Degradation of Extracellular Matrix by Mouse Trophoblast Outgrowths: A Model for Implantation

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ABSTRACT During implantation the embryo attaches to the endometrial surface and trophoblast traverses the uterine epithelium, anchoring in the uterine connective tissue. To determine whether trophoblast can facilitate invasion of the uterus by degrading components of normal uterine extracellular matrix, mouse blastocysts were cultured on a radio-labeled extracellular matrix that contained glycoproteins, elastin, and collagen. The embryos attached to the matrix, and trophoblast spread over the surface. Starting on day 5 of culture there was a release of labeled peptides into the medium. The radioactive peptides released from the matrix by the embryos had molecular weights ranging from >25,000 to <200. By day 7 there were areas where individual trophoblast cells had separated from one another, revealing the underlying substratum that was cleared of matrix. When trophoblast cells were lysed with NH_4OH on day 8, it was apparent that the area underneath the trophoblast outgrowth had been cleared of matrix. Scanning electron microscopy and time-lapse cinemicrography confirmed that the digestion of matrix was highly localized, taking place only underneath the trophoblast, with no evidence of digestion of the matrix beyond the periphery of the trophoblast outgrowth. The sharp boundaries of degradation observed may be due to localized proteinase secretion by trophoblast, to membrane proteinases on the surface of trophoblast, or to endocytosis. Digestion of the matrix was not dependent on plasminogen, thus ruling out a role for plasminogen activator. Digestion was not inhibited by a variety of hormones and inhibitors, including progesterone, 17β-estradiol, leupeptin, EDTA, colchicine, NH_4Cl, or ϵ-aminocaproic acid. This system of culturing embryos on extracellular matrix may be useful in determining the processes that regulate trophoblast migration and invasion into the maternal tissues during implantation.

Implantation is an invasive process that is limited in both time and space. In the mouse it begins on day 5 of gestation, with the initial apposition of the embryo to the endometrial surface, and extends to the time that the invading trophoblast taps into the maternal blood vessels. During this period the trophoblast traverses a uterine epithelium composed of cells and connective tissue matrix and penetrates the basement membrane before entering the uterine stroma (26). Although the actual mechanisms have not been elucidated, morphologic study (26) suggests that entry of trophoblast into the endometrium does not depend on the release of a cytolytic enzyme because maternal cells surrounding the implanting embryo remain largely intact.

Attachment of blastocysts onto glass or plastic and subsequent trophoblast outgrowth has been used as a model to study proteinase activity and the interaction of the embryo with other cell types in culture (10, 20, 30). Studies in culture with time-lapse cinemicrography have shown that there is contact inhibition between trophoblast and co-cultured dispersed cells (10). Trophoblast displaces but does not destroy the cells, and in the process occupies the space vacated by the cells. This type of interaction could partially explain the manner in which trophoblast passes through the epithelial layer in utero, but it does not reveal how trophoblast breaches the extracellular matrix that binds epithelial cells to one another.

Production of proteolytic enzymes by trophoblast would be a logical mechanism for this latter phenomenon. Plasminogen activator has been identified in the postimplantation mouse embryo (19, 34), and proteolytic activity has been demonstrated

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in the guinea pig and rat blastocyst (24). In addition, Denker (6) has described a gelatin-dissolving endopeptidase at the surface of the implanting rabbit blastocyst. To further characterize the proteolytic activity of the mouse embryo, we studied the ability of the postimplantation embryo in culture to digest an insoluble extracellular matrix composed of collagen, elastin, and glycoproteins. All three are constituents of the normal uterine connective tissue. We studied the degradation of mixtures of proteins rather than isolated proteins because the constituents of extracellular matrix may interact in vivo in ways that modify their accessibility to proteolytic enzymes (5, 16, 37).

MATERIALS AND METHODS

Embryo Culture

Randomly bred 6–10-wk old Dub:(ICR) mice (Dominion Labs, Inc., Dublin, VA) were induced to superovulate by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (Teikoku Zoki, Tokyo) followed 45 h later by 5 IU of human chorionic gonadotropin (Ayerst Laboratories, New York, NY). The females were caged overnight with males of proven fertility, and the presence of vaginal plugs was noted the next morning (gestation day 1). Blastocysts were flushed from the uteri on day 4 of gestation with flushing media II (31) supplemented with 10% fetal calf serum (Sterile Systems Inc., Logan, UT). The blastocysts were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air in organ culture dishes (Falcon 3037; Falcon Labware, Oxnard, CA) containing modified (31) Eagle's basal medium supplemented with 10% fetal bovine serum (complete medium, BME) (31, 32) for 16–24 h. After overnight culture, blastocysts were transferred to medium overlying matrix on coverslips (day 1 of culture). Control coverslips were incubated with medium but not with embryos. To study later stages of development in utero, embryos at the egg cylinder stage (7–8 d in utero) were dissected, freed of decidua, and cultured on matrix as described for the earlier stages.

Preparation of Radio-labeled Matrix from Smooth Muscle Cells

Radio-labeled extracellular matrices were prepared as previously described (17, 38). Rat vascular smooth muscle cells from fourth to ninth passage stock cultures of the R22 strain (18, 38) were seeded onto 12- or 25-mm glass or plastic coverslips (Thermanox, Flow Laboratories, Rockville, MD). The cells were grown in Eagle's minimal essential medium containing 10% fetal bovine serum and 2% tryptose phosphate broth. Ascorbic acid (50 μg/ml) was added to cultures daily and [^{3}H]proline was added 5 d after seeding. The cells were cultured for another 7 d with two changes of medium and then lysed by adding 0.5 ml of 0.25 M NH_4OH and incubated for 30 min at room temperature (17, 38). The insoluble extracellular matrix, which remained firmly anchored to the coverslips, was washed vigorously with distilled water followed by 70% ethanol, or stored in 10% ethanol at 4°C. By differential enzyme digestion, amino acid analysis, peptide analysis, radio-labeling, and morphologic studies, a typical insoluble extracellular matrix contained 25 μg matrix/cm^2 and was composed of 40% glycoproteins (of several types), 40% elastin, and 20% collagen (largely types I and III) (18, 37, 38). Matrices were sterilized with 70% ethanol for 4 min and then washed thoroughly with BME before the addition of BME and blastocysts.

