Placenta (1990), 11, 349–367

Human Trophoblast-Endometrial Interactions in an In Vitro Suspension Culture System

HARVEY J. KLIMAN[a,c], RONALD F. FEINBERG[b] & JULIA E. HAIMOWITZ[a]

[a] *Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA*

[b] *Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA*

[c] *To whom all correspondence should be addressed at: Department of Pathology and Laboratory Medicine, 6 Founders Pavilion, University of Pennsylvania School of Medicine, 3400 Spruce Street, Philadelphia, Pennsylvania 19104-4283, USA*

Paper accepted 19.3.1990

SUMMARY

We developed an in vitro suspension co-culture system to examine the interaction of 1st, 2nd and 3rd trimester purified cytotrophoblasts with human endometrium. Endometrium explants were added to cytotrophoblast cell suspensions and placed on an angled gyrating platform in a 37°C incubator. When endometrium was cultured alone it was able to remain viable for up to 3 days. When trophoblasts were cultured alone, they formed small and large aggregates, and occasionally spherical shells with hollow centers. When trophoblasts and endometrium were cultured together, the trophoblasts adhered to the exposed stromal surfaces of the tissue fragments. The surface epithelium was not receptive to trophoblast attachment except in one experiment when day 19 endometrium was used for the co-incubation, suggesting that surface attachment is usually restricted. A common finding was the presence of an acellular zone in the endometrium only adjacent to the attached trophoblasts. We speculate that this zone may be caused by proteolysis and resynthesis of ECM proteins by the trophoblasts. Based on our results, this in vitro suspension should prove useful for examining those factors which: (1) induce endometrial permissiveness, (2) promote paracrine effects on the endometrium, and (3) facilitate human trophoblast invasion.

INTRODUCTION

Human implantation depends on a series of specific interactions between the early trophoblastic cells of the blastocyst and the endometrium. By studying rare tissue sections of early human

0143–4004/90/040349 + 19 $05.00/0 © 1990 Baillière Tindall Ltd

pregnancies, Hertig attempted to describe histologically the events of blastocyst attachment to the endometrial surface and subsequent invasion and penetration of the basement membrane and underlying uterine stroma (Hertig and Rock, 1956). From these in vivo observations, it was hypothesized that the trophoblast layer of the developing blastocyst appeared to mediate both attachment to and invasion of the endometrium.

Trophoblasts are unique cells derived from the outer cell layer of the blastocyst which, in the human placenta, exist as undifferentiated cytotrophoblasts. Depending on their subsequent differentiation pathway, cytotrophoblasts can develop into hormonally active villous syncytiotrophoblasts, anchoring cell columns, or invasive intermediate trophoblasts (Kurman, Main and Chen, 1984). As human gestation progresses, invasive populations of trophoblasts attach to and interdigitate through the extracellular spaces of the endo and myometrium. The endpoint for this invasive behaviour is penetration of maternal spiral arteries within the uterus. Histologically, trophoblast invasion of maternal blood vessels results in disruption of extracellular matrix (ECM) components and development of dilated capacitance vessels within the uteroplacental vasculature. Biologically, trophoblast-mediated vascular remodeling within the placental bed allows for marked distensibility of the uteroplacental vessels, thus accommodating the increased blood flow needed during gestation. Abnormalities in this invasive process have been correlated with early and mid-trimester loss, pre-eclampsia, and intrauterine growth retardation (Pijnenborg et al, 1981; Roberts et al, 1989).

The cellular and biochemical basis of human implantation is currently not well understood because few in vitro model systems exist to study this fundamental process. Although trophoblasts derived from the human blastocyst are not generally available for investigation, we have theorized that cytotrophoblasts of the developed placenta have an undifferentiated phenotype and are capable of behaving in a similar fashion to the trophoblasts of the blastocyst. Human cytotrophoblasts prepared by the established method of Kliman et al (1986) undergo differentiation in culture to form syncytia and secrete the hormones human chorionic gonadotropin (hCG), human placental lactogen, and progesterone in a regulated fashion (Feinman et al, 1986; Kliman et al, 1986; Kliman, Feinman and Strauss, 1987). In addition, these unique cells attach to and invade ECM proteins (Kao et al, 1988; Kliman and Feinberg, 1990) and secrete urokinase-type plasminogen activator (Queenan et al, 1987). Plasminogen activator inhibitor types 1 and 2 are synthesized by trophoblasts both in vitro and in vivo (Feinberg et al, 1989), suggesting the capacity for autoregulation of invasive behaviour.

With this biochemical machinery, we wondered if normal human cytrotrophoblasts could mimic blastocyst interaction with the endometrial surface. Since these cultured cells exhibit attachment and invasive properties, we had the impetus to investigate trophoblast-endometrial interactions in vitro. Therefore, we developed an in vitro suspension model system utilizing purified human trophoblasts and explants of human endometrium. Using this system we have investigated: (1) trophoblast aggregation properties, (2) the specificity of trophoblast-endometrial attachment, (3) trophoblast-induced necrosis at sites of attachment, and (4) a paracrine effect of trophoblasts on endometrium. Our observations suggest this in vitro system may recapitulate some of the early cellular events of human implantation.

MATERIALS AND METHODS

Preparation and culture of human trophoblasts

The method of preparation and culture of human trophoblasts has been described previously (Kliman et al, 1986). The procedure works well for 1st trimester through term placental tissue.

Briefly, villous tissue was dispersed with trypsin and DNase. For first trimester placentae, digest times were reduced from 20 to 10 min. The dispersed cells were then purified on a Percoll gradient (5–70 per cent). A middle-band at density 1.040–1.060 g/ml is comprised of cytotrophoblasts. The cells isolated from this layer have the ultrastructural features of cytotrophoblasts, and contamination by macrophages (assessed by immunocytochemical localization of α_1-antichymotrypsin) and fibroblasts and endothelial cells (assessed by immunocytochemical detection of vimentin) is less than 5 per cent (Kliman et al, 1986). The cytotrophoblasts were cultured in Dulbecco's Modified Eagles' Medium (DMEM) containing 25 mM glucose and 25 or 50 mM HEPES (DMEM-HG) supplemented with gentamicin (50 μg/ml), glutamine (4 mM), and in some cases with 20 per cent (v/v) heat-inactivated fetal calf serum (FCS).

Endometrial co-culture

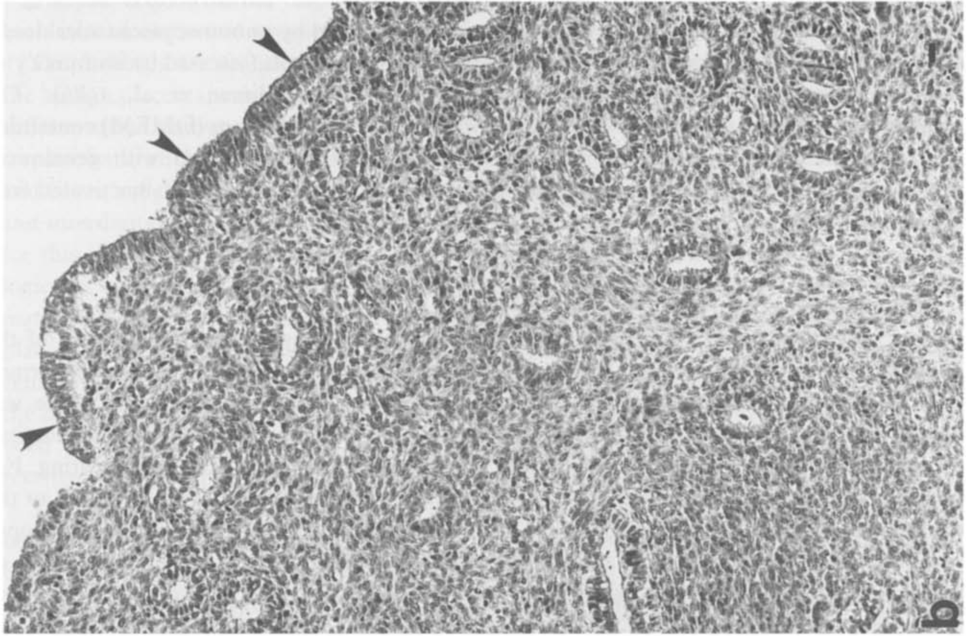
Endometrial tissue was collected in a sterile environment immediately after removal of the uterus or obtaining the specimen by dilation and curettage. The tissue was placed in warmed DMEM-HG supplemented with gentamicin (50 μg/ml). In the laboratory the tissue was minced into 1–2 mm cubes and transferred to DMEM-HG–20 per cent FCS–gentamicin (50 μg/ml). A small portion of each specimen was fixed in Bouin's for histological dating. For endometrial co-culture experiments, 1 ml of a 1×10^6 cells/ml suspension was added to the endometrial explants. Cells were cultured in loosely capped sterile 17×100 mm polypropylene snap-top tubes at 37°C in an atmosphere of humidified 95 per cent air–5 per cent CO_2 or 95 per cent O_2–5 per cent CO_2 while being gyrated on an angled ($\sim 30°$) rotator (Red Rotor, Hoefer Scientific Instruments, San Francisco, California) at a setting of five. Media was changed every 24 h by centrifugation of the tubes at low speed at room temperature, aspiration of the spent media, followed by replacement with fresh media.

