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Regulation of trophoblast beta1-integrin expression by contact with endothelial cells

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Abstract

Background: In human and non-human primates, migratory trophoblasts penetrate the uterine epithelium, invade uterine matrix, and enter the uterine vasculature. Invasive trophoblasts show increased expression of β1 integrin. Since trophoblast migration within the uterine vasculature involves trophoblast attachment to endothelial cells lining the vessel walls, this raises the possibility that cell-cell contact and/or factors released by endothelial cells could regulate trophoblast integrin expression. To test this, we used an in vitro system consisting of early gestation macaque trophoblasts co-cultured on top of uterine microvascular endothelial cells.

Results: When cultured alone, trophoblasts expressed low levels of β1 integrin as determined by quantitative immunofluorescence microscopy. When trophoblasts were cultured on top of endothelial cells for 24 h, the expression of trophoblast β1 integrin was significantly increased as determined by image analysis. β1 Integrin expression was not increased when trophoblasts were cultured with endothelial cell-conditioned medium, suggesting that upregulation requires direct contact between trophoblasts and endothelial cells. To identify endothelial cell surface molecules responsible for induction of trophoblast integrin expression, trophoblasts were cultured in dishes coated with recombinant platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), or αVβ3 integrin. Trophoblast β1 integrin expression (assessed by immunofluorescence microscopy and Western blotting) was increased when PECAM-1 or αVβ3 integrin, but not ICAM-1, was used as substrate.

Conclusions: Direct contact between trophoblasts and endothelial cells increases the expression of trophoblast β1 integrin.

Background

As part of the implantation process and development of the placenta in human and non-human primates, migratory trophoblasts penetrate the uterine epithelium, invade the uterine matrix, and enter the uterine vasculature [1-7]. These invasive trophoblasts show increased expression of β1 and α1 integrins and down-regulation of β4 integrin when compared to non-invasive villous trophoblast cells

[8-11]. Integrins are heterodimeric transmembrane proteins that function in cell-matrix and cell-cell adhesion. Integrins also function in cell signaling. Our previous studies suggest a role for trophoblast β1 integrin in trophoblast adhesion to endothelial cells [12]. Beta 1 integrins, and integrins in general, are also known to be involved in cell migratory activity [13-17]. The factors responsible for regulating the acquisition of the migratory trophoblast phenotype, and for controlling integrin expression in these cells, are poorly understood. Trophoblast integrin expression is increased when trophoblast cells are cultured on fibronectin or in the presence of TGF-β [18,19] and we recently showed that β1 integrin expression by macaque trophoblasts was increased when the cells were exposed to physiological levels of shear stress [11].

Since trophoblast migration within the uterine vasculature involves trophoblast attachment to endothelial cells lining the vessel walls, this raises the possibility that cell-cell contact and/or factors released by endothelial cells could regulate trophoblast integrin expression. This idea is supported by the analogous upregulation of leukocyte integrins by contact with endothelium [20,21]. In the present paper we have tested the notion that trophoblast-endothelial cell contact regulates trophoblast integrin expression. The studies use an in vitro system that we have previously described [12], consisting of macaque trophoblasts co-cultured with human uterine microvascular endothelial cells. The results show that cell-cell contact causes an upregulation of trophoblast β1 integrin. Other data presented here suggest that increased expression of trophoblast β1 integrin is mediated by interaction of trophoblasts with endothelial cell platelet endothelial cell adhesion molecule-1 (PECAM-1) and αVβ3 integrin.

Results

Trophoblast β1 integrin is upregulated by contact with endothelial cells

When early gestation (40–60 days) macaque trophoblasts were cultured for 24 h on fibronectin-coated slides under serum-free conditions, the cells attached to the substrate and remained rounded. A few small colonies were also present. When stained for β1 integrin, these cells showed a diffuse, punctate fluorescence (Fig. 1A). When trophoblasts were added to cultures of endothelial cells and incubated for 24 h, the trophoblasts attached to underlying endothelial cells. Some of these adherent trophoblasts were rounded whereas others appeared to have flattened and spread. We have previously described the kinetics and morphological characteristics of trophoblast adhesion to endothelial cells [12]. When the cocultures were stained for β1 integrin (Fig. 1B), the trophoblast cells showed a diffuse, punctate fluorescence that was much brighter than trophoblasts cultured in the absence of endothelial cells. The much larger and flatter uterine endothelial cells stained weakly for β1 integrin but can be seen beneath the more brightly stained and smaller trophoblasts. To confirm that the brightly fluorescent β1 integrin-positive cells were trophoblasts, other cocultures were double-stained with the anti-β1 integrin antibody and an antibody against cytokeratin. Trophoblasts are cytokeratin-positive whereas endothelial cells are negative for this intermediate filament protein. The double staining pattern showed that the brightly stained β1 integrin-positive cells (Fig. 1C) also co-stained for cytokeratin (Fig. 1D). To confirm that the changes in integrin expression were the result of direct contact between trophoblasts and endothelial cells and not due to soluble factors released by endothelial cells, trophoblasts were incubated with endothelial cell-conditioned medium for 24 h. When stained for β1 integrin (Fig. 1E), the fluorescence intensity was similar to that of trophoblasts cultured in the absence of conditioned medium (or the absence of endothelial cells). Figure 1F shows a culture stained with control matched mouse immunoglobulin and only a dull autofluorescence can be seen. As another control, trophoblasts were also co-cultured with REN mesothelioma cells and then stained for β1 integrin and cytokeratin. The results (Fig. 1G and 1H) show that, compared to trophoblasts cultured with endothelial cells (Fig. 1B), there was no increase in β1 integrin-associated fluorescence.

