Human Cytotrophoblasts Adopt a Vascular Phenotype as They Differentiate

A Strategy for Successful Endovascular Invasion?

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Abstract

Establishment of the human placenta requires that fetal cytotrophoblast stem cells in anchoring chorionic villi become invasive. These cytotrophoblasts aggregate into cell columns and invade both the uterine interstitium and vasculature, anchoring the fetus to the mother and establishing blood flow to the placenta. Cytotrophoblasts colonizing spiral arterioles replace maternal endothelium as far as the first third of the myometrium. We show here that differentiating cytotrophoblasts transform their adhesion receptor phenotype so as to resemble the endothelial cells they replace. Cytotrophoblasts in cell columns show reduced E-cadherin staining and express VE-(endothelial) cadherin, platelet-endothelial adhesion molecule-1, vascular endothelial adhesion molecule-1, and α4-integrins. Cytotrophoblasts in the uterine interstitium and maternal vasculature continue to express these receptors, and, like endothelial cells during angiogenesis, also stain for αVβ3. In functional studies, αVβ3 and VE-cadherin enhance, while E-cadherin restrains, cytotrophoblast invasiveness. Cytotrophoblasts expressing α4 integrins bound immobilized VCAM-1 in vitro, suggesting that this receptor-pair could mediate cytotrophoblast–endothelium or cytotrophoblast–cytotrophoblast interactions in vivo, during endovascular invasion. In the pregnancy disorder preeclampsia, in which endovascular invasion remains superficial, cytotrophoblasts fail to express most of these endothelial markers (Zhou et al., 1997. *J. Clin. Invest.* 99:2152–2164.), suggesting that this adhesion phenotype switch is required for successful endovascular invasion and normal placentation. (*J. Clin. Invest.* 1997. 99:2139–2151.) Key words: placentation • trophoblast • endothelium • integrins • cadherins

Introduction

Human placental development depends critically on the differentiation of the placenta's specialized epithelial cells, cytotrophoblasts (CTB).[1] Two differentiation pathways exist (see Fig. 1). In one, CTB remain in the fetal compartment and fuse to form multinucleate syncytiotrophoblasts that cover the floating chorionic villi. These villi, which are in direct contact with maternal blood in the intervillous space, perform nutrient and gas exchange for the fetus. In the other pathway, the focus of this paper, a subset of CTB in anchoring chorionic villi aggregate into cell columns that attach to the uterine wall. From there, CTB invade the uterine wall (interstitial invasion, see Fig. 1 *A*) and its blood vessels (endovascular invasion, see Fig. 1 *B*) as far as the first third of the myometrium. Anchoring villi thus attach the fetus to the uterus and establish the flow of oxygenated maternal blood to the intervillous space (1–4, reviewed in 5, 6).

CTB differentiation along the invasive pathway is a complex, multi-step process. CTB within cell columns lose the ability to divide (7; Genbacev and Fisher, unpublished data). As they invade the uterine wall, CTB upregulate expression of MMP-9, the 92-kD matrix metalloproteinase (8), HLA-G, a trophoblast-specific HLA class I molecule that is likely to be important in avoiding rejection of the conceptus by the maternal immune system (9, 10), and specific hormones, including human placental lactogen (hPL).

Relationships of differentiating CTB with extracellular matrix (ECM) and with other cells also change. For example, CTB intricately regulate expression of ECM ligands and their integrin receptors. CTB villus stem cells express the α6β4 integrin laminin receptor strongly. As they differentiate, CTB downregulate α6β4 and sequentially upregulate expression of fibronectin and the α5β1 fibronectin receptor in cell columns, and the α1β1 laminin/collagen receptor in the uterine wall (11, 12). CTB stem cells can be isolated from placental tissue and when plated on Matrigel, they recapitulate their differentiation program. Although we do not understand the signals that promote this differentiation program (see reference 13), we can use function-perturbing antibodies in conjunction with this in vitro model of invasion to determine which molecules are responsible for mediating the changes in migration and invasion. We have shown that interactions of α5β1 with fibronectin restrain invasion, and that interactions of α1β1 with collagen type IV and laminin promote CTB invasion in this system (13). We hypothesize that CTB balance invasion-restraining and invasion-promoting adhesion mechanisms as they differentiate,

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1. *Abbreviations used in this paper:* CTB, cytotrophoblasts; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells; MYO, myometrium; PECAM-1, platelet-endothelial cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VCAM-1, vascular cell adhesion molecule-1.

which may help regulate the depth of CTB invasion into the uterus (13).

The significance for normal placentation of this CTB differentiation program is highlighted by the fact that in the pregnancy disorder preeclampsia, in which both interstitial and endovascular invasion are abnormally shallow (14–17), CTB show significant defects in differentiation. For example, levels of MMP-9, HLA-G, and hPL in preeclamptic CTB are abnormally low (17, 18; Lim et al.).[2] Furthermore, CTB in preeclampsia increase expression of the α5β1 fibronectin receptor, but fail both to upregulate α1β1 and to downregulate α6β4 (Lim et al., 19). Thus, they appear arrested in their differentiation program and they express an ECM receptor phenotype that may not be optimal for invasion to the appropriate depth.

In continuing to search for fundamental insights into CTB differentiation during placentation, we have focused recently on the virtually unique ability of CTB to invade maternal blood vessels, to replace the maternal endothelium within the spiral arterial segments as far as the myometrium (MYO), and to remodel the muscular walls (tunica media) of these arteries. We hypothesized that to accomplish this, differentiating CTB stem cells must lose their epithelial phenotype and transform their cell–cell adhesion molecule phenotype dramatically, both to become invasive and to be able to interact with, and ultimately mimic certain behaviors of, the endothelial cells they displace. We therefore examined the phenotype of differentiating CTB for loss of epithelial adhesion receptors and the onset of expression of adhesion receptors characteristic of endothelium, or of leukocytes that are interacting with endothelium. Our results document that CTB undergo a comprehensive transformation of their adhesion molecule repertoire so as to mimic that of endothelial cells. Furthermore, in functional studies we show that the newly expressed adhesion receptors contribute to enhanced motility and invasiveness of differentiating CTB. Since CTB in preeclampsia are defective in endovascular invasion and colonization (16, 17, 19) and do not execute the switch to a vascular adhesion phenotype (20), we propose that this switch observed in normal CTB, which results in mimicry of vascular cells, is a fundamental requirement for normal placentation in humans.

Methods

Cell culture and isolation of CTB. JEG-3 choriocarcinoma cells were cultured in Dulbecco's MEM with 4.5 g/liter glucose (DME-high glucose), containing 10% FBS. Cultures of human foreskin keratinocytes and human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Randall Kramer and Dr. Kee-Hak Lim, UCSF, San Francisco, CA, respectively.

Normal CTB were isolated from chorionic villi by established procedures (8, 21). Briefly, placentas from normal uncomplicated pregnancies were obtained immediately after early gestation terminations (7–22 wk) or after delivery at term (34–40 wk). After initial purification of CTB on Percoll gradients, remaining leukocytes were removed with an antibody to CD-45 coupled to magnetic beads. Purified cells were used immediately or cultured on Matrigel-coated substrates (Collaborative Biomedical Products, Bedford, MA) for varying lengths of time in serum-free DME-high glucose, with 2% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN). Normal CTB from 7–22 wk placentas differentiate over 24–72 h, as shown previously by increased production of α1β1 (13, Lim et al.),[2] the MMP-9 metalloproteinase (8) and HLA-G (10), and they invade Matrigel in an established invasion assay (8).

To assure that endothelial cells in the maternal decidua do not express epithelial antigens in common with CTB stem cells (in particular E-cadherin), cultures of primary decidual endothelium were generated as described by Gallery et al. (22). Briefly, 0.5 mm^3 pieces of decidual tissue from placental bed biopsies were treated with trypsin and pronase, and then squeezed with the blunt end of a scalpel blade. The extruded cells were filtered through gauze and incubated on ice with magnetic beads coated with the ulex europaeus-1 lectin (E.Y. Laboratories, Inc., San Mateo, CA). Bound cells were plated on gelatin-coated petri dishes in DME-H21/Ham's F-12 (1:1), with 20% FBS supplemented with ITS (Collaborative Research, Inc., Lexington, MA). Cells were grown to confluency and passaged three times before performing immunocytochemistry.