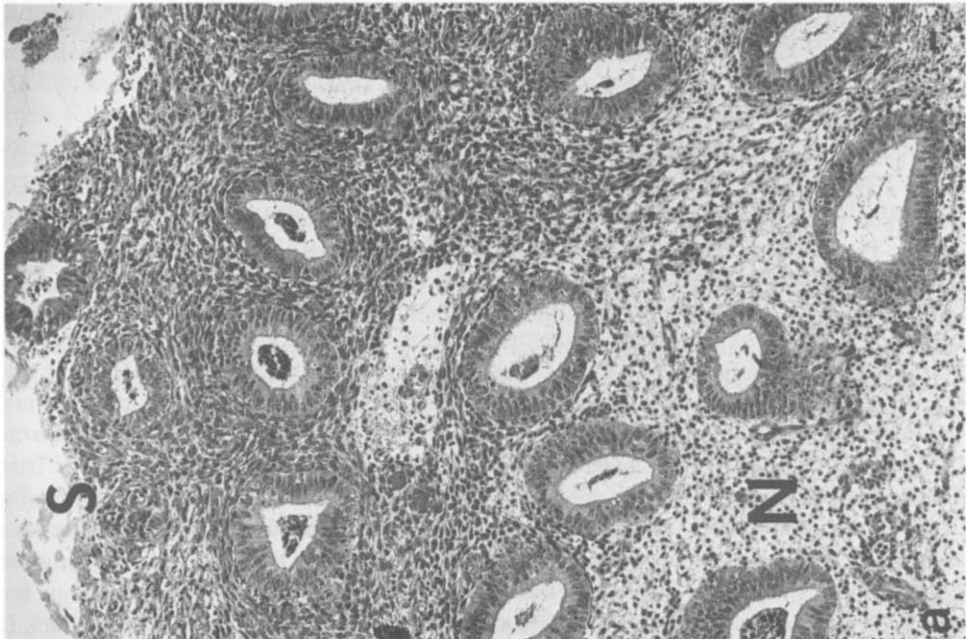
Preparation and culture of other tissues and cells

Endothelial cells were purified from human umbilical cords as previously described (Jaffe, Hoyer and Nachman, 1974), 14-49B melanoma cells, derived from a metastasis of human melanoma, were generously supplied by Dr Manard Herling of the Wistar Institute, Philadelphia, Pennsylvania. JEG-3 choriocarcinoma cells were obtained from the American Type Culture Collection, Rockville, Maryland. Normal human breast tissue, fallopian tube, and umbilical cord were collected after clinical examination of residual tissue from the Surgical Pathology Laboratory of the Hospital of the University of Pennsylvania under the auspices of the Cooperative Human Tissue Network, Eastern Division. All specimen collection protocols were approved by the Institutional Review Board of the University of Pennsylvania.

Histology

Tissue was fixed in Bouin's, embedded in paraffin and sectioned at 5-μm intervals. Twenty to 40 serial sections were made, with haematoxylin and eosin (H and E) staining of every other ten slides. H and E slides were assessed for the following: (1) trophoblast adhesion, including how many cells attached and whether they were attached to cut surfaces or surface epithelium, (2) tissue necrosis (superficial or central necrosis), and (3) the presence and depth from the surface of trophoblast invasion. All light level micrographs were taken from H and E stained material (unless otherwise noted) and photographed using Kodak Plus-X pan film on a Nikon Microphot FX microscope.




S
N

Immunocytochemistry

Since trophoblast invasion can not be readily assessed using H and E staining alone, immunocytochemistry against α-hCG subunit was employed. Three to four slides per co-culture were stained for α-hCG as previously described (Kliman et al, 1986). Rabbit anti-α-hCG was generously supplied by Dr Steven Birken of Columbia University.

Transmission electron microscopy (TEM)

Tissue was fixed with Karnovsky's fixative and processed for TEM as described previously (Kliman et al, 1986). Thick sections (1 μm) were cut and stained with Toluidine blue to evaluate the orientation of the specimen and to assess whether the section plane revealed appropriate trophoblast-endometrial interactions. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined at 50 or 75 kV with a Hitachi 600 electron microscope. Multiple micrographs were taken and assessed for presence of trophoblast-endometrial cell contacts, trophoblast stromal interactions, and the presence of cellular necrosis.

RESULTS

Endometrium-trophoblast suspension co-culture

When endometrium was cultured alone in a 95 per cent air–5 per cent CO_2 atmosphere it was able to remain viable for up to 3 days. When tissue necrosis was present, it was mainly in the central portion of the tissue [Figure 1(a)]. Culturing endometrium in a 95 per cent O_2–5 per cent CO_2 atmosphere led to a slight improvement in tissue viability [Figure 1(b)], but larger tissue fragments still, to a variable extent, exhibited central necrosis. When trophoblasts were cultured alone, they formed small and large aggregates. The large aggregates formed trabecular patterns and pseudo-villous structures [Figure 2(a)]. Occasional large aggregates formed spherical shells with hollow centers—reminiscent of the trophoblastic shell of an early blastocyst [Figure 2(b)].

When trophoblasts and endometrium were incubated together in suspension, trophoblasts adhered firmly to the tissue fragments [Figure 3(a)]. Utilizing antibodies against α-hCG, we confirmed by immunohistochemistry that these cells were trophoblasts [Figure 3(b)], and not, for example, other minor cell types from the trophoblast preparation or break down products of the endometrium itself. In several co-incubation preparations, we also noted α-hCG-positive trophoblasts which had actually penetrated into the endometrial explants [Figure 3(c)].

The concept of a 'window' for implantation on days 19–20 has been discussed by a number of workers (Navot et al, 1988). We therefore wondered if we would see different patterns of trophoblast-endometrial interactions depending on the date of the endometrium (Table 1). Trophoblast cells attached to the exposed endometrial stromal surfaces in 26 separate co-culture experiments [Figure 3(a)]. On one occasion when a day 19 endometrium was used, we observed trophoblast binding to the surface epithelium (Figure 4). We speculate that this asso-

Figure 1. Endometrial viability in suspension culture. Human endometrial explants were cultured for 24 h (a) or 48 h (b) in the presence of 95 per cent air–5 per cent CO_2 (a) or 95 per cent O_2–5 per cent CO_2 (b) in suspension culture, fixed and processed for histology, as described in the Materials and Methods section. (a) Proliferative glands can be seen within endometrial stroma. Note the pycnotic stromal nuclei (N) in the center of the biopsy as compared to the viable tissue near the cut surface (S). (b) Early secretory glands scattered in uniform endometrial stroma. Note that the surface epithelium (arrow heads) and deeper tissues are uniformly viable. The bars represent 20 μm.

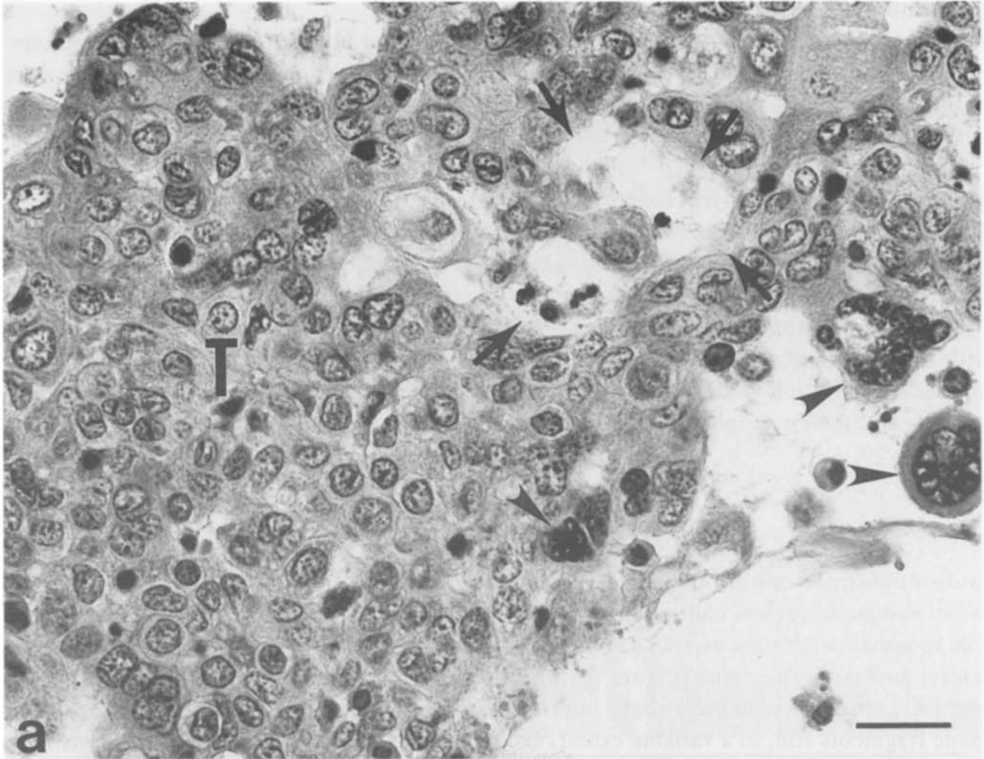


Figure 2. Trophoblast aggregation in suspension culture. Human trophoblasts were cultured for 24 h, fixed and processed for histology, as described in the Materials and Methods section. (a) Many of the trophoblasts have aggregated (T), and some have fused to form syncytiotrophoblasts (arrow heads), as has been described under two-dimensional culture conditions (Kliman et al, 1986). Within the aggregated trophoblasts, cavities can be identified (arrows), reminiscent of the trophoblast lining of a villous.

ciation with the endometrial surface epithelium with day 19 endometrium, although only observed on one occasion, may be related to the natural receptivity of the epithelium at this time of the cycle.