To confirm the visual impression that β1 integrin-associated fluorescence was increased when trophoblasts were cocultured with endothelial cells, multiple immunofluorescence images from three separate experiments were subjected to quantitative image analysis. The results of this analysis are shown in Fig. 2 where it can be seen that β1 integrin-associated fluorescence was significantly increased in trophoblasts cocultured with endothelial cells compared to trophoblasts cultured alone. Based on these analyses, 80% of the trophoblasts cocultured with endothelial cells showed an increase in β1 integrin-associated fluorescence that was at least two-fold greater than the mean value for trophoblasts cultured alone. No quantitative increase in fluorescence was seen for trophoblasts cultured in the presence of endothelial cell-conditioned medium.

Trophoblast β1 integrin upregulation is time- and temperature-dependent

Some additional control experiments were also performed to rule out the possibility that the putative upregulation of β1 integrin was not simply an artifact due to the selective attachment to endothelial cells of a population of strongly β1 integrin-expressing trophoblasts. When trophoblasts were incubated with endothelial cells for only 2 h, β1 integrin fluorescence was not increased and levels appeared similar to trophoblasts cultured alone (Fig 3A,

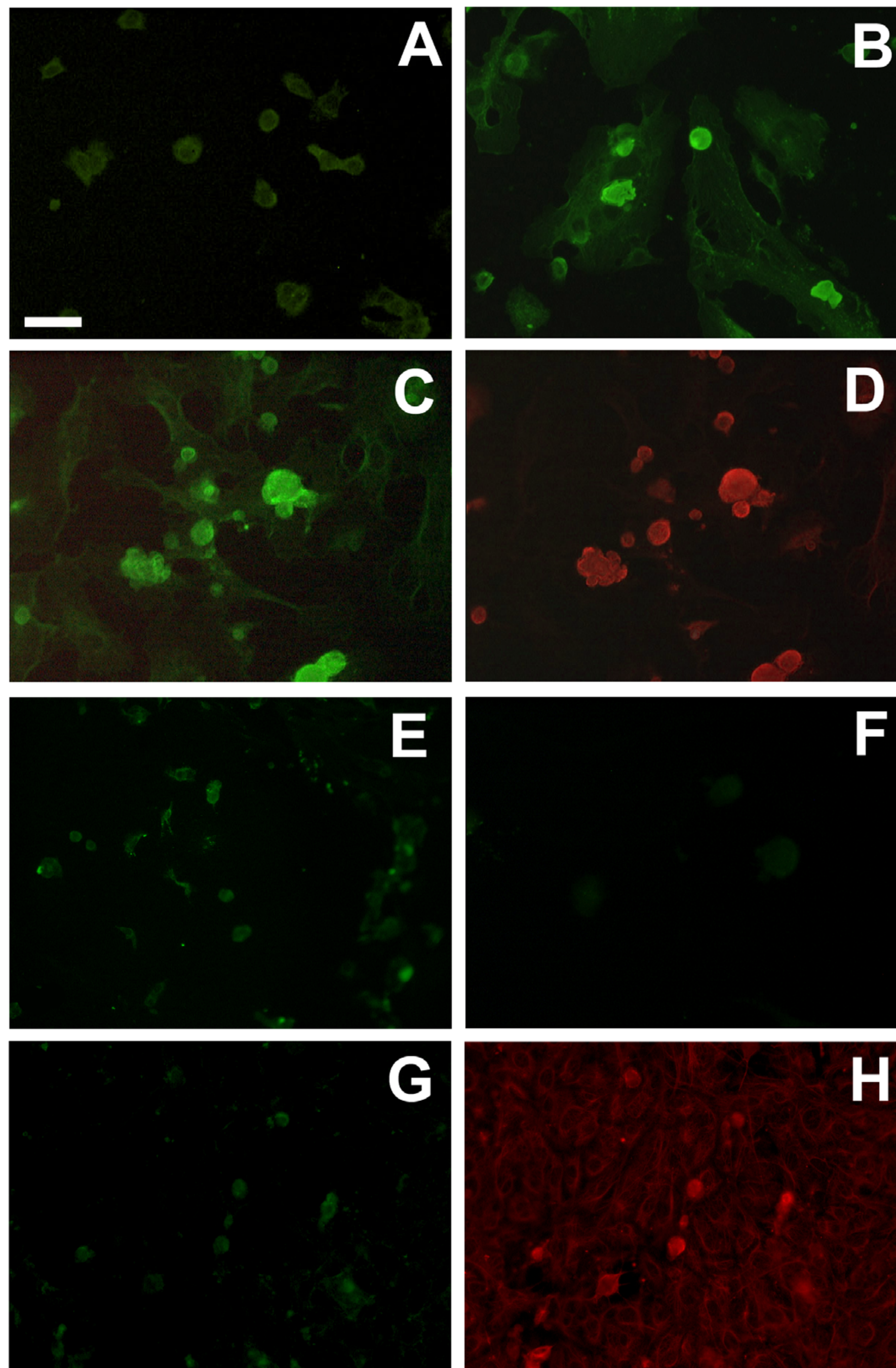

Figure 1

Expression of β1 integrin by trophoblasts cultured with uterine endothelial cells. Trophoblasts were added to confluent cultures of endothelial cells and cocultured for 24 h as described in Methods. The cultures were fixed in methanol and stained using antibodies against β1 integrin or cytokeratin. (A) Trophoblasts cultured alone and stained for β1 integrin. (B) Trophoblasts cultured on top of endothelial cells for 24 h then stained for β1 integrin. (C) Trophoblasts cultured on top of endothelial cells then fixed in methanol and stained for β1 integrin. (D) Same field as in C but viewed to show cytokeratin (red) staining. (E) Trophoblasts cultured alone in endothelial cell-conditioned medium then stained for β1 integrin. (F) Control culture incubated with isotype-matched mouse immunoglobulin. (G) Trophoblasts cocultured with REN cells and stained for β1 integrin. (H) Same field as G but viewed to show cytokeratin staining. These experiments were performed four times and representative images are shown. The horizontal bar represents 20 μm.

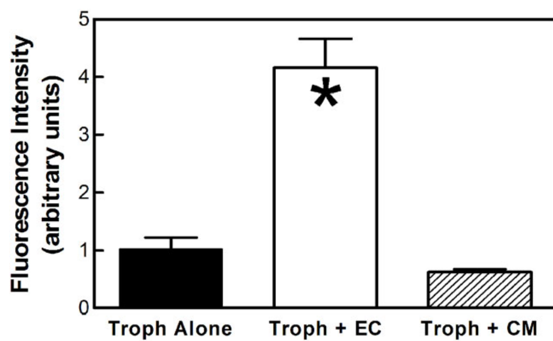

Figure 2
Quantitative image analysis of β1 integrin immunofluorescence. Immunofluorescence images of trophoblast/endothelial cell cocultures or trophoblasts cultured alone in the presence of endothelial cell-conditioned medium (CM) (see Fig. 1) were analyzed for fluorescence intensity as described in Methods. The results are mean values +/- SEM from three separate experiments. The asterisks indicate values that are significantly different ($p < 0.05$) from the control (trophoblasts incubated alone).

