The role of $\alpha_5\beta_1$-integrin in the IGF-I-induced migration of extravillous trophoblast cells during the process of implantation

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The role of integrins in the processes of adhesion and migration makes them attractive potential participants in the complex events of embryo implantation and placentation. Recently, the role of the $\alpha_5\beta_1$-integrin pathway was shown in the insulin-like growth factor-I (IGF-I)-stimulated migration of extravillous trophoblast (EVT) cells. This study was designed to investigate the role of $\alpha_5\beta_1$-integrin in this respect. Using cultured EVT cells, migration assays were carried out for IGF-I-treated or untreated cells in the presence or absence of the GRGDSP and GRGESP hexapeptides, $\alpha_1$IR3, and a blocking antibody against $\alpha_5\beta_1$-integrin. Immuno-electron microscopy and immunofluorescent staining were performed to localize the distribution of $\alpha_5\beta_1$- and $\alpha_3\beta_1$-integrins, Rab5a, paxillin, phospho-FAK (pFAK), and vinculin. The results showed that IGF-I-induced migration of EVT cells was abolished following treatment with GRGDSP hexapeptide, $\alpha_1$IR3, and a blocking antibody against $\alpha_5\beta_1$-integrin. Further, statistical analysis showed that the area-related numerical density of the $\alpha_5\beta_1$-integrin in the perinuclear regions was significantly higher than in the cell extensions. Immunocytochemical experiments demonstrated an up-regulation in $\alpha_5\beta_1$-integrin pathway was shown in the insulin-like growth factor-I (IGF-I), which promotes the migration of trophoblast cells in vitro (Lacey et al., 2002). There is clear evidence indicating that an interaction exists between the IGF-I receptor and integrins during cell migration (Clemmons and Maile, 2003). The role of $\alpha_5\beta_1$-integrin in IGF-I-induced migration of EVT cells has already been established (Kabir-Salmani et al., 2003), whereas the role of $\alpha_5\beta_1$-integrin in this context is a matter of some controversy (Damsky et al., 1994; Irving and Lala, 1995). The present study is designed to examine the significance of $\alpha_5\beta_1$-integrin in IGF-I-stimulated migration of EVT cells during the processes of embryo implantation and placentation. Furthermore, the effect of IGF-I on the expression of $\alpha_5\beta_1$-integrin and the co-localization of this integrin with the components of the focal adhesions (FA) of human EVT cells has been studied.

Key words: $\alpha_5\beta_1$-integrin/IGF-I/implantation/placentation/trophoblast migration

Introduction

During the process of embryo implantation, a highly migratory subpopulation of placental trophoblasts migrates and invades the maternal decidua and its vasculature to establish an adequate maternal decidua and its vasculature to establish an adequate

Materials and methods

Reagents

Recombinant human IGF-I was a gift from Fujisawa Pharmaceutical Co. (Japan). Monoclonal antibody against $\alpha$-subunit of IGF-IR (aIR3) was purchased from Calbiochem Oncogene Research Products (USA). $\alpha_5\beta_1$-
Integrin anti-mouse monoclonal IgG and α5β1-integrin anti-goat polyclonal IgG were from Chemicon International (USA). Paxillin anti-rabbit polyclonal IgG and α5-integrin anti-goat polyclonal IgG were purchased from Santa Cruz Biotechnology (USA). Phospho-FAK anti-rabbit polyclonal IgG was from Upstate Biotechnology (USA). Vinculin monoclonal anti-mouse IgG1 was from Sigma Chemical Company (USA). AMCA-conjugated donkey anti-rabbit IgG was purchased from Jackson Immuno Research Laboratories Inc (USA). Alexa 568-labelled donkey anti-mouse and anti-rabbit IgG were purchased from Molecular Probes (USA). Fibronectin (FN), FN inhibitor, Gly-Arg-Gly-Asp-Ser-Pro (RGD hexapeptide) and Gly-Arg-Gly-Glu-Ser-Pro (RGE hexapeptide) were synthesized by Iwaki Glass Co. (Japan). TAAB Epon 812, glutaraldehyde, and osmium tetroxide were from TAAB Laboratories Equipment Ltd (UK).