In 25 of 26 co-culture experiments the presence of 'contact necrosis' was observed at the points where the trophoblasts made contact with the endometrial explants [Table 1, Figures 3(a), 5 and 8]. Usually when endometrial explants were cultured alone in suspension they showed evidence of central necrosis, presumably because the central portions of the tissue were farthest away from the nutrients and oxygen in the media [Figure 1(a)]. When endometrium was co-cultured with trophoblasts, necrosis was usually not seen centrally, but peripherally, and always directly adjacent to the attachment sites of the trophoblasts. We speculate that this necrosis may be caused by the secretion of trophoblast proteases (Queenan et al, 1987), although stimulation of endometrial proteases by the trophoblasts can not be ruled out. It is also possible that this zone contains newly synthesized ECM proteins (Ulloa-Aguirre et al, 1987) in addition to the breakdown products of the endometrium.

It has been suggested by others that only first trimester placental trophoblasts have the capacity to degrade and invade into endometrium or ECM (Fisher et al, 1985, 1989). We therefore performed trophoblast-endometrium co-culture experiments using cytotrophoblasts derived from 1st trimester (8–12 weeks) (Figure 6), 2nd trimester (13 and 18 weeks, not shown) and

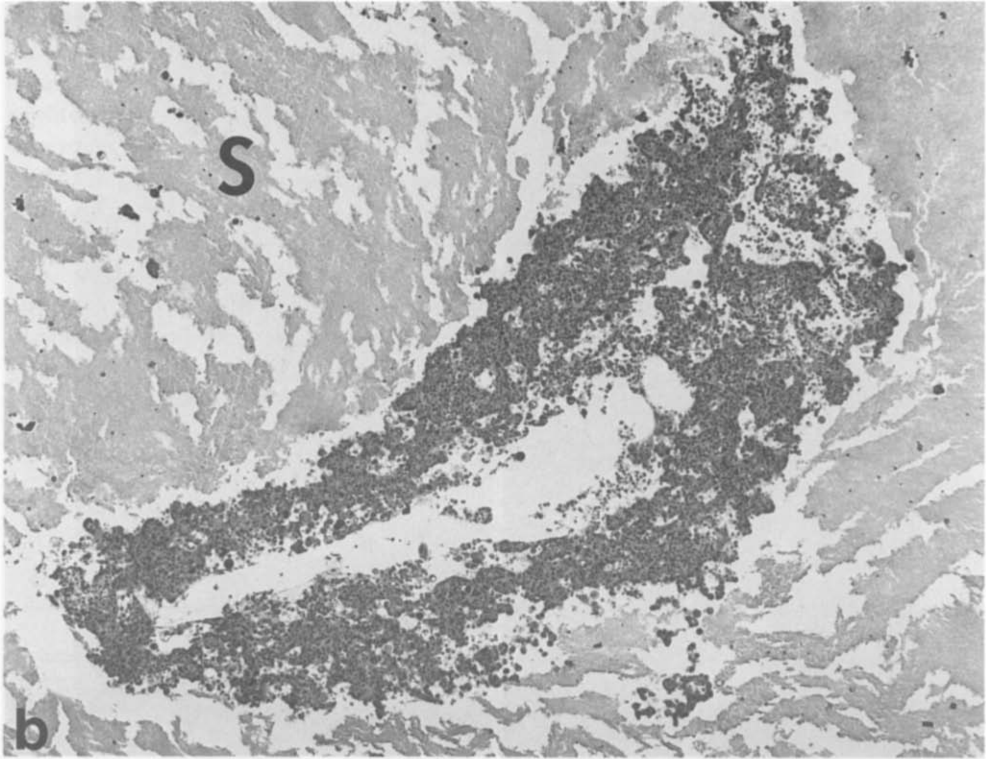
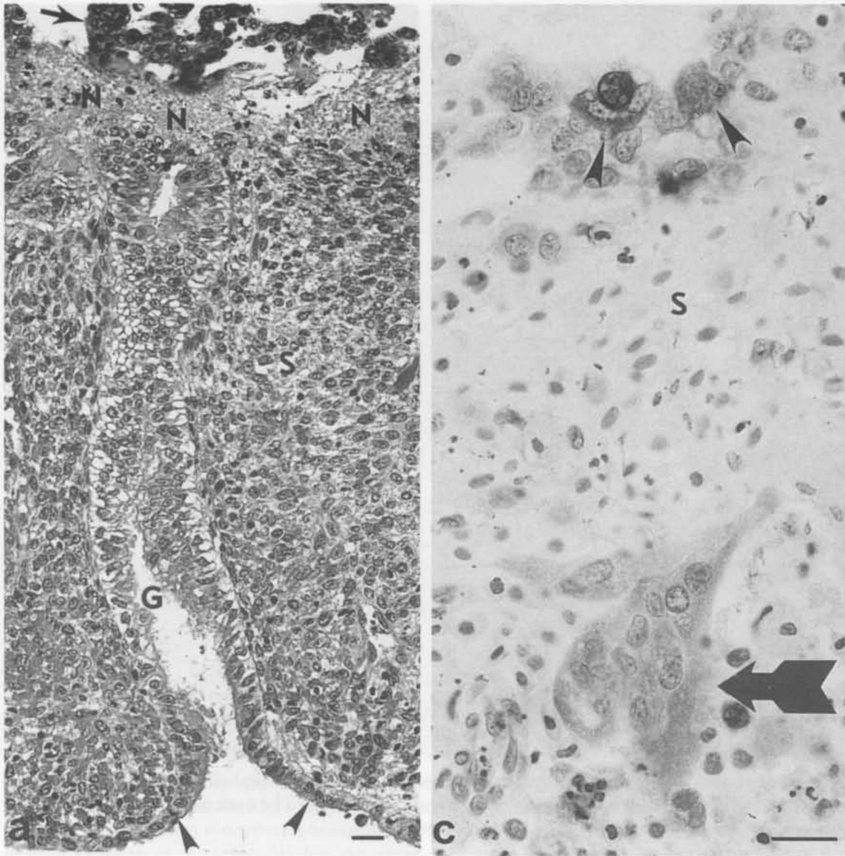


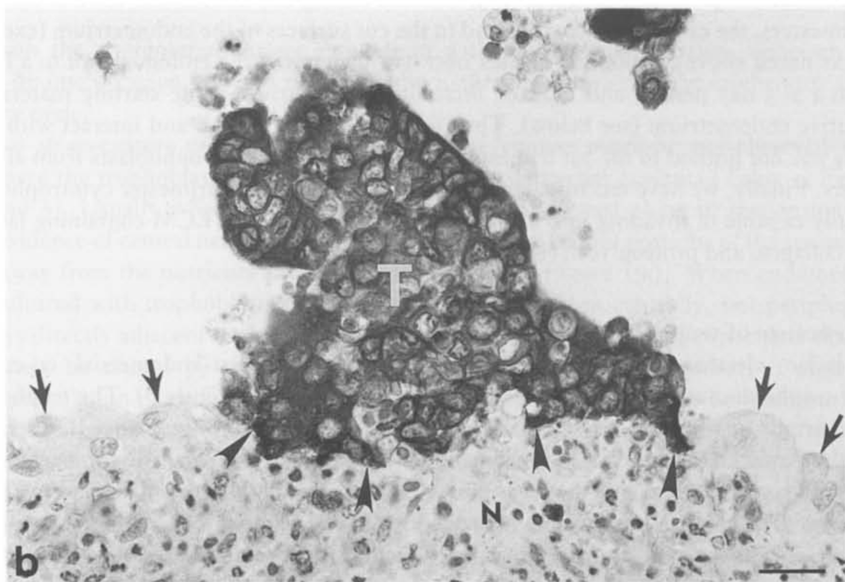
Figure 2. (b) Cross-sectioning through the center of a large aggregation of trophoblasts reveals it to have a hollow center, reminiscent of the trophoblastic shell of the developing blastocyst. The material around the aggregate represents fixed precipitated serum proteins (S). The bars represent 20 μm.

term placentae (>36 weeks) (Figures 3–5) without noting any substantial differences. In all three trimesters, the cytotrophoblasts bound to the cut surfaces of the endometrium (except on day 19, as noted above), produced contact necrosis, penetrated the endometrium to a limited extent in a 2–3 day period, and induced secretory endometrium if the starting material was proliferative endometrium (see below). Therefore, the ability to bind and interact with endometrium was not limited to the 1st trimester, but was exhibited by trophoblasts from all three trimesters. Finally, we have recently demonstrated that 1st and 3rd trimester cytotrophoblasts are equally capable of invading into and degrading Matrigel™, an ECM containing laminin, type IV collagen, and proteoglycan (Kliman and Feinberg, 1990).

