Effect of shear stress on migration and integrin expression in macaque trophoblast cells

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Abstract

During fetal development, trophoblast cells enter endometrial capillaries, migrate within the uterine vasculature, and eventually reside within spiral arteries of the uterus. This invasive activity is accompanied by upregulation of trophoblast β1 integrin expression. Fluid mechanical shear stress regulates migration and expression of adhesion molecules in vascular endothelial cells, but nothing is known about the effects of shear stress on trophoblast cells. We tested the hypothesis that shear stress regulates the motility and β1 integrin expression of trophoblast cells. Early gestation macaque trophoblast cells were cultured in 1 x 1-mm square cross-section capillary tubes within which the flow field was determined using three-dimensional computational fluid dynamic simulations. Trophoblast cells in the capillary tubes were exposed to a steady shear stress of 7.5, 15, or 30 dyn/cm² for up to 24 h. In the absence of flow, trophoblast cells were highly dynamic with constant nondirectional positional shifts but with no net cell migration. Exposure of the cells to shear stress within 24–72 h of cell plating significantly increased the level of this activity and led to net cell migration in the direction of flow. Shear stress also increased the expression and altered the topography of β1 integrin. These results suggest that shear stress regulates trophoblast motility and β1 integrin expression in vitro. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

During fetal development in higher primates, maternal blood is brought into direct contact with embryonic trophoblast tissue. This condition is established early in pregnancy when a specialized subset of trophoblast cells breach the uterine epithelium, migrate through endometrial connective tissue and become apposed to dilated endometrial capillaries. These invasive trophoblast cells then enter, and become locally established within, the lumens of maternal vessels. As a result of trophoblast intravasation, continuity is established between the uterine vasculature and the trophoblast-lined spaces that form the placenta, through which maternal blood circulates [1,2].

Morphological studies demonstrate that intravascular trophoblast cells adhere to the luminal surfaces of endothelial cells. These trophoblast cells are able to attach to endothelium with sufficient strength to resist displacement by blood flow, which suggests the involvement of intercellular adhesion molecules. Subsequently, through mechanisms not yet understood, trophoblast cells migrate extensively within small endometrial vessels and eventually reside within the spiral arteries of the uterus [2–7]. Some of these intravascular trophoblast cells eventually extravasate by migrating between arterial endothelial cells [2,8,9]. This process involves displacement of maternal endothelial cells and loss of their basal lamina followed by trophoblast cell migration into the tunica media, disruption of the smooth muscle, and synthesis of large amounts of extracellular matrix. Ultimately, trophoblast cells form the luminal surface of the invaded regions of affected maternal vessels. The extensive remodeling of the uterine vasculature that occurs as a result of this trophoblast activity is important in assuring an adequate blood supply to the developing fetal-placental unit [7–10]. Inadequate invasion of the spiral arteries is a characteristic of pregnancies complicated by hypertension and fetal growth retardation and has been implicated as a cause of miscarriages [11–20]. The factors that regulate the
invasive activity of intravascular trophoblast cells remain poorly understood.

Analyses of tissue sections and studies using isolated trophoblast cells suggest that trophoblast invasion is accompanied by changes in the expression of proteases, cadherins, and various integrins [21–24]. Increased expression of β1 integrin occurs as trophoblast cells enter the invasive pathway [22,23]. In vitro assays using human cytotrophoblast cells suggest invasion is regulated by EGF, TNF, and TGF-β [25–27]. Integrin expression by first trimester trophoblast cells can be altered by the composition of the extracellular matrix and by hypoxia [28].

Taking advantage of numerous similarities in placentalization between macaque monkeys and humans, we have previously used the macaque as a model system to study morphological aspects of trophoblast invasion [2,8,9,29]. To extend the usefulness of this model, we recently demonstrated for the first time that human and macaque trophoblast cells adhere to endothelial cells in vitro and that this attachment is partially mediated by αV and β1 integrins [30,31]. Our studies have also demonstrated that macaque trophoblast cells cultured in migration chambers preferentially migrate towards vitronectin (or serum) [31].