Antibodies. Antibodies against adhesion receptors were obtained from the following sources: Integrin α1, TS2/7, T Cell Sciences, Inc., Cambridge, MA; integrin α4, HP1/2, Dr. R. Lobb, Biogen, Cambridge, MA; integrin αVβ3, complex-specific function perturbing monoclonal antibody LM609, Dr. David Cheresh, Scripps Research Foundation, La Jolla, CA; integrin β3, II344; integrin αVβ5, complex-specific function perturbing monoclonal antibody, P3G2, Dr. Elizabeth Wayner, Fred Hutchinson Cancer Center, Seattle, WA; integrin β6, E7P6, Dr. Robert Pytela, UCSF, San Francisco, CA; E-selectin, S25, Dr. M. Gimbrone, Brigham and Women's Hospital, Boston, MA; E-cadherin, E9, (23), for immunoblotting and staining, and polyclonal anti-GP-80, made against purified 80 kD fragment of E-cadherin (24), for function perturbation; VE-cadherin, BV6, BV9 and TEA 1.31, Dr. E. Dejana (25, 26); P-cadherin, 6A9, Dr. M. Wheelock; platelet-endothelial cell adhesion molecule-1 (PECAM-1), 390, Dr. H.S. Baldwin, Univ. Pennsylvania, Philadelphia, PA; vascular cell adhesion molecule-1 (VCAM-1), 11/26 for staining, Dr. T. Yednock, (Athena Neurosciences, Inc., S. San Francisco, CA), and 4B9 for function-perturbation, Dr. R. Lobb, Biogen; cytokeratin, 7D3 (rat anti-human, 7D3;13) and K8.13 (mouse anti-human; Sigma Immunochemicals, St. Louis, MO); human von Willebrand Factor, F/8/86 (DAKO A/S, Glostrup, Denmark).

Immunocytochemistry. Chorionic villi with attached decidua were dissected from placentas immediately after elective terminations or delivery. Placental bed biopsy tissues were obtained from the site of implantation immediately after cesarean delivery (see description of informed consent procedures in 20). Tissues were processed for double indirect immunocytochemistry as described previously (11, 19). Briefly, tissues were fixed in 3% paraformaldehyde for 30 min, infiltrated with 5–15% sucrose followed by OCT, and frozen in liquid nitrogen. Sections (5–7 μm) were cut on a Hacker-Slee cryostat. Isolated CTB plated on Matrigel-coated coverslips for varying times were fixed in 3% paraformaldehyde for 10 min, and permeabilized with cold methanol. Fixed tissue sections or cell cultures were stained with a mixture of two primary antibodies (rat or mouse anti-human cytokeratin to mark CTB, and an antibody of a different species origin against individual adhesion receptors of interest) for 1 h to overnight, washed, and incubated with secondary antibodies conjugated to fluorescein or rhodamine (Jackson ImmunoResearch Labs, Inc., West Grove, PA). The secondary antibodies were cross-absorbed against nonimmune IgG of other species to eliminate cross-reactivity. Samples were viewed with the Zeiss Axiophot Epifluorescence microscope equipped with filters to selectively view the rhodamine and fluorescein images with no cross-contamination.

Cell extraction and immunoblotting. Immunoblotting was carried out to determine the steady state levels of cadherins present during CTB differentiation. Freshly isolated CTB or CTB cultured on Matrigel-coated tissue culture wells were washed 2× with PBS and extracted with 200 μl of lysis buffer (50 mM Tris buffer, pH 8.0, containing 0.1% SDS, 0.5% NP-40, 120 mM NaCl, 100 μm PMSF, 20 U/ml aprotinin, 10 μg/ml leupeptin). Cell extracts were centrifuged at

2. Lim, K.-H., Y. Zhou, M. Janatpour, M. McMaster, K. Bass, S.-H. Chun, and S.J. Fisher, manuscript submitted for publication.

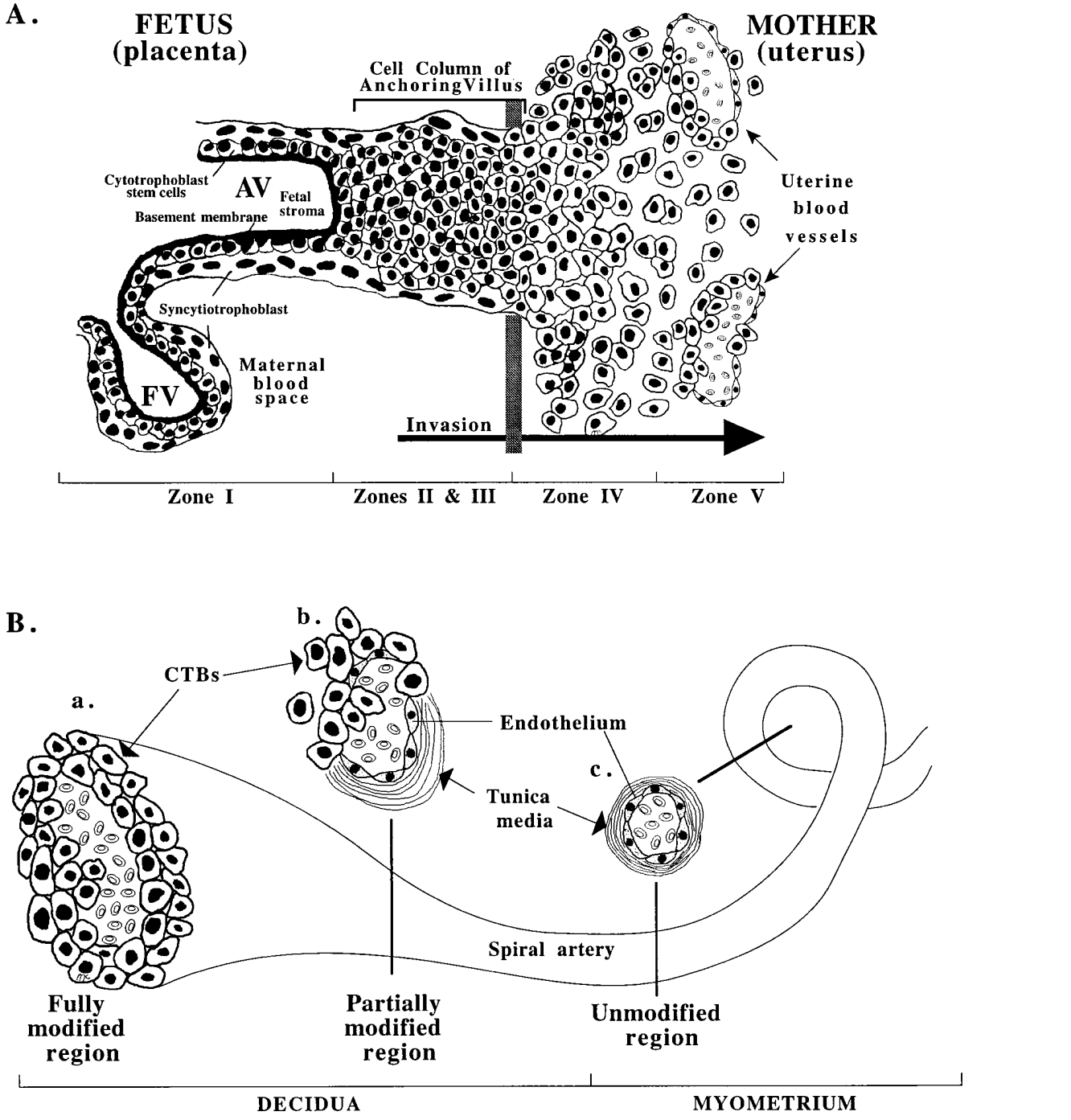

Figure 1. (*A*) Diagram of a longitudinal section of an anchoring chorionic villus (*AV*) at the fetal-maternal interface at ~ 10 wk gestational age. The anchoring villus (*AV*) functions as a bridge between the fetal and maternal compartments, whereas floating villi (*FV*) are suspended in the intervillus space and are bathed by maternal blood. CTB in AV (*Zone I*) form cell columns (*Zones II & III*). CTB then invade the uterine interstitium (decidua and first third of the myometrium, (*Zone IV*) and maternal vasculature (*Zone V*), thereby anchoring the fetus to the mother and accessing the maternal circulation. Zone designations mark areas in which CTB have distinct patterns of adhesion receptor expression as described in the text and in reference 11. (*B*) Diagram of a spiral artery in which endovascular invasion is in progress (10–18 wk gestation). Endometrial and then myometrial segments of spiral arteries are modified progressively. In fully modified regions (*a*) the vessel diameter is large. CTB are present in the lumen and occupy the entire surface of the vessel wall. A discrete muscular layer (*tunica media*) is not evident. (*b*) Partially modified vessel segments. CTB and maternal endothelium occupy discrete regions of the vessel wall. In areas of intersection, CTB appear to lie deep to the endothelium and in contact with the vessel wall (20). (*c*) Unmodified vessel segments in the myometrium. Vessel segments in the superficial third of the myometrium will become modified when endovascular invasion reaches its fullest extent (by 22 wk), while deeper segments of the same artery will retain their normal structure.

12,000 *g* for 5 min to remove insoluble material. Samples containing equal amounts of protein were mixed with SDS sample buffer, analyzed by SDS-PAGE under reducing conditions, and transferred to nitrocellulose. The nitrocellulose membranes were incubated with primary antibodies (E9, rat anti-human E-cadherin; BV9, mouse anti-human VE-cadherin, and 6A9, mouse anti-human P-cadherin) and peroxidase-conjugated secondary antibodies by standard procedures. Immune complexes were visualized using an enhanced chemiluminescence procedure and Hyperfilm (Amersham Life Sciences-USB, Arlington Heights, IL).