Primary trophoblast cell culture

Placental tissues between 6 and 10 weeks of gestation were obtained at legal, elective termination of pregnancy. All patients gave informed consent for collection and investigational use of tissues. This study was approved by the ethics committee of Kyorin University, School of Medicine, Tokyo, Japan. EVT were obtained by the method described previously (Kabir-Salmani et al., 2003). Briefly, the tissues were rinsed several times in cold medium 199 containing streptomycin (20 mg/ml) and penicillin (500 IU/ml). Selected tissues were then cut into small pieces and any obvious blood vessels or clots, membranes, and decidual tissues were removed. Then fragments were cultured in the same medium containing 10% FBS in tissue culture flasks, pre-coated with 20 µg of FN diluted in 1 ml phosphate-buffered saline (PBS, pH 7.4). Tissues were allowed to attach to the bottom of the flasks for 30–60 min before adding the medium. After 3–5 days, unattached cells were removed and culture was continued for an additional 1–2 weeks. The medium was changed every 48 h until cells were confluent and then the cells were passaged. Passages 2–4 were used for all experiments. The identity of cells as extravillous trophoblast cells was established by immunohistochemical staining as described previously (Kabir-Salmani et al., 2003).

Cell wounding and migration assay

Cells were plated at 1.2 ± 1.4 × 10⁵/cm² in medium 199 containing 10% FBS and allowed to grow to confluence over 3 days. Wounding was performed using a sterile razor blade to scrape cells off the culture plate, leaving a denuded area and a sharp visible demarcation line at the wound edge, as described in previous reports (Jones et al., 1996). The wounded monolayers were rinsed three times with serum-free medium 199 and were inspected immediately after wounding. Then sections of the wounds that were to be used for quantifying migration were selected according to the criteria described previously (Jones et al., 1996; Irving and Lala, 1995), marked, and numbered. The areas with technical problems such as extra lines made by razor blade, incomplete scraping or deep wounds were not selected as one of the experimental group. The cells were then incubated for 48 h with serum-free medium 199 with 0.01% bovine serum albumin (BSA) and one of the following: (i) 10 nmol/l IGF-I alone or in the presence of dR3, (ii) blocking antibody against α5β1-integrin alone or in the presence of 10 nmol/l IGF-I, (iii) 100 µmol/l GRGDSP hexapeptide alone or in the presence of 10 nmol/l IGF-I, or (iv) 100 µmol/l GRGESP hexapeptide as a control for GRGDSP alone or in the presence of 10 nmol/l IGF-I. Before addition of IGF-I, the cells were preincubated for 30–60 min with blocking antibodies and peptides. At the end of the preincubation period, cells were >95% viable as assessed by Trypan Blue dye exclusion. Then cells were rinsed in PBS, fixed and stained using Diff-Quick kit solutions and examined under phase-contrast microscopy. Migrated EVT cells were counted in sections 1.0 mm in length; allowing a 20 µm space from the demarcation line to minimize the possible physical effects of cell movement resulting from cell proliferation. A calibrated eyepiece grid was used to determine the mean number of migrated cells. Statistical analysis was calculated by averaging a mean of 10 sections per test substance for each experiment. The number of migrated cells was expressed as mean ± SEM. Statistical significance was evaluated using analysis of variance with Scheffé’s test and P < 0.01 was considered statistically significant. The experiments were repeated at least four times in each group to assess reproducibility.

