrix-subtrate (Fig. 2a, d). This migration was accompanied by the appearance of areas that had been totally cleared of matrix (Fig. 2b,e). Subsequently, the holes were gradually enlarged (Fig. 2c, f) by migration of trophoblastic cells from the original foci. These areas invariably formed in the wake of the migrating cells, with intact matrix clearly visible around the rest of the trophoblastic cells. This process continued until, at the end of 21 days, little of the original matrix remained in the culture dish (Fig. 2g). In addition, within the placenta, the ability to digest the matrix appeared to be a property only of the trophoblastic cells. In separate experiments (data not shown) placental fibroblasts, the other major cell type found in

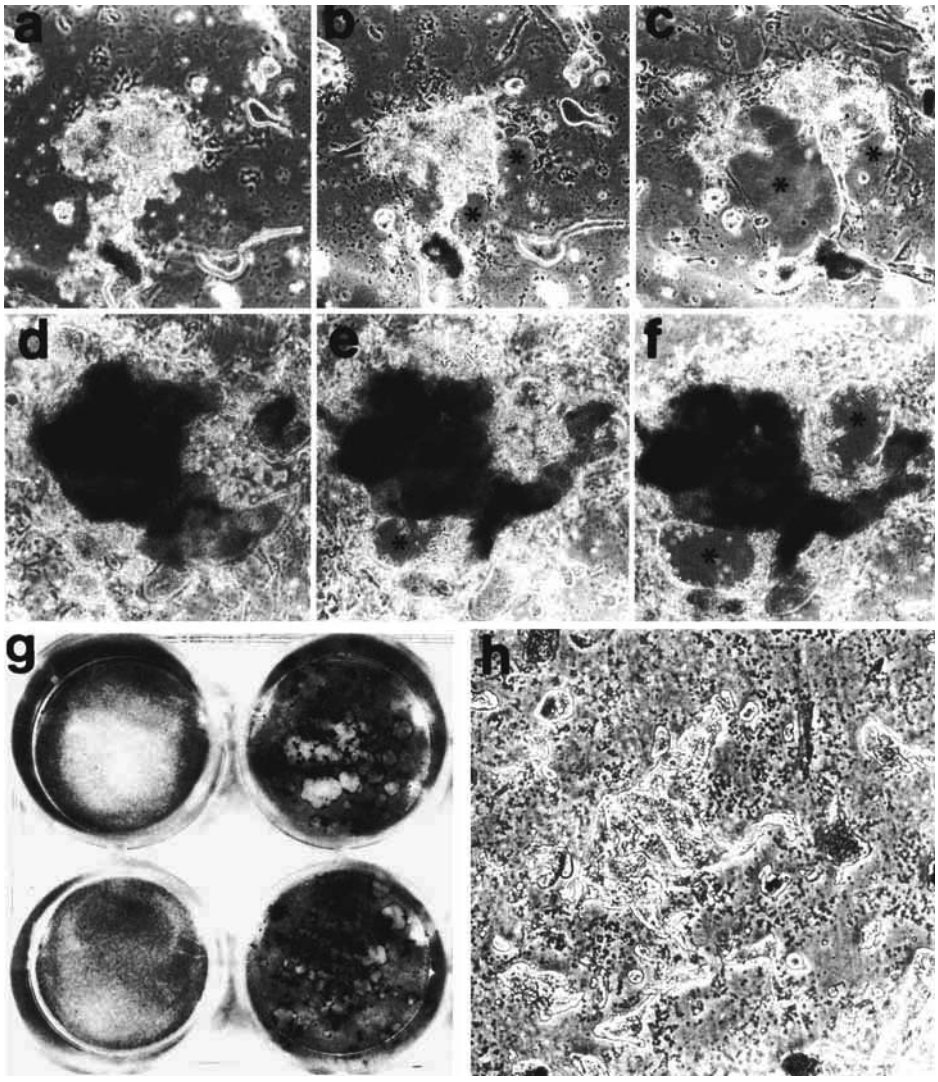

Fig. 2. Digestion of PF HR9 matrix by human trophoblastic cells (a–f). Photomicrographs taken at three (a, d), five (b, e), and seven (c, f) days depict the migration of trophoblast cells from the first-trimester human placental villi and the simultaneous dissolution of the matrix which occurred in the areas which are starred. First-trimester human placental villi also were cultured on matrix isolated from the PYS cell line (g). After three weeks the experiment was terminated and the placental cells, together with the remaining matrix, were stained with Toluidine blue. The wells at the left were controls that contained no villi. The two wells at the right show the large areas of matrix dissolution which were formed when the trophoblastic cells digested the matrix. Panel h shows that second trimester villi attached to the isolated PF HR9 matrix. After seven days, no areas of matrix dissolution were seen. Magnification, a–c, ×63; d–f, ×55; g, ×1.2; h, ×13.

