Integrin switching regulates normal trophoblast invasion

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SUMMARY

Cells invade extracellular matrices in a regulated manner at specific times and places during normal development. A dramatic example is trophoblast invasion of the uterine wall. Previous studies have shown that differentiation of trophoblasts to an invasive phenotype is accompanied by temporally and spatially regulated switching of their integrin repertoire. In the first trimester human placenta, α6 integrins are restricted to cytotrophoblast (CTB) stem cells and downregulated in invasive CTBs, whereas α5β1 and α1β1 integrins are upregulated in differentiating and invasive CTBs. The goal of the present study was to determine whether these changes have functional consequences for CTB invasiveness. Using an in vitro invasion model, we determined first that aggregates of invading first trimester CTBs in vitro undergo the same pattern of integrin switching as was observed in situ, thereby validating the utility of the model. We then showed that antibody perturbation of interactions involving laminin or collagen

type IV and their integrin α1/β1 receptor inhibited invasion by CTBs, whereas perturbing interactions between fibronectin and the α5/β1 fibronectin receptor accelerated invasion. Finally, we report that later gestation CTBs, which display greatly decreased invasive capacity, are also unable to upregulate α1β1 complexes, providing further evidence that this integrin is critical for CTB invasion. This gestational regulation is transcriptional. These data indicate that integrin switching observed during differentiation in situ has significant functional consequences for CTB invasion. The data suggest further that differentiating CTBs upregulate counterbalancing invasion-accelerating and invasion-restraining adhesion mechanisms. We propose that this contributes to regulating the depth of CTB invasion during normal implantation.

Key words: integrins, trophoblast invasion, implantation, human embryogenesis

INTRODUCTION

Cells invade extracellular matrices (ECM) in a regulated manner at specific times and places during normal development and as a physiological component of tissue remodeling in the adult. For example, cytotrophoblast (CTB) invasion of a precisely circumscribed region of the uterine wall and vessels takes place during a defined period in early pregnancy, resulting in formation of the hemochorial placenta. Failure to regulate inappropriate cell invasion is a fundamental aspect of many disease processes, including cancer and certain autoimmune diseases such as rheumatoid arthritis. As might be expected, all highly invasive cells, including CTBs, share many phenotypic properties, including an altered adhesion molecule phenotype (Plantefaber and Hynes, 1989; Giancotti and Ruoslahti, 1990; Albelda et al., 1990; Behrens et al., 1993; Oka et al., 1993) and elevated expression of matrix-degrading proteinases (Mignatti et al., 1986; reviewed by Alexander and Werb, 1991; Stetler-Stevenson et al., 1993). Enhanced understanding of how the invasive phenotype of trophoblasts is regulated during normal placentation is therefore not only important in its own right, but may provide insight into how such regulatory mechanisms are undermined in disease processes.

Examination of tissue sections of human placental bed biopsies (the fetal-maternal interface: Fig. 1) shows that differentiation along the invasive pathway occurs in a subset of CTBs present in those chorionic villi that anchor the embryo to the uterine wall. These cells switch their integrin phenotype at least twice during their differentiation and invasion (Damsky et al., 1992). The CTB stem cell population, a polarized epithelium expressing high levels of E-cadherin, is anchored to the trophoblast basement membrane and stains strongly for the α6β4 integrin. The first step in differentiation, formation of large nonpolarized aggregates (cell columns), is accompanied by upregulation of the α5β1 fibronectin (Fn) receptor and a Fn-rich cell-associated ECM. When individual CTBs leave the cell columns and invade the uterine wall, they express the α1β1 collagen/laminin (Ln) receptor as well as continuing to express the α5β1 Fn receptor. The α6β4 integrin is no longer detected, and E-cadherin staining is weak and discontinuous (Damsky et al., 1992, and unpublished observations). These in situ immunocytochemical data suggest that modulation of adhesion receptor phenotype is functionally important for the development of invasiveness. The reciprocal regulation of receptors for basement membrane constituents (α6β4 is downregulated, α1β1 is upregulated) is particularly striking.

To test the role of integrin switching in CTB differentiation and acquisition of invasiveness, we used an in vitro model system that recapitulates many aspects of CTB differentiation (Librach et al., 1991). In this model, highly purified CTBs isolated from first trimester human placentas are plated on a porous filter coated with the reconstituted basement membrane material, Matrigel. The isolated CTBs attach as a monolayer, migrate extensively and form large aggregates, which then penetrate the Matrigel. In contrast, CTBs isolated from late second trimester onward, like their counterparts in vivo, have greatly reduced capacity for invasion. Using this system, Librach et al. (1991) determined that the $92{\times}10^3$ M_r matrix metalloproteinase is critical for first trimester CTB invasion.

We report here that aggregates of isolated first trimester CTBs invading Matrigel undergo the same pattern of integrin switching as is observed in situ. We then show that antibody perturbation of interactions involving Ln or collagen type IV and their integrin $\alpha1/\beta1$ receptor inhibited invasion by CTBs, whereas perturbing interactions between Fn and the $\alpha5/\beta1$ Fn receptor accelerated invasion. In addition, we report that later gestation CTBs, which display greatly decreased invasive capacity, are also unable to express $\alpha1\beta1$ integrin complexes, providing further evidence that this integrin is critical for CTB invasion-competence. Thus, we suggest that regulation of integrins could play an important role both in enabling CTBs to invade and in temporally regulating their invasive capacity.

MATERIALS AND METHODS

Antibodies

The rat anti-cytokeratin monoclonal antibody (mAb) 7D3 stains CTBs at all stages of differentiation (Damsky et al., 1992). Antibodies against the hormone human placental lactogen (hPL) stain differentiated trophoblasts (Kurman et al., 1984): the syncytiotrophoblast layer that surrounds chorionic villi, and differentiated CTB within the uterine wall in situ (also called intermediate trophoblasts; Kurman et al., 1984; Yeh and Kurman, 1989), as well as isolated CTBs that have differentiated and invaded Matrigel (Librach et al., 1991). Polyclonal antibody against hPL was obtained from DAKO Corporation (Carpenteria, CA 93013). Monoclonal antibody (INNhPL-37) against hPL was obtained from Serotec Ltd. (Blackthorn Bicester, England). Antibody against the pan-leukocyte antigen CD45, used to remove bone marrow-derived cells from partially purified preparations of CTB, was purified from ascites fluid of the GAP 8.3 hybridoma (American Type Culture Collection, Rockville, MD).