One important potential regulator of trophoblast function within blood vessels that has not been examined is fluid mechanical shear stress. In endothelial cells, shear stress can initiate signaling pathways that result in altered gene expression and/or alterations in the surface distribution of adhesion molecules [32–38]. In the present paper, we hypothesized that flow-derived shear stress regulates the motility of macaque trophoblast cells and alters integrin expression. We have tested this hypothesis by examining the effect of controlled levels of steady shear stress on the migration of cultured trophoblast cells and the expression of β1 integrin in these cells. The results show that trophoblast cells cultured on extracellular matrix proteins migrate generally in the direction of fluid flow, that the extent of this migration is dependent on the magnitude of imposed shear stress, and that shear stress alters the distribution of trophoblast β1 integrin.

2. Materials and methods

2.1. Materials

A monoclonal antibody against β1 integrin (clone P4G11) and isotype-matched control mouse immunoglobulin were purchased from Chemicon (Temecula, CA). Oregon Green-labeled goat anti-mouse antibodies were purchased from Molecular Probes (Eugene, OR).

2.2. Trophoblast isolation

We have previously described in detail a procedure used to isolate trophoblast cells from term (165-day) macaque placentas[39]. The same procedure was used in the present case to isolate cells from 40–100-day placental villous tissue. Cell yields were approximately 3 × 10⁶ cells/g tissue (20–30 × 10⁶ cells per placenta). When the cells were cultured for 24 h on LabTek slides and then stained, at least 98% were positive for cytokeratin and negative for HLA-ABC/DR, factor VIII, and vimentin, consistent with trophoblast. The remaining cells were vimentin-positive and HLA-positive. The cells were subjected to an additional purification step using immunomagnetic microspheres coated with anti-HLA antibodies[40]. As we reported for human trophoblast cells [40], 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 (Fig. 1).

2.3. Trophoblast cell exposure to shear stress

Trophoblast cells were gently pipetted into 5 cm-long, 1 × 1-mm square cross-section borosilicate glass capillary tubes (VitroCom, Mountain Lakes, NJ) that had been coated with Type I rat tail collagen (Becton Dickinson, Bedford,
MA). Although the cell density was adjusted to minimize cell–cell interactions while still allowing a sufficient number of cells per field for analysis, trophoblast cells have a tendency to aggregate and the formation of some small colonies was unavoidable. The cells were allowed to attach to the inner walls of the tube for 5 h, after which the medium was replaced. All experiments were performed using a mixture of Ham’s F12 and Waymouth’s medium (50:50 vol/vol) containing 10% fetal bovine serum.

Cultured trophoblast cells were exposed to steady fluid shear stress using protocols that have been previously described [41–43]. Briefly, the capillary tube containing the cells was inserted into a recirculating flow loop. Cell culture medium, gently gassed with CO₂, was drawn from a reservoir using a peristaltic flow pump and passed through two smaller buffer reservoirs inserted between the pump and the capillary tube to dampen pulsatility. Flow out of the capillary tube was recirculated back into the feed reservoir. The temperature of the cell culture medium throughout the experiment was maintained at 37 °C by placing all reservoirs in a temperature-controlled water bath. Under static conditions, trophoblast viability as determined by Trypan blue staining was 84%. After exposure to shear stresses of 15 or 30 dyn/cm² for 24 h, viability was 96% and 90%, respectively.

2.4. Characterization of flow in capillary tubes

Capillary tubes were used for shear stress experiments rather than a parallel plate device because the former allowed us to use fewer cells. As previously described [41–43], the square cross-section capillary tubes used in the present experiments are a very practical system for studying the impact of fluid mechanical forces on various aspects of cell structure and function. However, flow within these capillary tubes cannot be approximated as that between infinitely wide parallel plates and must be characterized in detail for establishing the shear stress to which the cultured cells in these tubes are exposed. Indeed, the magnitude of this shear stress is maximum at the center of the face on which the cells are cultured and decreases progressively to zero at the tube side walls. An analytically derived expression for this shear stress variation along the width of the capillary tube has previously been given in the form of an infinite series [43].