Glycoprotein-depleted matrices were prepared by trypsin treatment of the matrices as described previously (16, 38). These matrices were typically composed of 70% elastin and 30% collagen (38).

Microscopy

Cultured embryos were examined daily by phase-contrast microscopy for trophoblast development (30) and clearing of the extracellular matrix. For histologic examination, embryos grown on matrix on plastic coverslips were fixed and embedded in Epon; thick sections were cut perpendicular to the plane of the coverslip and stained with toluidine blue as described previously (40). Serial sections were cut through each embryo to establish degradation patterns.

For time-lapse cinemicrography, glass coverslips containing matrix and embryos were placed in a Sykes-Moore chamber (Bellco Glass Co., Vineland, NJ) filled with BME on day 5 or 7 of culture. A constant temperature of 37°C was maintained by a proportional controller (Yellow Springs Instruments, Yellow Springs, OH) and a heated microscope stage (33). A continuous flow of 5% CO_2 in air through a polyethylene tube was directed against the silicone rubber O ring surrounding the medium. The specimens were photographed at 40-s intervals for 88 h with differential interference microscopy using a 16× objective and Kodak Plus X reversal film.

For scanning electron microscopy (1, 37), embryos cultured on extracellular matrix prepared on 12-mm glass coverslips were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 22°C, then dehydrated through graded alcohols, critical-point-dried with the use of "bone-dry" liquid CO_2 (Matheson Gas Products, East Rutherford, NJ) in a Bowmar apparatus, coated with ~20 nm of gold in a Hummer sputter coater (Lab-Tek, Naperville, IL), and examined by means of a Cambridge S 150 scanning electron microscope at 20 kV.

Plasminogen-depleted Serum

Plasminogen-depleted serum was prepared by the passage of fetal bovine serum through a lysine-Sepharose column and was verified to be zymogen-free using ^{125}I-fibrin plates with urokinase as activator (35).

Determination of Proteolytic Activity of Trophoblast

Aliquots (20–100 μl) of the conditioned medium were taken each day after the embryos attached to the matrix, and their radioactivity was determined by liquid scintillation spectrometry. Fresh medium (20–100 μl) was added each day to replace the medium that had been removed. In other experiments the entire medium was replaced each day by an equal volume of fresh medium. A portion of the removed medium was used to determine radioactivity and the rest was used to determine molecular size by gel filtration chromatography. Medium incubated on matrix without embryos was used as a control.

ANALYSIS OF DIGESTION PRODUCTS: Molecular size of digestion products was determined on a Sephadex G-25 column (26 x 1.8 cm) (38). The running buffer was 0.2 M Tris-HCl, pH 7.6, containing 0.1% NaN_3 and 0.1 mg/ml bovine serum albumin (BSA). Fractions of 1.5 ml each were collected and the radioactivity present in 0.5-ml samples was determined. The column was calibrated with blue dextran, which was recovered in fractions 12–13, and with [^{3}H]proline, which was recovered in fraction 21.

ANALYSIS OF THE COMPOSITION OF THE LABELED EXTRACELLULAR MATRICES: The composition of the complete and residual matrices was determined by sequential enzyme digestion with trypsin, pancreatic elastase, and bacterial collagenase (17, 38). The trypsin-sensitive, elastase-sensitive, and collagenase-sensitive peptides corresponded largely to glycoproteins, elastin, and collagen, respectively, as determined previously (17, 38).

Determination of Areas of Clearing under Trophoblast Outgrowths

Coverslips containing trophoblast outgrowths on matrix from culture days 4 through 9 were incubated in 0.25 M NH_4OH after removal of medium. The time course of the dissolution of the trophoblast cells was followed by viewing through a phase-contrast microscope, and the substratum was examined for presence or absence of matrix. Clearing was rated on a scale of 0–4: 0, no degradation; 1, <25% of area under trophoblast outgrowth cleared; 2, 25–50% of the area under trophoblast cleared; 3, 50–75% of the area under trophoblast cleared; 4, 75–100% of the area under the trophoblast cleared. In some experiments, clearing was measured by tracing areas cleared on photographs of the coverslip. Radioactivity in the NH_4OH solution was a measure of the radioactivity contained in the embryos.

To show the extent of elastin degradation, intact matrix and matrix that had been incubated with embryos and treated with NH_4OH on culture day 9 were stained with Verhoeff's elastica stain (21).

Treatment with Drugs, Enzyme Inhibitors, and Hormones

On culture day 5, the coverslips were removed from the medium and placed in fresh BME containing one of the following drugs or hormones at various concentrations: leupeptin-Pr from Vega Biochemicals (Tucson, AZ), soybean trypsin inhibitor, colchicine, EDTA, 2-deoxy-D-glucose, tetracaine, dimethyl sulfoxide (DMSO), progesterone, 17β-estradiol, ε-aminocaproic acid, dexamethasone, and indomethacin from Sigma Chemical Co. (St. Louis, MO), cytochalasin B (Aldrich Chemical Company), and NH_4Cl, ethanol, and NaN_3. Cytochalasin B was initially dissolved in DMSO (1 mg/ml) before dilution in BME. Cultures were examined each day through culture day 9 by phase-contrast microscopy.

RESULTS

Growth of Mouse Embryos on Extracellular Matrices

Blastocysts attached to the matrix on days 2–3 of culture, and their attachment was identical to that of embryos grown on glass coverslips. In studies with time-lapse cinemicrography, the trophoblast cells spread out as a sheet by active movement of the marginal cells. Maximal outgrowth, as measured by the mean area of the trophoblast, was achieved by days 6–7 of culture. A timed series of micrographs is shown in Fig. 1. Within a day of achieving maximal outgrowth (Fig. 1 *b*), trophoblast cells began to pull apart from each other and a few small spaces were formed by active contractile movement of at least one pair of separating cells (Fig. 1 *c*). The interstices continued to enlarge during days 8–9 (Fig. 1 *d*; Fig. 2). In this way the trophoblast sheet was gradually converted into a networklike structure consisting of open spaces enclosed by interconnecting trophoblast cells as observed previously (30). On days 7–8 of culture in the areas between the cells the substratum was bare and matrix was not visible (Fig. 1 *d*; Figs. 2 and 3). The only areas that were cleared of matrix were those under the trophoblast. By contrast, the matrix at the periphery of the trophoblast sheet remained intact.