Invasion assays. Invasion assays were conducted as described previously (7, 8, 13). Briefly, isolated CTB (2.5×10^5) were plated on Transwell inserts (6.5 mm; Costar Corp., Cambridge, MA), containing polycarbonate filters with 8-μm pores, that had been coated with Matrigel. After 48 h in the presence of 100 μg/ml control IgG or function-perturbing antibodies against αVβ3 (LM609: 50 μg/ml), E-cadherin (E9: 50 μg/ml), or VE-cadherin (BV6; 100 μg/ml), the cultures were fixed with paraformaldehyde, permeabilized with cold methanol, and stained with the 7D3 antibody against human cytokeratin. The filter inserts, with the CTB cultures, were excised with a scalpel blade and mounted on polylysine-coated slides, with the underside of the filter facing upward. The Matrigel-coated and underside surfaces of the filters were distinguishable when viewed under the Zeiss Axiophot Epifluorescence microscope, and therefore cytokeratin-positive

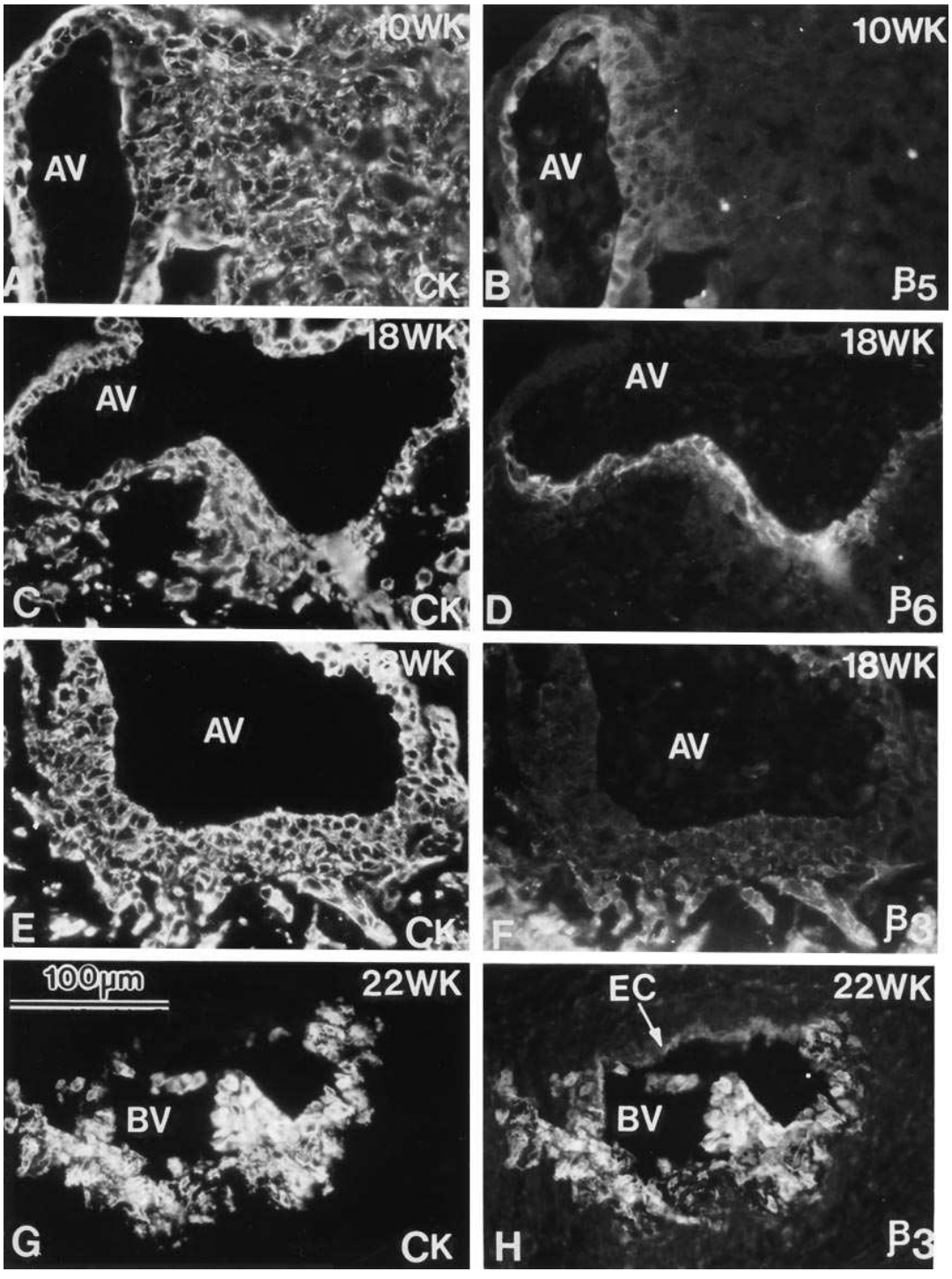

Figure 2. CTB switch the pattern of expression of αV-family integrin receptors during differentiation in vivo. Sections of 2nd trimester (18–22 wk) placental tissue were double stained with anti-cytokeratin (*CK*, 7D3) to mark CTB (*A*, *C*, *E*, and *G*), and with anti-αVβ5 (P3G2, *B*); anti-αVβ6 (E7P6, *D*); or anti-αVβ3 (II344, *F*, *H*). αVβ5 is detected on CTB in chorionic villi (*AV*), but not in other locations. αVβ6 is detected only on villus CTB at sites of column formation, and on the first layer of cell column. αVβ3 is detected on column CTB nearest the uterine wall and on interstitial and endovascular CTB (Zones III-V). BV, maternal blood vessel; EC, endothelial cells.

cell processes and whole cells that had penetrated the Matrigel could be counted. In all invasion assays, the total number of processes plus cells on the underside of each filter were counted. In the case of the anticadherin assays, three filters were used for each condition in each experiment. The VE-cadherin assay was repeated five times, and the E-cadherin assay was repeated four times. Data are expressed as percent control. In the anti-αVβ3 invasion assays, four filters were used for each experimental condition. The experiment was repeated two times. The statistical significance of the data was analyzed by a Student's *t* test.

Adhesion assay. A substrate of immobilized VCAM-1 was prepared by incubating 8-well chamber slides overnight at 4°C with 200 μl protein A (Sigma Chemical Corp., St. Louis, MO) at 1 μg/ml in PBS, pH 7.4. After nonspecific staining was blocked with 1% BSA in PBS, 200 μl/well of purified soluble chimeric VCAM-1–IgGFc (1.5 μg/ml in PBS: gift of Dr. Ted Yednock, Athena Neurosciences, Inc.) was added to the protein A-coated wells for 5 h at 24°C, or overnight at 4°C. This results in the binding of the VCAM-1 chimera to protein A via its Fc end, leaving the adhesive domains of VCAM-1 available for interaction with counter-receptors. CTB (10^5) were then added to the VCAM-1–coated wells in 250 μl serum-free DME containing control IgG or either anti-VCAM-1 (100 μg/ml) or anti-integrin α4 (80 μg/ml) IgG (four samples of each condition/experiment), and incubated at 37°C for 1h. The samples were washed with PBS to remove nonadherent cells, fixed and adherent cells counted. The experiment was repeated three times. The statistical significance of the data was analyzed by a Student's *t* test.

Isolation of RNA and Northern blotting. RNA was extracted from freshly isolated or cultured CTB by the method of Chomczynski and Sacchi (27), as described previously (13). Total RNA (10 μg/lane) was separated by formaldehyde-agarose gel electrophoresis, transferred to Nytran membranes (Schleicher & Shuell, Keene, NH) and analyzed by Northern blot hybridization as described previously (28). Probes were synthesized by random priming of a 2.4 kb fragment of human VE-cadherin and a 2.5 kb fragment of E-cadherin (gift of Dr. D. Rimm, Yale University, New Haven, CT) using α[^{32}P]dCTP and the Klenow fragment of DNA polymerase-1 according to standard methods. Final posthybridization was carried out in 0.3× SSC (45 mM NaCl, 15 mM sodium citrate) and 0.1% SDS at 65°C (13). In all experiments, gels were stained with acridine orange prior to transfer to ensure integrity of the RNA samples, and to confirm that equal amounts of RNA had been loaded onto each lane.

Results

There are several critical stages in the differentiation of CTB stem cells along the invasive pathway: (*a*) conversion of chorionic villus CTB from a monolayer to a cell column; (*b*) their invasion of the uterine wall and its vascular bed (Fig. 1 *A*); and (*c*) their interaction with maternal endothelium in spiral arterioles (Fig. 1 *B*). It is likely that significant switches in adhesion receptor phenotype are important for enabling CTB to execute this complex morphogenetic program. In this study, we tested the hypotheses that CTB mimic broadly the adhesion phenotype of the endothelial cells they replace, and that the changes in adhesion phenotype have the net effect of enhancing CTB motility and invasiveness. To test these hypotheses, we first stained tissue sections of the fetal-maternal interface for specific integrins, cadherins, and immunoglobulin family adhesion receptors that are characteristic of endothelial cells and leukocytes. We then tested the functional consequences for CTB adhesion and invasion of expressing the particular adhesion receptors that were upregulated during CTB differentiation.