Antibodies against extracellular matrix components were obtained from the following sources: mouse mAbs specific for the A chains of Ln (4C7 and 11D5/C4) and of merosin (2G9, 1F9/G6), the B1 chain of Ln (3E5/H2E7), and the B2 chain of Ln (2E8/A11), Dr Eva Engvall, La Jolla Cancer Foundation (La Jolla, CA) (Leivo and Engvall, 1988; Engvall et al., 1990); mouse mAbs against the B2 subunit of Ln (D18), Dr Joshua Sanes, Washington University (St. Louis, MO) (Sanes et al., 1990); polyclonal rabbit anti-mouse Ln, Drs Deborah Hall and Louis Reichardt, UCSF (San Francisco, CA); rabbit polyclonal anti-Fn, Collaborative Research; mouse anti-Fn mAbs specific for the alternatively spliced A and B type III repeats (IST-9 and BC-1, respectively) and for a site common to all Fns (IST-4), Dr L. Zardi, University of Genova, Italy (Carnemolla et al., 1989); a second common-site mouse anti-Fn mAb, Telios Pharmaceuticals (La Jolla, CA); mouse mAb against collagen type IV (no. M785), DAKO Corporation; polyclonal anti-collagen type IV (no. AB748), Chemicon International Inc. (El Segundo, CA).

Antibodies against integrin adhesion receptors were obtained as follows: rat mAbs against the integrin $\beta1$ (AIIB2), $\alpha5$ (BIIG2) and $\alpha6$ (J1B5) subunits, and a mouse mAb against the $\alpha1$ subunit (S2G3) were produced in this laboratory (Werb et al., 1989; Hall et al., 1990; Damsky et al., 1992). All except the anti-$\alpha6$ perturb function. The GoH3 adhesion-perturbing rat anti-$\alpha6$ mAb was kindly provided by Dr Arnoud Sonnenberg, Amsterdam (Sonnenberg et al., 1986, 1990; Hall et al., 1990); a rat mAb against the integrin $\beta4$ subunit (439-9B) was kindly provided by Dr Steven Kennel, Oak Ridge National Laboratories (Oak Ridge, TN) (Kennel et al., 1989); a mouse anti-$\alpha1$ mAb, originally designated TS2/7 was available commercially under the name ACT-T-SET, T-Cell Sciences (Cambridge, MA). The rat monoclonal antibody BIVF2, against an $80{\times}10^3$ M_r cell surface antigen on CTB of unknown function was used as a control in the invasion assays. Fluorochrome-conjugated secondary antibodies that had been absorbed to reduce interspecies cross reactivity (rhodamine- or fluoroscein-conjugated goat anti-rat and -mouse, and donkey anti-rabbit), were purchased from Jackson ImmunoResearch Lab. Inc. (West Grove, PA).

Isolation of first trimester CTBs

CTBs were isolated from first trimester placenta by a two-step enzymatic digestion procedure followed by Percoll gradient fractionation as described previously (Fisher et al., 1989, 1990 and Librach et al., 1991). For all experiments, the CTB-enriched fraction from the Percoll gradient was purified further by negative selection with the common leukocyte antigen CD45 bound to magnetic beads (Advanced Magnetics Inc., Cambridge, MA) as described by Librach et al. (1991). This procedure removed cells of bone marrow origin. The resulting purified CTBs were washed and plated in high-glucose DMEM-ND (Dulbecco's minimal essential medium with 4 g/l glucose, 50 µg/ml gentamycin and 2% Nutridoma, a serum substitute that lacks attachment factors).

Assessment of ECM and integrin expression by invading CTB in vitro

Plugs of Matrigel (Collaborative Research, Bedford, MA) were used to assess, by immunocytochemistry, the expression of ECM components and integrins by invading CTBs in vitro. Plugs were prepared as described by Librach et al. (1991). Briefly, 2 parts ice cold Matrigel were diluted with 1 part DMEM-ND (final Matrigel protein concentration 6-8 mg/ml) and polymerized at 37°C in small capsules. CTBs were plated on top of the solidified plug at a density of $3{\times}10^5$ cells in 250 µl of DMEM-ND, and cultured for up to 72 hours. During this time, CTBs aggregated and invaded the Matrigel plug. Matrigel plugs with invading CTBs were fixed for 30-60 minutes at 4°C in 3% paraformaldehyde in PBS/CaCl$_2$. The plug was removed from the capsule and washed in PBS containing 0.1 M glycine to block remaining aldehyde groups. For histology, samples were dehydrated in ethanol and embedded in JB4 resin (Polysciences, Warrington, PA). 1 µm sections were stained with hematoxylin and eosin and photographed with a Nikon HFX-IIA (Garden City, NY). For immunocytochemistry, paraformaldehyde-fixed and washed plugs were embedded in OCT (Miles, Scientific Division, Naperville, IL) and frozen in liquid nitrogen (Fisher et al., 1989; Librach et al., 1991). 5 µm sections were cut in an HR cryostat (Slee International Inc., Tiverton, RI), fixed in 3% paraformaldehyde for 20 minutes, and rinsed in PBS containing 0.15 M glycine to quench unreacted aldehyde groups. Nonspecific staining was blocked by incubating samples for 20 minutes with 0.2% BSA in PBS (PBS-BSA). Samples were exposed for 1-2 hours to individual primary antibodies or to a mixture of two primary antibodies of different species origin, diluted in PBS. Samples were washed with several changes of PBS-BSA for 15 minutes, incubated for 30 minutes with the appropriate secondary antibodies conjugated to either rhodamine isothiocyanate or FITC, rinsed in several changes of PBS, and mounted with Gelvatol (Air Products, Allentown, PA), which contained P-phenylenediamine (Sigma, St. Louis, MO) to inhibit quenching of the fluorescein signal.

Normal or nonrelevant rabbit, rat or mouse IgG were substituted for primary antibodies as controls for nonspecific staining. Samples were examined with a Zeiss Axiophot phase/epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with the appropriate filters and 40× and 63× planapo oil immersion objectives. Sections were photographed with Kodak T max 400.

Invasion assay

To quantify the effects of adhesion-perturbing antibodies on invasion, CTBs were cultured in Matrigel-coated Transwell filter chambers (6.5 mm; Costar, Cambridge, MA; Librach et al., 1991). 15 μl of Matrigel diluted as described above was applied to the Nucleopore filter and allowed to polymerize in a humidified incubator at 37°C. Cells (3×10^5 in 0.2 ml DMEM-ND) were added to the top of the filter and 1 ml of medium was added to the culture well. Samples were incubated for 24-48 hours to permit invasion, and then fixed and processed for scanning electron microscopy (Librach et al., 1991). Where indicated, nonimmune control or function-perturbing polyclonal antibodies against extracellular matrix constituents (100 μg/ml IgG), or mono-clonal antibodies against integrin subunits or against a nonrelevant 80×10^3 M_r cell surface antigen BIVF2 (75 μg/ml IgG or IgM), were diluted into the culture medium and into the Matrigel and were present during the invasion assay period. The antibody concentrations used were chosen after preliminary experiments in which several dilutions were tested; the concentration used produced the maximum effect on CTB invasion. Samples were viewed in a JEOL (JSM-840) scanning electron microscope. Invasion was quantified by morphometric analysis of the surface area of the bottom of the filter covered by invading cells (described in detail by Librach et al., 1991 and based on the principles established by Weibel and Bolender, 1973). For each condition tested, at least two filters were examined per experiment and the experiments were repeated at least three times.