Although matrix clearing could be observed first in areas between cells, it was not restricted to these areas. To further investigate the disappearance of matrix, trophoblast sheets or networks were treated at various stages of culture with NH_4OH, which dissolved cells but not the insoluble matrix. Trophoblast spreading preceded matrix clearing. Clearing of matrix was first visible in small areas under the trophoblast on day 5 (Fig. 4), and progressed until days 8–9 when the entire area under the trophoblast was cleared. The removal of matrix, first noted morphologically with time-lapse cinemicrography, scanning electron microscopy, and phase-contrast microscopy, was further demonstrated by use of an elastin stain that showed absence of staining in the areas where trophoblast had been located before dissolution in NH_4OH (not shown). Serial sections taken through embryos growing on matrix revealed that the matrix was intact under the peripheral trophoblast on day 5 (Fig. 5 *a* and *b*). In the center of the outgrowth the matrix was intact under the inner cell mass of the embryo while beginning to clear under the adjacent trophoblast (Fig. 5 *c*).

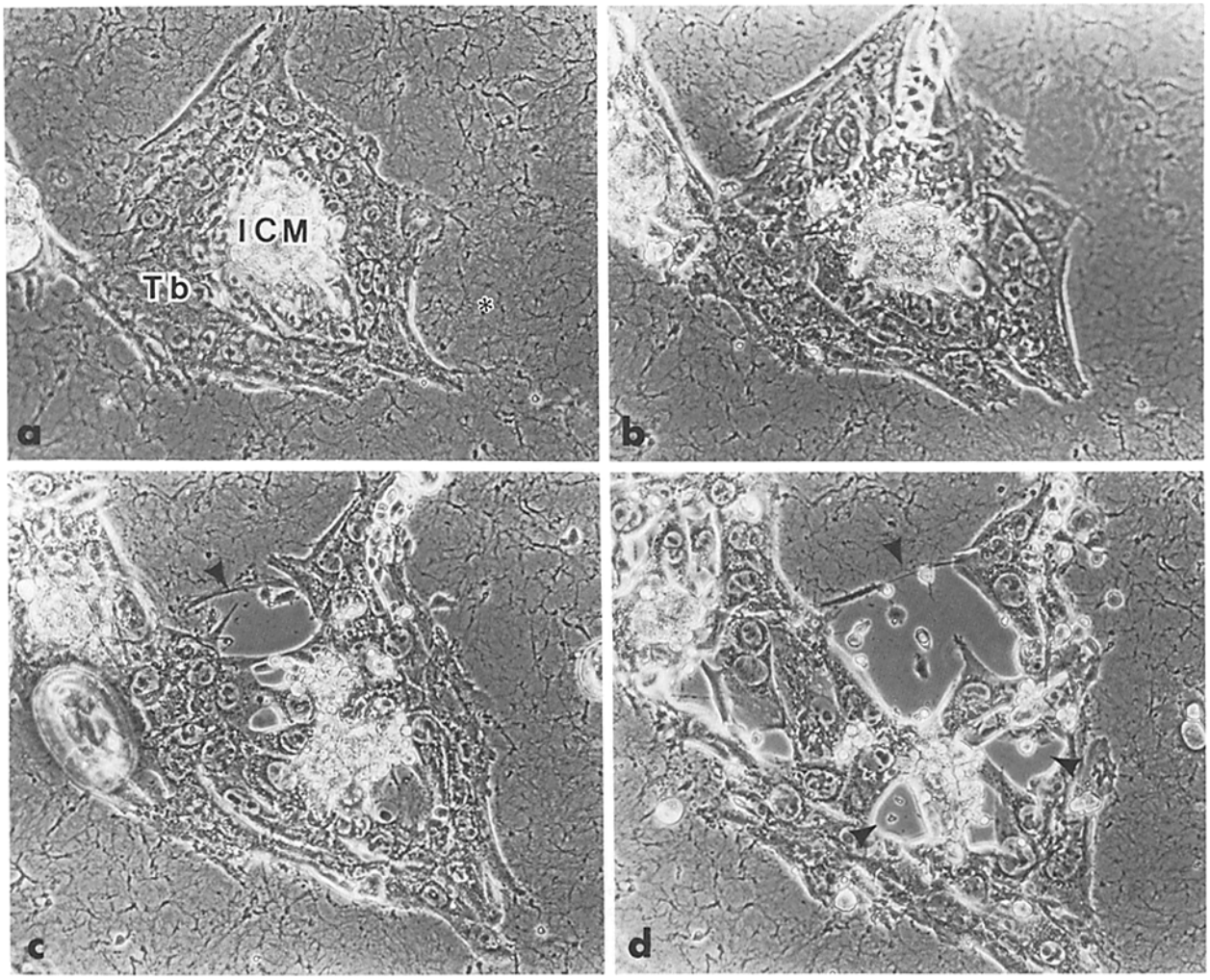

FIGURE 1 Time course of mouse embryo development on extracellular matrix. Appearance of a living embryo (*a*) 5 d in culture; (*b*) 6 d; (*c*) 7 d; (*d*) 8 d. *ICM*, inner cell mass; *, intact matrix; *Tb*, trophoblast. Arrowheads indicate areas where trophoblast cells have separated from one another and the area that has been under the cells is visible. Note that the matrix has been cleared in these areas and the surface of the dish is seen. Phase contrast, × 100.