compare with cells cultured for 24 h in Fig. 1A). Figure 3B shows the same field as in 3A but viewed to show cytokeratin staining. At this early time point, trophoblasts were rounded and showed little or no evidence of spreading. We have already shown that trophoblast adhesion to endothelial cells is complete by 2 h [12]. When trophoblasts were incubated with endothelial cells for 24 h at 4°C, it was more difficult to find adherent trophoblasts. Those that could be identified (examples are shown in Fig. 3C and 3D) were rounded and showed the same level of β1 integrin fluorescence (Fig. 3C) as trophoblasts incubated alone at 37°C (compare with Fig. 1A). These data confirm that we are not selecting for a population of strongly β1 integrin-expressing trophoblasts and that the increase in immunofluorescence staining after co-culture is time-dependent and a consequence of coculture at 37°C.

Effect of solid-phase recombinant adhesion molecules on trophoblast β1 integrin expression

To identify endothelial cell surface molecules that might be triggering the upregulation of trophoblast β1 integrin, studies were carried out in which trophoblasts were cultured in dishes that had been coated with recombinant forms of adhesion molecules known to be expressed by endothelial cells. Compared to control trophoblasts cultured on bovine serum albumin (BSA)-coated dishes (Fig. 4A), trophoblasts cultured for 24 h on recombinant PECAM-1 (Fig. 4B) showed increased β1 integrin-associated fluorescence. When trophoblasts were cultured on recombinant intercellular adhesion molecule-1 (ICAM-1) (Fig. 4C) the overall fluorescence intensity appeared similar to trophoblasts cultured on BSA. Increasing the ICAM-1 coating concentration (as much as 4-fold) did not change the results (not shown). Trophoblasts cultured in dishes coated with recombinant αVβ3 integrin (Fig. 4D), showed increased β1 integrin-associated fluorescence compared to the control.

Quantitative analysis of multiple images from the above study confirmed that the fluorescence intensity was significantly increased for trophoblasts cultured with PECAM-1 or αVβ3 integrin (Fig. 5), but not with ICAM-1. Trophoblasts incubated in dishes coated with a mixture of PECAM-1 and αVβ3 integrin showed significantly increased fluorescence compared to cells cultured on BSA. While the increase was greater than that seen in cells cultured with either substrate alone, the increase was not strictly additive. Increasing the concentration of PECAM-1 or αVβ3 integrin (up to 40 μg/ml) did not result in any further increase in β1 integrin immunofluorescence (results not shown).