Immunocytochemical analysis of the time course of α1β1 expression in vitro

To determine the time and rate of onset of α1 expression in vitro, CTBs were plated (2.5×10^5/15 mm diameter well) in DMEM-ND on 11 mm glass coverslips that had been coated for 2 hours with a thin layer of Matrigel or with a mixture of Ln (10 μg/ml), collagen type IV (10 μg/ml) and Fn (5 μg/ml). CTBs formed clusters of partially spread cells within several hours. Samples were fixed at several time points over 36 hours. To obtain data for the zero time point, freshly isolated CTB were immobilized on coverslips by a 1 minute cytospin and fixed in 3% paraformaldehyde for 20 minutes. Samples were double stained for both α1 integrin and cytokeratin and processed as described above for Matrigel sections. The percentage of cytokeratin-positive cells that were also α1 positive was scored. Slides were labeled with a letter code and scored blindly. Values represent the mean in three separate experiments with at least three filters per experiment.

RNA extraction

RNA was extracted from first trimester and term CTBs. Isolated CTBs were cultured at a concentration of 1.0×10^6 cells/ml of serum free DMEM-ND in 24 mm culture wells coated with Matrigel, as described by Librach et al. (1991). The cells were harvested at several time points: after isolation but prior to culture (0 time), and after 12, 24 and 48 hours in culture. Total RNA was extracted according to published methods (Chomszynski and Sacchi, 1987). Briefly, $2-5\times10^7$ cells per sample were homogenized in 500 μl guanidine buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl), followed by the addition of 50 μl 2 M sodium acetate (pH 4), 500 μl water-saturated phenol and 100 μl chloroform. After centrifugation the RNA was pelleted from the aqueous phase by the addition of 500 μl isopropanol, extracted with 4 M LiCl, and reprecipitated from a solution containing 10 mM Tris (pH 7.5), 1 mM EDTA, 0.5% SDS. The pellet was then washed with 70% ethanol, vacuum dried and dissolved in sterile water. The concentration of RNA was determined by measuring the absorbance at 260 nm.

Northern blotting

Total RNA (10 μg) was separated by formaldehyde-agarose gel elec-trophoresis, transferred to Nytran membranes (Schleicher and Schuell, Keene NH) and analyzed by northern blot hybridization as described previously (Lehrach et al., 1977; De et al., 1989). The probe was synthesized by random priming of full length α1 integrin cDNA (the gift of Dr E. Marcantonio, Columbia University) using [^{32}P]CTP and the Klenow fragment of DNA polymerase I according to standard methods. Probes had a specific activity of 2×10^9 dpm/μg. The final posthybridization wash was carried out in 0.3× SSC (0.15 M NaCl, 20 mM sodium citrate) and 0.1% SDS at 68°C. 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

Fig. 1 shows the microscopic anatomy of an anchoring villus at the fetal-maternal interface (10 weeks gestation). A polarized CTB stem cell monolayer is attached to the tro-phoblast basement membrane that surrounds the villus stroma. At selected sites, CTBs leave the basement membrane and form a large aggregate (cell column) that attaches to and invades the uterus and its spiral arteries (Fig. 1, inset), thereby physically joining maternal and fetal tissues and establishing the placental circulation. CTBs in different zones of the anchoring villus express distinct patterns of integrins and ECM ligands, as indicated in the figure (see also Damsky et al., 1992, 1993).

Our previous work determined that when CTBs isolated from first trimester placental villi are plated on Matrigel, they aggregate and invade this basement membrane-like matrix (Librach et al., 1991). Fig. 2A shows a section of a Matrigel plug invaded by CTBs over a 3-day period. The CTBs invaded as an aggregate, in most cases retaining their cell-cell associ-ations as they do in cell columns in situ. At the invasive front, small groups of cells appeared to be dissociating from the main aggregate, similar to the situation that occurs when cells from the columns enter the external wall. The vacuolated appear-ance of the cells also resembles that of differentiating CTBs in situ. Invasive CTBs contain high levels of glycogen, which is then washed out during tissue processing, creating this char-acteristic morphology (Enders, 1968). The cells within the Matrigel were identified as CTBs by staining with antibodies against both cytokeratin (Fig. 2B) and hPL (Fig. 2C), a hormone specific for differentiated CTBs (Kurman et al., 1984; Librach et al., 1991).

CTBs within the Matrigel produced an extensive pericellu-lar extracellular matrix that stained strongly for Fn, type IV collagen, and the LnB2 chain, but not for merosin, an alterna-tive A chain (Fig. 3). It also stained for the Ln B1 and A chains (not shown). This staining pattern is characteristic of the matrix produced by CTBs that are about to enter the uterine wall in situ (transition from zones III-IV; Fig. 1 and Damsky et al., 1992). In contrast, undifferentiated villus CTBs in situ (zone I, Fig. 1) do not stain for Fn but do stain for merosin, as well as other Ln chains (Damsky et al., 1992). CTBs within the Matrigel also expressed an integrin repertoire like that on
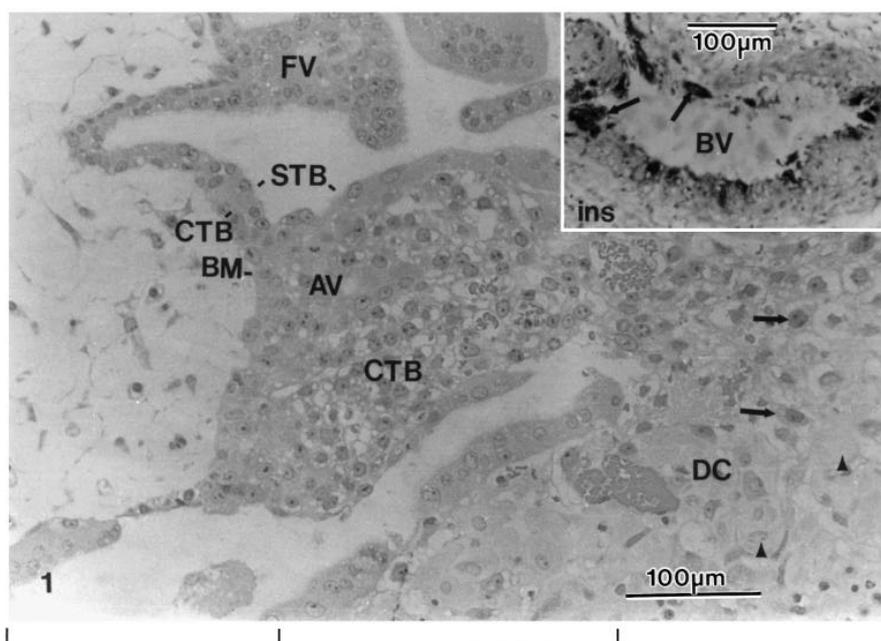
Fig. 1. Section of a 10 week first trimester placenta showing the morphology of an anchoring villus at the fetal-maternal interface. Expression of integrins, laminins, fibronectins, and collagen type IV by differentiating cytotrophoblasts (CTB) in situ is tightly regulated, as indicated by the adhesion molecule profile characteristic of CTB in four zones (data summarized from Damsky et al., 1992). Zone I: monolayer of CTB on the trophoblast basement membrane (BM); Zones II and III: the cell aggregate (column) formed by differentiating CTB. Zone IV: decidua (DC) into which individual or small clusters of CTB have invaded. STB, syncytiotrophoblast; AV, anchoring villus; FV, floating villus; BM, basement membrane; arrow, CTB; arrowhead, DC cell. Inset (ins). Higher magnification of a region from the myometrium of a second trimester placenta that contains a maternal blood vessel (BV). Note that the vessel is lined with cytokeratin-positive CTB.

