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Human trophoblast–extracellular matrix (ECM) interactions *in vitro*: ECM thickness modulates morphology and proteolytic activity

(invasion/placenta/choriocarcinoma/Matrigel/cyclic AMP)

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ABSTRACT **Trophoblast invasion of the uterine extracellular matrix, a critical process for human implantation and uteroplacental vascular development, is a striking example of controlled invasiveness. To examine cellular behavior relevant to this process, human trophoblasts were cultured on (*i*) Millicell filters prelayered with Matrigel and (*ii*) coverslips precoated with a gentle slope of Matrigel (Matribeach). Histologic sections of the Millicell system demonstrated significant invasion. However, on Matribeach the cells exhibited markedly different characteristics depending on the thickness of the Matrigel. On zone 1 (1–4 μm thick), flat aggregates and syncytia were seen. In contrast, cells on zone 2 (4–14 μm) formed rounded aggregates with intercellular processes. In this zone, prominent degradation of pericellular Matrigel proteins was assessed by both light microscopy and scanning electron microscopy. Treatment with 8-bromo-cAMP inhibited this proteolytic process. On zone 3 (14–60 μm), unicellular trophoblasts or small aggregates caused minimal matrix degradation. JEG-3 human choriocarcinoma cells exhibited similar morphologic and degradative properties on Matribeach, but zone 2 proteolysis was not affected by 8-bromo-cAMP. Our results suggest that extracellular matrix thickness has profound effects on cellular morphology and proteolytic activity. Furthermore, while both normal and malignant human trophoblasts can degrade extracellular matrix proteins, only normal trophoblast extracellular matrix degradation is inhibited by 8-bromo-cAMP.**

During human gestation, trophoblasts invade the uterine extracellular matrix (ECM), remodel the maternal vasculature, and line vascular channels in the placental bed (1). The biological repertoire necessary for these unique trophoblast–ECM interactions is unknown. Although trophoblast behavior has been compared to that of tumor cells with invasive activity, trophoblasts generally exhibit tightly regulated growth and invasion *in vivo*.

Certain features of cytotrophoblast differentiation *in vivo* can be recapitulated *in vitro* when human cytotrophoblasts are purified and cultured from term placentae (2). Once isolated from the placental villi, these mononuclear cells undergo morphologic as well as biochemical differentiation *in vitro*. Cytotrophoblasts form multinucleated syncytia and carry out *de novo* secretion of human chorionic gonadotropin (hCG), human placental lactogen, and progesterone (2–4). During this differentiation process cytotrophoblasts also attach to ECM proteins (5) and synthesize fibronectin (6) and laminin (7). Urokinase-type plasminogen activator (u-PA) is secreted by cytotrophoblasts in culture (8), as are the plasminogen activator inhibitor (PAI) types 1 and 2 (9). cAMP has profound effects on cytotrophoblasts in culture by affecting their intracellular morphology and synthesis of hCG, fibronectin, and u-PA (4, 6, 8).

Since cytotrophoblasts in culture are capable of exhibiting multiple facets of the trophoblast differentiation process, we wished to determine whether cytotrophoblasts cultured on ECM could express an invasive phenotype. Our goal was to establish a reliable method for assessing trophoblast–ECM interactions, morphology, and proteolytic activity, since it is likely that these parameters are relevant to both trophoblast and tumor cell invasion (10). Assays of tumor cell invasion have recently utilized Matrigel, the laminin-type IV collagen-proteoglycan ECM, for quantitating cellular invasiveness and migration (11, 12). We therefore developed a Matrigel assay system that allowed us to examine trophoblast–ECM interactions *in vitro* at the light and electron microscopic level. Using a thickness gradient of Matrigel with an ≈8° slope (Matribeach), we discovered that ECM thickness affects trophoblast morphology as well as the ability of these cells to degrade the ECM. Furthermore, in contrast to a previously described trophoblast–ECM assay (13), we found remarkably similar cell behavior on Matribeach when we compared normal term and first trimester cytotrophoblasts with JEG-3 choriocarcinoma cells.

METHODS

Trophoblast and JEG-3 Choriocarcinoma Cell Culture. The method of preparation and culture of term human cytotrophoblasts has been described (2). For first trimester placentae, the digest times were reduced to 10 min. JEG-3 choriocarcinoma cells were obtained from the American Type Culture Collection and were cultured under the same conditions as trophoblasts. cAMP-treated cultures received 1.5 mM 8-bromo-cAMP (Sigma).