β1 Integrin expression by trophoblasts that had been cultured on BSA, recombinant PECAM, or αVβ3 was also analyzed by Western blotting. The anti-β1 integrin antibody used for detection revealed two bands at 116 and 109 kD, respectively (Fig. 6A). Examination of blots from several experiments by densitometry using tubulin as an internal loading control showed that the intensity of the β1 integrin bands was significantly higher for cells cultured on PECAM-1 or αVβ3 integrin compared to the BSA control (Fig. 6B). There was no significant difference in β1 integrin band intensity between cells plated on PECAM-1 or αVβ3 integrin. Since the intensity of the two integrin bands changed in parallel, the values for β1 integrin intensity in Fig. 6B represent the sum of both band intensities. Cells cultured on recombinant ICAM-1 showed no significant increase in β1 integrin band intensity. Blots incubated with control mouse immunoglobulin showed no protein bands.

Discussion

The results presented here support the idea that the expression of trophoblast β1 integrin is upregulated by direct contact with endothelial cells. We found no evidence that integrin expression was regulated by soluble factor(s) released by endothelial cells or that we were selecting for a population of strongly β1 integrin-expressing trophoblasts. It is well known that the expression of β1 integrin by human and macaque trophoblasts is increased as trophoblasts acquire a migratory phenotype and enter the invasive pathway [8-11]. Invasive trophoblasts migrate within the maternal uterine stroma, although this occurs to a greater extent in the human than

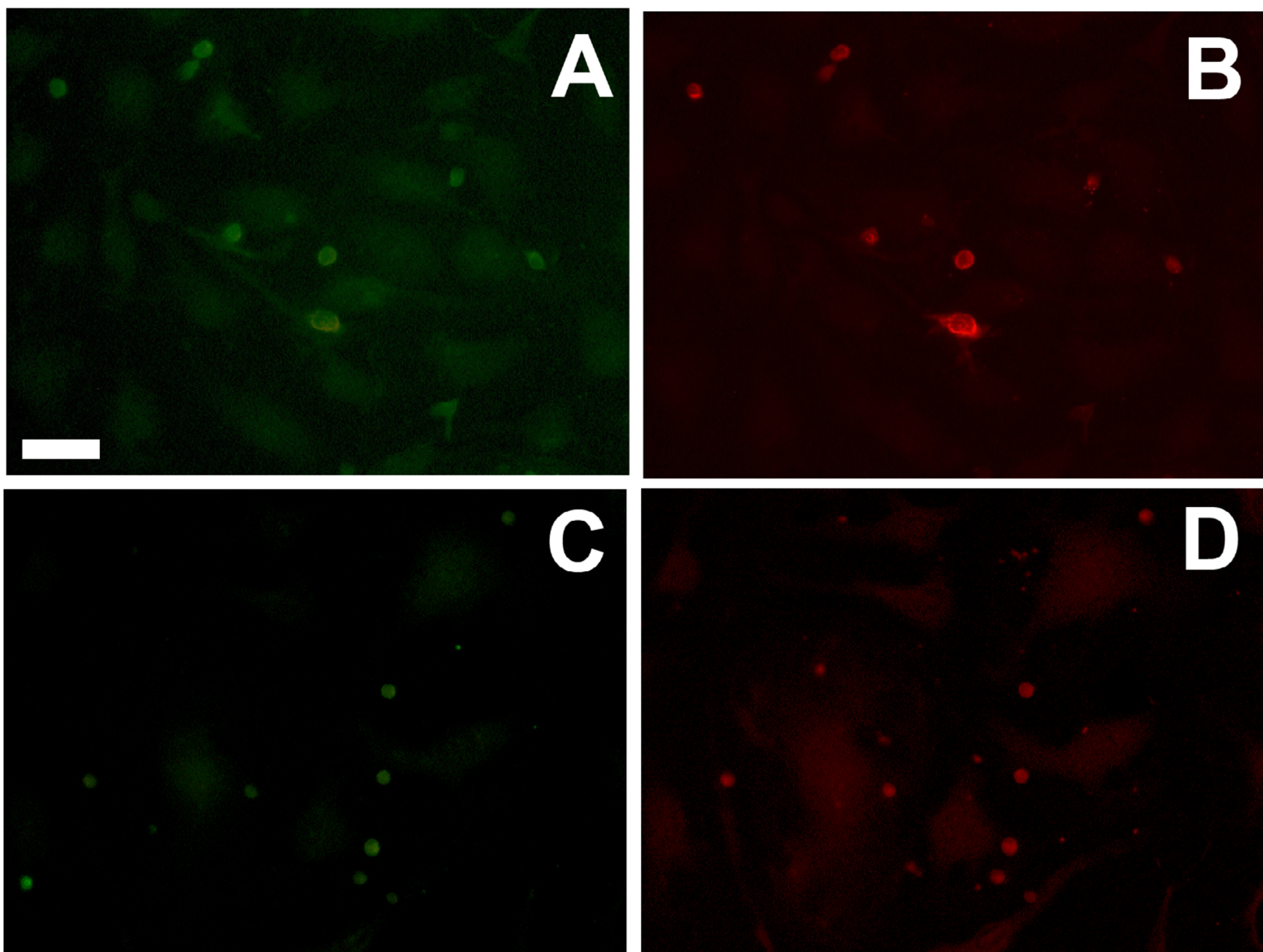

Figure 3
Effect of temperature and incubation time on trophoblast β1 integrin expression. Trophoblasts were cocultured with endothelial cells at 37°C for 2 h (A and B) or at 4°C for 24 h (C and D). Staining for β1 integrin is shown in (A) and (C). Respective identical fields viewed to show cytokeratin staining (red) are shown in (B) and (D). The bar represents 20 μm.