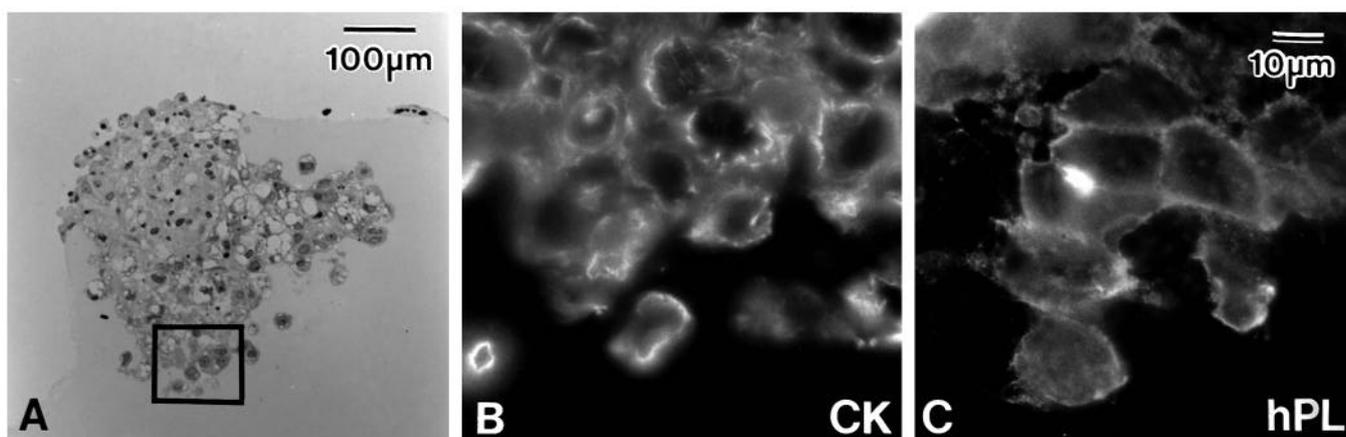|	Zone I	Zones II and III		Zone IV
Integrins	α6/β4	α6/β4	α6/β4↓	–
	–	–	α5/β1↑	α5/β1
	–	–	–	α1/β1↑
Matrix	Ln, Mer	Ln	Ln	Ln$_{B1, B2}$
	A+, B+ Fn	–	A+, B+ Fn↑	A+, B+ Fn
	Col IV	–	Col IV↑	Col IV

Fig. 2. Isolated CTBs that invade Matrigel express a CTB-specific differentiation antigen. (A) Section of a JB-4-embedded aggregate of CTB that have invaded Matrigel over a 48-hour period. Cells that have invaded have the large nuclei and vacuolated appearance typical of invading CTBs in situ. (B) Higher magnification of a frozen section of the invasive front of a CTB aggregate, stained with anti-cytokeratin (CK) antibody. Area depicted is similar to that indicated by box in A. (C) Similar section of an invading CTB aggregate, stained for the hormone human placental lactogen (hPL), which is expressed specifically on those CTBs that have invaded the uterine wall in situ.

CTBs within the uterine wall in situ (zone IV, Fig. 1): they all stained strongly for the β1, α1 and α5 subunits (Fig. 4A-C), whereas staining for the β4 and α6 subunits was heterogeneous, with many cells negative and others weakly stained (Fig. 4D,E). Taken together, these results indicate that this culture model supports the acquisition by CTBs of both an invasive phenotype and an integrin repertoire similar to that of differentiated CTBs within the uterine wall in situ. CTBs can also undergo integrin switching when plated on a mixed substrate of collagen IV and Ln (not shown) indicating that other factors in Matrigel besides matrix constitutents are not responsible for this alteration in integrin repertoire.

To determine what role CTB-ECM interactions have in regulating CTB invasion, we used the Matrigel-coated Transwell filter invasion assay described by Librach et al. (1991) in conjunction with adhesion-perturbing monoclonal antibodies against the integrins and the ECM components whose expression was closely regulated during CTB differentiation in situ. Since CTBs produce an ECM (Fig. 3), this assay should reflect their interaction with endogenous matrix constituents as well as with matrix components in the Matrigel. Invasion was quantified morphometrically by determining the surface area of the underside of the filter occupied by CTBs that had arrived there in 24-48 hours by crossing the Matrigel barrier (see Librach et al., 1991). Fig. 5 displays graphically the results of assays investigating CTB interactions with collagen type IV and Ln. Polyclonal antibodies against Ln and collagen type IV blocked CTB invasion by over 80%. The anti-α6 IgG, GoH3, did not inhibit CTB invasion when added by itself. However, the S2G3 anti-α1 IgM reduced invasion to less than 50% of control, and when combined, the anti-α1 and anti-α6 antibodies acted synergistically to reduce CTB invasion to 20% of the control level. Since the GoH3 antibody does not block interactions with α6β4, this indicates that a low level of α6, probably complexed with β1, contributed to CTB-Ln interactions. These results demonstrated that CTB interactions with Ln and collagen, mediated primarily through α1β1, were critical for CTB invasion in this system.

CTB interactions with Fn had the opposite effect (Fig. 6). Since Matrigel has a very low Fn content, we are likely measuring CTB interactions with endogenous Fn (Fig. 3C). Inclusion of anti-Fn IgG in the invasion assay did not block CTB invasion and even had a small stimulatory effect. More striking was the observation that addition of anti-α5 IgG in the assay stimulated CTB invasion more than twofold. These results are consistent with the idea that CTB interactions with Fn restrain invasion. This possibility was tested further by incorporating exogenous Fn into the Matrigel layer. The presence of the additional Fn reduced CTB invasion to 20% of the control level. However, addition of anti-α5 IgG as well as the Fn overcame this restriction and showed that the CTB interaction with Fn was mediated through the α5β1 integrin. Control antibodies, including nonimmune rabbit IgG, mouse monoclonal anti-integrin α2 IgG, rat monoclonal antibody J1B5, which recognizes the

integrin α6 subunit but does not perturb function, and BIVF2 against an 80×10³ M_r cell surface glycoprotein on CTB, had no effect on CTB invasion. Taken together, the data indicate that CTB-Fn interactions and CTB-Ln/collagen interactions have opposing effects on CTB invasion, suggesting that the net invasive capacity of CTBs depends significantly on the relative contributions of these distinct sets of adhesive interactions.