Matribeach Production. Flame sterilized no. 1½ glass coverslips (22 × 22 mm) were placed in Nunclon (Nunc) six-well multidishes, which were angled at ≈8° on metal surgical trays. One hundred microliters of ice-cold Matrigel (solubilized basement membrane components from mouse Engelbreth-Holm–Swarm tumor; ref. 14) at 10–12 mg/ml (Collaborative Research) was applied to the middle of the coverslips to allow the liquid to run to the lower edge, forming a beach with a thick portion (≈60 μm) and a slope that tapered down to a thin edge (1–2 μm)—Matribeach. The plates were placed

Abbreviations: ECM, extracellular matrix; hCG, human chorionic gonadotropin; u-PA, urokinase-type plasminogen activator; PAI, plasminogen activator inhibitor; SEM, scanning electron microscopy.

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at 37°C for 2–3 hr to allow for complete gelling before cell suspensions were applied to the coverslips. Approximately 0.75 ml of cytotrophoblast cell suspension at 1×10^6 cells per ml, or JEG-3 at 2×10^4 cells per ml, was added and cultured for 24–72 hr.

Matrigel Supports. Approximately 100 μl of ice-cold Matrigel was layered into 12-mm Millicell-HA (Millipore) inserts to form an ≈0.9-mm-thick bed of undiluted Matrigel on a mixed cellulose ester filter. The Matrigel was allowed to gel completely at 37°C for 2–3 hr. From 50 to 100 μl of a suspension (1×10^6 cells per ml) of trophoblasts was added and incubated at 37°C in an atmosphere of humidified 95% air/5% CO_2 for 1–7 days. Medium surrounding the inserts was changed daily. The inserts were immersed in Bouin's solution and fixed overnight. The filters were excised from the plastic assemblies with a pointed blade, processed, embedded in paraffin, and sectioned at 5-μm intervals.

Immunocytochemistry and Scanning Electron Microscopy (SEM). For immunocytochemical analysis, the coverslips with Matribeaches were washed three times with 10 mM phosphate-buffered saline (150 mM NaCl, pH 7.4) (PBS), fixed in Bouin's solution for 1 hr, washed three times with PBS, and stored at 4°C in PBS/0.1% sodium azide until immunocytochemistry was performed as described (2). Rabbit anti-mouse laminin and rabbit anti-mouse type IV collagen (Collaborative Research) were used at 1:500, rabbit anti-human α-hCG (generously supplied by Steven Birken, Columbia University) was used at 1:1000, and rabbit anti-human β-hCG (Dako, Santa Barbara, CA) was used at 1:5000. SEM was performed as described (3).

Quantification. After incubation for 24–72 hr, the cultures were fixed, immunocytochemically stained for mouse laminin, and then counterstained with hematoxylin. The Matrigel slope was divided into three zones: zone 1, edge of slope to 4-μm-thick Matrigel, containing mostly flat cells; zone 2, between 4- and 14-μm-thick Matrigel, containing mostly invasive cells; and zone 3, >14-μm-thick Matrigel, containing mostly noninvasive single cells or aggregates. Matrigel thickness was determined by first calibrating the fine focus dial of a Riechart Microstar V microscope to the edges of a coverslip of known thickness (0.18 mm) with a ×40 objective and ×10 eyepieces, and then by focusing on the bottom and top edges of the Matrigel at different points on the slope. Determinations from eight different experiments with a total of 32 measurements were made to determine the thickness at the edge of each zone. The percentage of cells (or nuclei within a single syncytial cell) that were flat, rounded, or surrounded by zones of matrix lysis was calculated after counting a total of 100 cells for each zone. Three counts were made on each coverslip at each time point. The standard error of the mean was calculated when data from several different experiments were pooled.

RESULTS

Trophoblast Invasion of Matrigel-Coated Millicell Inserts. Our initial approach to assessing trophoblast invasion utilized a 900-μm-thick bed of undiluted Matrigel layered on a Millicell insert (15). When trophoblasts were added to the inserts, the cells attached to the Matrigel surface, showed evidence of surface erosion over the first 72 hr, and eventually, after 96–120 hr, invaded to depths of 10–50 μm into the Matrigel (Fig. 1). Although histologic examination revealed invading trophoblasts in cross section, we could not easily quantify this invasive behavior. Therefore, a thickness gradient of Matrigel was created to examine and quantify trophoblast–ECM interactions as a function of time and Matrigel thickness.