in the macaque [22]. In both species, trophoblasts enter superficial venule-like, non-arteriolar vessels [1,23,24] and then attach to, and eventually remodel, uterine blood vessel walls. Endovascular trophoblasts express high levels of β1 integrin and α1 integrin compared to villous cytotrophoblasts but show reduced levels of β4 integrin. The in vitro results described here suggest that attachment of trophoblasts to the endothelial surface could contribute to the upregulation of β1 integrin expression seen in vivo. Examination of sections of early gestation human and macaque implantation sites indicates that increased expression of trophoblast β1 integrin begins before the cells enter the vasculature [8-11]. Thus, factors in addition to attachment to endothelial cells are involved in regulating trophoblast integrin expression.

Previous studies have shown that β1 integrin expression by cultured trophoblasts can be increased by extracellular matrix components and by TGF-β [19]. We have also recently shown that trophoblast β1 integrin expression can be increased by fluid flow-derived shear stress [11]. Integrins are involved in cell-cell and cell-extracellular matrix attachment and facilitate cell migration [25-30] and so increased trophoblast β1 integrin expression is likely related to trophoblast adhesion and motility. It is not unreasonable to speculate that the ability of trophoblasts to withstand, and indeed to migrate against, the flow

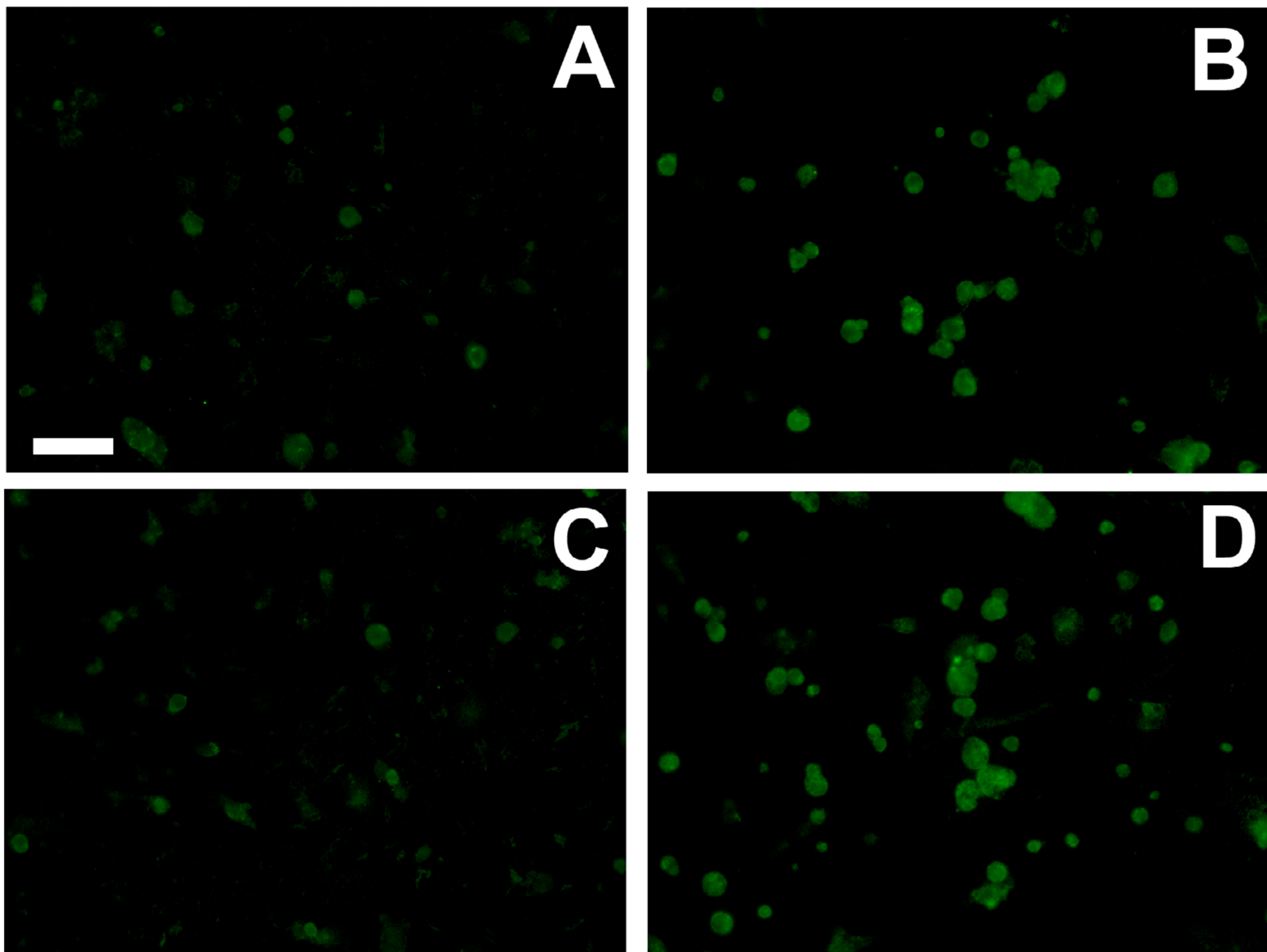

Figure 4
Effect of recombinant adhesion molecules on trophoblast β1 integrin expression. Trophoblasts were cultured for 24 h in dishes precoated with BSA (A) or recombinant forms of (B) PECAM-1, (C) ICAM-1, or (D) αVβ3 integrin. The cells were fixed in methanol and then stained for β1 integrin as described in Methods. Representative images from 3 separate experiments are shown. The bar represents 20 μm.

of blood requires a sufficiently high level of integrin expression. Factors that regulate trophoblast integrin expression within the uterine stroma may not be present within the vasculature and so the combined effects of endothelial contact and shear stress would ensure that high levels of integrin expression are maintained in the vascular environment. Since integrins are also involved in signal transduction [31-34], it is possible that increased integrin expression facilitates signaling events that are important for invasive trophoblast survival and function.