The αVβ3 integrin is spatially regulated on differentiating CTB and promotes their invasiveness. The distribution patterns of αV integrin family members were examined because of their regulated expression on endothelial cells during angiogenesis (29) and their upregulation on some types of metastatic tumor cells (e.g., melanoma; 30, 31). αV family members displayed unique and highly specific spatial staining patterns on CTB in anchoring villi and the placental bed (Fig. 2). The αVβ5-complex-specific antibody (Fig. 2, *A* and *B*) stained the CTB stem cell monolayer in chorionic villi. Staining was uniform over the entire cell surface. At sites of column initiation in first trimester tissue (10 wk), αVβ5 staining extended to the first 2–3 layers of column CTB. The syncytiotrophoblast layer, and CTB in more distal layers of cell columns and in the placental bed, did not stain for αVβ5. In contrast, anti-αVβ6 did not stain most villus CTB: only villus CTB that were at sites of column formation stained (Fig. 2, *C* and *D*). The CTB layer still in contact with basement membrane at sites of column initiation stained brightly for αVβ6. In second trimester tissues (18 wk; Fig. 2, *C* and *D*), the next layer of the CTB within the column showed reduced staining. In first trimester tissues (10 wk), the first 2–3 layers of CTB in cell columns also stained strongly for β6 (not shown), as was observed for αVβ5. The rest of the CTB stem cells in chorionic villi, CTB in more distal regions of cell columns, and CTB within placental bed and vasculature did not stain for αVβ6, documenting a specific association of this integrin with initiation of column formation.

In yet a different pattern, staining for anti-αVβ3 was weak or not detected on villus CTB or on CTB in the initial layers of cell columns. However, strong staining was detected on CTB within the uterine wall and vasculature (Fig. 2, *E* and *H*). Thus, individual members of the αV family, like those of the β1 family, are spatially regulated during CTB differentiation. Of particular relevance to this study is the observation that αVβ3 integrin, whose expression is stimulated on endothelial cells by angiogenic factors (29, 32), is enhanced on CTB that have invaded the uterine wall and maternal vasculature.

Blocking αVβ3 function suppresses endothelial migration during angiogenesis (29). To determine whether perturbing the function of αVβ3 also affects CTB invasion in vitro, freshly isolated first trimester CTB were plated for 48 h on Matrigel-

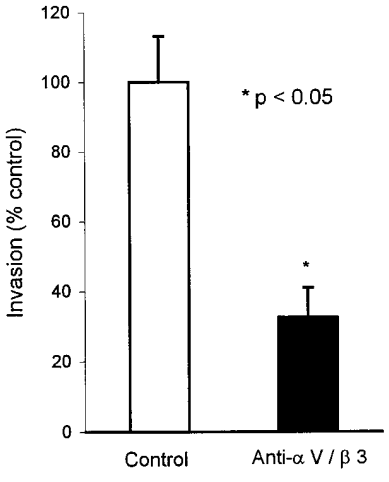

Figure 3. Antibody against αVβ3 strongly inhibits invasion of isolated CTB. CTB isolated from first trimester placental villi were cultured for 48 h on Matrigel-coated Transwell inserts in the presence of control mouse IgG or complex-specific anti–αVβ3 antibody LM609 (50 μg/ml) as described in Methods. Cells and processes that had invaded the Matrigel, and were visible on the undersides of the filters, were counted. Four wells of each condition were counted in each experiment. The experiment was conducted twice. Data were analyzed by the Student's *t* test. Bars indicate standard error of the mean.

coated Transwell filters in the presence of control mouse IgG or the complex-specific anti–αVβ3 IgG, LM609. CTB invasion was evaluated by counting cells and cellular processes that had invaded the Matrigel barrier and extended through the holes in the Transwell filters (7, 13). LM609 reduced CTB invasion by more than 75% in this assay (Fig. 3), indicating that this receptor, like the α1β1 integrin (13), contributes significantly to the invasive phenotype of CTB.

Cadherin switching accompanies CTB differentiation in vivo. In first and second trimester chorionic villi, the CTB epithelial monolayer stained strongly for the ubiquitous epithelial cadherin, E-cadherin, in a polarized pattern (Fig. 4). Staining was strong on the surfaces of CTB in contact with one another and with the overlying syncytiotrophoblast layer, and was absent at the basal surface of CTB in contact with basement membrane (most evident in Fig. 4 *F*). In cell columns, E-cadherin staining intensity was reduced on CTB near the uterine wall and on CTB within the decidua (Fig. 4, *A*, *B*, *E*, and *F*). This reduction in staining was particularly pronounced in second trimester tissue (Fig. 4 *F*). At this stage, E-cadherin staining was also very weak or undetectable on CTB that had colonized maternal blood vessels and on CTB in the surrounding myometrium (Fig. 4, *G* and *H*). All locations of reduced E-cadherin staining were areas in which invasion is active during the first half of gestation. Interestingly, staining intensity of E-cadherin was strong on CTB in all locations in term placentas (34–40 wk, not shown), at which time CTB invasive activity is poor (8, 13). With the exception of uterine gland epithelium, maternal cells in these tissues, including decidual endothelium, did not stain for E-cadherin at any stage of gestation. Taken together, these data are consistent with the idea that CTB transiently reduce E-cadherin function at times and places of their greatest invasive activity.

Cadherin switching occurs frequently during embryonic de-

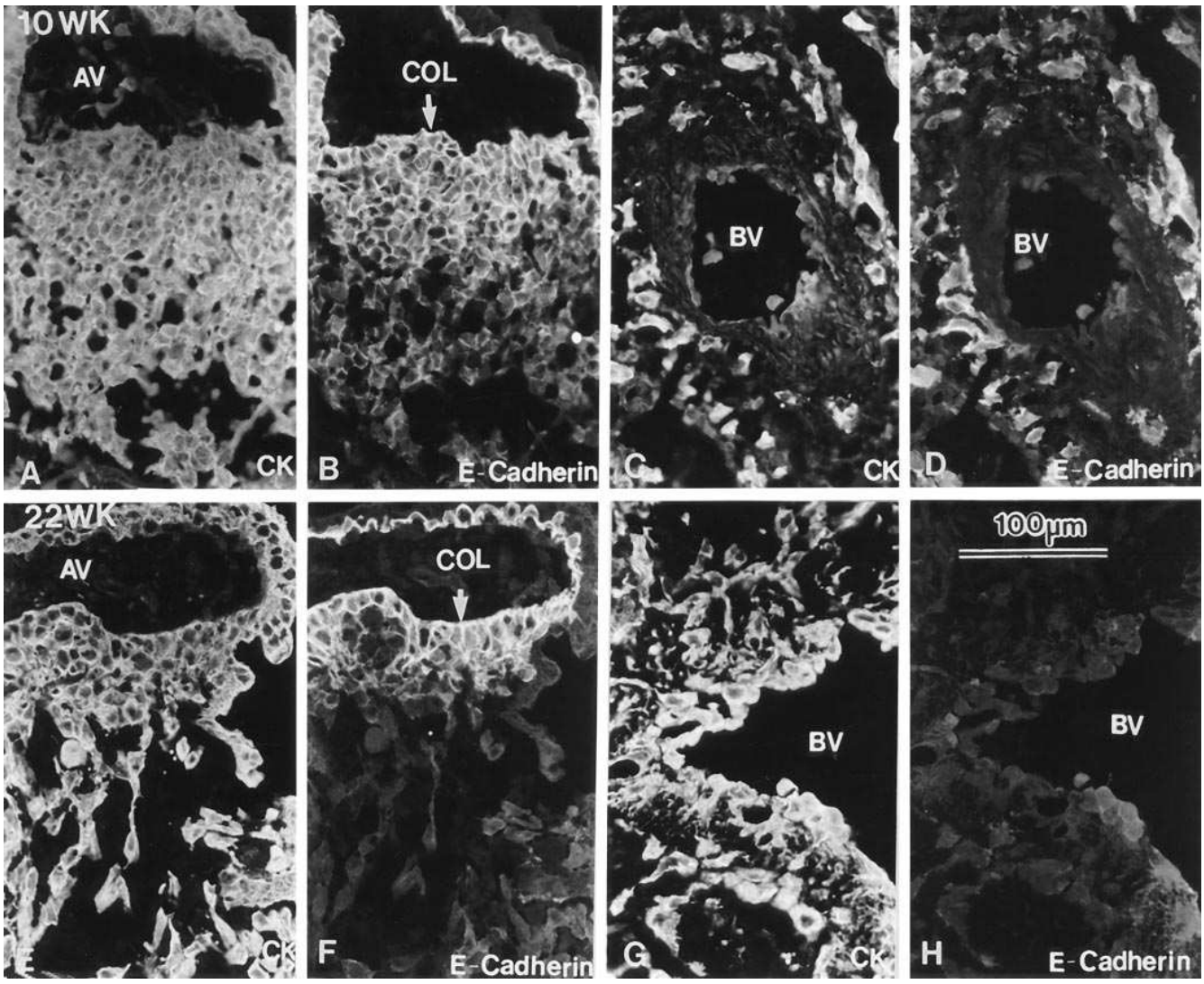

Figure 4. E-cadherin staining is reduced in normal differentiating 1st and 2nd trimester CTB. Sections of 1st trimester (*10 wk*, *A–D*) and 2nd trimester (*22 wk*, *E–H*) placental bed tissue were stained with antibody against cytokeratin (*CK*, 7D3, *A*, *C*, *E*, and *G*) or E-cadherin (E9, *B*, *D*, *F*, and *H*). E-cadherin staining was strong on anchoring villus (*AV*), CTB, and syncytium, and on CTB in the proximal portion of cell columns (Zone II). Staining was sharply reduced on CTB in the distal column (Zone III) and in the uterine interstitium (Zone IV). Staining was not detected on CTB within maternal vessels (Zone V). COL, column; BV, blood vessel.