Another potential concern with the square cross-section tubes is the possibility of the presence of significant secondary flow motion within the tubes due to streamline curvature. If significant secondary flows are present, then they might be expected to influence the shear stress levels to which the cells are exposed. In order to more comprehensively characterize the flow field within the square cross-section capillary tubes, we have performed three-dimensional computational fluid dynamic simulations of the steady flow field using the commercially available computer program FLUENT (Fluent, Lebanon, NH). FLUENT is a finite volume code capable of efficiently solving the governing three-dimensional mass and momentum conservation (Navier–Stokes) equations [44]. In FLUENT, the computational domain is subdivided into polyhedra within which the governing equations, cast in control-volume form, are solved. The code is capable of handling multidimensional, unstructured meshes, and the solution yields the complete three-dimensional velocity distribution within the tube as well as derived quantities such as the wall shear stress. The results of these simulations were used to guide the specification of the flow rate within the flow loop in order to generate the desired values of shear stress. A flow rate of 6, 12, or 24 ml/min was used in the present study. According to both the FLUENT computations performed here and the analytical expression given by Weisner et al. [43], these flow rates generated a steady shear stress at the center of the tube (i.e. a maximum wall shear stress) of 7.5, 15, or 30 dyn/cm², respectively.

2.5. Analysis of flow-induced trophoblast cell motility

Trophoblast cells were divided into two groups: “early culture” cells which were exposed to shear stress within 24–72 h of initial plating and “late culture” cells which were cultured in the capillary tubes for periods exceeding 72 h prior to flow exposure. Trophoblast motility was examined separately in each of the two groups. In each case, the capillary tube was positioned on the stage of an inverted phase microscope (Nikon TE300, Tokyo, Japan). When a suitable field of view was identified, flow was initiated to subject the cells to the desired shear stress level. Trophoblast cell images within the selected field of view were captured every hour for 24 h using a digital CCD camera (SenSys, Photometrics, Tuscon, AZ) interfaced with a computer. Similar recordings were performed on no-flow control cells.

Following the 24-h recording period, individual trophoblast cell contours were traced using an electronic graphics tablet and pen (artZII, Wacom, Vancouver, WA), and the data were quantitatively analyzed using image analysis software (Scion Image version beta 3, http://www.scion-corp.com). For each cell, the image analysis involved quantitation of cell area and perimeter as well as x- and y-coordinates of the center of gravity (cog) at the end of each hour during the 24-h recording period. Average cell migration velocities were computed as the total distance traversed by the cell (based on the changes in the x- and y-coordinates of the cog) during the 24-h period divided by 24. Net cell displacement in the direction of flow (x-direction) during the 24-h recording period was determined from the difference between the x-coordinates of the cell cog at the zero and 24-h time points.

2.6. Immunocytochemistry and FACS analysis

Trophoblast cells cultured in the glass capillary tubes were exposed in the flow loop to a shear stress of 15 dyn/
cm² for 30 min, 6, and 24 h. Following cessation of flow, the cells were immediately fixed in either ice-cold methanol or 3% paraformaldehyde and stained with monoclonal antibodies against β1 integrin subunits for immunocytochemical analysis as described previously [30]. Primary antibodies were detected using Oregon Green-labeled goat anti-mouse Ig. Controls consisted of cells that were not subjected to shear stress but which were stained identically. Antibody controls were also included. For these, cells were incubated with isotype-matched mouse Ig followed by Oregon Green-labeled secondary antibody. 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-β1 antibody-associated fluorescence. The software was calibrated using the InSpeck Image Intensity Calibration Kit (6-μm beads; Molecular Probes). 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 isotype-matched control mouse immunoglobulin instead of the anti-β1 integrin antibody) was subtracted from experimental values.

FACS analysis of cytotrophoblasts with anti-β1 integrin antibody was performed as described previously [31].

2.7. Immunohistochemistry

Placentas and attached decidua basalis were obtained from three animals on day 30 of pregnancy. Representative blocks of tissue were immersed in cryoprotectant (O.C.T. Compound, Miles Diagnostics), rapidly frozen in liquid nitrogen, and stored at −70 °C. Sections (6-μm thick) were prepared with a cryostat, mounted on poly-L-lysine coated slides, and air-dried at room temperature.