Trophoblast Interactions with Matribeach. Human cytotrophoblasts from first or third trimester cultured on Matrigel slopes (Matribeaches) yielded a typical pattern of trophoblast morphology (Fig. 2A). After 48 hr, the trophoblasts on the glass surface flattened, aggregated, and formed syncytia (Fig. 2B) as described (2). These cells seemed to be unaffected by the presence of Matrigel elsewhere on the glass. On the thin part of the slope, zone 1 (defined as 0–4 μm in Matrigel thickness), the cells flattened and spread, as they did on glass (Fig. 2C). On zone 2 (4–14 μm thick), the cells promoted clearing of the Matrigel by creating pericellular zones of lysis around the trophoblast aggregates (Fig. 2 D and E). This lysis was particularly pronounced after the Matribeaches were immunocytochemically stained for mouse laminin or type IV collagen, which resulted in multiple areas of negative staining around the cells on zone 2. The cells on zone 3 (Matrigel >14 μm thick) remained spherical, mostly single, and exhibited little or no zones of lysis (Fig. 2F). This pattern was repeated in multiple studies (Fig. 3). Time course studies revealed that the basic separation of zones occurred by 24 hr and progressed through 72 hr of culture. When Matribeaches were incubated with medium alone, there was no change noticed at the light microscopic level for as long as 72 hr.

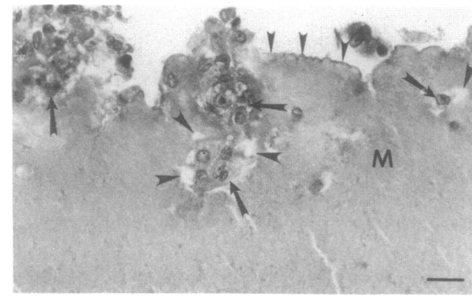

FIG. 1. Penetration of thick Matrigel (M) by trophoblasts. Term trophoblasts were plated onto a 0.9-mm-thick layer of Matrigel in a Millicell support, cultured for 120 hr, fixed, sectioned, and stained with hematoxylin and eosin. Note the trophoblast aggregates that have penetrated into the Matrigel (arrows). Note the clearing of the Matrigel (large arrowheads) around these trophoblast aggregates. The trophoblasts have penetrated ≈50 μm from the surface (small arrowheads) of the Matrigel. (Bar = 20 μm.)

The width of each zone depended on the steepness of the beach. When the slope was gradual (7–8°), the zones were wide and clearly distinguishable; when the slope was steep (30–40°), the zones were compressed but still apparent. This result suggests that it is the actual thickness of the Matrigel that determines cell morphology and biological behavior and not, for example, the distance of the cells from the edge of the beach.

SEM. As the trophoblasts degraded the Matrigel on zone 2, they formed concentric circles of clearing (Fig. 2 A, D, and E). To confirm this result, we performed SEM on the Matribeaches at 24, 48 (Fig. 4A), and 72 (Fig. 4B) hr. The SEM revealed several important points. First, the Matrigel appeared as a woven mesh of filamentous elements. Second, the cell processes extended into and merged with the surrounding matrix elements. Finally, as time went on, the trophoblast groups on zone 2 progressively eroded the Matrigel until the glass surface was exposed (Fig. 4B). These micrographs give compelling evidence for the proteolytic potential of these cells and suggest that this degradative process is time dependent.

Effect of 8-Bromo-cAMP. When treated with 1.5 mM 8-bromo-cAMP, the normal trophoblasts remained round on all zones of the Matrigel, without any evidence of ECM degradation, even on zone 2 (Fig. 5). Immunohistochemical staining of these cells revealed strong cytoplasmic induction of α- and β-hCG staining, as has been shown previously (4), indicating that the cells were biochemically responsive to 8-bromo-cAMP. As has been shown (6), this concentration of