The identity of the endothelial cell surface component(s) that are responsible for the induction of trophoblast integrin expression is obviously an important question. While other as yet unidentified molecules could be involved, the studies presented here using recombinant proteins indicate that PECAM-1 and αVβ3 integrin, both of which are major endothelial cell surface molecules, play a role in regulating trophoblast integrin expression. The Western blot data suggest that trophoblast β1 integrin protein amount is increased by contact of trophoblasts with these molecules. Increased protein amount most likely reflects increased protein synthesis but could also reflect decreased degradation. We found no evidence that ICAM-1, another endothelial cell adhesion molecule, is involved in regulating trophoblast integrin expression. PECAM-1 is a member of the immunoglobulin superfamily of adhesion molecules and is expressed by

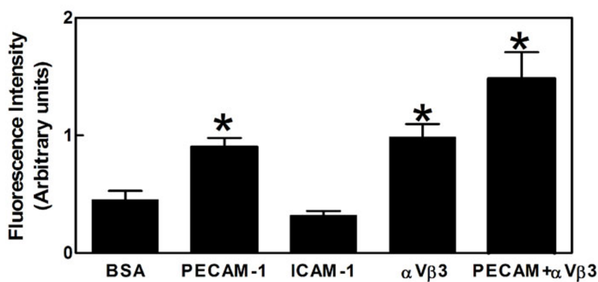

Figure 5
Quantitative image analysis of β1 integrin immunofluorescence in trophoblasts cultured on recombinant proteins. β1 integrin immunofluorescence images from the experiments described in Fig 4 were subjected to intensity analysis (see Methods). The results are mean values +/- SEM from three separate experiments. The asterisks indicate values that are significantly different ($p < 0.05$) from the BSA control.

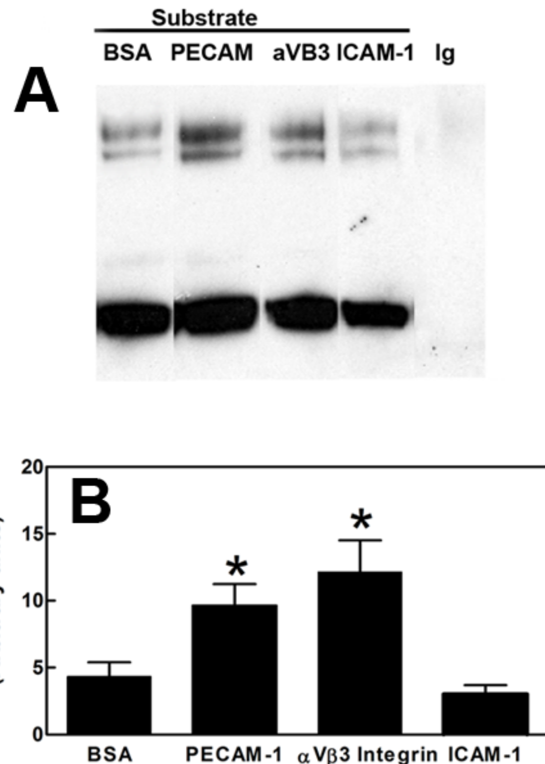

Figure 6
Western blot analysis of β1 integrin expression in trophoblasts cultured with recombinant adhesion molecules. Trophoblasts were cultured for 24 h in dishes precoated with BSA or recombinant adhesion molecules as described in Methods. The cells were then subjected to Western blot analysis using an antibody against β1 integrin. The results of a typical chemiluminescence detection assay are shown in (A). The graph in (B) shows the results of densitometric analyses of the integrin bands from three experiments. The asterisk indicates a value that is significantly different ($p < 0.05$) from the BSA control culture. While values for PECAM-1 and αVβ3 were significantly different from the control they were not significantly different from each other. The lane labeled Ig shows the result of incubating the blot with control mouse immunoglobulin instead of anti-β1 integrin antibody.

endothelial cells, platelets, and leukocytes. PECAM-1 is believed to play roles in leukocyte extravasation, angiogenesis, cell migration, and cell signaling [35-40]. PECAM is capable of homophilic (PECAM-PECAM) binding as well as heterophilic binding to other molecules such as αVβ3 or CD38. Homophilic interaction between PECAM expressed by neutrophils and endothelial cells is reported to cause upregulation of neutrophil integrin expression [21]. Antibody cross-linking of PECAM also results in activation of several integrins [41]. Homophilic PECAM interactions could also be responsible for the increased expression of β1 integrin seen in the present coculture study since migratory trophoblasts in both the macaque and the human express PECAM-1 [42-44].