Prior to immunostaining, sections were fixed in cold (−20 °C) acetone for 10 min and air-dried. Each step in staining was succeeded by thorough rinsing in phosphate buffered saline. Sections were stained with anti-β1 integrin antibody or isotype-matched mouse Ig followed by Oregon Green-labeled secondary antibodies as described under Immunocytochemistry (above). Some sections were stained with an anti-cytokeratin antibody to positively identify trophoblasts.

2.8. Statistical analysis

Experiments were repeated at least three times using cells from different placentas in each case. Statistical analyses were performed by ANOVA followed by Tukey multiple comparison and linear trend post-tests using the Instat software program (GraphPad, San Diego, CA). Differences in means were considered significant if \( P < 0.05 \).

3. Results

3.1. Macaque trophoblast cell culture in glass capillary tubes

As illustrated in Fig. 2, early gestation (40–100 day) macaque trophoblast cells cultured in collagen-coated square cross-section glass capillary tubes exhibited variable morphology, some being fusiform and others more ovoid. This is similar to the appearance of these cells in conventional plastic culture dishes. Despite plating at relatively low densities (approximately 150,000 cells/ml; the cells do not replicate), small colonies of cells were often observed; however, all quantitation of cell motility was based on data derived from single cells. Trophoblast cells were studied 24 to 144 h after plating.

3.2. Computational determination of the flow field within capillary tubes

In order to determine the flow field within the glass capillary tubes, we have used the computational fluid dynamic code FLUENT for solving the three-dimensional Navier–Stokes equations within models of these tubes. Of particular interest in these simulations is the wall shear...
Fig. 3. (A) Schematic of the square cross-section capillary tube demonstrating the simulated region as well as the flow boundary conditions. (B) Wall shear stress distribution in the capillary tube determined either from the FLUENT computational fluid dynamic simulations or from the analytical expression given by Wiesner et al. [43] for flow rates of 6, 12, and 24 ml/min.
stress distribution as well as the possible presence of significant secondary flow motion. Because of symmetry, only one quarter of the square cross-section capillary tube needed to be modeled, while the entire 5-cm length was simulated. As shown in Fig. 3A, a no-slip boundary condition was imposed on all solid walls, while a symmetry boundary condition (i.e., zero flux for all flow quantities) was specified at the fluid boundaries. At the flow inlet, a steady flow rate with a flat (uniform) velocity profile was specified. Simulations were performed for three flow rates—6, 12, and 24 ml/min, matching the flow rates used in the experiments. At the flow outlet, a zero-pressure boundary condition was imposed. All results shown here are for a mesh consisting of 1000 × 35 × 35 computational nodes (in the x, y, and z directions, respectively); therefore, the conservation equations were solved at a total of 1,225,000 discrete points within the computational domain. All solutions have been verified to be mesh-independent—a finer mesh than the one given leads to velocities and shear stresses that are within 1% of those shown here. Numerical convergence was achieved when the residual in the three components of the velocity vector between successive

Fig. 4. Effect of shear stress on trophoblast cell motility. (A) No-flow (control) cells. (B) Cells subjected to a shear stress of 7.5 dyn/cm². (C) Cells subjected to a shear stress of 15 dyn/cm². (D) Cells subjected to a shear stress of 30 dyn/cm². In all cases, data points correspond to the x- and y-coordinates of the center of gravity (cog) of trophoblast cells at every hour during the 24-h recording period. Coordinates of the cog at the beginning of the recording (t=0) are taken as (0,0). Flow in panels B–D is from left to right (−x to +x).
iterations became smaller than a prescribed small number $\varepsilon$ (taken to be $10^{-7}$ in the present simulations). The corresponding residual in overall mass conservation was approximately $10^{-2}$. All computations were performed on a personal computer (Pentium 450 MHz with 512 Mbytes of RAM), and convergence was always achieved within 1000 iterations.