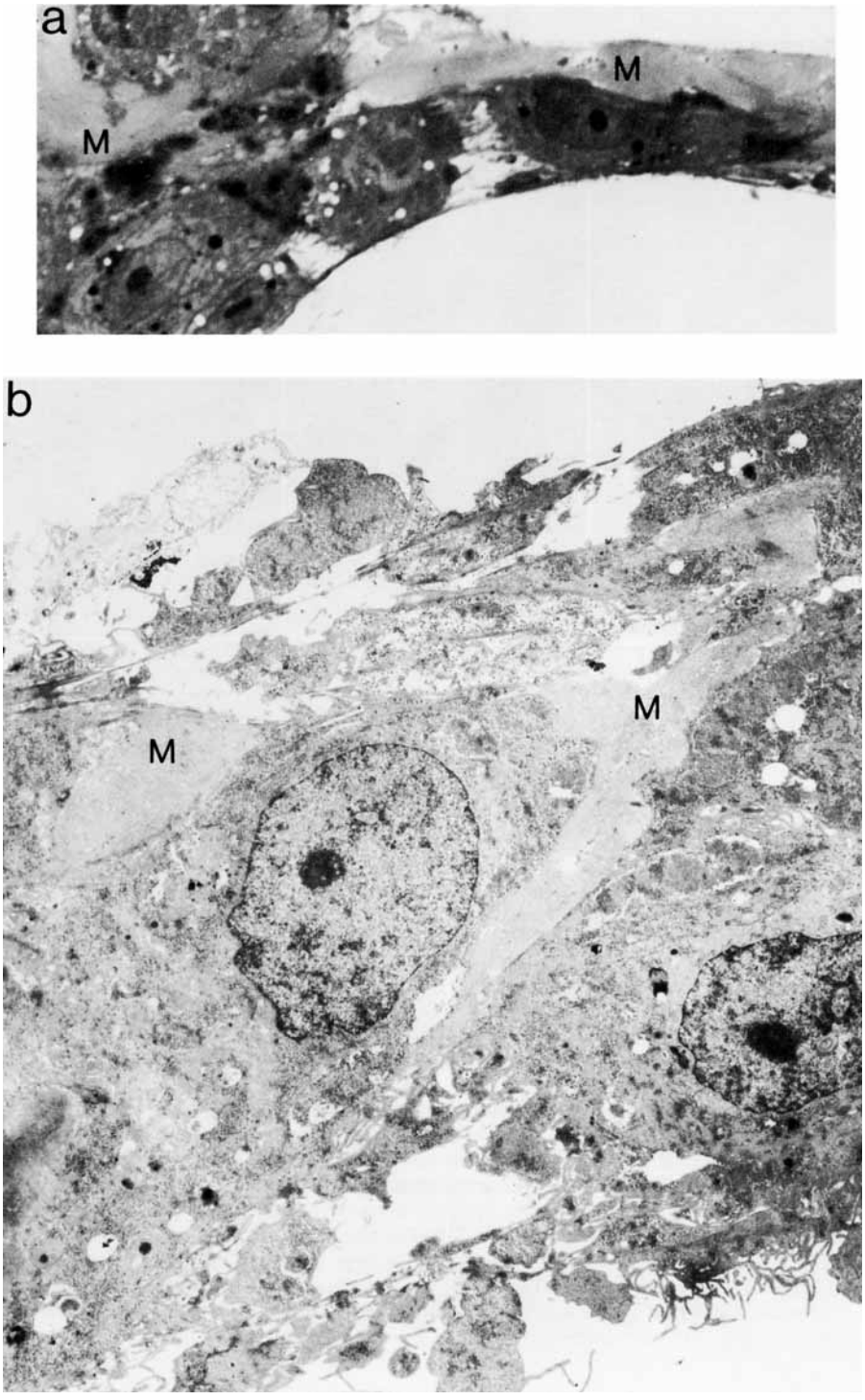

Fig. 3 (a,b)