αVβ3 integrin is a cell adhesion molecule expressed by several cell types including endothelial cells. αVβ3 integrin binds to vitronectin, fibronectin, osteopontin, and PECAM-1 [45-49]. Upregulation of trophoblast β1 integrin could therefore be the result of interaction between endothelial cell αVβ3 and trophoblast PECAM-1. It could also be the result of endothelial αVβ3 interaction with another as yet unidentified ligand on the trophoblast surface. Clearly, the identity of the signaling pathways responsible for the cell-mediated regulation of integrin expression in trophoblasts warrants further attention.

The Western blot data indicate that trophoblast β1 integrin exists in two forms, distinguishable by slight differences in molecular mass. Studies using various cancer and normal cell lines have demonstrated two different molecular mass forms of β1 integrin [50-54] that appear to be the result of differences in glycosylation. While we have not confirmed that the different β1 integrin forms found in macaque trophoblasts result from differences in glycosylation, the molecular masses correspond to those reported for other cell types. Furthermore, Moss et al [55] showed that differences in electropheretic mobility of β1 integrin from early and term human cyotrophoblasts resulted from differences in glycosylation. The function of

these different β1 integrin isoforms in trophoblasts remains to be elucidated.

Conclusions

The results presented here support the idea that the expression of trophoblast β1 integrin is upregulated by direct contact with endothelial cells. Upregulation was time- and temperature- dependent and no evidence was found to suggest that integrin expression was regulated by soluble factor(s) released by endothelial cells. While additional molecules could be involved, the studies using recombinant proteins indicate that interaction of trophoblast cells with PECAM-1 and/or αVβ3 integrin, both of which are major endothelial cell surface molecules, could play an important role in regulating endovascular trophoblast β1 integrin expression.

It is not unreasonable to speculate that the ability of trophoblasts to withstand, and indeed to migrate against, the flow of blood requires a sufficiently high level of integrin expression. The effects of endothelial contact (as demonstrated here) combined with shear stress would ensure that high levels of trophoblast integrin expression are maintained in the vascular environment. The in vitro data presented here may provide an explanation for the increased expression of β1 integrin that is observed for endovascular trophoblasts in both the human and the macaque. Since integrins are also involved in signal transduction [31-34] it is possible that increased integrin expression facilitates signaling events that are important for invasive trophoblast survival and function.

Methods

Trophoblast isolation and culture

We have previously described in detail a procedure used to isolate trophoblast cells from term (165-day) macaque placentas [56]. The same procedure was used in the present case to isolate cells from 40–60 day placental/endometrial tissue. Yields were approximately 3×10^6 cells/g tissue ($20–30 \times 10^6$ cells per placenta). The cells were subjected to an additional purification step using immunomagnetic microspheres coated with anti-HLA antibodies [57]. This step removes contaminating HLA-positive cells leaving pure (i.e., 100% cytokeratin-positive, HLA-ABC/DR-negative, vimentin-negative) trophoblast cells. FACS analysis of this purified trophoblast population revealed that 75% of the cells were β1 integrin-positive [11].

Endothelial cells and co-culture conditions

Human uterine myometrial endothelial cells (UtMVEC, passage 4) were purchased from Clonetics Corporation (San Diego, CA) and maintained in endothelial basal medium-2 (Clonetics) supplemented with human recombinant epidermal growth factor, human fibroblast growth factor, vascular endothelial growth factor, ascorbic acid (Vitamin C), hydrocortisone, human recombinant insulin-like growth factor, heparin, gentamicin, amphotercin, and 5% fetal bovine serum. Cells were plated into 8-chamber LabTek slides that had been coated with fibronectin (Becton Dickinson, Bedford, MA). The chambers were incubated at 37°C in humidified 95% air and 5% CO_2 for 12–24 hours to allow formation of a near confluent UtMVEC layer. Prior to the addition of trophoblasts, the endothelial cells were incubated for 12 h under serum-free conditions. Trophoblasts were then added and the cocultures were incubated for 24 h. The cocultures were then analyzed by immunocytochemistry as described below. As a control, other trophoblasts were cultured on slides coated with fibronectin and without endothelial cells.