Figure 1. (A) Migration of extravillous trophoblast (EVT) cells following wounding of the cells and incubating with serum-free medium 199 containing 0.01% bovine serum albumin and one of the following: 10 nmol/l insulin-like growth factor-I (IGF-I) alone or in the presence of antibody against α-subunit of IGF-IR (dR3; 10 nmol/l), functional-blocking antibody against α5β1-integrin (10 µg/ml) alone or in the presence of 10 nmol/l IGF-I, 100 µmol/l RGD hexapeptide alone or in the presence of 10 nmol/l IGF-I, and 100 µmol/l RGE hexapeptide as a control for RGD alone or in the presence of 10 nmol/l IGF-I. Before addition of IGF-I, the cells were preincubated with blocking antibodies and peptides for 60 min. Preincubation of cells with 10 nmol/l dR3, RGD peptide, and antibody against α5β1-integrin significantly decreased the number of migrated cells in the 10 nmol/l IGF-I-treated and untreated cells. Scale bar = 50 µm. (B) Morphometric evaluation of EVT cell migration stimulated with or without 10 nmol/l IGF-I in the above-mentioned groups. A calibrated eyepiece grid was used to calculate the mean number of migrated EVT cells/1.0 mm section of wound. Statistical evaluation was calculated by averaging a mean of 10 sections per test substance for each experiment. The numbers of migrated cells were expressed as mean ± SEM. *The number of migrated cells shows significant difference versus control (P < 0.01); †the number of migrated cells shows no significant difference versus control; ‡the number of migrated cells shows significant difference versus 10 nmol/l IGF-I (P < 0.01); §the number of migrated cells shows no significant difference versus 10 nmol/l IGF-I.
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![Figure 2.](https://academic.oup.com/molehr/article-abstract/10/2/91/984281)

**Figure 2.** (A) Immunogold labelling for $\alpha_5\beta_1$- and $\alpha_3\beta_1$-integrin in ultrathin sections of extravillous trophoblast (EVT) cells incubated for 2 h in serum-free medium 199 containing 10 nmol/l insulin-like growth factor-I (IGF-I) to localize their distribution pattern in different areas of EVT cells after IGF-I stimulation. Scale bar = 0.5 μm. Distribution of $\alpha_5\beta_1$- (a and c) and $\alpha_3\beta_1$- (b and d) integrins in the perinuclear and cell extension areas: arrowheads point to the lamellipodia. (B) Morphometric analysis of area-related numerical densities of gold particles labelling $\alpha_5\beta_1$- and $\alpha_3\beta_1$-integrin in ultrathin sections of EVT cells treated with 10 nmol/l IGF-1. *Significant difference in the number of gold particles; $P < 0.001$. PR = perinuclear region; CE = cell extension.

**Results**

**Migration assay**

The results of the migration assays showed that the treatment with 10 nmol/l IGF-I significantly ($P < 0.01$) stimulated the migration of EVT cells, which was ~2-fold higher than control (Figure 1). Inhibition of IGF-IR by IR3 antibody demonstrated that the number of migrating cells was significantly ($P < 0.01$) decreased compared with its corresponding control. The preincubation of EVT cells with a functional blocking antibody against $\alpha_5\beta_1$-integrin significantly decreased the migration of EVT cells in both IGF-I-treated and untreated cells. Moreover, the migration of IGF-I-treated EVT cells was abrogated by preincubation of these cells with GRGDSP hexapeptide but not GRGESP hexapeptide. GRGDSP hexapeptide decreased the number of migrating cells compared with its corresponding control, which was treated with GRGESP hexapeptide. These data further showed that preincubation of EVT cells with an appropriate primary antibody (anti-$\alpha_5\beta_1$-integrin IgG; 1.5 μg/ml and anti-$\alpha_3\beta_1$-integrin IgG; 1.5 μg/ml). After washing several times with PBS, the cells were incubated overnight with their appropriate colloidal gold-conjugated anti-goat, anti-rabbit and anti-mouse IgG (using 6–12 nm gold particles). The cells were then fixed in cacodylate buffer (100 mmol/l sodium cacodylate, pH 7.2, 120 mmol/l CaCl$_2$), containing 2.5% glutaraldehyde overnight at 4°C. After washing with cacodylate buffer, the cells were post-fixed in 1% OsO$_4$ for 2 h, washed and dehydrated by successive 10 min incubations with 30, 50, 70, 90 and 100% ethanol. Following dehydration, the cells were detached and embedded in Epon resin. Ultrathin sections were cut using a diamond knife and the staining procedure was performed. Grids were observed using a JEM, 1010 JEOL transmission electron microscope (Tokyo, Japan) operated at 80 kV. This experiment was repeated four times in four different preparations. For morphometric analysis of immunogold-labelled integrins, 200 cells per test group were chosen randomly by an observer who was blind to the identity of these grids and four to six photomicrographs were taken from body and cell extension regions of each cell. Area-related numerical density of immunogold particles was calculated using NIH Image software (Version 1.61). Morphometric analysis was performed to quantify these data as described previously (Glasbey and Roberts, 1997). To determine the co-localization of $\alpha_5\beta_1$-integrin with Rab5a, the same procedures were carried out.