Ultrastructure of trophoblast-endometrial interaction

Transmission electron microscopic examination of trophoblast-endometrial co-cultures revealed trophoblast attachment to and penetration of the ECM (Figure 7). The trophoblasts attached firmly to the exposed stromal surfaces of the endometrial explants [Figure 7(a)]. Where they attached to the ECM, there appeared to be fewer microvilli compared to the opposite cell surface, suggesting the beginning of polarization of the trophoblast. Some trophoblasts showed cytoplasmic extensions penetrating the endometrial ECM [Figure 7(b)], possibly representing the cellular mechanism by which these cells degrade and invade into this substrate.


N
N
N
S
G
a
S
c


T
N
b

Paracrine effect of trophoblasts on proliferative endometrium

In addition to the observations noted above we also observed the induction of secretory changes on proliferative endometrium when co-cultured with trophoblasts (Table 1). When proliferative phase endometrial explants [Figure 8(a)] were cultured for 24 h in the absence of exogenous steroids, little change was noted [Figure 8(b)]. As with the 0-h tissue, the 24-h explant showed a few subnuclear vacuoles in the glands, and the stromal cells showed only scant cytoplasm, consistent with predecidual stroma. When exogenous progesterone was added at physiological levels (50 ng/ml), secretory phase endometrium consistent with day 17 (Blaustein, 1985) could be induced [Figure 8(c)], as has been shown previously (Kohorn and Tchao, 1969). In addition, the progesterone appeared to induce expansion of the stromal cell cytoplasm, consistent with early decidual changes. When this proliferative endometrium was concurrently co-cultured in parallel with only cytotrophoblasts for as little as 24 h, secretory endometrium consistent with day 18–19 was induced [Figure 8(d)]. Similar secretory changes

Table 1. Effect of endometrial date on endometrial-trophoblast co-culture

Endometrial date		Binding to ECM	Binding to surface	Contact necrosis	Paracrine effect
Proliferative	(n = 15)	15/15	0/15	15/15	13/15
Secretory	(n = 11)	11/11	1/11	10/11	N/A
day 16	(n = 1)	1/1	0/1	1/1	
19	(n = 2)	2/2	1/2	2/2	
19	(n = 1)	1/1	0/1	1/1	
23	(n = 2)	2/2	0/2	1/2	
25–27	(n = 5)	5/5	0/5	5/5	
Total	(n = 26)	26/26	1/26	25/26	13/15

Endometrial explants from 26 different patients were co-incubated with different preparations of cytotrophoblasts as described in the Materials and Methods section. The endometrial date was determined by histological examination at 0 h by standard dating methods (Blaustein, 1985). Binding to ECM was defined as trophoblasts attached to cut, exposed endometrial surfaces without evidence of surface epithelium. Binding to surface was defined as trophoblasts clearly attached to intact surface epithelium. Contact necrosis was defined as any disruption of stromal cellular architecture or death at the points where trophoblasts were attached. Paracrine effect was defined as induction of secretory changes in the glandular epithelium, which, therefore, was not applicable (N/A) to initially secretory endometrium.

Figure 3. Trophoblast-endometrium co-incubation. Human term trophoblasts were co-incubated with endometrial explants for 24 h, fixed and processed for histology and either stained with H and E (a) or immunocytochemically stained using antibodies against α-hCG (b) and (c) as described in the Materials and Methods section. (a) Trophoblast aggregates (arrow) have attached to the cut surface of the endometrial explant, producing a zone of necrosis (N). Note that the surface epithelium (arrow heads) and glandular epithelium (G) show no evidence of trophoblast attachment. The stromal cells (S) away from the trophoblasts are viable. (b) A strongly α-hCG positive group of trophoblasts (T) is firmly attached to exposed endometrial stroma (arrow heads). Note the zone of necrosis (N) adjacent to the attached trophoblasts. On either side of the trophoblasts, surface epithelium can still be seen (arrows). (c) Endometrial explant with α-hCG positive trophoblasts attached at the cut surface (arrow heads) and an α-hCG positive syncytiotrophoblast within the endometrial stroma (s) (large arrow). The bars represent 20 μm.

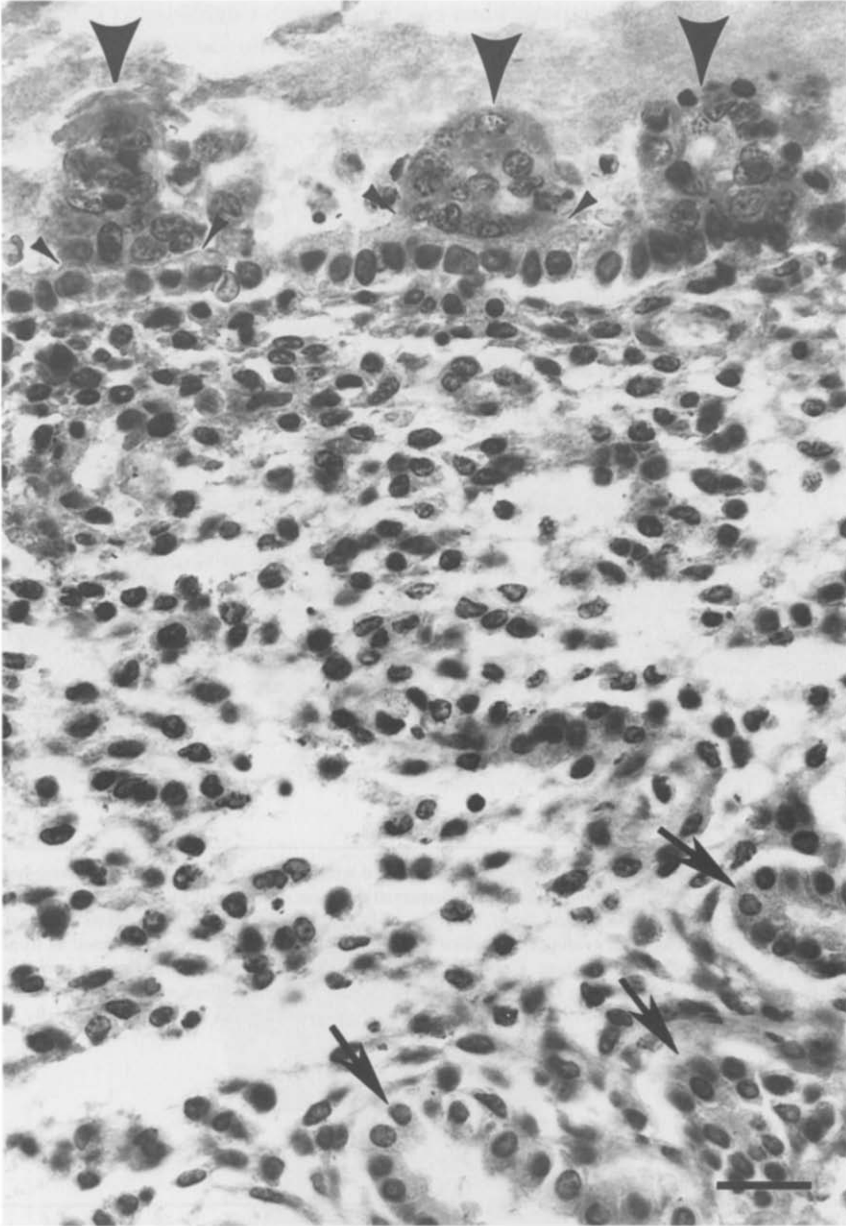


Figure 4. Co-incubation with day-19 endometrium. Human term trophoblasts were co-incubated with day-19 endometrial explants for 24 h, fixed and processed for histology as described in the Material and Methods section. Three trophoblast aggregates (large arrow heads), can be seen attached (small arrow heads) to the endometrial surface epithelium. A portion of a convoluted endometrial gland can be seen within the endometrium (arrows). The bar represents 20 μm.