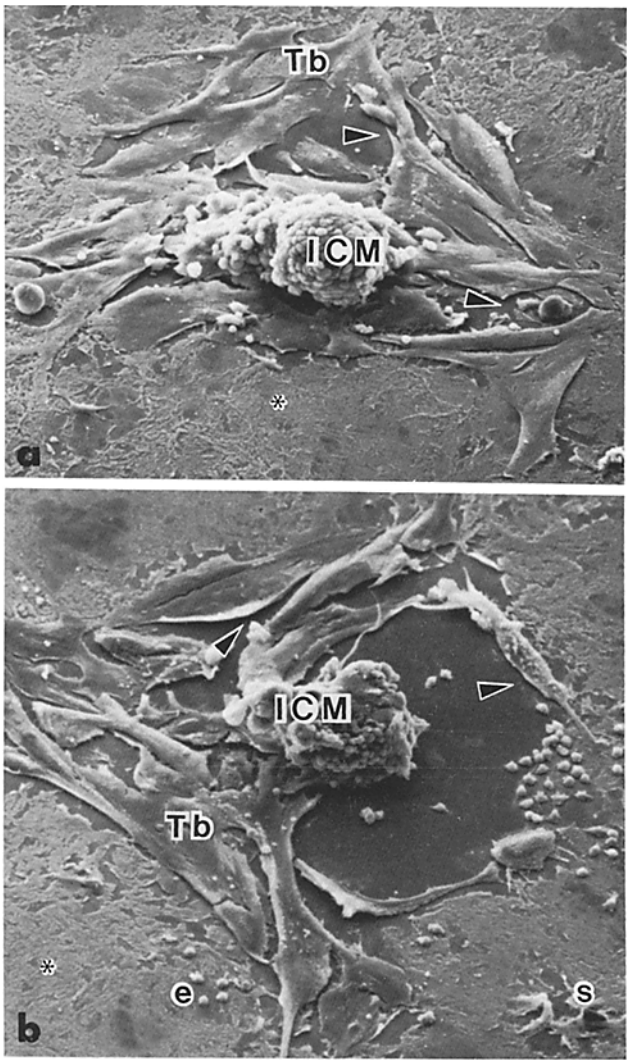

FIGURE 2 Scanning electron micrographs of mouse embryos grown on extracellular matrix for (*a*) 7 d in culture; (*b*) 9 d in culture. Note areas of clearing (arrowheads), where trophoblast (*Tb*) cells have separated from one another. Matrix clearing can also be seen under secondary trophoblast cells (s), but not under parietal endoderm cells (*e*). *ICM*, inner cell mass; *, intact matrix. × 150.

However, the matrix was thin or absent under the entire trophoblast on day 8 (Fig. 5 *d-f*).

In some areas the peripheral matrix of the advancing trophoblast outgrowth was slightly thickened as though the trophoblast had pushed matrix before it, showing a possible mechanical phase to the migration through the matrix (Fig. 5 *d*). Matrix located in other areas was normal in thickness, as was the matrix under the inner cell mass.

Biochemical Analysis of Matrix Degradation

The time sequence of release of radioactive peptides into the medium correlated with the morphologic changes. The initial release of radioactivity from the matrix was detected on day 5 of culture, when a clearing of 1 was seen, and increased progressively through day 8, when a clearing of 4 was seen (Fig. 6). Although the radioactive peptides were largely released into the medium, when trophoblast cells were lysed with NH_4OH, ~10% of the radioactivity was associated with the cells (Table I).

The extent of matrix solubilization, determined by the release of radioactive peptides, correlated well with the area of clearing determined morphometrically (Table I). Most of the released fragments were acid insoluble and were excluded by Sephadex G-25 in medium that was replaced each day (Fig. 7 *b*). The median molecular weight of the excluded fragments was 25,000.

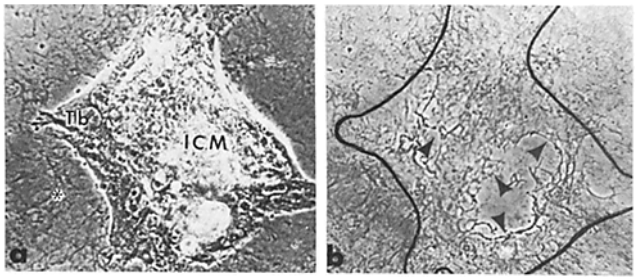

FIGURE 4 Demonstration of initial areas of clearing of matrix under spreading trophoblast outgrowth from embryo at 5 d in culture. (*a*) Appearance of living embryo showing inner cell mass (*ICM*), trophoblast (*Tb*), and intact matrix beyond edge of outgrowth (*). (*b*) View of same field after NH_4OH lysis of the embryonic cells. Black line indicates extent of outgrowth and arrowheads show areas where matrix has been cleared. Phase contrast, × 70.

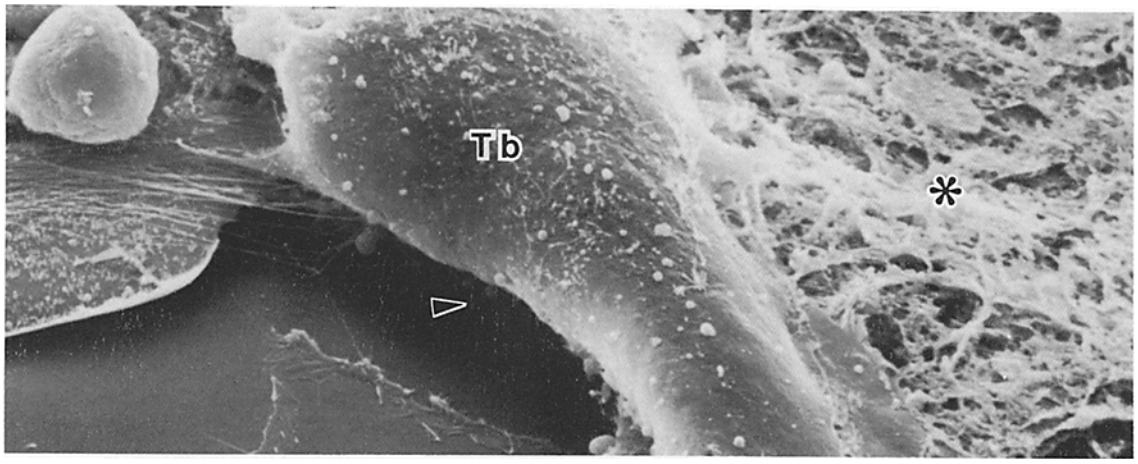

FIGURE 3 Scanning electron micrograph of the edge of the trophoblast outgrowth of a mouse embryo grown on extracellular matrix for 9 d. The matrix in this preparation was much thicker than the matrix shown in Fig. 2. The trophoblast (*Tb*) cell has broken away from the glass dish, and under the cell (arrowhead) the matrix has been completely cleared, whereas beyond the edge of the outgrowth, the matrix is intact (*). × 1,500.