velopment when significant morphogenetic events take place (33). We therefore stained sections of first and second trimester placental tissue with antibodies to other classical cadherins. These tissues did not stain with antibodies against P-cadherin (not shown). However, they did stain with three different monoclonal antibodies that recognize the endothelial cadherin, VE–cadherin (cadherin-5; 26, 34, 35). In chorionic villi, antibody to VE-cadherin did not stain villus CTB, although it stained the endothelium of fetal blood vessels within the villus stroma. In contrast, anti–VE-cadherin stained CTB in cell columns and in the decidua (Fig. 5, *A*, *B*, *E*, and *F*). These are just the areas in which E-cadherin staining was reduced. VE-cadherin staining was stronger in second trimester tissues in these areas. In maternal vessels that had not yet been modified by CTB, VE-cadherin stained the endothelial layer strongly (i.e., at 10 wk gestation, Fig. 5, *C* and *D*). To confirm that maternal decidual endothelial cells expressed VE-cadherin, but not E-cadherin, these cells were isolated from decidual tissue fragments as described previously (22), and stained with antibodies against E-cadherin, VE-cadherin, and the endothelial marker, von Willebrand Factor (vWF). The decidual endothelial cultures stained strongly for both VE-cadherin and vWF, but not for E-cadherin (data not shown). Following endovascular invasion, CTB lining maternal blood vessels stained strongly for VE-cadherin (Fig. 5, *G* and *H*). They did not stain for vWF (not shown; see companion paper, 20). Thus, CTB that invade the uterine wall and vasculature express a cadherin characteristic of endothelial cells.

Normal CTB upregulate VE-cadherin in vitro. To determine the functional consequences for CTB differentiation/invasion of expressing VE-cadherin, it was important to show that CTB could modulate their cadherin repertoire in vitro. CTB were

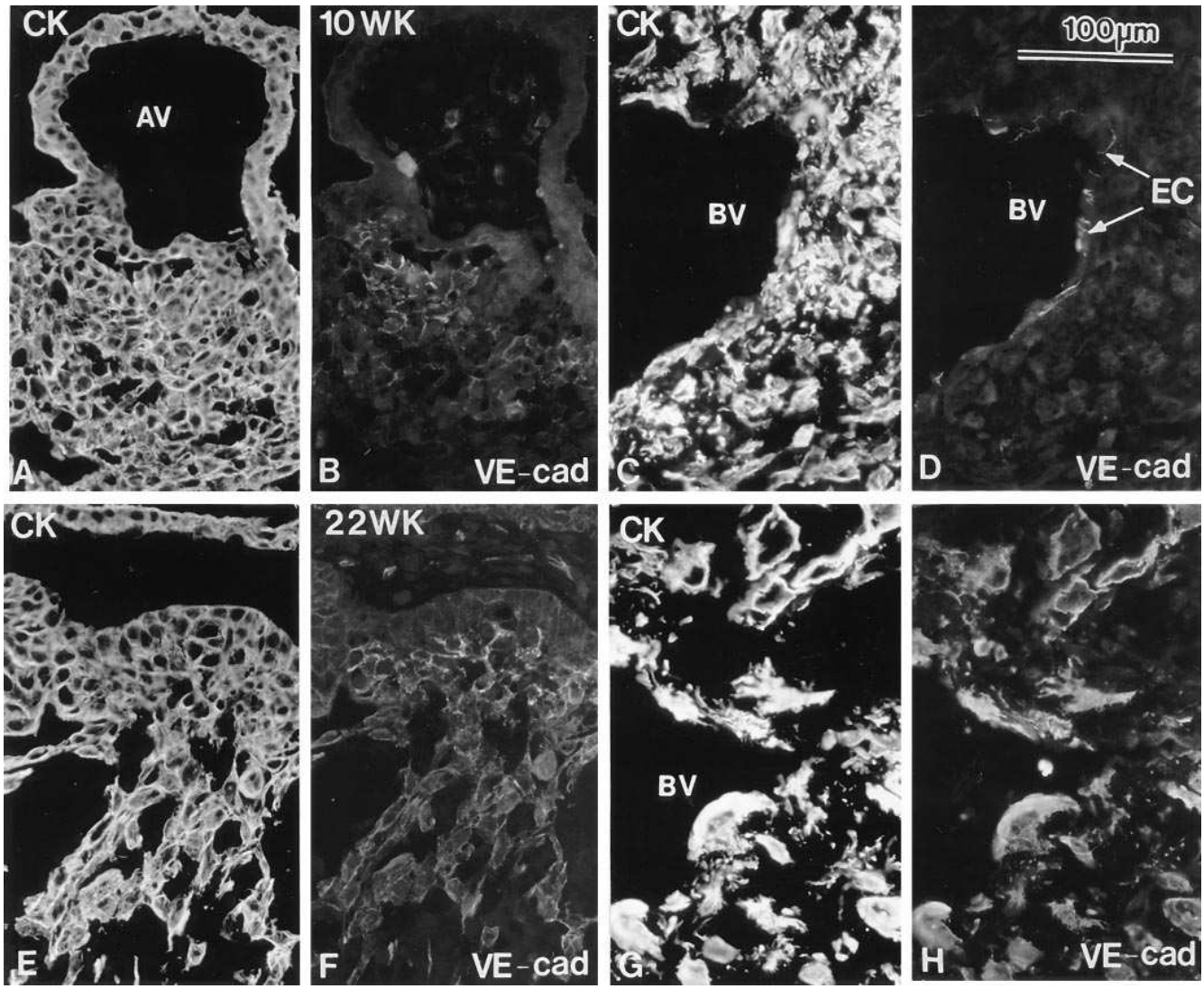

Figure 5. VE-cadherin *(VE-cad)* staining is detected on differentiating and endovascular CTB. Sections of 1st trimester (*10 wk*, *A–D*) and 2nd trimester (*22 wk*, *E–H*) placental bed tissue stained with antibody against cytokeratin (*CK*, *A*, *C*, *E*, and *G*) or VE-cadherin (BV6, *B*, *D*, *F*, and *H*). VE-cadherin is not detected on CTB in AV(although fetal blood vessels in villus stromal core are stained). VE-cadherin is detected on column CTB (*B* and *F*) and on interstitial and endovascular CTB (*G* and *H*). VE-cadherin is also detected on maternal endothelial cells (*EC*) in vessels that have not been modified by CTB (*C* and *D*). VE-cadherin staining was more intense on 2nd trimester CTB than 1st trimester column CTB, and in the uterine wall VE-cadherin staining was more intense on endovascular than interstitial CTB.