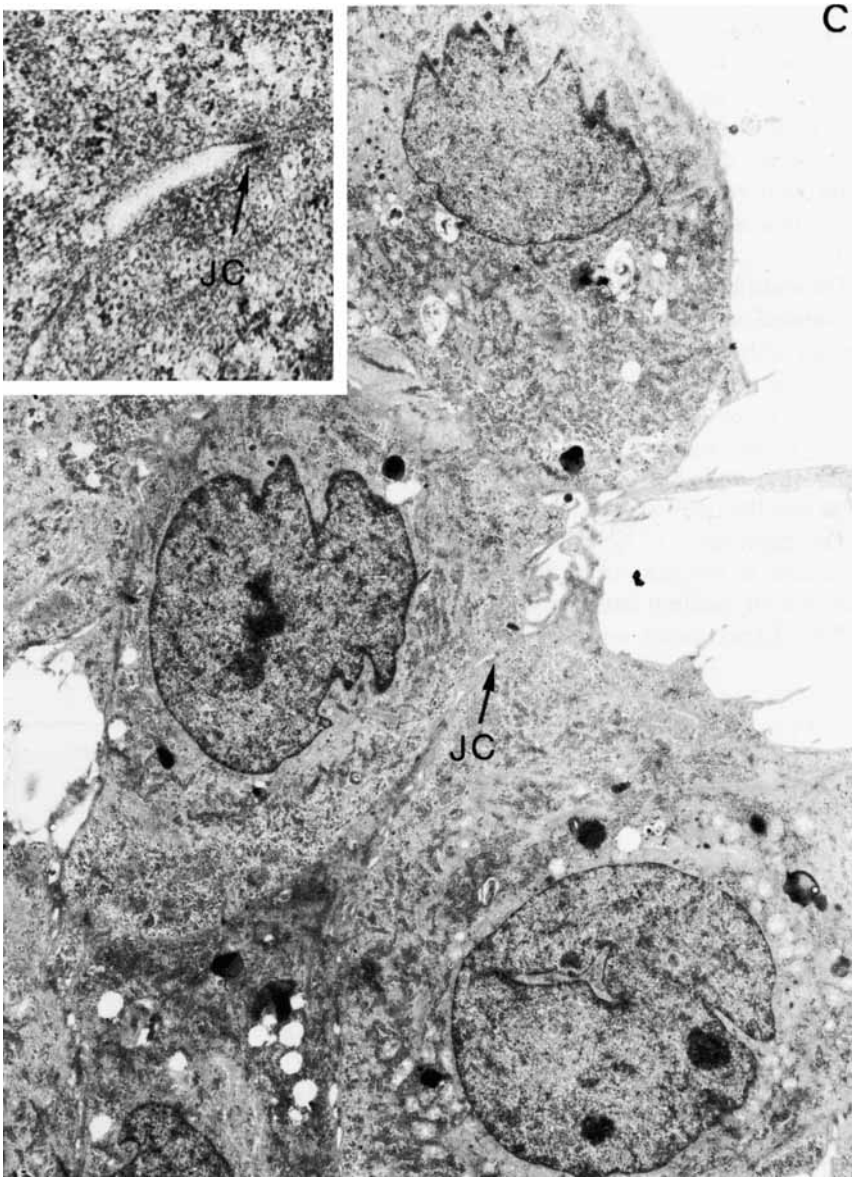

Fig. 3. Morphological observations of the interface of matrix dissolution at the light (a) and electron microscopic (b and c) levels. Cells and adjacent matrix were sectioned parallel to the dish. Intact matrix (M) was visible only at the convex outer edge of the migrating trophoblastic cells (a) or between cells (a and b). These cells were epithelial-like in appearance (c), forming specialized junctional complexes (JC) with one another, one of which is indicated by the arrow and enlarged in the accompanying insert. Magnification, panel a, ×1000; panel b, ×3680; panel c, ×3296; insert, ×24,000.

the villi, were cultured on isolated extracellular matrix. These cells did not degrade the matrix.

To confirm and extend the observations that were made by phase contrast microscopy, the circular areas devoid of matrix, together with the cells at the periphery, were sectioned parallel to the culture dish and examined by both light and electron microscopy. Intact matrix was clearly visible in both the light micrographs (Fig. 3a) and the electron micrographs (Fig. 3b) as an amorphous substance found either between the cells or just preceding them. However, no matrix was visible in areas through which the cells had migrated, indicating that migration of the trophoblastic cells is accompanied by focal dissolution of the matrix with which they are in contact.

The morphology of the cells comprising the interface of matrix dissolution also was examined carefully. Electron micrographs (Fig. 3b, c) showed cells that appeared to contain only one nucleus and thus were probably not of syncytiotrophoblastic origin. These cells also displayed an epithelial-like appearance, forming numerous specialized junctions (Fig. 3c). In addition, these cells appeared to contain large quantities of glycogen, a particular characteristic of the cytotrophoblastic cells that comprise the implanting cell columns [17]. Taken together, this evidence strongly suggests that the cells which digest the matrix are of cytotrophoblastic origin.

The appearance of holes in the PF HR9 matrix was accompanied by a concomitant increase in the amount of ^{3}H-leucine radioactivity released into the medium, as compared with medium from the control cultures that contained no trophoblastic cells (Fig. 4A). Experiments with radiolabeled matrices isolated from both the PF HR9