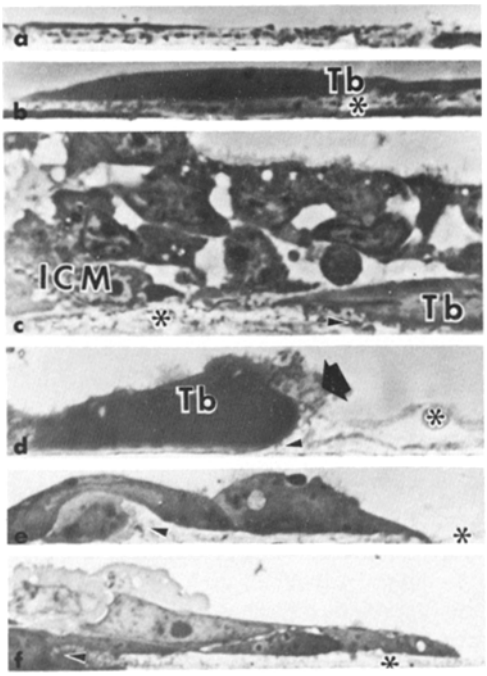

FIGURE 5 Cross-sectional appearance of mouse embryo outgrowths on extracellular matrix. Plastic sections from oriented blocks were cut perpendicular to the plastic coverslip surface that appears at the bottom of each micrograph. (*a*) Control matrix. Note the multilayer appearance of the matrix. The distinct globules correspond to amorphous elastin: (*b*) section from periphery of trophoblast growth at 5 d in culture showing intact matrix; (*c*) section through the center of the embryos at 5 d in culture showing area of matrix thinning under the trophoblast (arrowhead), whereas matrix under the inner cell mass is intact; (*d*) section through the edge of the trophoblast outgrowths at 8 d in culture. Matrix has been completely cleared to the edge of the cell (arrowhead), but remains intact beyond this. An area of matrix thickening (arrow) is seen at the edge of the embryo; (*e* and *f*) sections through the edge of the trophoblast outgrowth at 8 d in culture. Here, the edge of the trophoblast extends over an area of apparently intact matrix. A sharp boundary of the extent of matrix clearing (arrowheads) is seen in a pocketlike area in the trophoblast outgrowth. *Tb*, trophoblast; *ICM*, inner cell mass; *, intact matrix. Toluidine blue stained. × 570.

In contrast, when the medium was left in the cultures continuously over the course of a week, up to 50% of the radioactivity migrated with free [^{3}H]proline (Fig. 7*a*). We were unable to demonstrate any proteolytic activity against the matrix in media conditioned by embryos for 8 d in culture. At neutral pH, Triton-X-100 lysates of cultured embryos failed to degrade the matrix.

The glycoprotein, collagen components and elastin components were all degraded coordinately by the trophoblast, in keeping with the morphology of complete clearing of all visible components in the areas under the trophoblast (Table II). Extracellular matrix that contained only elastin and collagen, as well as the complete matrix (Tables I, II), and layers of pure type I collagen, (not shown) were all degraded.

Role of Plasminogen in Matrix Degradation

Trophoblast has been shown to make plasminogen activator (34). Accordingly, we examined the role of plasminogen in matrix degradation by the embryo. Replacement of the 10% fetal bovine serum in Eagle's medium with 10% plasminogen-depleted serum did not prevent breakup of the trophoblast sheet, and the network structures formed normally. The matrix under the trophoblast in plasminogen-depleted medium was cleared to the same extent as in medium containing plasminogen. Similarly, in the presence of plasminogen, soybean trypsin inhibitor (100 μg/ml), leupeptin (10 μg/ml), and ε-aminocaproic acid (100 mM), all inhibitors of plasmin activity, had no morphologic or biochemical effect on matrix clearing (Table III).

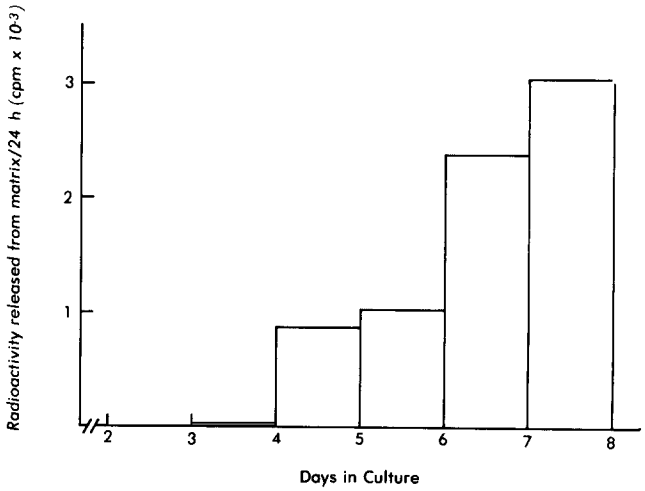

FIGURE 6 Time course of release of radioactive peptides from extracellular matrix by embryos. Medium was changed daily and radioactivity was determined in a portion of the removed medium.