**Immunocytochemistry**

For immunogold transmission electron microscopy preparations, serum-starved EVT cells were incubated with serum-free medium 199 with or without 10 nmol/l IGF-I for 2 h to study the effect of IGF-I on the distribution patterns of $\alpha_5\beta_1$- and $\alpha_3\beta_1$-integrins. Cells were then fixed using 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked by 5% BSA for 1 h at 4°C. Thereafter, the cells were incubated overnight at 4°C with an appropriate primary antibody (anti-$\alpha_5\beta_1$-integrin IgG; 1.5 μg/ml and anti-$\alpha_3\beta_1$-integrin IgG; 1.5 μg/ml). After washing several times with PBS, the cells were incubated overnight with their appropriate colloidal gold-conjugated anti-goat, anti-rabbit and anti-mouse IgG (using 6–12 nm gold particles). The cells were then fixed in cacodylate buffer (100 mmol/l sodium cacodylate, pH 7.2, 120 mmol/l CaCl$_2$), containing 2.5% glutaraldehyde overnight at 4°C. After washing with cacodylate buffer, the cells were post-fixed in 1% OsO$_4$ for 2 h, washed and dehydrated by successive 10 min incubations with 30, 50, 70, 90 and 100% ethanol. Following dehydration, the cells were detached and embedded in Epon resin. Ultrathin sections were cut using a diamond knife and the staining procedure was performed. Grids were observed using a JEM, 1010 JEOL transmission electron microscope (Tokyo, Japan) operated at 80 kV. This experiment was repeated four times in four different preparations. For morphometric analysis of immunogold-labelled integrins, 200 cells per test group were chosen randomly by an observer who was blind to the identity of these grids and four to six photomicrographs were taken from body and cell extension regions of each cell. Area-related numerical density of immunogold particles was calculated using NIH Image software (Version 1.61). Morphometric analysis was performed to quantify these data as described previously (Glasbey and Roberts, 1997). To determine the co-localization of $\alpha_5\beta_1$-integrin with Rab5a, the same procedures were carried out.

For immunofluorescent preparations, EVT cells were cultured on FN-coated Lab-Tek chamber slides and incubated for 18–24 h with serum-free medium 199. To stimulate assembly of FA in these cells, the cells were treated with 10 nmol/l IGF-I for 30 min. Cells were then fixed and permeabilized as described above. For double-staining, the cells were incubated with the appropriate primary antibodies (anti-vinculin IgG; 1 μg/ml, anti-paxillin IgG; 1.5 μg/ml, anti-phospho-FAK IgG; 1.5 μg/ml, anti-Rab5a IgG; 1.5 μg/ml, and anti-$\alpha_5\beta_1$- integrin IgG; 1.5 μg/ml) for 1–3 h at room temperature. For control, the coverslips were incubated at room temperature for 1–3 h with the same concentration of corresponding antibodies, including monoclonal anti-mouse IgG (substituted for mouse anti-vinculin), polyclonal anti-rabbit IgG (substituted for paxillin IgG, Rab5a IgG, and phospho-FAK IgG), and polyclonal anti-goat IgG (substituted for $\alpha_5\beta_1$-integrin IgG). Then cells were rinsed in PBS extensively and counter-stained with proper fluorescent-labelled secondary antibodies (Alexa 568-labelled donkey anti-mouse IgG, 1 μg/ml; FITC-labelled donkey anti-rabbit IgG, 1 μg/ml; and AMCA-labelled donkey anti-goat IgG, 1 μg/ml). After washing with PBS, rinsing in deionized water and mounting, cells were observed using an AX-80 fluorescence microscope (Olympus Optical, Japan). These experiments were repeated three times in three different preparations in each group.
**Immunocytochemistry**