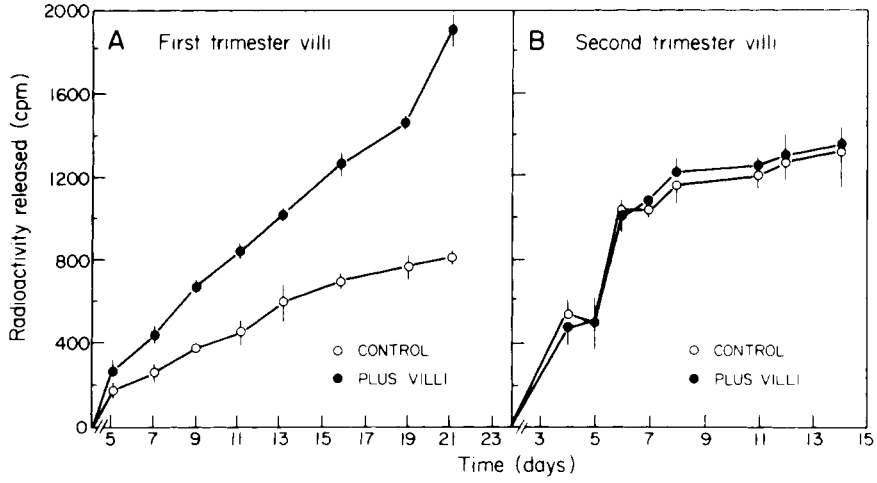

Fig. 4. Time course of the release of radioactivity into the medium. First-trimester villi (panel A) were cultured on ^{3}H-leucine-labeled matrix (labeling media contained 5 μCi/ml) isolated from the PF HR9 cell line. The medium was changed on days 6, 12, and 18. The data points represent the mean of radioactivity released from triplicate dishes. Bars represent standard error of the mean. Panel B shows the results of similar experiments in which second-trimester villi were cultured on ^{3}H-leucine-labeled PF HR9 matrix (labeling media contained 10 μCi/ml). Medium was changed on days 5, 7, and 11. Panels A and B depict results that were typical of five and three separate experiments, respectively. In both cases, the experiment was terminated when 50% of the total radioactivity incorporated into the matrix was released.

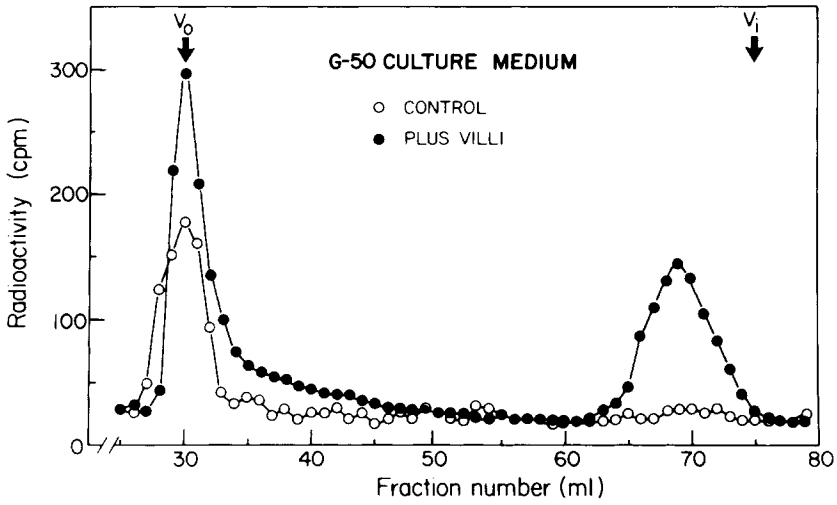

Fig. 5. Degradation of extracellular matrix by first trimester cytotrophoblastic cells, as demonstrated by chromatography of culture medium on Sephadex G-50. Only the medium from cultures that contained both first-trimester placental villi and labeled matrix (PYS) contained low-molecular-weight components. The column was calibrated by determining the elution volumes of ^{14}C-bovine serum albumin (Vo) and ^{3}H-leucine (Vi). More than 90% of the radioactivity applied to the column was recovered.

and the PYS-2 cells yielded similar results. As evidenced by the enlargement of the holes and the release of radioactivity into the medium, the solubilization of the matrix was progressive up to 21 days, at which time the experiments were terminated.