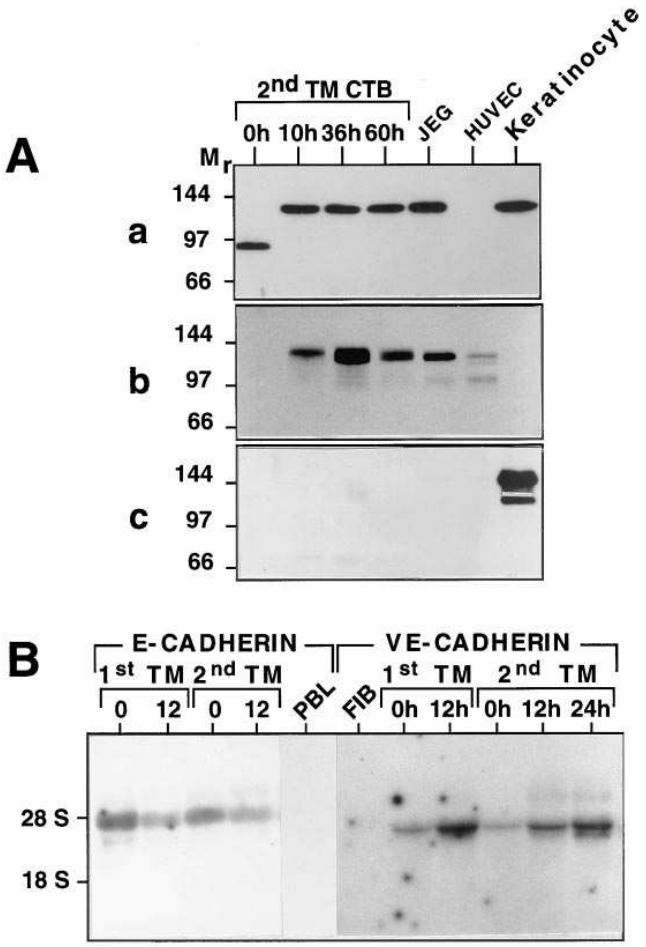

Figure 6. VE-cadherin protein and mRNA are upregulated in CTB differentiating in vitro. (*A*) CTB were isolated from 2nd trimester chorionic villi and lysed immediately or after culture for the times indicated. Lysates of CTB, human umbilical vein endothelial cells (*HUVEC*), JEG-3 choriocarcinoma cells and primary human foreskin keratinocytes were analyzed by immunoblotting using (*a*) anti–E-cadherin (E9); (*b*) anti–VE-cadherin (BV9); or (*c*) anti–P-cadherin (6A9). (*B*) RNA was isolated from first and second trimester CTB immediately after isolation from chorionic villi or after culture for the times indicated. The RNA samples were analyzed by Northern blotting using $\alpha[^{32}P]$dCTP-labeled cDNA probes for E-cadherin or VE-cadherin. PBL, peripheral blood lymphocytes (negative control used for E-cadherin); FIB, placental fibroblasts (negative control used for VE-cadherin). Lines indicate positions of 28S and 18S RNA.

therefore isolated from first and second trimester placentas. At several time points after plating, extracts of isolated CTB were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to E- and VE-cadherin (Fig. 6 *A*). Anti–E-cadherin recognized an 80 kD band in freshly isolated CTB and a band of similar density at 120 kD at all subsequent time points (Fig. 6 *A*, *a*). These data indicate that CTB express similar levels of total E-cadherin protein throughout their differentiation. The 80-kD band observed at 0 time reflects the presence of a large, stable E-cadherin fragment that is generated when cells are exposed to trypsin in the presence of Ca^{2+} (e.g., 24), as occurs for a brief period during the cell isolation period. The anti–VE-cadherin antibody did not bind to anything in extracts of freshly isolated CTB. However, by 10 h, this antibody recognized a single band at 140 kD, which increased in intensity at 36 h (Fig. 6 *A*, *b*). This band comigrated with a 140-kD band recognized by anti–VE-cadherin in extracts of human umbilical vein endothelial cells (HUVEC; Fig. 6 *A*, *b*). Both the full length VE-cadherin and a large proteolytic fragment at about 100 kD, analogous to the 80 kD E-cadherin fragment, were detected in HUVEC. However, neither HUVEC (Fig. 6 *A*, *a*) nor cultured decidual endothelial cells (not shown) expressed E-cadherin. Interestingly, both E-cadherin and VE-cadherin were detected in extracts of the JEG-3 choriocarcinoma cell line (Fig. 6 *A*, *a* and *b*). Neither CTB, nor JEG-3 and HUVEC expressed P-cadherin, although this cadherin was expressed prominently by keratinocytes (Fig. 6 *A*, *c*).

To determine whether cadherin modulation by differentiating CTB was reflected at the mRNA level, Northern blotting was carried out on RNA extracted from cultured first and second trimester CTB (Fig. 6 *B*). Steady state levels of E-cadherin mRNA were either somewhat reduced (as shown in Fig. 6 *B*) or remained unchanged during the first 12 h of culture. In contrast, when the same blots were stripped and reprobed for VE-cadherin, the VE-cadherin mRNA levels were consistently very low in freshly isolated CTB and increased over the next 24 h. 28S ribosomal RNA was monitored by acridine orange prior to transfer of the gel to assure uniform loading of samples (not shown). Taken together, these results indicate that normal CTB continue to produce E-cadherin mRNA and protein as they differentiate, but upregulate expression of VE-cadherin at both mRNA and protein levels.

E-cadherin and VE-cadherin have opposing effects on CTB invasion in vitro. Next, we used function-perturbing anti–cadherin antibodies, in conjunction with the Matrigel invasion assay, to assess the functional consequences of cadherin modulation for CTB invasiveness (Fig. 7). We plated isolated second trimester CTB for 48 h on Matrigel-coated filters in the presence of control IgG or function-perturbing antibodies against VE-cadherin or E-cadherin. By 48 h significant invasion was

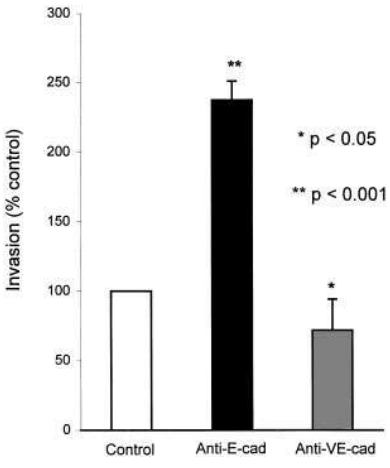

Figure 7. E-cadherin and VE-cadherin have opposing effects on CTB invasion. CTB isolated from 2nd TM chorionic villi were plated on Matrigel-coated filters in Transwell inserts and cultured for 48 h in the presence of control IgG (100 μg/ml), anti–VE-cadherin IgG (BV6, 100 μg/ml) or anti–E-cadherin IgG (anti-GP80, 50 μg/ml). Cells and processes that had invaded the Matrigel and were visible on the undersides of the filters were counted. Triplicate wells were counted in each experiment. The experiment was repeated four times for E-cadherin and five times for VE-cadherin. Data were analyzed by the Student's *t* test. Bars indicate standard error of the mean.

Table I. Staining Patterns of Adhesion Molecules in Normal CTB at the Fetal–Maternal Interface In Situ

Adhesion receptor	Normal 10–18 wk placental tissue: Zone I Villus CTB	Zone II Column CTB Proximal	III Column CTB Distal	Zone IV Placental bed CTB Interstitial	V Placental bed CTB Endovascular	Maternal endothelium
β4 integrin*	+	+	+/−	−	−	−
β6 integrin‡	−/+‡	−/+‡	−	−	−	−
β3 integrin	−	−	+	+	+	+
β5 integrin	+	−	−	−	−	−
β1 integrin*	−/+§	−/+§	+	+	+	+
α1 integrin*	−	−	−	+	+	+/−
E-cadherin	+	+	+/−	+/−	−	−
VE-cadherin‖	−	+	+	+	+	+
α4 integrin	+	+	+	+	+	+/−
VCAM-1	−	+/−	+	+	+	+/−
PECAM-1	−	−	+	+	+	+
E-selectin¶	+	+	+	+	+	+/−

*Data from refs 11, 19. Antibodies against all other receptors are listed in Methods. ‡Stains villus CTB only at sites of column formation in 2nd trimester tissue. In 1st trimester tissue, β6 staining was also detected on the first 2–3 layers of column CTB. §Not detected in 1st trimester villus or proximal column CTB: staining for β1 was detected at these sites in 2nd trimester and term tissue. ‖Staining pattern indicated was for 2nd TM tissue. Not all column and uterine wall CTB stained for VE-cadherin in 1st trimester tissue. ¶P- and L-selectin were not detected on CTB.

evident in control CTB. In cultures treated with anti–E-cadherin, CTB invasiveness increased more than threefold, suggesting that the E-cadherin normally has a restraining effect on CTB invasiveness. In contrast, antibody against VE-cadherin reduced the invasion of CTB to about 60% of control (Fig. 7). This suggests that the presence of VE-cadherin normally facilitates CTB invasion. Taken together, these functional data suggest that as they differentiate, CTB modulate their cadherin repertoire to one that contributes to their increased invasiveness.

Differentiating CTB undergo a comprehensive switch in cell–cell adhesion molecule expression such that they mimic the adhesion phenotype of endothelial cells. Our data thus far indicate that, as they differentiate, CTB downregulate adhesion receptors highly characteristic of epithelial cells (integrin α6β4 [11] and E-cadherin) and upregulate analogous receptors that are expressed on endothelial cells (integrins α1β1 [11, 13] and αVβ3, and VE-cadherin). These observations support our hypothesis that normal CTB undergo a comprehensive switch in phenotype so as to resemble the endothelial cells they replace during endovascular invasion. To investigate this possibility further, we stained sections of first and second trimester normal placental bed biopsies with antibodies against cell–cell adhesion molecules characteristic of quiescent or activated endothelial cells, or against leukocyte cell adhesion molecules that are involved in transendothelial trafficking. The data are summarized in Table I.