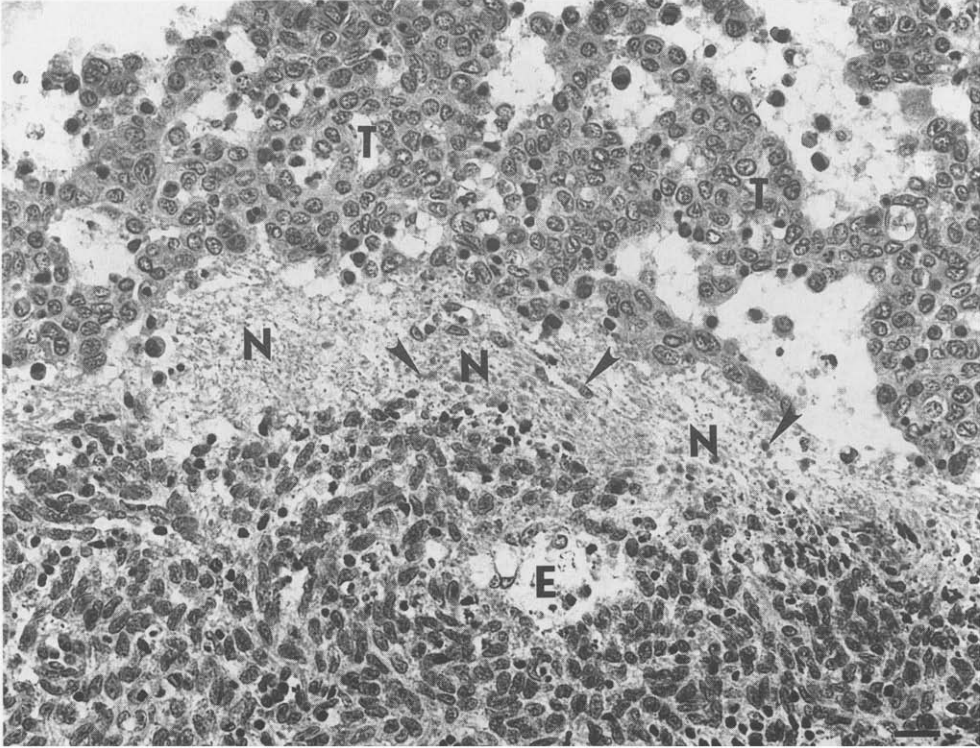


Figure 5. Zone of contact necrosis. Human term trophoblasts were co-incubated with an endometrial explant for 24 h, fixed, and processed for histology. The aggregated trophoblasts (T) have attached to the cut surface of the endometrium (E), forming a zone of necrosis (N). Cell remnants (arrow heads) can be seen within this zone, indicating that it is not an acellular zone. The bar represents 20 μm.

were noted in 13 out of 15 proliferative endometrial co-culture experiments (Table 1). We have shown previously (Feinman et al, 1986; Kliman et al, 1986) that cultured cytotrophoblasts secrete progesterone, suggesting that trophoblast derived progesterone is active in stimulating the secretory changes we see in these co-culture experiments. These results also suggest that localized hormonal effects of the implanting blastocyst may direct morphologic and biochemical changes in the specific area of the endometrium where trophoblast attachment and invasion is occurring.

Co-culture results as a function of tissue and cell type

We also investigated the specificity of our observations by co-culturing cytotrophoblasts with explants of breast tissue, fallopian tube, and umbilical cord; and incubating endometrium with human endothelial cells, JEG-3 choriocarcinoma cells, and malignant melanoma cells. When cytotrophoblasts were co-cultured with breast tissue they adhered to the cut surfaces, but showed no evidence of contact necrosis [Figure 9(a)]. When incubated with umbilical cord explants, the trophoblasts attached to the cut surfaces of umbilical cord [Figure 9(b)], and again showed no evidence of contact necrosis. Co-culture experiments performed with JEG-3 cells (a malignant trophoblast cell line) revealed that they attached to endometrial cut surfaces, but not to the surface epithelium, and induced necrosis at the sites of attachment [Figure 9(c)], much like normal trophoblasts. When endothelial cells were co-cultured with endometrium,

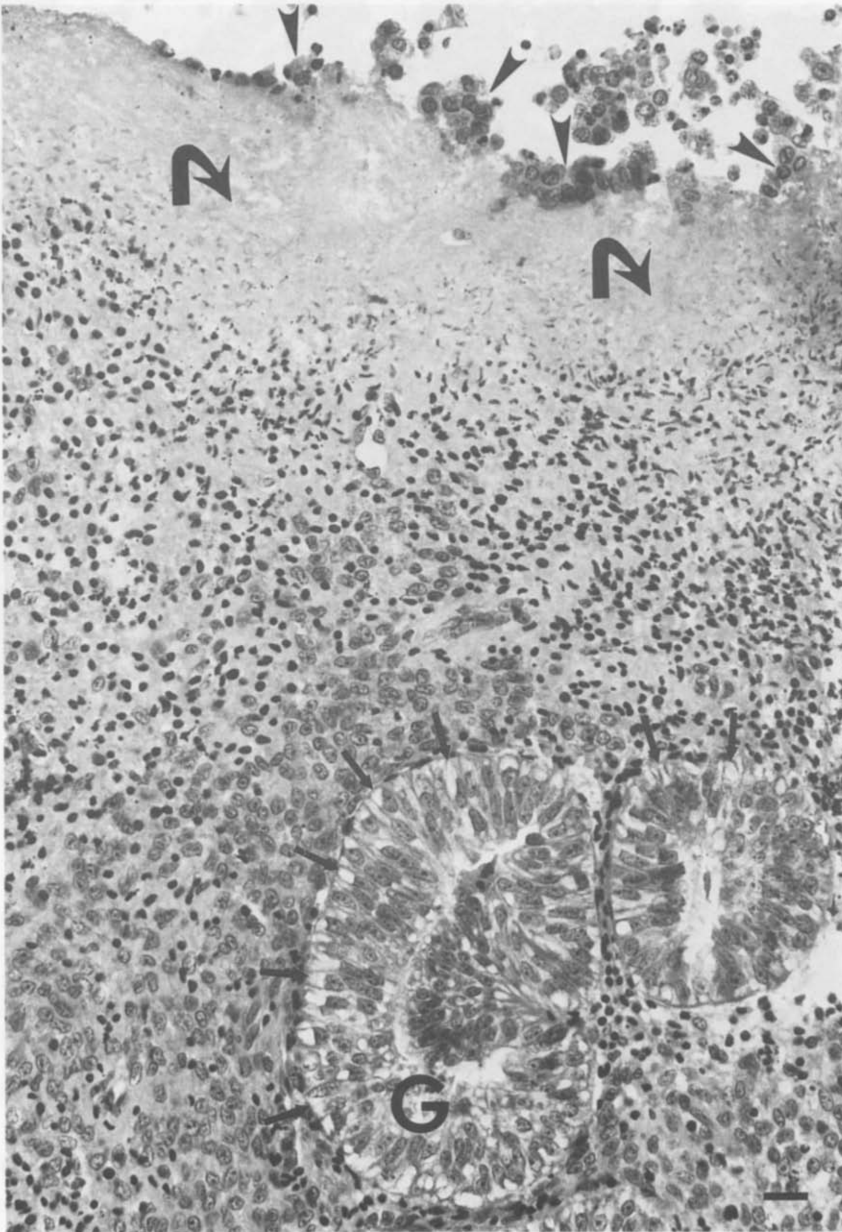


Figure 6. Co-incubation of endometrium with 1st trimester trophoblasts. Human 1st trimester trophoblasts were co-incubated with proliferative endometrial explants for 24 h, fixed and processed for histology as described in the Materials and Methods section. Multiple trophoblast aggregates have attached to the exposed ECM surface of the endometrial explant (arrow heads). Beneath the trophoblasts is a zone of necrosis (bent arrows) similar to that seen when term trophoblasts are utilized (see Figure 5). The glands (G) have been induced to produce secretory vesicles (arrows), as can be seen when term trophoblasts are co-incubated with proliferative endometrium [see Figure 8(d)]. The bar represents 20 μm.

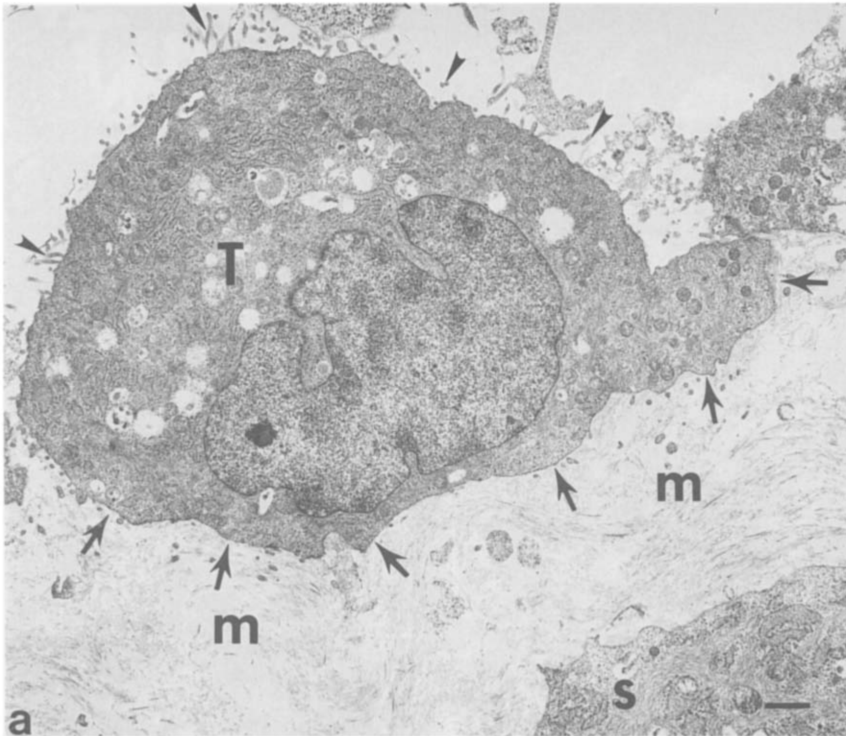


Figure 7. Ultrastructure of trophoblast-endometrium interaction. Human term trophoblasts were co-incubated with endometrial explants for 24 h, fixed and processed for transmission electron microscopy as described in the Materials and Methods section. (a) Trophoblast (T) attached firmly (arrows) to exposed endometrial ECM (m). A portion of a stromal cell can be seen (s), as well as collagen fibers of the ECM. Note the considerable number of microvilli (arrow heads) on the unattached cell surface as compared to the paucity of microvilli on the cell surface attached to the endometrial ECM (arrows).