Mesothelioma cells

REN mesothelioma cells were provided by S. Albelda (University of Pennsylvania, Philadelphia, PA)

Incubation of trophoblasts with recombinant proteins

A recombinant form of intercellular adhesion molecule-1 (ICAM-1) and a recombinant form of the extracellular domain of human PECAM-1 were obtained from R&D Systems Inc. Minneapolis, MN, and maintained as aqueous stock solutions in PBS. Recombinant αVβ3 integrin was obtained from Chemicon as a stock solution in octylglucoside. The surfaces of LabTek culture chambers were coated with recombinant proteins (10 μg/ml) for 1 h at 37°C. Coating with higher concentrations (up to 40 μg/ml) did not alter the results obtained and so 10 μg/ml was routinely used. The solution was then removed and the chambers were allowed to air dry. The coated chambers were then blocked using bovine serum albumin (BSA; 10 mg/ml) for 1 h at 37°C. Trophoblasts (350,000 cells per cm^2) were added to the precoated chambers in Ham's/Waymouth's medium containing BSA (10 mg/ml) and incubated for 24 h. Controls consisted of chambers coated only with BSA. Other control experiments (not shown) confirmed that octylglucoside (carrier for recombinant αVβ3) did not affect trophoblast integrin expression.

Immunocytochemistry and image analysis

Monoclonal antibodies against β1 integrin (clone P4G11) were purchased from Chemicon, Temecula, CA. A polyclonal antibody against cytokeratin (pan) (cat #18-0059) was purchased from Zymed, San Francisco, CA. Oregon Green-labeled goat anti-mouse Ig antibody and TRITC-labeled goat anti-rabbit Ig antibody were purchased from Molecular Probes, Eugene, OR.

Cells in LabTek culture chambers were fixed and permeabilized in ice-cold methanol or fixed in 2% paraformaldehyde (without permeabilization) then stained with

primary antibody. Primary antibodies were detected using Oregon Green-labeled or TRITC-labeled goat anti-mouse or goat anti-rabbit Ig. Antibody controls in which cells were incubated with isotype-matched mouse Ig or non-immune rabbit Ig were also included. The stained cells were examined using a Nikon Eclipse E800 epifluorescence microscope. Multiple images from random fields were captured using an Optronics DEI750 CCD camera and Adobe Photoshop software. Identical exposure and brightness level settings were used for test and control samples. Captured digitized images were imported into Image Pro Plus software to determine cellular levels of anti-integrin antibody-associated fluorescence. The software was calibrated using the InSpeck fluorescence Image Intensity Calibration Kit (6 :m beads; Molecular Probes, Eugene OR). Relative cellular fluorescence intensity was determined by reference to a standard curve generated using the calibration beads and is expressed as mean density normalized by area. Background fluorescence (calculated using cells treated with control mouse immunoglobulin instead of the anti-integrin antibody) was subtracted from experimental values. At least 4 random microscope fields were analyzed for each sample well and experiments were repeated at least 3 times.

Western blotting
Cultures were washed with Dulbecco's Modified PBS containing Ca^{2+} and Mg^{2+}. The cells were then lysed on ice by the addition of M-PER Mammalian Protein Extraction Reagent (Pierce) supplemented with 1% Protease Inhibitor Cocktail (Sigma). The lysate was homogenized by repeated passage through a 27 gauge needle, then mixed with an equal volume of Laemmli sample buffer (BioRad) containing 5% β-mercaptoethanol and heated in a boiling water bath for 5 minutes. The samples were immediately chilled on ice and loaded on to an 8 % SDS-polyacrylamide gel (Gradiopore) at 20 μg per lane. Electrophoresis was performed at 200 V for 45 minutes after which proteins were transferred to PVDF membrane (BioRad) at 100 V on ice for 1 hour. The membrane was blocked for 1 hour in 1% non-fat dried milk (NFDM) followed by overnight incubation with a 1/1000 dilution of mouse anti-β1 integrin antibody (clone JB1A; Chemicon) and 1/2000 dilution of mouse monoclonal antibody cocktail against tubulin (clones DM1A, DMA18, migG1; RDI). Tubulin was used as an internal loading control. The membrane was washed 6X in TBS containing 1%Tween-20 after which it was incubated with goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (BioRad) diluted 1/50,000 in 1% NFDM for 1 h at room temperature. After washing, the membrane was incubated with chemiluminescent substrate (SuperSignal West Dura; Pierce) for 5 min at room temperature. The membrane was then exposed to X-ray film (Pierce). Scanned images of exposed X-ray film were analyzed using Kodak 1D gel analysis software. Band densities were obtained and corrected for background. Densities of bands of interest were expressed relative to the intensity of the loading control (tubulin).

Statistical analyses
Experiments were repeated at least 3 times using cells from different placentas in each case. Cells from different placentas were not pooled. Statistical analyses were performed by ANOVA followed by Tukey-Kramer multiple comparison post-test using the Prism software program (GraphPad Inc., San Diego, CA). Differences in means were considered significant if $p < 0.05$.

Competing interests
None declared.

Authors' contributions
TLT isolated primary cultures, carried out cell adhesion assays, and Western blotting. SH carried out adhesion assays and immunostaining. AS carried out immunostaining and optimized endothelial cell culture conditions. NM carried out immunostaining and Western blotting. AIB participated in experimental design and analysis, and manuscript preparation. GCD was responsible for the overall study concept and experimental design, immunofluorescence microscopy, Western blot analyses, and statistical analyses.

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