The inhibitory activity of the anti-α1 antibody in the invasion assay (Fig. 5) together with the increased staining for the α1/β1 integrin on CTBs within the uterine wall in situ (Damsky et al., 1992), pointed to a particularly important role for the upregulation of this collagen/Ln receptor in promoting CTB invasion competence. To test this idea further, we investigated the onset of expression of α1β1 in cultured first trimester CTBs and then asked whether the onset of expression of this integrin and invasive capacity were regulated in parallel.
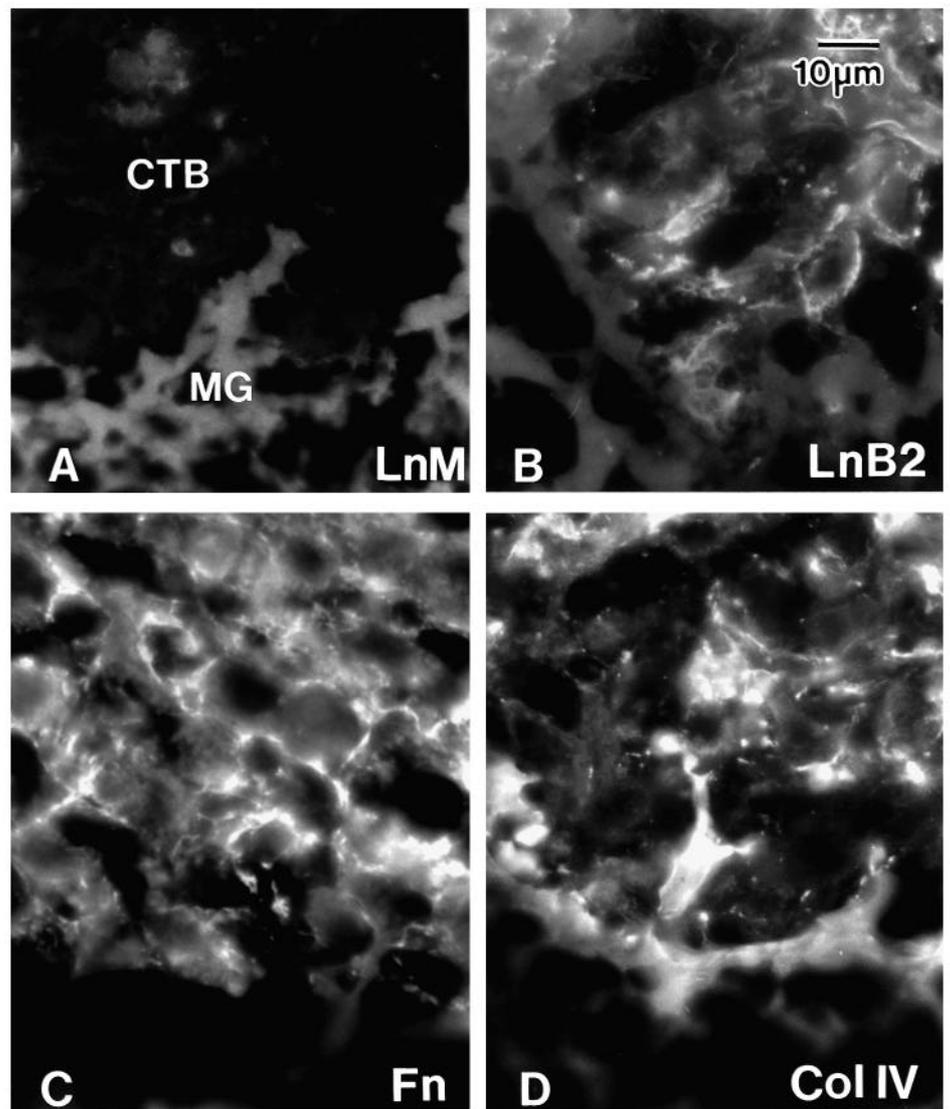

Fig. 3. Expression of extracellular matrix components by CTB aggregates that have invaded Matrigel in vitro. Sections were stained with antibodies to (A) merosin (LnM); (B) laminin B2 (LnB2); (C) fibronectin (Fn); (D) collagen IV (Col IV). The absence of merosin and the presence of the other ECM components is typical of CTB in situ that have left the chorionic villi and are differentiating along the invasive pathway. MG, Matrigel.
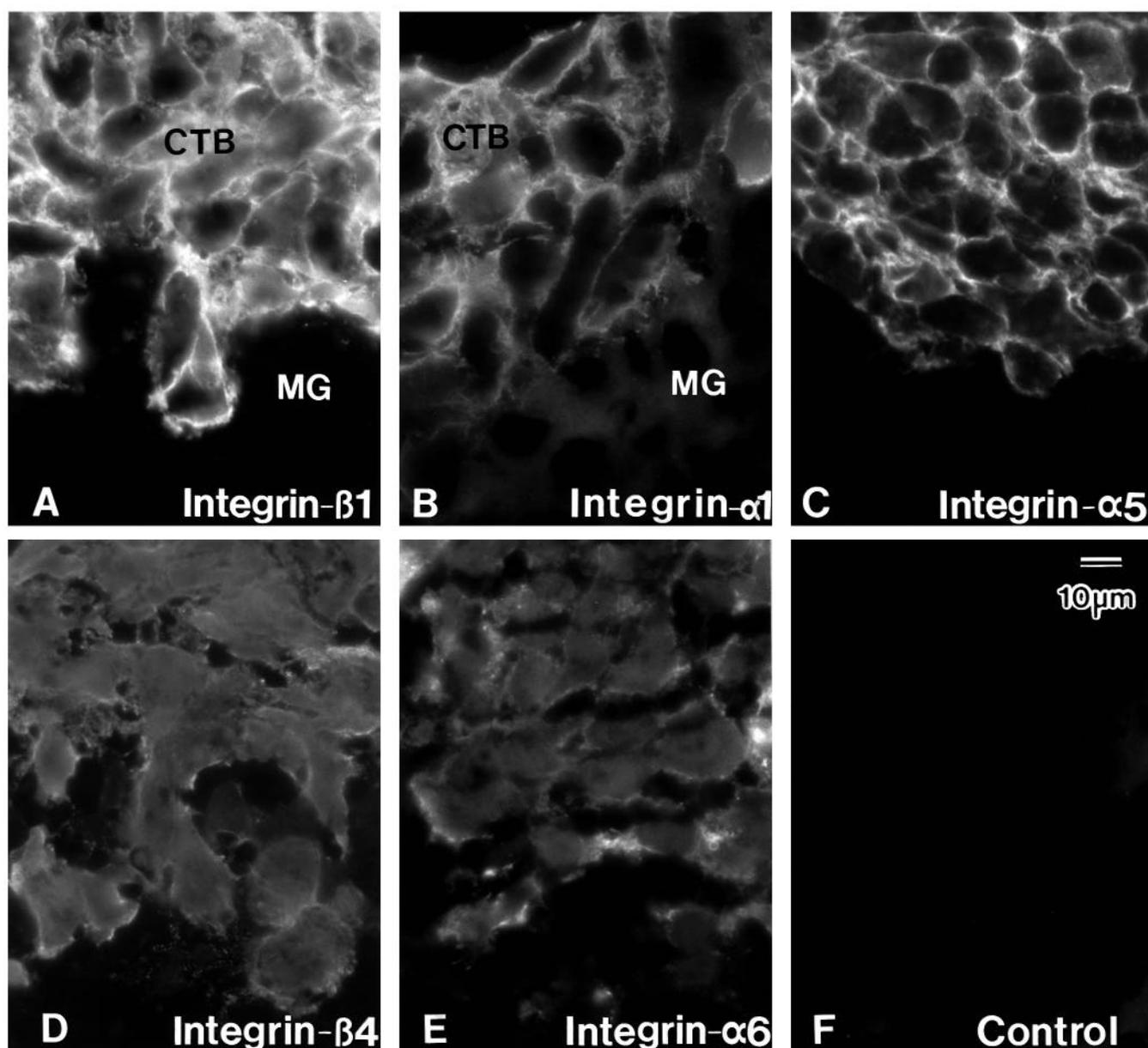
Fig. 4. Expression of integrins by CTB aggregates that have invaded Matrigel in vitro. Sections were stained with antibodies to integrin subunits: (A) β1; (B) α1; (C) α5; (D) β4; (E) α6. (F) Control section, reacted with nonrelevant IgG. Like invading CTBs in situ, CTBs invading Matrigel express α1/β1 and α5/β1 complexes.