E-selectin was expressed constitutively by normal first and second trimester CTB in all compartments, including CTB stem cells in the chorionic villi (Table I). However, chorionic villus CTB did not stain for most of the other vascular adhesion molecules examined. In contrast, CTB in cell columns stained for VCAM-1 (Fig. 8, *A–D*), and the α4 subunit of its integrin counter-receptors (α4β1 and α4β7; Fig. 8, *E–H*), as well as PECAM-1 (Fig. 9, *A–D*). These receptors were also detected on CTB within the uterine wall and staining for all three was particularly strong on endovascular CTB (Fig. 8, *C*, *D*, *G*, and *H* and Fig. 9, *C* and *D*). These three regulated adhesion molecules, like VE-cadherin, were all detected on isolated CTB within 12 h of culture in vitro (not shown). Furthermore, the α4-containing complexes on CTB were functional, as determined by the ability of isolated CTB to adhere to a substrate coated with VCAM-1–IgGFc in an in vitro adhesion assay. This interaction was blocked by both anti-α4 and anti-VCAM-1 (Fig. 10). These data suggest that CTB could use their α4-integrins to interact either with VCAM-1 on other CTB, or with VCAM-1 expressed by maternal endothelial cells.

Discussion

During the first half of gestation, a subset of chorionic villus CTB leave the fetal compartment and invade the uterine wall and its vascular network, thereby anchoring the fetus to the mother and colonizing the maternal spiral arteries as far as the first third of the myometrium. CTB replace the maternal endothelium on the vessel walls in the process. In order to accomplish this extraordinary feat, it is likely that this subset of CTB must not only acquire an invasive phenotype (8, 13, 21), but must also transform their adhesion molecule phenotype in a comprehensive manner so as to mimic that of cells of the vascular system, particularly endothelial cells. Our data, both in vivo and in vitro, strongly support this idea. First, we found that CTB in vivo show reduced staining for adhesion receptors characteristic of stable epithelial monolayers (e.g., α6β4 [11] and E-cadherin) and show enhanced staining of adhesion molecules characteristic of endothelial cells and certain leukocytes. These include the integrins α1β1 (11, 19) and αVβ3, VE-cadherin, the receptor–counter-receptor pair integrin α4β1 and VCAM-1, and PECAM-1. Second, invasion and cell adhesion assays using isolated CTB in conjunction with function-perturbing antibodies indicated that the newly expressed adhesion receptors are functional and enhance the net invasiveness of the differentiating CTB.

This tilt toward greater invasiveness is well illustrated for both cadherins and integrins. Antibodies against E-cadherin enhanced CTB invasion, indicating that this cadherin normally restrains invasion. This result is consistent with a large body of literature indicating that E-cadherin and its associated catenin complexes function to stabilize epithelia (reviewed in 36, 37). In contrast, antibodies against VE-cadherin reduced CTB invasiveness. This suggests that the downstream consequences of adherence via VE-cadherin are distinct from those of E-cadherin, even though they are closely related proteins. In support of this, Navarro et al. (38) reported that when transfected into CHO cells, which do not express cadherins, VE-cadherin can promote cell–cell recognition and cell aggregation, even when expressed as a truncated protein lacking the COOH-terminal portion of the cytoplasmic domain required for interaction with catenins. This is the case, even though full-length VE-cadherin, like other classical cadherins, is able to interact with the cytoplasmic complexes containing α- and β-catenin. In contrast, E-cadherin requires interaction of its cytoplasmic do-

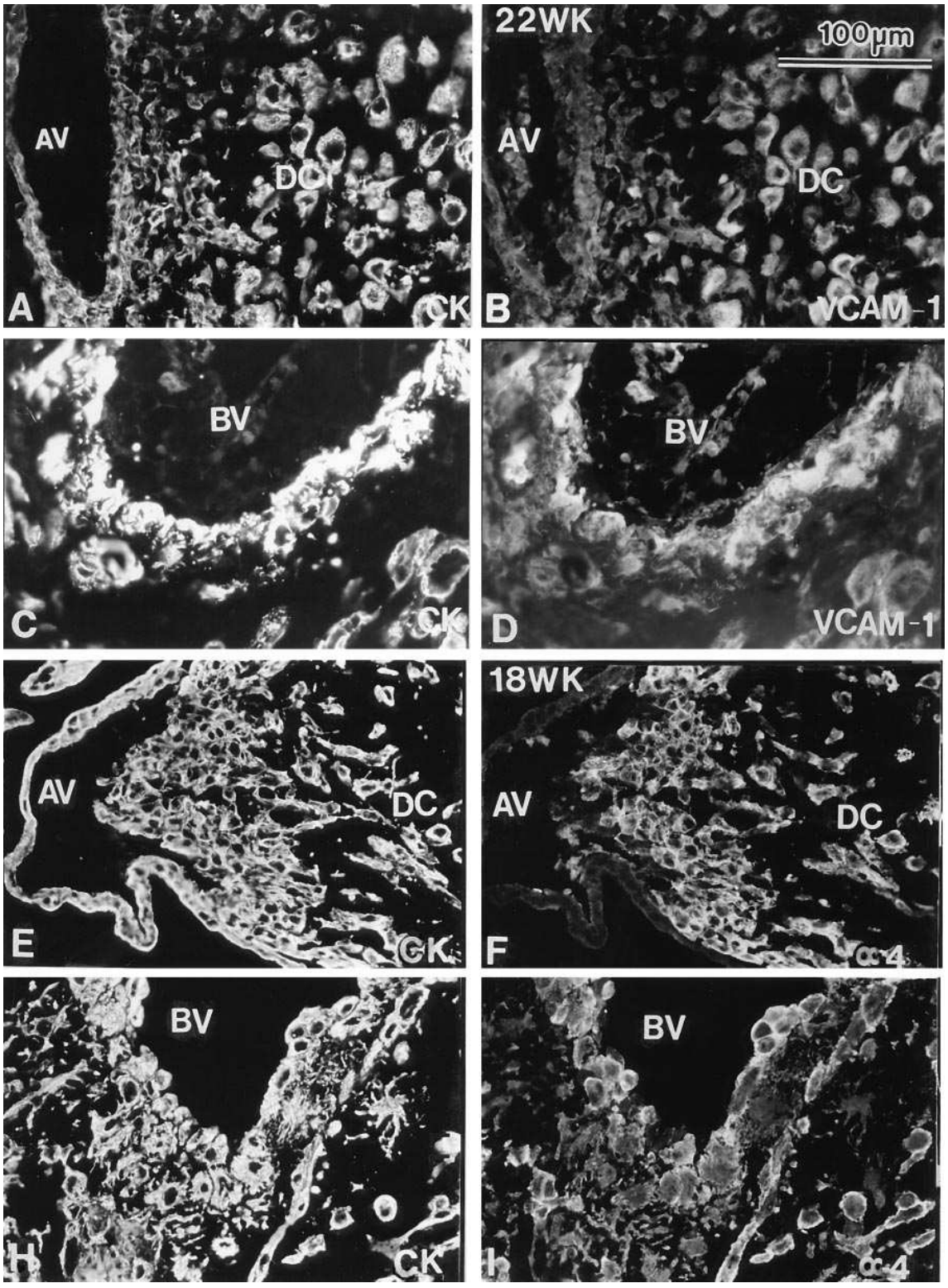

Figure 8. CTB stain for VCAM-1 and the α4 subunit of its integrin counter-receptors. Sections of placental tissue from 22 wk (2nd trimester) anchoring villi (*A*, *B*, *E*, and *F*) and placental bed biopsies (*C*, *D*, *G*, and *H*) stained with antibodies against cytokeratin (*CK*, *A*, *C*, *E*, and *G*), VCAM-1 (11/26, *B* and *D*) or integrin-α4 (HP1/2, *F* and *H*). Villus CTB did not stain for VCAM-1 or integrin α4, but both were expressed by CTB in cell columns, and by interstitial and endovascular CTB. Both were expressed on some, but not all, maternal vessels before modification by CTB (not shown). *DC*, decidua.