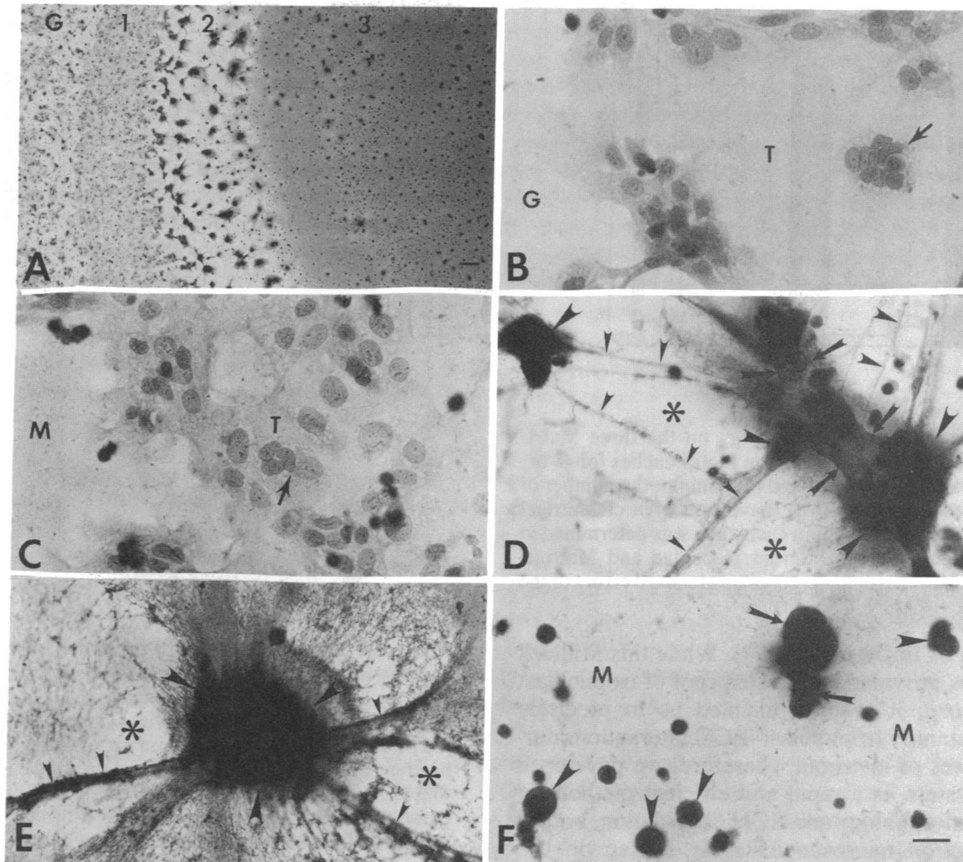

FIG. 2. Trophoblast morphology on a Matrigel slope (Matribeach). Term human trophoblasts (*A–D* and *F*) or first trimester trophoblasts (*E*) were plated onto Matribeaches, cultured for 48 hr, fixed, and immunocytochemically stained for laminin as described. (*A*) Panoramic view of the Matribeach with clearly defined zones 1, 2, and 3. G, glass surface. (*B*) Trophoblasts on the glass surface (G) showing the typical morphology of a mixed syncytia aggregate, as has been described (5). Note the large syncytial trophoblast (T) with the central multiple nuclei (arrow). (*C*) Trophoblasts on zone 1 (1–4 μm thick) of the Matrigel (M) slope. Note that these cells have flattened out and formed syncytia (T) with multiple nuclei (arrow) in a manner very similar to that found on the glass surface alone. (*D*) Term trophoblasts on zone 2 (4- to 14-μm-thick Matrigel) showing very distinct circumferential areas of Matrigel degradation (asterisks). Within the area of degradation, fine cytoplasmic extensions (small arrowheads) can be seen emanating from the central trophoblast aggregate (arrows). Note the thick zone of laminin-positive material directly around the trophoblast aggregates (large arrowheads). (*E*) First trimester trophoblasts on zone 2 showing the same area of Matrigel degradation (asterisks) and cytoplasmic extensions (small arrowheads). Again, note the dense zone of laminin-positive material directly around the trophoblast aggregate (large arrowheads). (*F*) Term trophoblasts on zone 3 (>14-μm-thick Matrigel) showing scattered individual cells (arrowheads) and small aggregates (arrows) without evidence of flattening or Matrigel (M) degradation. Similar results were observed in 10 experiments. In some experiments, a small amount of surface degradation of the Matrigel was noted on zone 3 after 48–72 hr. First trimester trophoblasts behaved similarly on zones 1 and 3 as described above for the term trophoblasts. (*A*, bar = 200 μm; *B–F*, bar = 20 μm.)