To determine the percentage of freshly isolated CTBs expressing α1β1 integrin, some CTBs were immobilized on coverslips by cytospin and fixed immediately. The remainder of the cells were plated on Matrigel-coated coverslips, fixed at the times indicated over a 36-hour period, and stained for both cytokeratin and the α1 subunit. At zero time, about 20% of the first trimester CTBs (cytokeratin-positive cells) stained for α1. The percentage of α1-positive CTBs increased steadily over the next 36 hours and reached a plateau of 70% at about 24 hours (Fig. 7). First trimester CTBs could also upregulate expression of the α1β1 integrin to almost the same degree when plated on a substrate composed of a mixture of purified Ln, collagen IV and Fn, suggesting that additional components in Matrigel

were not primarily responsible for modulating integrin expression (not shown). Interestingly, the rate of onset of expression of the α1 subunit by second trimester cells was considerably slower, although a similar percentage of these cells ultimately stained positively for α1 by 36 hours. In contrast, only about 2% of CTBs from term placentas stained positively for the α1 subunit, and this level did not increase over the 36 hour sampling period, suggesting that term CTBs were unable to upregulate expression of this molecule.

To determine whether expression of the α1 subunit was being regulated at the transcriptional or post-transcriptional level, the levels of α1 mRNA present in CTBs isolated from placentas of different gestational ages, and cultured for

Human Cytotrophoblast Invasion
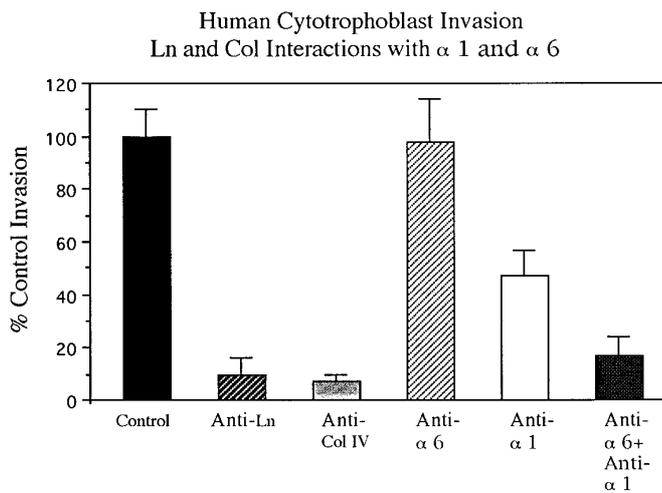### Ln and Col Interactions with α 1 and α 6

Fig. 5. Blocking CTB interactions with laminin and collagen IV inhibits CTB invasion. Control invasion (obtained using nonimmune rabbit serum or nonrelevant monoclonal antibodies) is set at 100%. Cells were plated on Matrigel-coated Transwell filters, but also express endogenous ECM components in vitro (Fig. 3). Control or function-perturbing antibodies to ECM components or integrin subunits were added to Matrigel and culture medium. Integrin α6β1 is a Ln receptor and α1β1 a Ln/collagen receptor.

Human Cytotrophoblast Invasion
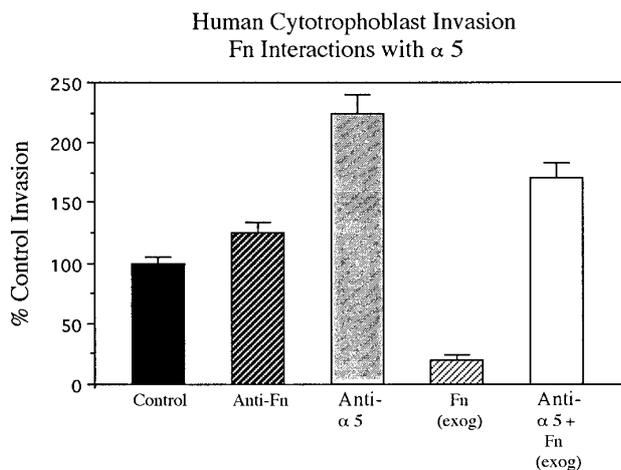### Fn Interactions with α 5

Fig. 6. Blocking CTB interactions with fibronectin stimulates CTB invasion. Control invasion (obtained using nonimmune rabbit serum or nonrelevant monoclonal antibodies) is set at 100%. Integrin α5β1 is a fibronectin receptor. Since Matrigel is very low in Fn content, CTB were interacting primarily with endogenous Fn. In some experiments exogenous Fn (100 μg/ml) was added to Matrigel.