At this time the remaining culture media was harvested from the control and the experimental dishes and analyzed by gel filtration on Sephadex G-50 (Fig. 5). All of the radioactivity present in the medium of the control dishes chromatographed in the void volume, suggesting that those labeled matrix components that were passively released had an estimated molecular weight of more than 10,000. In contrast, nearly half of the solubilized radioactivity from the culture dishes containing first trimester placental villi and labeled PYS-2 matrix components chromatographed near the inclusion volume of the column. Chromatographic analysis of the culture medium from dishes containing first-trimester placental villi and labeled PF HR9 matrix gave similar results (data not shown).

There was also a marked difference in the ability of villi from first, second, and third trimester human placentas to interact with the isolated extracellular matrices in vitro. As described previously, the first-trimester villi rapidly adhered to and degraded the matrices which were tested. There was no qualitative difference in the number of second-trimester villi as compared with those from first-trimester placentas that attached to dishes containing matrix. However, no holes were formed (Fig. 1 h), and after 14 days no significant release of radioactivity into the media had occurred, as compared with controls (Fig. 4b). In contrast to both first- and second-trimester villi, term placental villi adhered poorly to the matrix. In three separate experiments, less than 5% of the villi added to each culture dish attached to the matrix substratum. Furthermore, as with the second-trimester villi, no evidence of matrix degradation (appearance of holes or release of radioactivity into the medium) was observed (data not shown).