8-bromo-cAMP induces specific changes in gene expression. Therefore, the effect of this agent on zone 2 normal trophoblasts is likely to be mediated by a limited number of gene products.

We also cultured JEG-3 choriocarcinoma cells on Matribeach in the absence or presence of 1.5 mM 8-bromo-cAMP. Under unstimulated conditions, JEG-3 cells had the same basic behavior on Matribeach as normal trophoblasts: they were flat on zone 1, they aggregated and showed Matrigel degradation on zone 2, and they lost cohesiveness and remained as individual cells on zone 3 (Fig. 6). In the presence of 8-bromo-cAMP, the JEG-3 cells still demonstrated typical zone 1, 2, and 3 morphologies, with evidence of clear degradation of the Matrigel on zone 2. Therefore, JEG-3 and normal trophoblasts are distinctly different in their invasive and degradative potential when cultured on Matribeach in the presence of 1.5 mM 8-bromo-cAMP (Fig. 5*B*).

DISCUSSION

Normal human trophoblasts, like tumor cells, are capable of invasive behavior. For this complex process to occur, Liotta *et al.* (10) have proposed that invasive cells must (*i*) attach to ECM proteins, (*ii*) secrete proteases capable of degrading these proteins, and (*iii*) demonstrate migration through the degraded ECM. During implantation and subsequent placentation in the human, populations of trophoblasts invade the endometrium and maternal vasculature within the uterus. Once reaching the spiral arteries within the myometrium, trophoblast invasiveness ceases. Therefore, unlike tumor cells, which typically exhibit uncontrolled invasion, trophoblast invasion is tightly regulated. Suboptimal trophoblast invasion within the placental bed has been demonstrated in the pregnancy disease states of preeclampsia and intrauterine growth retardation (1). Malignant transformation of normal trophoblasts results in choriocarcinoma, a tumor with a particularly aggressive phenotype (16). To understand the pathophysiology of these diseases, it is critical to examine the regulatory mechanisms of human trophoblast invasion and to compare normal and malignant trophoblast behavior.

Purified human cytotrophoblasts, not only from first trimester but also from term placentae, provide a unique *in vitro* model system for studying ECM attachment, proteolysis, and invasion. Once attached to a thick layer of ECM, trophoblasts burrow into the matrix and, by 5 days of culture, invade

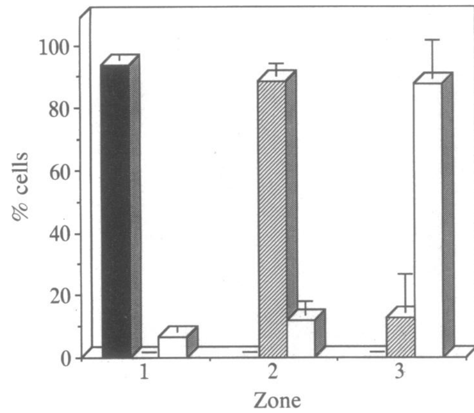

FIG. 3. Quantification of cell morphology on the three Matribeach zones. Trophoblasts were cultured on Matribeaches for 48 hr, fixed, and immunocytochemically stained for laminin; the number of cells that were flat (■), invasive (▨) (having an area of Matrigel destruction around it), or round (□) in each zone was determined in four separate experiments using different placentae and Matrigel preparations as described. Error bars represent standard errors of the mean.

Matrigel to depths of 10–50 μm (Fig. 1). While this Millicell support system has provided histologic proof of trophoblast invasiveness *in vitro*, it has not enabled us to precisely characterize and quantify trophoblast–ECM interactions during the initial phases of invasion. Therefore, we developed the Matribeach system as a rapid and efficient method for assessing cellular morphology and ECM degradation, both at the light and electron microscopic levels. Based on this assay, we discovered that cytotrophoblasts from first trimester or term placentae form three distinct zones of biological behavior, depending on the Matrigel thickness beneath the cells. In comparison, malignant JEG-3 trophoblastic cells form an identical three zone pattern of behavior, with zone 2 cells promoting the most pronounced Matrigel proteolysis.