different time periods, were compared by northern blotting using a full length human α1 cDNA probe. The human α1 integrin mRNA ran as a sharp band at about 4.1-4.2 kb, just slightly faster than 28S RNA. A slower moving, more diffuse band was present at about 5.2-6.5 kb. The samples from CTB and placental fibroblasts had a similar doublet profile (Fig. 8), as did positive samples from other tissues, such as spleen (not shown), while neither band was present in negative tissues, such as PBL (Fig. 8) or heart and lung (not shown). Data from first trimester CTBs showed that some α1 mRNA was present
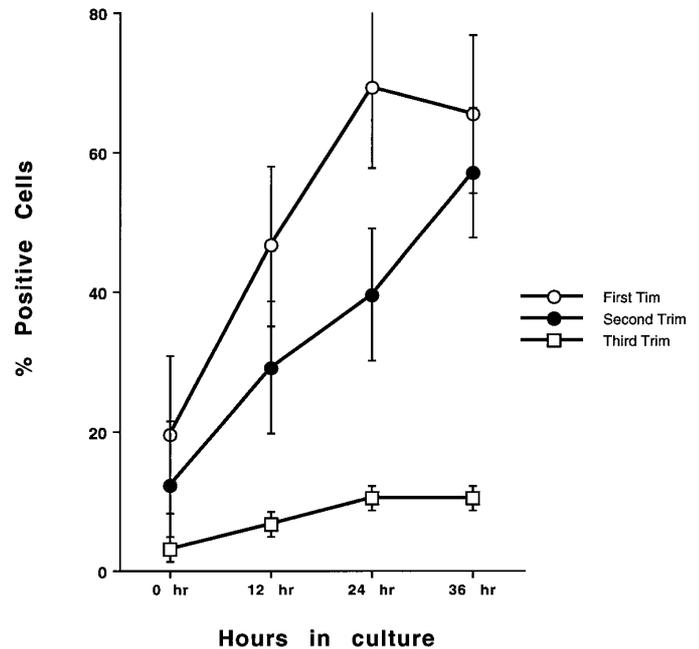
Integrin Alpha 1 Expression

Fig. 7. First and second trimester, but not term CTB upregulate α1/β1 integrin protein production in vitro. Time course of induction of α1/β1 integrin in vitro by CTBs isolated from different periods of gestation. CTBs were plated on Matrigel-coated coverslips, and the percentage of cytokeratin-positive cells that also stained with an anti-integrin α1 antibody was determined over a 36-hour period. Data are expressed as the mean ± standard error.

in freshly isolated cells, but that the level increased during the course of the experiment (0-48 hours; Fig. 8A). In contrast, α1 mRNA was not detected in CTBs isolated from term placentas at any time during the culture period, in agreement with the observation that they were not able to express the α1 protein (Fig. 8B). These data show that the dramatic alteration in integrin α1 protein expression that occurs after culture and as a function of gestational age is accompanied by corresponding differences in integrin α1 mRNA levels. Thus, gestational regulation of CTB expression of this adhesion molecule appears to be transcriptional.

DISCUSSION

The experiments reported here were undertaken to test directly the role of cell-ECM interactions in the developmentally regulated acquisition of an invasive phenotype by CTBs. The data in Figs 2-4 document that cultured CTBs not only formed aggregates that invaded the Matrigel (Librach et al., 1991), but also produced a pericellular ECM and executed the switch in adhesion molecule phenotype characteristic of differentiated CTBs invading the uterine wall in vivo. In particular, they stained weakly for α6β4 and strongly for α5β1 and α1β1 in reactions using the same antibodies as were used for our previous immunocytochemical studies of normal and preeclamptic placental tissue (Damsky et al., 1992; Zhou et al.,
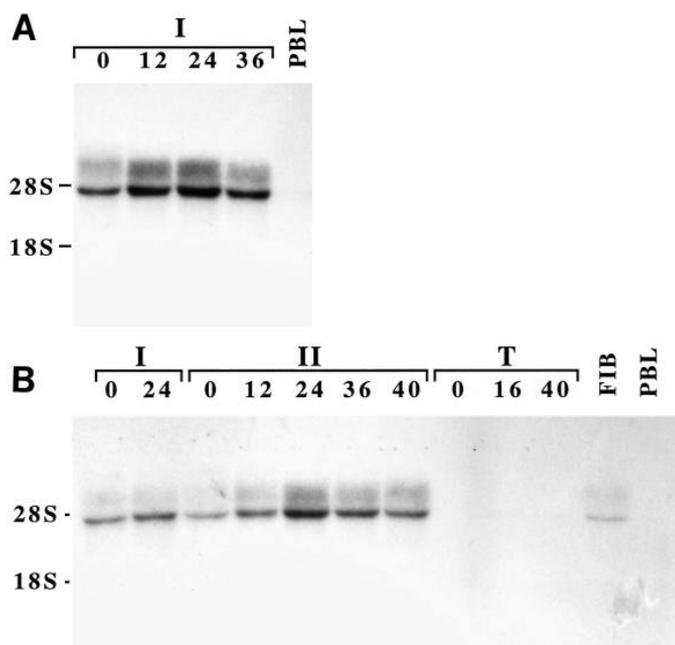
Fig. 8. First and second trimester, but not term CTB upregulate α1β1 mRNA production in vitro. Analysis of expression of mRNA for the integrin α1 subunit by northern blotting. Blots were hybridized with a full length human α1 cDNA probe. (A) Northern blot of RNA extracted from first trimester (I) CTB immediately after isolation, or following culture for the times indicated (in hours). Integrin α1 subunit mRNA was detected in freshly isolated first trimester CTB, and the level increased with time in culture. PBL (peripheral blood lymphocyte) RNA was used as a negative control for α1 expression. (B) Northern blot of RNA extracted from CTB isolated from first (I) and second (II) trimester or term (T) placental villi and cultured for the times indicated (in hours). Integrin α1 subunit mRNA was detected in freshly isolated first (I) and second trimester (II) CTB. The levels increased over the first 24 hours in culture in both trimesters. Integrin α1 subunit mRNA was not detected in term (T) CTB at any of the times tested. Placental fibroblasts (FIB), shown previously to express α1 protein (Hall et al., 1990), and PBL were included as positive and negative controls, respectively. Positions of the 28S and 18S ribosomal RNAs are indicated and were obtained from acridine orange staining of the gels prior to transfer.

1993). These results validated our use of the Matrigel model to investigate further the specific contributions of individual adhesive interactions to the invasive behavior of CTBs.

We used an antibody-perturbation approach to determine the functional consequences of these changes for CTB invasion. The data suggested an invasion-promoting role for α1β1 and an invasion-restraining role for α5β1. This interpretation implies that CTBs upregulate expression of adhesion mechanisms with opposing effects on invasion as part of their normal differentiation program, and that CTBs might regulate the depth of their invasion in part by titrating the net effects of these counterbalanced interactions. We also considered the possibility that the α5 antibody might stimulate CTB invasion by signaling enhanced motility or metalloproteinase (MMP) production directly, through the α5β1 Fn receptor, rather than by overcoming an adhesive interaction between Fn and its receptor that restrained CTB invasion. There is precedent for

this, as the same α5 antibody used in this study, BIIG2, enhances expression of interstitial collagenase and stromelysin in synovial fibroblasts (Werb et al., 1989). However, first trimester CTBs cultured in the absence of antibodies already express high levels of activated forms of MMP, particularly the 92×10^3 M_r type IV collagenase (Librach et al., 1991). We could not demonstrate any further elevation in the levels or activity of MMP in response to the anti-α5 antibody (not shown). Furthermore, the addition of exogenous Fn to the Matrigel invasion barrier reduced CTB invasion, and this effect was overcome by addition of the anti-α5 antibody (Fig. 6). These last results show that promoting increased CTB interaction with Fn had the effect of restraining invasion in an α5β1-dependent manner. There is precedence for proposing that cell interactions with Fn via α5β1 restrain invasiveness, especially if the Fn is present as part of an endogenous fibrillar pericellular network (discussed by Ruoslahti, 1991), as it is in differentiating CTB in situ (Damsky et al., 1992) and in vitro (Fig. 3). Taken together, these experiments strongly support the hypotheses that upregulation of the α1β1 integrin is critical for acquisition by CTB of an invasive phenotype during early gestation and that the net invasiveness of CTBs is determined, at least in part, by independent regulation of integrin-mediated adhesion mechanisms that accelerate or restrain invasion.