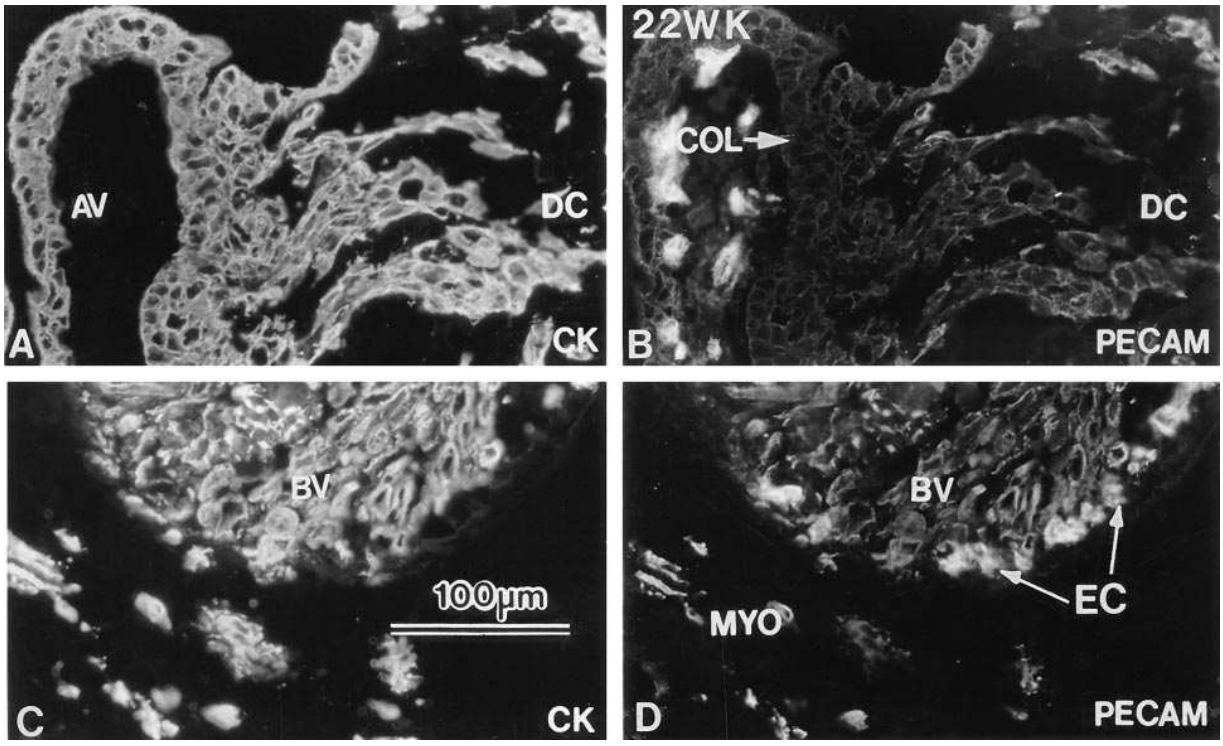

Figure 9. CTB stain for PECAM-1. Sections of placental tissue from 22 wk (2nd trimester) anchoring villi (*A* and *B*) and placental bed biopsy (*C* and *D*) were stained with antibody against CK (7D3, *A* and *C*) and PECAM-1 (390, *B* and *D*). PECAM-1 was detected on fetal blood vessels within the AV, but on not villus CTB. (*B*) Arrow marks site of column initiation. PECAM-1 was detected on CTB in the uterine decidua (*DC*, *B*), myometrium (*MYO*, *D*), and on CTB within maternal blood vessels: the wall of the vessel in (*C* and *D*) contains endothelial cells (*EC*) as well as CTB. BV, blood vessel; COL, cell column.

main with catenins in order to promote tight adhesion. The presence of significant levels of VE-cadherin in differentiating CTB may interfere with the ability of E-cadherin to establish the appropriate interactions with catenins, thereby undermining its ability to establish tight adhesion. Reduction of E-cadherin anchorage to the cytoskeleton is likely to enhance its turnover rate (39). Enhanced E-cadherin turnover may in turn account for the reduced staining for E-cadherin observed in column and placental bed CTB in situ, despite the similar levels of E-cadherin protein observed throughout CTB differentiation in vitro. Whatever the mechanism, however, upregulation of VE-cadherin in differentiating CTB helps to tip the balance towards increased invasiveness. Thus, cadherin modulation, like β1-integrin modulation (13), contributes to the acquisition of an invasive phenotype by differentiating CTB.

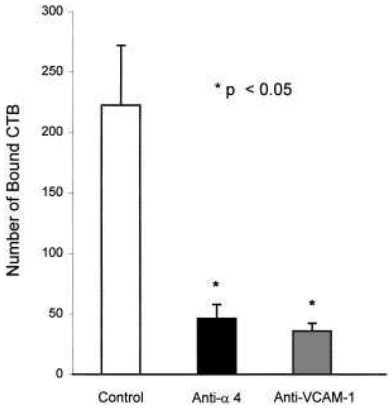

Figure 10. α4β1 on CTB interacts with immobilized chimeric VCAM-1/IgGFc. Freshly isolated CTB were plated in serum free medium, containing control IgG or anti-VCAM-1 (4B9) or anti–integrin α4 (HP1/2) IgG, on VCAM-1/IgGFc that had been bound to a Protein A-coated substrate. After 1 h at 37°C, wells were washed with PBS and fixed. Bound cells were counted. Triplicate samples were analyzed for each condition and the experiment was repeated three times. Error bars show standard error of the mean. Statistical significance was determined using the Student's *t* test.

Switching the profile of αV-associated integrins during their differentiation also supports increased invasiveness of CTB. In this case, expression of αVβ5 is reduced and that of αVβ3 is enhanced as CTB differentiate. Since treatment of isolated CTB with anti-αVβ3 suppresses their invasion significantly, its increased expression by differentiating CTB in vivo is highly likely to stimulate motility and invasiveness. This observation is consistent with studies implicating αVβ3 in endothelial cell migration in response to angiogenic stimuli both in vitro and in vivo (29, 31), and in the transition of melanoma to an invasive phenotype in vivo (30, 31). Since several αVβ3 substrates, including fibrinogen, are expressed by differentiating CTB (12), the enhanced levels of αVβ3 detected in placental bed CTB are likely to affect their motility or invasiveness in vivo as well as in vitro.

In addition to their contributions to CTB invasiveness, the integrins, VE-cadherin and other vascular adhesion receptors detected on differentiated CTB are likely to play significant roles in the process by which CTB replace the endothelium in the maternal spiral artery network. Based on studies of those primates in which placentation is most similar to the human, CTB invade the post-capillary venule and arteriole networks

in the superficial decidua and, in the case of arteries, but not veins, travel upstream through the endometrial and myometrial vessel segments, replacing the maternal endothelium and remodeling the tunica media of the vessel wall. This process results in conversion of these highly muscular, high-resistance arterial vessels into large-bore, low-resistance vessels that will conduct maternal blood efficiently to the intervillous space (4, 40, 41). Although the endothelial replacement process is poorly understood mechanistically, immunocytochemical data suggest that CTB and endothelium can transiently coexist in discrete patches on the walls of partially modified vessels (see companion paper; 20). If an early step of the replacement process involves direct binding of CTB to endothelial cells, CTB could utilize integrin α4β1 or PECAM-1 to interact, respectively, with VCAM-1 or with PECAM-1 or αVβ3 on the maternal endothelium. In support of this idea, we have now shown that the integrin α4β1 on CTB can bind to immobilized VCAM-1 in vitro. Instead, or in addition, these same receptors, along with VE-cadherin, could be involved in mediating CTB–CTB interactions, as these cells form a monolayer on the subendothelial ECM of the vessel wall after displacing the maternal endothelium. Once maternal endothelial cells are displaced, the α1β1 integrin collagen/laminin receptor, which is upregulated in interstitial and endovascular CTB (11, 19), could be critical for the attachment of CTB to subendothelial ECM and subsequent invasion of the tunica media (42).

Having described this remarkable switch in adhesion receptor phenotype executed by CTB, it is now critical to understand how this program is regulated. CTB express endothelial adhesion receptors (e.g., PECAM-1 and VE-cadherin) that are expressed at very early stages of endothelial cell differentiation in the yolk sac and early embryo (vasculogenesis, reviewed in 43–45), and during angiogenesis (αVβ3; 29, 32). This suggests that receptors for vasculogenic/angiogenic factors, such as vascular endothelial growth factor (VEGF) family members, may also be expressed by differentiating CTB. Interestingly, there are several reports that CTB in cell columns and in the placental bed express the Flt-1 VEGF receptor, and that VEGF itself is expressed by CTB, as well as by fetal macrophages in chorionic villi and by maternal macrophages in the uterine wall (46–48). Thus, CTB could respond in either a paracrine or autocrine fashion to VEGF. We hypothesize that the wave of endovascular invasion and blood vessel colonization, which peaks during second trimester, resembles a vasculogenic/angiogenic response on the part of CTB differentiating along the invasive pathway. This most unusual response, together with the highly invasive behavior of these CTB, may account for the virtually unique ability of CTB to enter blood vessels, displace resident endothelial cells, and colonize and remodel the arterial wall. In contrast, metastatic tumor cells enter and then extravasate, leaving the vessel wall relatively intact.

In summary, we have described a remarkable transformation in adhesion phenotype for those CTB that differentiate along the invasive pathway during formation of the functional human placenta. Together with our earlier studies documenting the intricate regulation of β1 integrins (11, 19, Lim et al.),[2] the present studies demonstrate the profound importance of regulating the adhesion phenotype of the cells that are involved in interstitial and endovascular invasion. Since important pregnancy disorders in the human, including preeclampsia, are associated with defects in the placenta in general, and with faulty CTB invasion in particular, we also have the opportunity to test the in vivo relevance of the changes reported herein. In the accompanying paper (20), we document that the switch to a vascular adhesion phenotype that accompanies the differentiation of CTB in normal pregnancy is defective in preeclampsia, suggesting that this switch is part of the strategy used by CTB for successful endovascular invasion.

Acknowledgments

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