Fig. 3B illustrates the FLUENT results for wall shear stress distribution halfway down the tube length (i.e., $x = L/2$) for 6, 12, and 24 ml/min. Also shown are the analytical predictions of Weisner et al. [43] when the first 10 terms in the infinite series are considered. The figure clearly demonstrates that the numerical and analytical results are in very close agreement. The peak wall shear stress for a flow rate of 6 ml/min was 7.5 dyn/cm², while the equivalent values for 12 and 24 ml/min were 15 and 30 dyn/cm², respectively. Furthermore, the results demonstrate that the wall shear stress is relatively flat (a variation of less than 10%) within 200 µm of the tube centerline but decreases sharply as the side walls are approached. Because of these results, all assessment of flow-induced trophoblast cell motility was limited to cells within 200 µm of the tube centerline. Therefore, the cells whose motility was studied were exposed to a shear stress of approximately 7.5 dyn/cm² at 6 ml/min, 15 dyn/cm² at 12 ml/min, and 30 dyn/cm² at 24 ml/min. Secondary flow velocities were very small throughout the capillary tube at all flow rates studied. The ratio of peak secondary flow velocity to peak primary flow velocity was $4 \times 10^{-5}$ for the 6 ml/min simulations, $7 \times 10^{-7}$ for the 12 ml/min simulations, and $7 \times 10^{-5}$ for the 24 ml/min simulations.

3.3. Effect of steady shear stress on trophoblast cell motility

Trophoblast cell motility in response to controlled levels of shear stress was assessed by using image analysis to track changes in the coordinates of the cog of individual cells over a period of 24 h. Initial analyses suggested that the dynamics of trophoblast cell motility may depend on time in culture; therefore, we have separately studied the motility of trophoblast cells that had been plated 24–72 h prior to motility recordings (“early culture” cells) and cells cultured for periods of 72–144 h (“late culture” cells).

Fig. 4 illustrates the effect of flow on changes in the $x$- and $y$-coordinates of the cog of representative early culture trophoblast cells. In each case, the position of the cell at the beginning of the recording ($t=0$) is taken at the origin of the coordinate system (0,0), and the ($x$, $y$) coordinates of the cog are shown for every hour during the 24-h recording period. Under no-flow (control) conditions (Fig. 4A), trophoblast cells were highly dynamic exhibiting constant positional shifts. However, these shifts were nondirectional and appeared to be associated with slow and seemingly random morphological changes and hence did not lead to net cell migration. When early culture trophoblast cells were exposed to a steady shear stress of 7.5 dyn/cm² (Fig. 4B), similar dynamic behavior was observed, but the cells now exhibited limited flow-induced migration. When cells were exposed to a steady shear stress of 15 or 30 dyn/cm² (Fig. 4C and D), significant migration was observed in most cells, and this migration usually followed complex trajectories. Under 7.5, 15, and 30 dyn/cm², cell migration was generally in the direction of flow. In most cases and as evident in Fig. 5.

![Fig. 5](image_url)
3C and D, flow-induced trophoblast cell motility had a significant component normal to flow (i.e. y-direction). Considering that the cells studied were not exposed to significant variations in wall shear stress in the y-direction (all cells were within \( \pm 200 \mu m \) of the tube centerline), the reason for this y-direction movement remains unclear. These results suggest that fluid mechanical shear stress can regulate trophoblast cell motility in vitro and that the extent of the flow-induced migration increases with an increase in the magnitude of the applied shear stress. Interestingly, a shear stress of either 7.5 or 15 dyn/cm\(^2\) failed to elicit significant migration in late culture cells (data not shown). These results suggest that time in culture is an important determinant of trophoblast cell responsiveness to shear stress in vitro.

Fig. 5 shows sequential phase-contrast images of representative early culture single cells taken at 0, 7, 14, and 21 h after initiation of flow at a shear stress of 15 dyn/cm\(^2\). Also shown is a similar sequence for a control (no-flow) cell. The cell exposed to flow exhibit clear migration in the direction of flow as well as shape changes that involve extension and retraction of filipodia at its leading edge. The no-flow cell, on the other hand, shows no significant net displacement and no major change in morphology for the duration of the experiment.

The effect of shear stress on trophoblast cell migration velocity and displacement for early culture cells is more quantitatively illustrated in Fig. 6. Fig. 6A demonstrates that the average trophoblast cell migration velocity (defined, as

![Average Migration Velocity](image1)

Therefore, the average migration velocity (\( \mu m/hr \)) increases with increasing shear stress (dyne/cm\(^2\)).