Morphometric analysis of the distribution of gold particles over body and cell extension areas of EVT cells revealed that area-related numerical density of $\alpha_5\beta_1$-integrin is higher in the perinuclear region compared with the cell extensions and lamellipodia, whereas $\alpha_5\beta_3$-integrin was found to have a higher area-related numerical density in the cellular extensions compared with the cell body (Figure 2). Further, area-related numerical density of $\alpha_5\beta_1$-integrin in the cell extensions and lamellipodia showed a relatively higher density compared with $\alpha_5\beta_1$-integrin.

Semi-quantitative analysis of immunogold-labelled $\alpha_5\beta_1$-integrin in ultrathin EVT cell sections demonstrated that IGF-I treatment increased their internalization ~3-fold above control (Figure 3). Aggregations of $\alpha_5\beta_1$-integrin showed co-localization with Rab5a (Figure 4), a member of the Rab subfamily small GTPases, which is considered to be one of the key regulators of intracellular transportation (Horiuchi et al., 1997).

Images from immunofluorescent staining further confirmed the co-localization of $\alpha_5\beta_1$-integrin with Rab5a in the EVT cells (Figure 4). Double-staining for $\alpha_5\beta_1$-integrin with $\alpha_5\beta_3$-integrin, pFAK, paxillin, and vinculin demonstrated that $\alpha_5\beta_1$-integrin did not co-exist with these components at the FA of EVT cells (Figure 5). No staining of these structures could be seen without primary antibodies in the control group (results not shown). These confocal images support the results obtained from immunogold labelling, indicating a perinuclear distribution of $\alpha_5\beta_1$-integrin, whereas $\alpha_5\beta_1$-integrin showed a peripheral localization corresponding to the FA.

**Discussion**

Explants of placental villi from first trimester pregnancy have been shown to provide an appropriate *in vitro* model for studying migratory events during the process of human embryo implantation and placentation (Vicovac et al., 1995). Employing propagated EVT cells in this study, the results of the migration assays exhibited an essential role for $\alpha_5\beta_1$-integrin during the migratory function of EVT cells in both control and IGF-I-treated EVT cells. This is consistent with previous studies in a variety of cell types suggesting that the binding of the $\alpha_5\beta_1$-integrin to the FN substrate enhanced cell migration and the addition of anti-$\alpha_5$ and anti-$\beta_1$-integrin blocking antibodies inhibited the trophoblast migration on FN (Irving and Lala, 1995). Further, it has been reported that $\alpha_5$-integrin affects the rate of tumour metastasis (Tani et al., 2003). In another set of experiments using a villous explant organ culture assay, it has been demonstrated that treatment of EVT cells with anti-$\alpha_5$-integrin antibody interfered

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**Figure 3.** Immunogold localization of $\alpha_5\beta_1$-integrin aggregations in the ultrathin sections of extravillous trophoblast (EVT) cells incubated for 2 h with serum-free medium 199 containing 10 nmol/l insulin-like growth factor-I (IGF-I) (a), and serum-free medium 199 (B). Scale bar = 0.5 μm.