DISCUSSION

The results of the present study show that first-trimester human trophoblastic cells are capable of solubilizing and degrading matrices rich in basement membrane-specific macromolecules in vitro. The morphology of basement membranes varies, depending both on their location within the body and on the cell type with which they are associated. However, all are composed of basement membrane-specific macromolecules, which include type IV collagen, laminin glycoprotein, heparan sulfate-rich proteoglycans, and entactin [18–22]. The two extracellular matrices tested in this study (PF HR9 and PYS-2) have been shown both here [10–11] and by other investigators [23–29] to contain all these components and to have the biochemical characteristics of basement membrane in vivo. In addition, fluorograms of the labeled matrix components from both cell lines were virtually identical to those published by other investigators using the same two cell lines [23–29].

Interestingly, the cells involved in degrading the matrix in vitro strongly resemble, by morphological criteria, the cytotrophoblastic cells of the intact placenta. The early placental villi that were cultured on the matrix were comprised of three major cell types: a fibroblast core, surrounded by cytotrophoblastic cells within the covering of a syncytiotrophoblastic shell. Presumably, the trophoblast syncytium, of which we found no evidence in our cultures, is involved in adhesion of the villi to the matrix but degenerates in vivo, allowing for an outgrowth of the then exposed cytotrophoblastic cells. Thus, the in vitro events herein described may be analogous to certain aspects of implantation in vivo where the cytotrophoblastic cell columns appear to break through the syncytiotrophoblastic layer and erode the decidua [16,29,30]. Rarely, some cultures were overgrown by a proliferation of fibroblasts which, unlike the trophoblast cells, grew well on the exposed areas of the plastic dishes but slowly on matrix. These cells were readily identified by their morphology. As control experiments in which fibroblasts isolated from early human placentas did not show degradation of the matrix (data not shown), we have concluded that this is a property of the cytotrophoblast cells alone.

In addition, two of our results indicate that the degradation of matrix by the trophoblastic cells is an active process and not the result of simple mechanical disruption or penetration. First, the gradual focal dissolution of the matrix that accompanied trophoblastic migration was a consistent observation in virtually all of our cultures. Second, low molecular weight ^{3}H-leucine-labeled components were simultaneously released into the culture medium. In order for this to occur, these cells presumably must produce a wide variety of proteolytic and glycosidic enzymes that are degrading the components of the two matrices tested in this study. These reactions appear to have been limited to areas of the culture dish that had direct contact with the trophoblastic cells. A similar result occurs when mouse blastocysts are cultured on smooth muscle extracellular matrix, suggesting that many of these enzymes are membrane bound rather than secreted into the medium [6].

In a previous study, the authors found that metastatic melanoma cells were unable to cause a significant solubilization or degradation of the HR9 matrix proteins, although the tumor cells did degrade the matrix-associated heparan sulfate proteoglycans via the elaboration of an endoglycosidase [11]. Furthermore, although the tumor cells did attach to the matrix, there was never any evidence of matrix penetration or zones of matrix lysis as is observed in the current study. Apparently, the cytotrophoblastic cells, which are highly specialized for erosion of the uterus in vivo, maintain this differentiated function in vitro.

The present study suggests that the culture of first-trimester human placental villi on isolated basement membrane is an extremely useful model for the study of the embryonic mechanisms involved in human implantation. In addition, we found that only trophoblastic cells from first trimester villi were capable of solubilizing and degrading the matrix. This suggests that some aspects of the in vitro phenomenon that were observed closely mimic the in vivo situation, as implantation and growth of the placenta is limited to early pregnancy. As a result, this system may be applicable also to the study of the mechanisms that circumscribe invasion of maternal tissues by the trophoblastic cells.

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