Effects of Inhibitors and Hormones on Outgrowth and Degradation of Matrix

We studied the effect of several proteinase inhibitors, hormones, and membrane-active drugs on the trophoblast outgrowth and degradation of matrix. Morphologic analysis of the areas of clearing between and under embryos revealed that only NaN_3 inhibited degradation (Table III). Colchicine, cytochalasin B, 2-deoxy-D-glucose, and tetracaine limited the outgrowth of the trophoblast and caused eventual disintegration. Despite this disintegration, the matrix under the embryo was lysed to the full extent of the trophoblast cells, and there was still no dissolution of matrix at the periphery of the trophoblast cells. At nontoxic concentrations effective in other systems, leupeptin, ε-aminocaproic acid, EDTA, soybean trypsin inhibitor, NH_4Cl, dexamethasone, and indomethacin did not cause disintegration of the embryo and did not prevent digestion of matrix under the trophoblast. Progesterone, at a concentration of 10 μg/ml, caused disintegration of some trophoblast cells, but was not toxic at 5 μg/ml. At both these concentrations of progesterone, trophoblast was still able to lyse the matrix. 17β-Estradiol had no effect on either trophoblast outgrowth or degradation of matrix. In contrast, NaN_3 poisoned the trophoblast without producing detachment of the cells, and clearing did not proceed under the cells. When some inhibitors were evaluated for matrix degradation by the release of radioactive peptides, the effects (Table III) were essentially the same as those observed morphologically.

Matrix Clearing by Trophoblast from Later Stages of Development

Trophoblast attachment and migration in utero occur not only during the initial interaction with the endometrium at the fifth day of gestation, but also extend to the time that tropho-

TABLE I

Comparison of Matrix Degradation (Measured Biochemically) and Clearing (Determined Morphologically)

Experiment number	No. of embryos	Days in culture	Type of matrix	Matrix clearing	Matrix degradation			
					^{3}H cpm in medium		^{3}H cpm associated with embryo	
				% total area	× 10^{-3}	% total	× 10^{-3}	% total
1	20	9	Complete	3	4.2	3.7	0.3	0.3
2	30	9	Complete	8	10.4	7.0	1.2	0.8
3	40	9	Complete	8	12.0	7.7	1.2	0.8
4	40	8	Complete	ND*	7.3	8.3	1.3	1.5
5	50	9	Complete	ND	11.5	11.6	ND	ND
6	30	9	Glycoprotein-depleted	5	5.2	6.1	ND	ND

Different batches of matrix were used in these experiments.
* ND, not determined.

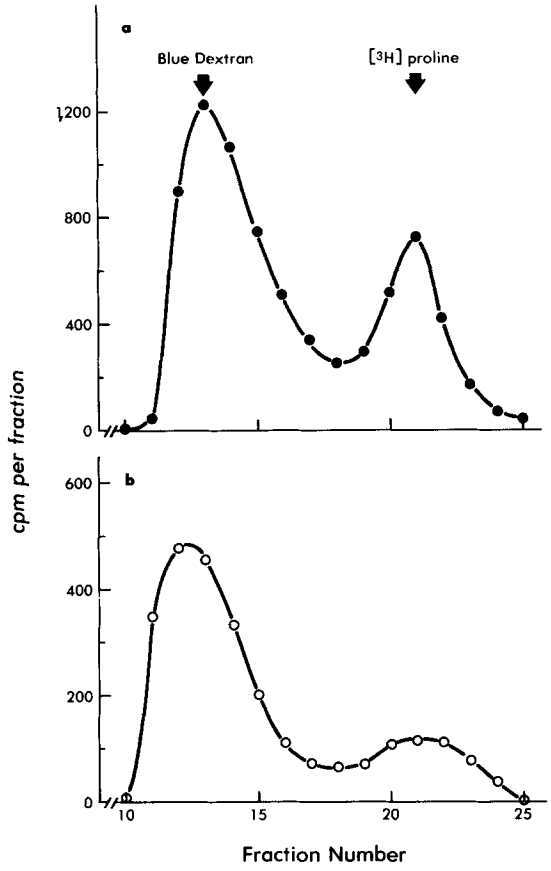

FIGURE 7 Sephadex G-25 separation of peptides released by embryos cultured on matrix for 7 d. (*a*) Chromatogram of medium that was continuously present from days 1-7. (*b*) Chromatogram of medium at day 7 from cultures in which medium was changed daily.

blast breaches the basement membrane and enters the stroma (8). This is achieved both by mural trophoblast, which gives rise to the outgrowths seen in culture, and by polar trophoblast-derived cells in the ectoplacental cone (days 7–8 in utero). Therefore, we studied the capacity of embryos at the egg cylinder stage to degrade extracellular matrix in culture. Within 24–48 h, matrix had been degraded in areas where the trophoblast was attached (Fig. 8). As in the case of early trophoblast, all matrix components were cleared completely.

DISCUSSION

Cultured blastocysts undergo both morphologic and biochemical transitions that are characteristic of their development in vivo. Because culturing allows a separation of embryonic and maternal elements, and continuous accessibility for observation, it is a useful means of isolating embryonic mechanisms involved in the peri-implantation period (3, 10, 11–14, 19, 20, 28–30, 40, 41). The dissimilarities between development in utero and in culture cannot be ignored—the hormonal and anatomic milieu provided by the maternal organism is absent in culture (8). Nevertheless, we believe that the outgrowth of mouse trophoblast on matrix in culture is a useful model for implantation.

In the present study we observed that within one day of maximum trophoblast outgrowth, the trophoblast sheet was converted into a network as the cells began to pull apart from each other. Before this stage there was no discernible morphologic effect of the embryo on the matrix. When the trophoblast cells separated from one another it was apparent that matrix under the trophoblast had been cleared, but there was no effect beyond the periphery. Figure 9 summarizes the correlation between trophoblast development and matrix clearing.