As gestation proceeds, the invasive capacity of CTBs declines, both in vitro (Librach et al., 1991) and in vivo (Zhou et al., 1993). Data in Fig. 7 show that the ability of CTBs to upregulate expression of the α1β1 integrin complex also declines, such that CTBs from term placentas can neither invade nor upregulate α1β1 within the assay period. This result indicates that downregulation of adhesion receptors, such as α1β1, that promote invasion could contribute to the temporal as well as spatial regulation of CTB invasion. Analysis using northern blotting suggested that α1 expression is being regulated primarily at the transcriptional level. Although some α1 integrin subunit mRNA is detected in freshly isolated first trimester CTBs, when fewer than 20% of the cells show α1 staining, the level is higher 24 hours later, when 70% of the cells stain. Furthermore, α1 subunit mRNA was undetectable in term CTB at all times during their culture. Thus, the gestational decline in the ability of CTB to express α1 protein is accompanied by a parallel decline in the presence of α1 mRNA.

Our findings suggest that a critical balance of adhesion-promoting and adhesion-restraining mechanisms contributes to the rapidly progressing, but highly regulated trophoblast invasion characteristic of normal human placentation. Examination of certain disease processes suggests that disruption of such a counterbalanced mechanism for normal turnover and remodeling in placenta and many other tissues could lead to serious consequences. For example, selective upregulation of adhesion mechanisms in the invasion arm of this kind of program could contribute to the invasive properties of tumor cells. Although the particular integrins altered in specific types of tumors may differ, the integrin phenotype of cells in invasive tumors is usually distinct from that of their normal or noninvasive counterparts. In a well-studied example, elevated expression of the αVβ3 integrin is positively correlated with the invasiveness of melanoma (Albelda et al., 1990; Felding-Habermann et al., 1992). Other studies have associated elevated expression of the α5β1 Fn receptor with a noninva-

sive or nonmetastatic phenotype (Giancotti and Ruoslahti, 1990). If the balanced expression of adhesion molecules is necessary for normal CTB invasion, then trophoblast tumors (choriocarcinomas) may selectively upregulate integrins that promote invasion (e.g., $\alpha1\beta1$) and/or downregulate those that restrain invasion (e.g., $\alpha5\beta1$). Unfortunately, long established cell lines of choriocarcinoma origin (Jar, BeWo) do not provide a suitable model for investigating this. Therefore, a test of this prediction awaits analysis of tissue samples and cells isolated from these tumors.

Conversely, failure to upregulate $\alpha1\beta1$, combined with maintenance of the adhesion mechanisms that restrain invasion, would be expected to reduce CTB invasion. This situation in fact occurred in studies of placental tissue taken from preeclamptic pregnancies. Preeclampsia is characterized by abnormally shallow invasion of CTBs into the uterine wall and the virtual absence of blood vessel invasion by CTBs. This results in reduced flow of maternal blood to the fetus, subsequent intrauterine growth retardation, and a myriad of maternal physiological changes. When integrin profiles displayed by CTBs in the basal plate (superficial region of the uterine wall) from gestational age-matched normal and preeclamptic placentas are compared (Zhou et al., 1993), it is clear that the CTBs from the preeclamptic pregnancies have not undergone the normal pattern of integrin switching. They continue to stain strongly for the $\alpha6\beta4$ integrin complex characteristic of undifferentiated normal CTBs, whereas staining for the $\alpha1\beta1$ integrin complex is barely detectable. Of particular interest is the observation that CTBs present in basal plate tissue from preeclamptic pregnancies do display the $\alpha5\beta1$ integrin that we hypothesize restrains invasion. Thus, a probable proximal (although not necessarily primary) defect in preeclampsia is that CTBs upregulate the invasion-restraining, but not the invasion-accelerating, arm of their normal differentiation program and therefore do not adopt an adhesion phenotype that is optimal for invasion to the appropriate depth.

Our present data, together with our immunocytochemical studies of normal and preeclamptic placental tissue, imply that the regulation of integrin expression and function plays a critical role in determining the invasive capacity of CTBs. However, appropriate regulation of other classes of molecules is also clearly essential to normal CTB differentiation and invasion. For example, we have shown that production of the 92×10^3 M_r metalloproteinase by isolated CTBs of different gestational ages parallels their invasive capacity (Fisher et al., 1989) and, like the $\alpha1\beta1$ integrin, is rate limiting for CTB invasion in vitro (Librach et al., 1991). We also postulate that cell-cell adhesion molecules are important in this regard. E-cadherin is expressed by all epithelia, and its function is required for the initial formation of the trophectoderm (Damsky et al., 1993; reviewed by Takeichi et al., 1991). Initial studies show that staining for E-cadherin in situ is strong on the polarized CTB stem cell monolayers and weak in differentiating and invading CTBs. Similarly, E-cadherin is not detected in vitro in isolated CTB monolayers at sites of active invasion (Fisher et al., 1989) or in CTB aggregates invading Matrigel (Fitzgerald, Fisher and Damsky, unpublished data). This is consistent with studies showing that downregulation of E-cadherin expression or function in tumor cells of epithelial origin is strongly correlated with tumor invasiveness (Behrens et al., 1993; Oka et al., 1993), and has led to the suggestion

that the cadherin-catenin cell adhesion system functions as a tumor suppressor (reviewed by Birchmeier et al., 1991).

Our observations that a normal pattern of integrin switching and correlated increased invasiveness of CTBs take place in vitro suggests that this differentiation program is largely under autocrine control, with the maternal environment playing primarily a modulatory role. This theory is corroborated by recent evidence that a similar stepwise pattern of integrin switching takes place during mouse trophoblast differentiation and outgrowth in vitro ($\alpha6\beta1$ is downregulated and $\alpha1\beta1$ is upregulated on migrating trophoblast; Sutherland et al., 1993). In this case the embryo is cultured from the two-cell stage onward, minimizing the potential for maternal influence. We are currently determining which growth factors and cytokines are produced by CTBs in a developmentally regulated manner, and are thus candidate effector molecules to regulate integrin expression during CTB differentiation.

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