The mechanism by which ECM thickness might modulate trophoblast behavior is unknown. However, we hypothesize that trophoblasts distinguish varying ECM thicknesses based on their differential ability to make contact with the underlying glass surface. Since the Matrigel is very thin in zone 1, the cells probably have very early contact with the underlying surface and therefore flatten and spread as they do on glass. On zone 2, the cells may make contact with the glass by extending fine cytoplasmic extensions. This contact may trigger matrix degradation, perhaps via expression and specific localization of activated proteases. The cells on zone 3 may initially extend cellular processes, but when they fail to make contact with the underlying surface, they are not stimulated to degrade the ECM during the first 72 hr of culture. Alternatively, trophoblasts could secrete autocrine factors that modulate their motility and invasiveness (17). The cells on zone 3, therefore, might be limited in their invasive capabilities because the large bed of ECM under the cells could adsorb such factors away, thus reducing the autocrine stimulation for invasion.

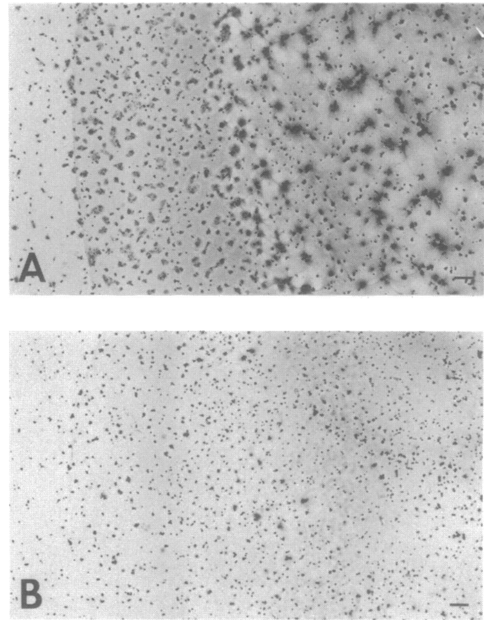

FIG. 5. Effect of 1.5 mM 8-bromo-cAMP on trophoblast morphology on Matribeach. Trophoblasts were cultured on Matribeaches for 24 hr in the absence (*A*) or presence (*B*) of 8-bromo-cAMP. (*A*) Low power view of glass surface and zones 1–3 of the Matribeach showing the same pattern described in Fig. 2*A*. At 24 hr, the extent of lysis of the Matrigel on zone 2 is somewhat less than that at 48 hr but is still easily appreciated. (*B*) Low power view of a parallel culture in the presence of 8-bromo-cAMP at the same magnification showing complete absence of differential trophoblast morphologies. The trophoblasts are all round (like the cells on zone 3) and there is no evidence of Matrigel destruction in the region that represents zone 2. Both micrographs are at the same magnification. (Bars = 100 μm.)

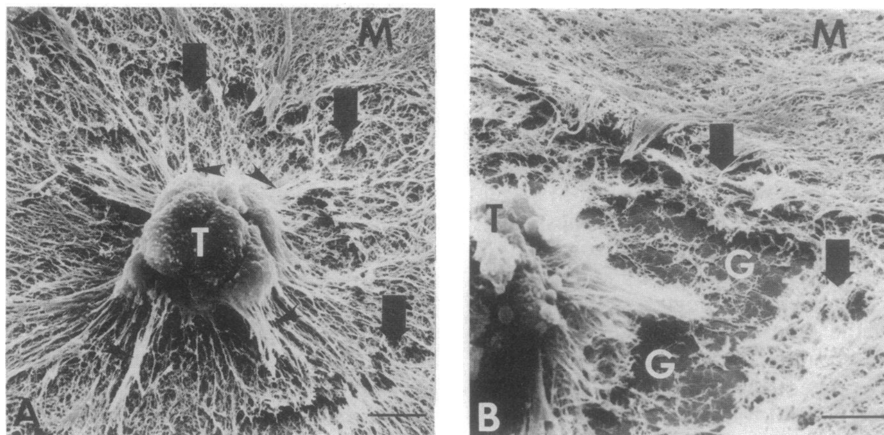

FIG. 4. SEM of trophoblasts on zone 2 at 48 (*A*) and 72 (*B*) hr. (*A*) An aggregate of trophoblasts (T) is shown with a circumferential area of Matrigel lysis (arrows). Thin cytoplasmic processes (arrowheads) emanate from the cells and merge into the surrounding Matrigel. Note the flat filamentous nature of the undigested Matrigel (M). (*B*) At 72 hr, this trophoblast aggregate (T) has completely degraded the Matrigel (arrows) so that the surface of the underlying coverslip is exposed (G). Note again the flat surface of the undegraded Matrigel (M). (Bars = 10 μm.)