The sharp boundaries of matrix clearing observed here may be due to a combination of the active mechanical invasion of the matrix and proteolysis mediated by localized proteinase secretion, localized excess of proteinase, physical containment of enzymes by trophoblast outgrowth, or membrane proteinases on the surface of the cells. Trophoblast in culture actively displaces other cells (10), and cell migration through connective tissue matrices may, in some cases, be independent of proteolysis (27). Although there appeared to be some physical displacement of matrix as the trophoblast migrated out from the embryo, this did not account for a significant part of the clearing observed (Table I). Despite the endocytic capacity of trophoblast (9), it is unlikely that endocytosis and intracellular digestion (36) are the primary route or the rate-limiting step of matrix degradation by trophoblast cells because morphologically identifiable matrix components were not seen intracellularly in thin sections of embryos (data not shown), and because inhibitors of lysosomal digestion had no effect on degradation. The labeled fragments found associated with the embryo after NH_4OH lysis were probably derived from matrix in areas under the trophoblast inaccessible to usual washing procedures.

Reactions localized to the pericellular area have been noted in other studies using extracellular matrix (16, 37). Human fibrosarcoma cells do not digest the collagen or elastin components unless there is direct contact between the cells and the matrix (16). Cultured macrophages have a surface-bound plasminogen-dependent fibrinolytic system (2), and they digest glycoproteins and elastin of the matrix to a greater extent when they are in proximity (37). Similarly, a surface-bound collagenase has been found on *Entamoeba histolytica* (23).

TABLE II

Components of Extracellular Matrix Degraded by Trophoblast Outgrowths after 8 Days in Culture

Experiment	No. of embryos	Type of matrix	Matrix component degraded		
			Glycoproteins	Elastin	Collagen
			% total	*% total*	*% total*
1	50	Complete	26	23	19
2	30	Complete	8	7	6
3	30	Glycoprotein-depleted	0	8	8

Complete matrices or matrices depleted of glycoproteins by trypsin treatment (see Materials and Methods section) were used. Total radioactivity solubilized was determined at 8 d in culture. Amounts of solubilized radioactivity from control matrices cultured without embryos (~2%) were subtracted from the values. Composition of matrix components degraded was determined by calculation of the composition of components remaining in the matrix after embryos were removed by NH_4OH treatment.

TABLE III

Effects of Inhibitors on Matrix Degradation

Inhibitor	Concentration added	Effect on trophoblast spreading*	Matrix cleared‡	Radioactivity solubilized§
				% control
None	—	None	4	100
Leupeptin	10 μg/ml	None	4	ND
ε-Aminocaproic acid	10 mM	None	4	105
	100 mM	None	4	100
EDTA	1 mM	None	4	ND
Soybean trypsin inhibitor	100 μg/ml	None	4	98
Colchicine	10 μM	Toxic	4	ND
Cytochalasin B	5 μg/ml	Toxic	4	ND
2-Deoxy-D-glucose	100 mM	Toxic	4	ND
NaN_3	125 μg/ml	Stops	0	2
NH_4Cl	5 mM	None	4	ND
Ethanol	3%	None	4	ND
Tetracaine	1 mM	Toxic	4	ND
Dimethyl sulfoxide	0.5%	None	4	ND
Progesterone	5 μg/ml	None	4	100
	10 μg/ml	Toxic	4	ND
17β-Estradiol	5 μg/ml	None	4	ND
Dexamethasone	1 μM	None	4	95
Indomethacin	1 μM	None	4	ND

* Toxicity was noted as partial disintegration of trophoblast cells.

‡ Matrix clearing was scored at 8 d in culture on a scale of 0–4 as described in Materials and Methods.

§ Radioactivity solubilized from the matrix by 10–40 embryos in different experiments was determined as percentage of counts released compared to same number released from control embryos. Results are from at least duplicate wells. *ND*, not determined.

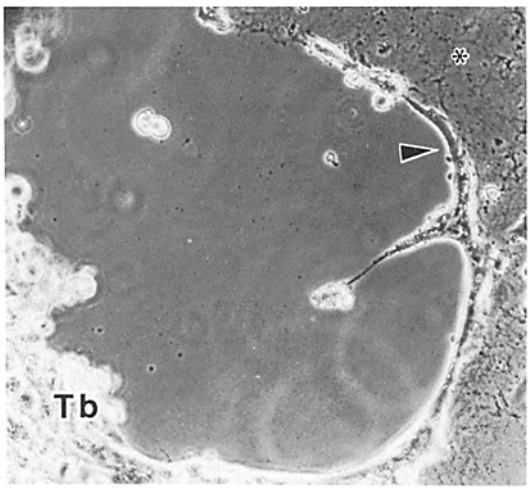

FIGURE 8 Area of matrix clearing produced by culturing egg cylinder stage embryos on matrix for 48 h. The living outgrowing cells (*Tb*) were retracted from the matrix by incubating at 4°C for 1 h. Clearing is seen over the entire area where the cells had been present (arrowhead), and intact matrix (*) is seen beyond the outgrowth. Phase contrast, × 100.

Trophoblast and parietal endoderm have been shown to produce plasminogen activator in large amounts, and plasmin produced by embryos may be involved in the attachment phase of development (34). Embryos cultured with fibrin overlay create zones of clearing that extend beyond the periphery of the trophoblast outgrowth (19, 34), as distinct from the localized clearing of matrix. Furthermore, unlike the matrix digestion observed here, the destruction of fibrin by trophoblast is plasminogen-dependent. Removal of plasminogen decreases hydrolysis of extracellular matrix by human fibrosarcoma cells (16) and macrophages (17, 37, 38), in contrast with lysis of matrix by trophoblast. Martin and Arias (22) showed that the fibrinolytic activity of human trophoblast in vitro is inhibited by progesterone and they suggest that progesterone might act to limit the invasiveness of trophoblast. In the current study, however, progesterone did not interfere with trophoblast digesting the matrix, and plasminogen-dependent fibrinolytic activity was not required for digestion of matrix.