they exhibited attachment to the endometrial cut surfaces, but not the surface epithelium, and there was no evidence of contact necrosis [Figure 9(d)]. Malignant melanoma cells attached to the endometrial cut surfaces, did not attach to the surface epithelial layer, and did not induce necrosis at the sites of attachment [Figure 9(e)]. Interestingly, when these same melanoma cells were incubated with fallopian tube explants, the tumour cells again only attached to the cut surfaces [Figure 9(f)]. We conclude from these studies that attachment to the cut surfaces of tissue fragments, i.e. stromal ECM components, is not specific to trophoblasts, but is a behaviour shared by a wide variety of normal and malignant cells. What appears to be specific is that: (1) it is the surface epithelium of both the endometrium and fallopian tube which allows or prevents cell attachment, (2) normal human trophoblasts bind to the endometrial surface epithelium only at a specific time in the menstrual cycle, and (3) when trophoblasts bind to endometrium, they induce a band of necrosis similar to the fibrinoid layer (Nitabuch's layer) noted at implantation sites in vivo.

DISCUSSION

Studies which focus on early blastocyst behaviour and nidation have typically utilized animal

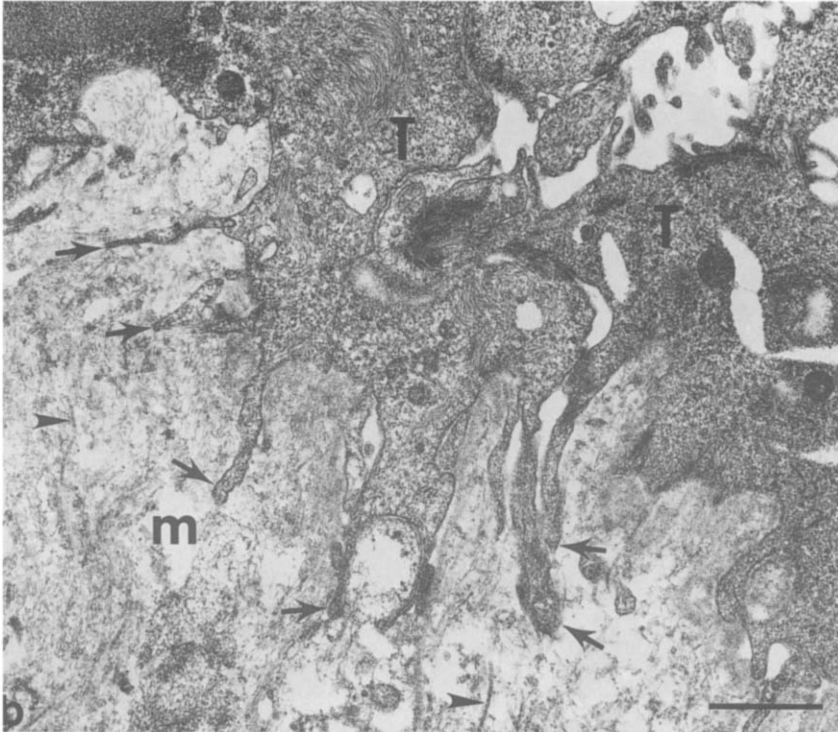
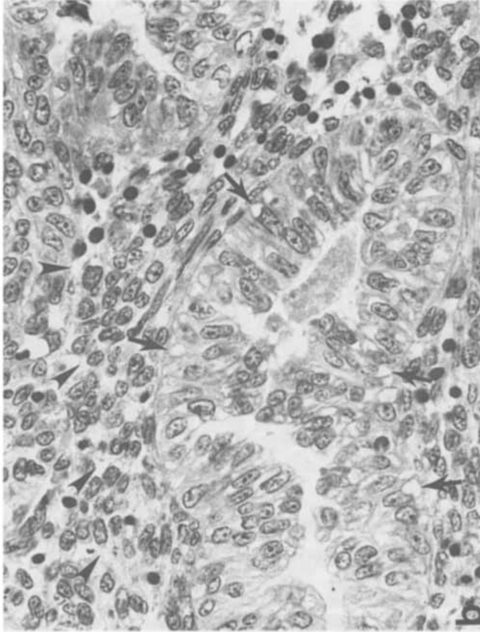


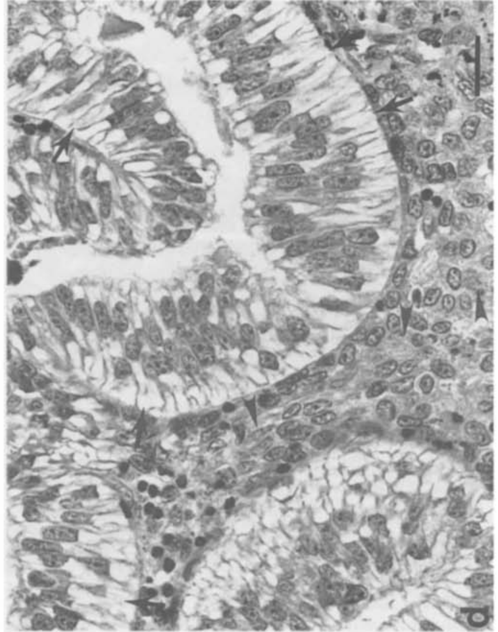
Figure 7. (b) Higher power of a different trophoblast (T) at the point of attachment to the ECM (m). Multiple fine trophoblast cytoplasmic extensions (arrows) can be seen penetrating into the endometrial ECM. Note the collagen fibers (arrow heads) within the matrix. Bars represent 1 μm.

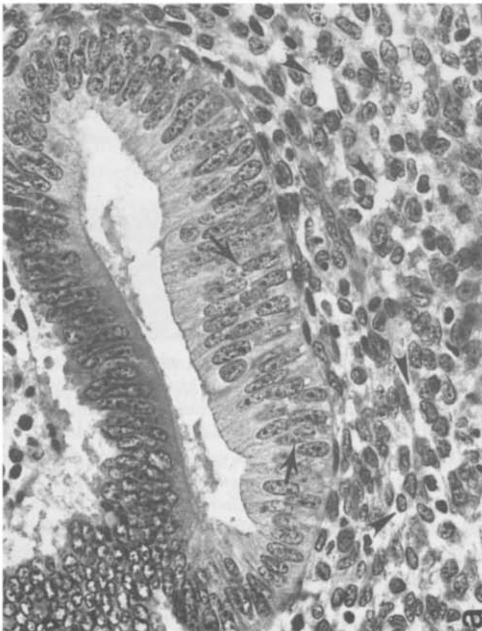
blastocyst outgrowth models (Enders, Chavez and Schlafke, 1981; Armant, Kaplan and Lennarz, 1986; Farach et al, 1987; Carson, Tang and Gay, 1988). When murine blastocysts are isolated and plated onto various surfaces, they proceed to hatch, attach to the substratum, and initiate spreading of their trophoblastic shell. This work has permitted the study of early developmental processes of peri-implantation blastocysts, such as initial trophoblastic attachment and outgrowth, in the absence of endometrial influences. Others have modified these ani-

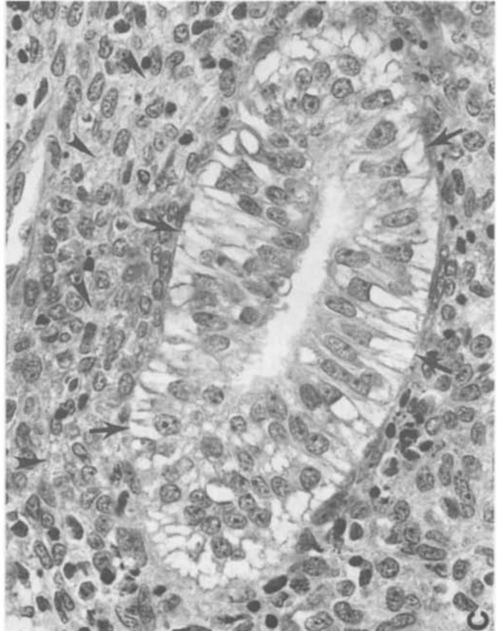
---

Figure 8. Induction of secretory endometrium by trophoblasts. Results of a representative experiment showing the effects of a 24-h suspension culture incubation of late proliferative human endometrium (a) in the presence of media alone, (b), media with 50 ng/ml of progesterone (c), and media with 1 + 10^6 trophoblasts/ml (d). (a) Late-proliferative human endometrium showing stratification of the glandular nuclei (arrows) with scanty cytoplasm in the stromal cells (arrow heads). (b) After 24 h of suspension culture in media alone, the endometrial glands still show stratified glandular nuclei, but a few distinct subnuclear vacuoles can be seen (arrows). The stromal cells still have indistinct cytoplasms (arrow heads). (c) In the presence of 50 ng/ml of progesterone, the glandular cells show classic changes consistent with day 17 secretory endometrium: loss of stratification of the nuclei, rows of distinct sub-nuclear vacuoles (arrows) with occasional supranuclear vacuoles. The stromal cells now have evidence of distinct cytoplasms (arrow heads), suggestive of early decidual change. (d) In the presence of trophoblasts without exogenous progesterone, the tissue has been converted to classic days 18–19 endometrium: Large columnar glandular cells with extensive subnuclear and supranuclear vacuoles (arrows). Secretory material is not noted within the glandular lumens (characteristic of days 20–21 endometrium). The stromal cells appear to have developed distinct cytoplasms (arrow heads), possibly denser than that seen with progesterone alone (c). These results were observed with seven additional endometrial specimens when co-cultured with seven different trophoblast preparations from first and third trimesters. All micrographs are at the same magnification. The bar represents 20 μm.