![Net x-direction Displacement](image2)

Fig. 6. Effect of shear stress on (A) the average migration velocity and (B) the net displacement in the direction of flow in trophoblast cells. Flow is in the \( +x \)-direction. Data are presented as mean \( \pm \) S.E. for the number of cells indicated in parentheses.

![Histograms](image3)

Fig. 7. Histograms showing percentage of total trophoblast cells having average migration velocities within the indicated ranges. Note distribution shift towards higher velocities as the shear stress level is increased.

![Expression of \( \beta_1 \) integrin](image4)

Fig. 8. Expression of \( \beta_1 \) integrin in early gestation macaque placental tissue. Frozen sections of macaque placental/endometrial tissue were incubated with anti-\( \beta_1 \) integrin antibody (A and C), control mouse Ig (B and D) and viewed by fluorescence microscopy as described in Section 2. The bars represent 100 \( \mu m \). Images are representative of sections from four different animals. VC, villous cytotrophoblast; EVC, extravillous cytotrophoblast; S, syncytiotrophoblast.
described in Section 2, as the total distance traversed by the cell during the 24-h period divided by 24) increased in response to increasing levels of shear stress. A Trend Analysis post-test of the data revealed a significant ($P<0.05$) increasing linear trend. Cell migration velocity at 30 dyn/cm$^2$ was significantly different ($P<0.05$) from the static value. Fig. 6B illustrates similar conclusions for net trophoblast cell displacement in the direction of flow. At the end of the 24-h recording period, net displacement of early culture trophoblast cells subjected to 0, 7.5, 15, or 30 dyn/cm$^2$ showed a significant increasing linear trend ($P<0.001$), and this displacement was generally in the direction of flow (+$x$-direction). Late culture cells exposed to either 7.5 or 15 dyn/cm$^2$ did not exhibit significant increases in either average migration velocity or net displacement in response to flow (data not shown).

Fig. 7 shows histograms of the fraction of early culture trophoblast cells having average velocities within selected ranges. The results demonstrate the progressive shift in the distribution towards higher velocities in these cells as the value of the applied shear stress is increased. This shift did not occur in late culture cells (data not shown), consistent

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**Fig. 9. Effect of fluid mechanical shear stress on $\beta$1 integrin expression in trophoblast cells.** Trophoblast cells were maintained under static conditions ($t=0$) or subjected to shear stress (15 dyn/cm$^2$) for 30 min, 6, or 24 h and then fixed/permeabilized using methanol and stained using an anti-$\beta$1 integrin antibody and an Oregon green-labeled second antibody (see Section 2). Parallel cultures were incubated with an isotype-matched mouse immunoglobulin (antibody control). The stained cells were viewed by epifluorescence microscopy. The horizontal bar represents 50 $\mu$m. Representative images from three separate experiments are shown. The graph summarizes the image analysis of the antibody-associated fluorescence intensity (see Section 2). Data are means $\pm$ S.E. for the number of cells indicated in parentheses.
with the lack of responsiveness of these cells to the applied shear stress. Interestingly, the distribution for cells exposed to 30 dyn/cm² appeared bimodal so that larger fractions of cells exhibited relatively low (2–3 μm/h) or very high velocities (5+ μm/h) than intermediate velocities (3–5 μm/h). Reasons for this behavior remain unclear but may reflect heterogeneities in trophoblast cellular responses to relatively high shear stresses.

3.4. Effect of shear stress on trophoblast β1-integrin expression

Integrins are involved in regulating the adhesion and migration of various types of cells on extracellular matrix [45–49] and are implicated in trophoblast invasion in the human and the macaque [22,23,31,50,51]. We have recently demonstrated that the adhesion of human and macaque trophoblast cells to endothelial cells in vitro is mediated by αV and β1 integrins [30,31]. As with the human, low levels of β1 integrin expression occur in villous cytotrophoblasts and levels are higher in extravillous cytotrophoblasts (see Fig. 8A and C). Syncytiotrophoblast did not express β1 integrin (Fig. 8C). To determine if exposure of villous trophoblast cells to shear stress affected integrin expression, we exposed early culture trophoblast cells in capillary tubes to a steady shear stress of 15 dyn/cm² for periods of 30 min, 6, and 24 h. The cells were then fixed and permeabilized using methanol, and stained for β1 integrin.