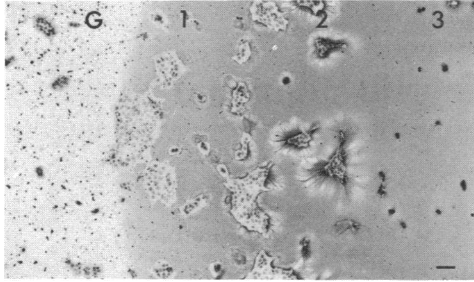

FIG. 6. JEG-3 choriocarcinoma growth on Matribeach. JEG-3 cells were cultured on Matribeach for 24 hr and processed as described. Like normal trophoblasts, these malignant trophoblasts exhibited three different morphologies on the Matrigel depending on the thickness they were growing on. The choriocarcinoma cells on the glass (G) and zone 1 formed flat aggregates. The cells on zone 2, like the normal trophoblasts, aggregated into rounded structures or cords and induced circumferential areas of Matrigel lysis. On the thicker Matrigel of zone 3, the cells formed small aggregates or remained as single cells and did not show evidence of Matrigel destruction within the 24-hr period examined. (Bar = 100 μm.)

Matrigel clearing around the trophoblasts on zone 2 appears to be promoted by trophoblast proteases, which, either directly or indirectly, degrade the ECM proteins laminin and type IV collagen. The biochemical basis of trophoblast-mediated proteolysis is unknown but may be initiated by u-PA, which converts plasminogen to the active protease plasmin. In culture, trophoblasts produce both u-PA (8) and PAI types 1 and 2 (9). Additional evidence for the role of the plasminogen activator system in regulating trophoblast invasion is based on the finding of PAI type 1 within trophoblasts that have invaded the placental bed *in vivo* (9). The potential role of trophoblast-secreted collagenases in the invasive process is not well understood. In zymographic analysis of serum-free medium from normal term trophoblasts, we find that one active gelatinase in the 90- to 92-kDa range is secreted (unpublished observations), in agreement with the findings of Fisher *et al.* (13). Since plasminogen activators are proposed to be activators of procollagenases (18), these enzyme systems may interact and have a pivotal role in controlling trophoblast-mediated ECM proteolysis and invasion.

Specific effects of 8-bromo-cAMP on normal cytotrophoblast gene expression have been previously studied, including induction of hCG synthesis and secretion (4, 5) and down-regulation of fibronectin mRNA and protein production (6). cAMP also transiently affects u-PA expression (8) and may regulate other trophoblast proteases or protease inhibitors. JEG-3 cells are also sensitive to 8-bromo-cAMP, as measured by cAMP induction of hCG mRNA and protein in these cells (19). Based on previous results with this major intracellular regulator, we wondered what effect cAMP would have on the interaction of normal and malignant trophoblasts with Matribeach. Interestingly, we found that 8-bromo-cAMP completely eliminated any evidence of zone 2 matrix degradation by normal trophoblasts and induced a cellular morphology similar to zone 3 cells. JEG-3 choriocarcinoma cells in zone 2 were unaffected, suggesting that malignant trophoblasts do not respond to regulatory agents as do normal trophoblasts. The precise mechanism to explain this cAMP effect is unknown, but it could involve fibronectin, which is known to facilitate normal trophoblast attachment *in vitro* (5). Since significant down-regulation of normal trophoblast fibronectin expression occurs in response to 8-bromo-cAMP (6), this glycoprotein, as well as other cell-adhesion molecules (CAMs), could mediate trophoblast–ECM interactions. Regulation of fibronectin and other CAMs by JEG-3 cells has not yet been studied.

Although it had been previously suggested by Fisher *et al.* (13) that term cytotrophoblasts are incapable of invasive behavior, our findings indicate that placental cytotrophoblasts from first or third trimester exhibit remarkably similar biological behavior to malignant JEG-3 choriocarcinoma cells when cultured on the Matribeach substrate. Furthermore, our results demonstrate that ECM thickness has a major impact on the invasive phenotype of both normal and malignant trophoblasts in culture. Therefore, cytotrophoblasts isolated from the more easily obtainable term placenta and cultured on Matribeach represents an ideal *in vitro* system for studying trophoblast morphology and differentiation (2), cell–cell interactions (20), and the biochemistry and regulation of ECM proteolysis and invasion.

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