b


d


a


c

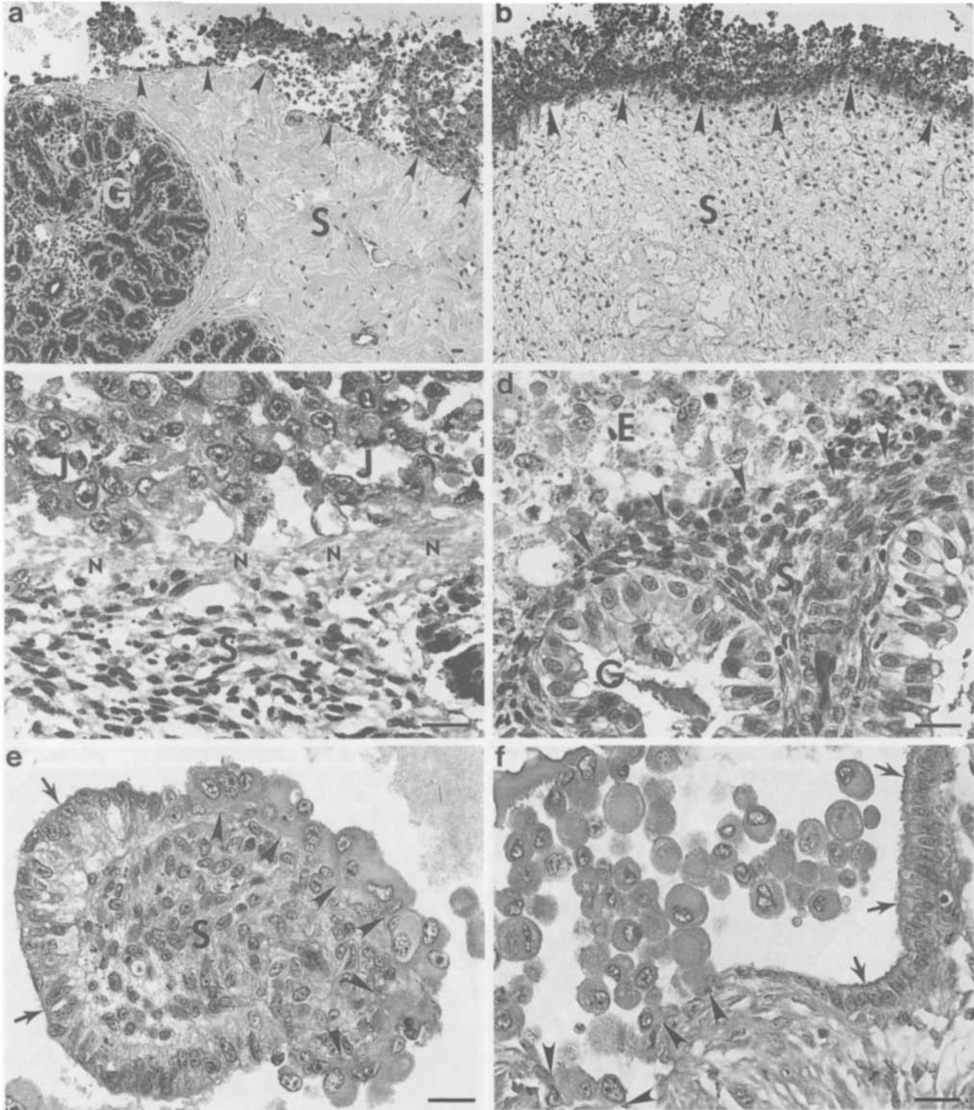


Figure 9. Tissue and cell co-culture controls. A variety of human tissues were co-incubated with various cell types (1×10^6 cells/ml) in suspension culture for 24 h. The cultures were fixed and processed for histology as described in the Materials and Methods section. (a) Term trophoblasts were co-cultured with explants from normal human breast. The trophoblasts have attached firmly (arrow heads) to the exposed ECM of the breast stroma (S). There is no evidence of contact necrosis. Note the glandular elements of the breast (G). (b) Term trophoblasts were co-cultured with pieces of human umbilical cord. The trophoblasts have coalesced onto the umbilical cord surface (arrow heads). The underlying myxoid stroma (s) appears unaffected by the trophoblasts. (c) JEG-3 choriocarcinoma cells were co-cultured with human endometrium. The JEG-3 (J) cells have attached to the endometrium and produced a zone of necrosis (N). Near this zone of contact, the stromal cells (S) appear more pycnotic than usual, but are not frankly necrotic. (d) Human umbilical vein endothelial cells were co-cultured with human endometrium. Like the trophoblasts, these cells have attached to the exposed cut surfaces of the endometrium (arrow heads), but unlike the trophoblasts, the endothelial cells have not induced a zone of necrosis in the stromal cells (S) at the contact points. Note the endometrial gland (G). (e) Malignant melanoma cells co-cultured with human endometrium. The malignant cells have firmly attached to the cut surface of the endometrium (arrow heads), but have not attached to the surface epithelium (arrows). Note the lack of necrosis in the stroma (S). (f) Malignant melanoma cells co-cultured with human fallopian tube. As with the endometrium, the melanoma cells have attached to the exposed stromal surfaces (arrow heads), but have not attached to the epithelial surfaces (arrows). There is also a lack of necrosis at the contact points. Bars represent 20 μm.

mal outgrowth models by plating blastocysts onto endometrial monolayers (Glass, Spindle and Pedersen, 1979) or onto whole endometria (Glenister, 1961; Grant, Ljungkvist and Nilsson, 1975) in an attempt to examine the role that the endometrium plays in regulating implantation. The endometrial monolayer studies have offered insight into specific cell adhesion, and the studies utilizing endometrial organ culture demonstrate the three-dimensional relationships which exist during actual implantation. Although these model systems have contributed to progress in animal implantation, the parallels to human implantation are unknown.

Histological studies of both animal and human implantation sites suggest that it is the trophoblastic shell of the blastocyst that plays a critical role in the initiation and progression of implantation (Hertig and Rock, 1956). Therefore, we speculated that purified cytotrophoblasts from placentae of different gestational ages could be co-cultured with endometrium in order to elucidate implantation events in the human. Our initial goal was to observe specific attachment of trophoblasts to the epithelial surface of cultured endometrial explants. By utilizing a shaking suspension culture system, we have shown that we are able to maintain viable endometrial explants for at least 3 days [Figure 1(a), (b)]. However, when trophoblasts were purified from placentae of any trimester and co-cultured with proliferative or late secretory endometria, they attached primarily to the exposed ECM of the explants [Figures 2(a), 2(b), 3(a), 3(b) and 5]. Once attached, α-hCG positive trophoblasts penetrated the endometrial ECM [Figure 3(c)]. A striking feature of these experiments was that trophoblasts did not attach to the epithelial surfaces of the explants, except in one experiment when trophoblasts were co-cultured with day 19 endometrium. In this experiment, we speculate that the trophoblasts did bind to the surface epithelium, possibly because of the presence of menstrual cycle-dependent cell adhesion molecules (Nose and Takeichi, 1986).

Our results suggest that the intact endometrium has a critical role in determining whether trophoblast attachment and subsequent implantation will occur. The restrictive effect of the endometrial surface, which we have demonstrated, is convincing since cultured trophoblasts will readily bind to basement membrane proteins and to surfaces that contain the requisite cell adhesion molecules (Kao et al, 1988; Kliman and Feinberg, 1990). Although trophoblasts did not typically attach to the surface endometrial epithelium of our explant cultures, trophoblast-endometrial gland interactions can be observed in vitro (Kliman et al, 1989). In this system, cytotrophoblasts will intermingle with cultured human glandular epithelial cells by attaching to endometrial cell islands and then penetrating into them. Thus, glandular epithelium, as opposed to the surface epithelium, may produce factors which promote trophoblast chemotaxis and attachment.