As shown in Fig. 9, control (no-flow) cells exhibited a punctate β1 integrin staining pattern. Nuclei were clearly revealed as areas of negative staining (black) in the cells. However, after 30 min of shear stress, the fluorescence was more diffuse and negatively stained nuclei were not observed. After 6 h of shear stress, many, but not all, cells had discernable nuclei. After 24 h of shear stress, the fluorescence pattern was similar to that seen in nonsheared cells and nuclei were identified as negatively stained regions within cells. Fig. 9 also shows a culture incubated with control mouse immunoglobulin which exhibits only a weak and diffuse autofluorescence. In order to determine whether these changes in integrin distribution were accompanied by quantitative changes in integrin expression, the antibody-associated fluorescence was quantitated for multiple random cells using image analysis software. The graph in Fig. 9 shows the results of this analysis, and it can be seen that fluorescence levels in cultures subjected to shear stress (15 dyn/cm²) were significantly increased (P<0.05; ANOVA with Tukey post-test) compared to the static cultures at all time points measured. It is also apparent that after 24-h exposure to shear stress, fluorescence levels were significantly lower (P<0.05) than the levels after either 30-min or 6-h exposure.

![Fig. 10. Effect of fluid mechanical shear stress on the cell-surface expression of β1 integrin. Trophoblast cells were maintained under static conditions (t=0) or subjected to shear stress (15 dyn/cm²) for 30 min, 6, and 24 h. The cells were then fixed using paraformaldehyde (without permeabilization) and stained using an anti-β1 integrin antibody and an Oregon green-labeled second antibody (see Section 2). The cells were viewed by epifluorescence microscopy. The horizontal bar represents 50 μm. Representative images from two separate experiments are shown.](image-url)
In order to examine the effects of shear stress on the surface expression of β1 integrin, trophoblast cells were exposed to shear stress as above but at the end of the experiment were fixed in paraformaldehyde without permeabilization (Fig. 10). Cells that had not been subjected to shear stress showed a weak diffuse staining pattern. On close examination a dark, a negatively stained region could be discerned on most cells. After 30 min of shear stress, a bright diffuse fluorescence was associated with the cells and negatively stained central regions were less obvious. Bright staining was maintained after 2 and 6 h of shear stress. After 6-h exposure to shear stress, the fluorescence appeared brighter at cell peripheries. After 24 h of shear stress, staining appeared to be restricted to cell peripheries and negatively stained regions were visible at the centers of cells.

4. Discussion

Trophoblast cell invasion of, and migration within, uterine vasculature are essential processes in the development of the placenta. In this paper, we have used a glass capillary culture system to demonstrate that fluid mechanical shear stress regulates the motility of early gestation macaque trophoblast cells as well as the expression and topography of β1 integrin in these cells. The wall shear stresses to which trophoblast cells in the capillary tubes were exposed were determined using three-dimensional computational fluid dynamic simulations. Under no-flow culture conditions, trophoblast cells were highly dynamic with continuous nondirectional shifting in their centers of gravity and no net migration. A steady shear stress of 7.5 dyn/cm² significantly increased cellular average migration velocity and net displacement in the direction of flow. Shear stresses of 15 and 30 dyn/cm² elicited further increases in both average migration velocity and net displacement in the direction of flow. The migratory response of trophoblast cells to shear stress was found to be dependent on the length of time that the cells were in culture prior to being exposed to flow. Trophoblast cells exposed to shear stress within 72 h of initial plating (early culture cells) migrated in response to flow, while cells maintained in culture for 72–144 h prior to exposure to shear stress (late culture cells) did not exhibit significant migratory activity. Reasons for this difference are not clear. One possibility is that in the absence of any stimulus, cultured cells follow a different (noninvasive) differentiation pathway that does not express the shear stress sensing system or in which this system is not responsive.