Light microscopy and biochemistry revealed that the entire thickness of the matrix underneath the trophoblast was cleared, and all matrix components were degraded coordinately. Other cell types studied on extracellular matrices have not displayed

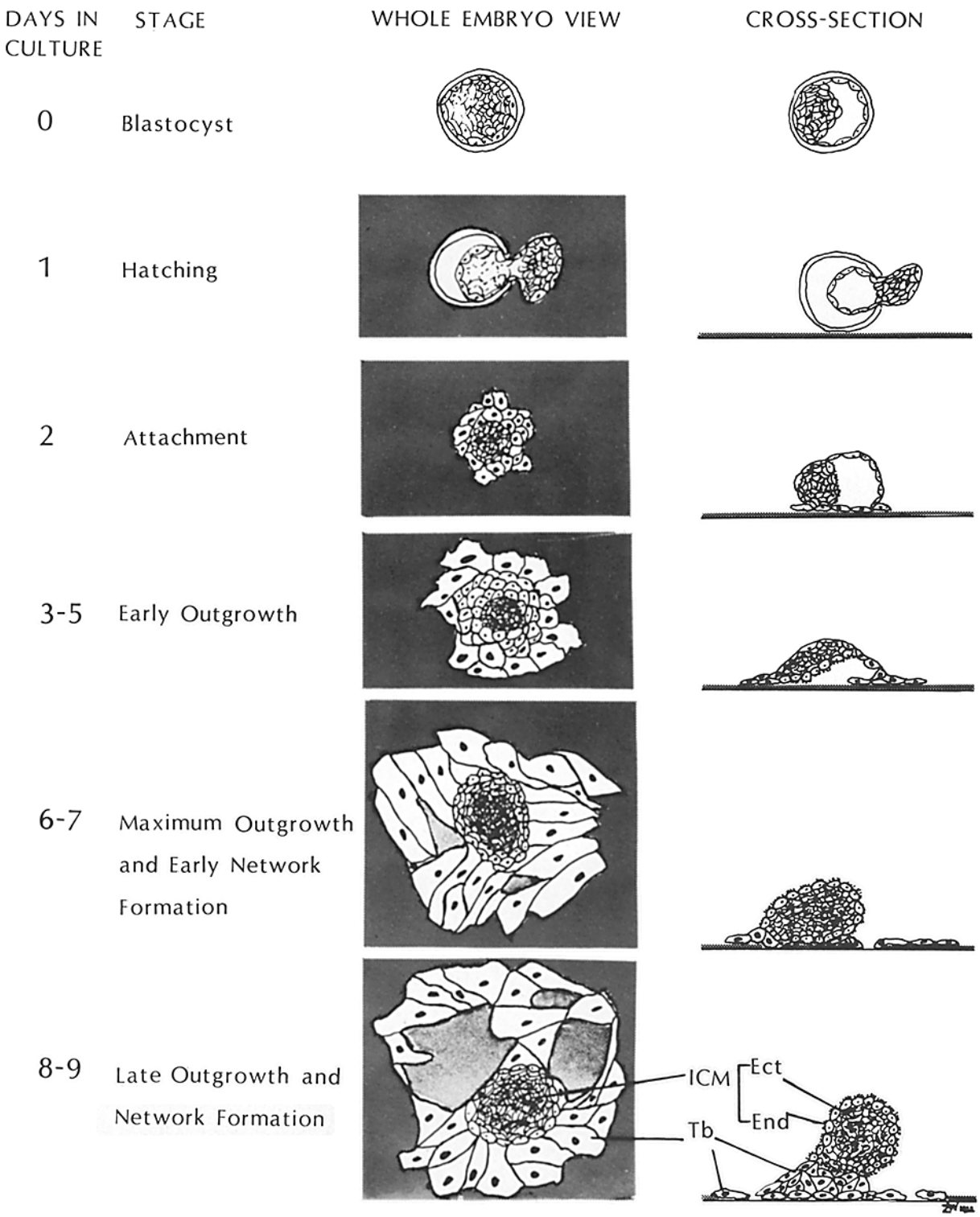

FIGURE 9 Diagram summarizing embryo development and matrix degradation in culture. Dark screened areas show intact matrix. Light gray screened areas show where matrix has been cleared. *Tb*, trophoblast; *ICM*, inner cell mass; *End*, endoderm cells; *Ect*, ectoderm cells.

an equal ability to digest all componens of the intact matrix—glycoproteins, elastin, and collagen (5, 16, 37). Thus, studies with purified single proteins in vitro may be less reliable guides to activity in vivo, and the relative degradation of the proteins of a complex matrix may provide information on degradation mechanisms. Studies with a native in vivo organized stroma, such as human amnion basement membrane (25), may shed further light on the proteolytic potential of trophoblast.

Our work suggests that proteinases play a role in implantation and, therefore, interference with proteinase activity would be expected to disrupt implantation. Accordingly, the chloromethyl ketone proteinase inhibitors derived from L-phenylalanine and L-lysine, and basic pancreatic trypsin inhibitor decreased the number of implanted embryos at days 12–18 of pregnancy when they were inserted into the uterine cavity of mice on days 2–4 post coitum (4). A fibrinolytic inhibitor, ϵ-

aminocaproic acid, did not block implantation in the rat when it was injected into the uterine lumen, although it did exert a deleterious effect on attachment and outgrowth in culture (7, 20). Although the trophoblast appears to have both diffusible and highly localized proteinase activities, the actual nature of the enzyme systems used by the trophoblast to effect degradation remains obscure. We were unable to show any effect of a spectrum of proteinase and metabolic inhibitors on trophoblast ability to digest matrix. The inhibitors may not have been specific for the trophoblast enzymes, or they may not have gained access to the active sites of enzyme digestion, either because the trophoblast was growing in direct contact with the matrix or because surface-bound enzymes are relatively resistant to fluid-phase inhibitors (15, 37). NaN_3 completely inhibited degradation although the cells remained attached to the matrix, which suggests that matrix degradation is a dynamic process requiring metabolically active cells.

Successful implantation may thus require a combination of cellular processes, including contact inhibition, mechanical movement through the uterine stroma, and proteolytic activity by trophoblast. Plasminogen activator may be crucial during the early attachment stages (20), whereas enzymes that digest glycoproteins, elastin, and collagen could be important as the embryo traverses the stroma. The localized nature of the latter activity may be vital for preserving the implantation site and preventing widespread disruption of the endometrium.

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