How do our in vitro results correlate with early human nidation in vivo? Interestingly, it appears that the human endometrial surface is generally not permissive to implantation, and may only promote trophoblast attachment and penetration during a relatively short time in the menstrual cycle. Clinical results with in vitro fertilization patients have shown that pregnancies occur only when blastocysts are returned to the uterus on days 16–20, with no successful pregnancies occurring after day 20 (Navot et al, 1988). Even during this purported 'implantation window', successful pregnancy following assisted reproduction occurs less than 25 per cent of the time (Navot and Laufer, 1989).

Although it is not possible to precisely replicate the complex in situ environment of the implanting human blastocyst, our trophoblast-endometrium co-culture system does recapitulate some important physiological facets of human nidation: (1) trophoblast attachment to the intact endometrial surface epithelium is extremely restricted, and may only occur with day 19 endometrium, (2) at trophoblast contact points with the exposed endometrial stroma, an eosinophilic zone of degraded and/or newly synthesized ECM protein is noted, possibly analogous

to the in vivo Nitabuch's layer, (3) occasional trophoblasts can be identified which have penetrated into the endometrial ECM, similar to the invading trophoblasts of the normal placental bed (Pijnenborg et al, 1981; Feinberg et al, 1989), and (4) the trophoblasts stimulate a marked paracrine effect on the endometrium, inducing a histologic conversion from proliferative to secretory endometrium. Our results suggest that this model system will be useful for studying those factors which induce endometrial permissiveness, promote paracrine effects on the endometrium, and facilitate human trophoblast invasion.

ACKNOWLEDGEMENTS

The authors wish to thank Drs C. Coutifaris and J. Strauss for helpful discussions, and Dr D. Cines for supplying us with the human endothelial cell cultures. We also wish to thank the histotechnologists of the Department of Pathology and Laboratory Medicine, and Ms Justina Minda and Mr Lewis Johns of the Electron Microscopic Laboratory for helping to process the numerous samples required for this study. Our appreciation is also extended to the nurses and residents in the Labor and Delivery Suite of the Hospital of the University of Pennsylvania who helped us procure normal placentae for our trophoblast preparations. Finally, we wish to thank Dr Virginia LiVolsi and Ms Elizabeth Freeman, of the Cooperative Human Tissue Network, Eastern Division, who helped to procure the endometrial material used in these studies. Supported by USPHS Grant HD-00715 and a McCabe Fund Award (HJK), and a Basil O'Connor Award (5-672) from the March of Dimes (RFF).

REFERENCES

Armant, D. R., Kaplan, H. A. & Lennarz, W. J. (1986) Fibronectin and laminin promote in vitro attachment and outgrowth of mouse blastocysts. *Developmental Biology*, **116**, 519–523.

Blaustein, A. (1985) *Interpretation of Endometrial Biopsies. Biopsy interpretation series* (2nd edn). 28 pp. New York: Raven Press.

Carson, D. D., Tang, J. P. & Gay, S. (1988) Collagens support embryo attachment and outgrowth in vitro: Effects of the Arg-Gly-Asp sequence. *Developmental Biology*, **127**, 368–375.

Enders, A. C., Chavez, D. J. & Schlafke, S. (1981) Comparison of implantation in utero and in vitro. In *Cellular and Molecular Aspects of Implantation* (Ed.) Glasser, S. R. & Bullock, D. W. pp. 365–382. New York: Plenum Medical.

Farach, M. C., Tang, J. P., Decker, G. L. & Carson, D. D. (1987) Heparin/heparin sulfate is involved in attachment and spreading of mouse embryos in vitro. *Developmental Biology*, **123**, 401–410.

Feinberg, R. F., Kao, L.-C., Haimowitz, J. E., Queenan, Jr., J. T., Wun, T.-C., Strauss, J. F. III & Kliman, H. J. (1989) Plasminogen activator inhibitors types 1 and 2 in human trophoblasts: PAI-1 is a marker of invading trophoblasts. *Laboratory Investigation*, **61**, 20–26.

Feinman, M. A., Kliman, H. J., Caltabiano, S. & Strauss, J. F. III. (1986) 8-Bromo-3′5′-adenosine monophosphate stimulates the endocrine activity of human cytotrophoblasts in culture. *Journal of Clinical Endocrinology and Metabolism*, **63**, 1211–1217.

Fisher, S. J., Leitch, M. S., Kantor, M. S., Basbnaum, C. B. & Kramer, R. H. (1985) Degradation of extracellular matrix by the trophoblastic cells of first-trimester human placentas. *Journal of Cell Biology*, **27**, 31–41.

Fisher, S. J., Cui, T., Zhang, L., Hartman, L., Grahl, K., Guo-Yang, Z., Tarpey, J. & Damsky, C. H. (1989) Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. *Journal of Cell Biology*, **109**, 891–902.

Glass, R. H., Spindle, A. I. & Pedersen, R. A. (1979) Mouse embryo attachment to substratum and interaction of trophoblast with cultured cells. *Journal of Experimental Zoology*, **208**, 327–336.

Glenister, T. W. (1961) Organ culture as a new method for studying the implantation of mammalian blastocysts. *Proceedings of the Royal Society B*, **154**, 428–431.

Grant, P. S., Ljungkvist, I. & Nilsson O. (1975) The hormonal control and morphology of blastocyst invasion in the mouse uterus *in vitro*. *Journal of Embryology and Experimental Morphology*, **34**, 299–310.

Hertig, A. T. & Rock, J. (1956) A description of 34 human ova within the first 17 days of development. *American Journal of Anatomy*, **98**, 435–494.

Jaffe, E. A., Hoyer, L. W. & Nachman, R. L. (1974) Synthesis of von Willebrand factor by cultured human endothelial cells. *Proceedings of the National Academy of Sciences USA*, **71**, 1906–1909.

Kao, L.-C., Caltabiano, S., Wu, S., Strauss, J. F. III & Kliman, H. J. (1988) The human villous cytotrophoblast: interactions with extracellular matrix proteins, endocrine function, and cytoplasmic differentiation in the absence of syncytium formation. *Developmental Biology*, **130**, 693–702.

Kliman, H. J. & Feinberg, R. F. (1990) Human trophoblast-extracellular matrix (ECM) interactions *in vitro*: ECM

thickness modulates morphology and proteolytic activity. *Proceedings of the National Academy of Sciences USA*, 87, 3057–3061.

Kliman, H. J., Feinman, M. A. & Strauss, J. F. III. (1987) Differentiation of human cytotrophoblasts into syncytiotrophoblasts in culture. *Trophoblast Research*, 2, 407–422.

Kliman, H. J., Nestler, J. E., Sermasi, E., Sanger, J. M. & Strauss, J. F. III. (1986) Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology*, 118, 1567–1582.

Kliman, H. J., Coutifaris, C., Babalola, G. O., Soto, E. A., Kao, L.-C., Queenan, J. T., Feinberg, R. F. & Strauss, J. F. III (1989) The human trophoblast: Homotypic and heterotypic cell-cell interactions. *Progress in Clinical and Biological Research*, 294, 425–434.

Kohorn, E. I. & Tchao, R. J. (1969) Conversion of proliferative endometrium to secretory endometrium by progesterone in organ culture. *Journal of Endocrinology*, 45, 401–405.

Kurman, R. J., Main, C. S. & Chen, H.-C. (1984) Intermediate trophoblast: a distinctive form of trophoblast with specific morphological, biochemical and functional features. *Placenta*, 5, 349–370.

Navot, D. & Laufer, N. (1989) Assisted reproductive technology. A clinical appraisal. *Journal of Reproductive Medicine*, 34, 3–9.

Navot, D., Droesch, K., Liu, H. C., Keriner, D., Veeck, L., Steingold, K., Muasher, S. & Rosenwaks, Z. (1988) Efficiency of human conception in vitro related to the window of implantation. *Abstracts of the 35th Annual Meeting of the Society for Gynecologic Investigation*. Baltimore, Maryland, 1988.

Nose, A. & Takeichi, M. (1986) A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *Journal of Cell Biology*, 103, 2649–2658.

Pijnenborg, R., Robertson, W. B., Brosens, I. & Dixon, G. (1981) Trophoblast invasion and the establishment of hemochorial placentation in man and laboratory animals. *Placenta*, 2, 71–92.

Queenan, Jr., J. T., Kao, L.-C., Arboleda, C. E., Ulloa-Aguirre, A., Golos, T. G., Cines, D. B. & Strauss, J. F. III. (1987) Regulation of urokinase-type plasminogen activator production by cultured human cytotrophoblasts. *Journal of Biological Chemistry*, 262, 10903–10906.

Roberts, J. M., Taylor, R. N., Musci, T. J., Rodgers, G. M., Hubel, C. A. & Mclaughlin, M. K. (1989) Preeclampsia—An Endothelial Cell Disorder. *American Journal of Obstetrics and Gynecology*, 161, 1200–1204.

Ulloa-Aguirre, A., August, A. M., Golos, T. G., Kao, L.-C., Sukuragi, N., Kliman, H. J. & Strauss, J. F. III. (1987) 8-Bromo-3′5′ adenosine monophosphate regulates expression of chorionic gonadotropin and fibronectin in human cytotrophoblasts. *Journal of Clinical Endocrinology and Metabolism*, 64, 1002–1009.