We have previously shown that macaque trophoblast cells express β1 integrins and that these adhesion molecules play a role in the adhesion of trophoblast cells to endothelial cells [30,31]. β1 integrin has been shown to be essential to the migratory function of trophoblast cells derived from human first trimester villous tissue explants [26]. In the present paper, we have demonstrated that shear stress alters the distribution of β1 integrin in trophoblast cells. The punctate pattern of β1 integrin staining and the fact that nuclei appeared as negatively stained structures in nonpermeabilized cells suggested that nuclei are masked. This finding is consistent with the localization of β1 integrin to focal adhesions as has been described for other cell types. The finding that nuclei were no longer readily discernible in permeabilized cells as negatively stained structures after 30 min and up to 6 h of shear stress indicates a change in expression or redistribution of β1 integrin. This idea is supported by the similar change in β1 integrin staining seen after 30 min of shear stress using nonpermeabilized cells. The alteration in the distribution of β1 integrin appears to be transient and was maximal between 30 min and 6 h of shear stress. One explanation for the disappearance of negatively stained nuclei at these times is that there is increased surface expression of β1 integrin such that nuclei are masked. This conclusion is consistent with the increased surface expression of β1 integrin found in the paraformaldehyde-fixed (i.e. nonpermeabilized) cultures.
Image analysis of the digitized cell-associated fluorescence in permeabilized cultures showed significant increases in fluorescence intensity in cells subjected to shear stress. This is suggestive of a shear stress-induced increase in total levels of immunoreactive β1 integrin. While changes in integrin distribution and/or avidity can occur very rapidly (within seconds), changes in levels of integrin protein expression are dependent on alterations in rates of synthesis and/or degradation that generally occur more slowly. Induction of integrin mRNA expression in mouse lung endothelial cells was detected within 1 h [54]. Our conclusion that shear stress increases total β1 integrin expression in trophoblast cells is not inconsistent with this finding. However, further studies will be required to confirm this conclusion and to better understand the altered distribution/expression of β1 integrin in response to shear stress in this system.

Immunohistochemical and in vitro studies in the human indicate that villous cytotrophoblasts express low levels of β1 integrin but that expression increases in trophoblasts within cell columns as the cells enter the invasive pathway [22,23]. High levels of β1 integrin expression are maintained in interstitial trophoblasts and intravascular trophoblasts. The immunohistochemical and FACS analyses described here show a similar pattern of trophoblast β1 integrin expression in the macaque. Some of the factors that control trophoblast integrin expression and invasion are beginning to be identified [23,26,28,52,55]. When first trimester villous trophoblasts are exposed to different extracellular matrices, the cells adopt a migratory phenotype characterized by changes in integrin expression and increased migratory behavior [23]. The results presented here show that trophoblasts derived from early gestation macaque villous tissue acquire a migratory phenotype when exposed to shear stress. The shear stress-induced change in the expression/distribution of β1 integrin in macaque trophoblast cells raises the possibility that expression of this molecule by intravascular trophoblasts in vivo could be regulated by blood flow-derived shear forces. In particular, shear stress may be responsible for maintaining high levels of β1 integrin in intravascular trophoblasts. Increased surface expression of β1 integrin could be involved in the initial burst of trophoblast migratory activity in response to shear stress. Increased integrin expression may also assist in keeping the cells adherent and preventing detachment under flow conditions. It should be noted that the effects of flow-derived shear stress are not restricted to cells within the vasculature. Subendothelial smooth muscle cells are thought to respond to the effects of interstitial fluid flow [56,57]. Thus, it is possible that interstitial fluid flow could affect integrin expression in interstitial trophoblasts immediately adjacent to, but not yet within, uterine vessels.

The mechanisms involved in shear stress regulation of migration and integrin expression in trophoblast cells are entirely unknown. In endothelial cells, shear stress has been shown to increase or decrease the expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [34, 58,59]. In other cell types, most notably vascular endothelial cells, shear stress sensing and transduction has been postulated to occur through a cascade of events involving the initial sensing of the shear force by cell-surface structures that act as primary flow sensors, followed by transmission and transduction of this force to the nucleus via second messenger pathways and/or the cytoskeleton [32,60]. Candidate flow sensing mechanisms in endothelial cells include activation of shear stress-sensitive ion channels, stimulation of G protein-linked receptors or cell-surface integrins, and alterations in cell membrane fluidity. It would be particularly interesting to investigate the possible presence of similar flow sensing structures in trophoblast cells.

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