**Figure 4.** Immunofluorescent images of double-staining of $\alpha_5\beta_1$-integrin (a) with Rab5a (b) revealed a co-localization of $\alpha_5\beta_1$-integrin with Rab5a (c). Scale bar = 10 μm. Rab5a is considered an important regulator for intracellular transport. Immunogold double-staining for $\alpha_5\beta_1$-integrin and Rab5a in the ultrathin sections of extravillous trophoblast (EVT) cells incubated for 2 h in serum-free medium 199 containing 10 nmol/l insulin-like growth factor-I (IGF-I) (d). Scale bar = 0.1 μm.
with the outgrowth of EVT cell column and caused rounding of the spindle-shaped EVT cells (Aplin et al., 1999), which could indicate a loss of cell motility in these cells. In contrast to the above findings, it has been previously reported that the adhesion of trophoblast cells to FN via \(\alpha_5\beta_1\)-integrin generates an invasion-restraining signal. The addition of the exogenous FN to a three-dimensional matrigel invasion assay system inhibited the trophoblast invasion, whereas the addition of the anti-\(\alpha_5\)-integrin monoclonal antibody accelerated invasion (Damsky et al., 1994). These conflicting results may be due, at least in part, to the type and concentrations of different ECM proteins used in each assay system. ECM concentration has been shown to be crucial to the cellular responses, as lower concentrations of FN have been observed to support cellular migration, whereas high concentrations retarded migration (Madri et al., 1988). It is of interest that the invasiveness of the trophoblast cells in molar pregnancies is almost unrestricted and in this pre-malignant condition, villous trophoblast, which does not normally express the \(\alpha_5\)-subunit, becomes strongly positive for this subunit and thus resembles the extravillous invading trophoblast phenotype in expressing \(\alpha_5\beta_1\)-integrin (Bischof et al., 1993).

In our migration assay system, the GRGDSP hexapeptide and a blocking antibody against \(\alpha_5\beta_1\)-integrin were used to clarify the importance of the binding of \(\alpha_5\beta_1\)-integrin to the RGD domain of the FN substrate during the IGF-I-stimulated migration of the EVT cells. Upon treatment of EVT cells with GRGDSP hexapeptide or blocking antibody against \(\alpha_5\beta_1\)-integrin, the number of migrating cells showed a statistically significant reduction in both IGF-I-treated and untreated cells. This is in agreement with a previous report showing that the treatment of EVT cells with GRGDSP hexapeptide abolished IGF-II-stimulated migration of EVT cells in vitro (Irving and Lala, 1995). It is

Figure 5. Confocal Immunofluorescent microscopy to detect the co-localization of \(\alpha_5\beta_1\)-integrin with the components of the focal adhesions of insulin-like growth factor-I (IGF-I)-treated extravillous trophoblast (EVT) cells. Double-staining for pFAK, paxillin, and vinculin with \(\alpha_5\beta_1\)-integrin revealed that \(\alpha_5\beta_1\)-integrin was absent from the core of focal adhesions of the migrating EVT cells. Scale bar = 10 \(\mu\)m.
also consistent with another study that showed the importance of \( \alpha_5 \beta_1 \)-integrin binding to the RGD domain of FN during IGF-binding protein-1-stimulated migration of EVT cells (Jones et al., 1996). Considering that highly migratory tumour cells secrete RGD-containing FN into their surroundings and use it as a platform for their migration (Humphries, 1990), secretion of FN by EVT cells (Burrows et al., 1996) may suggest that these cells use the similar substrate during their integrin-dependent migration as well. Notably, binding to FN affects the terminal differentiation of EVT cells, which can influence their migration. Tenascin, an ECM protein, has been demonstrated to interfere with the attachment of EVT cells to FN (Erickson and Bourdon, 1989). Strong expression of this protein has been observed in human uterine myometrium, particularly in the areas where trophoblast migration ceased (Burrows et al., 1996). This further indicates that the binding of the migrant EVT cells to the FN substrate is essential to their migratory function.

As cell migration requires the efficient, regulated formation and breakdown of cell–ECM adhesions and integrin recycling following internalization, we next examined the effects of IGF-I on the internalization rate of \( \alpha_5 \beta_1 \)-integrin. Semi-quantitative analysis of immunogold-labelled \( \alpha_5 \beta_1 \)-integrin in the ultrathin sections of EVT cells demonstrated that IGF-I treatment increased the internalization rate of \( \alpha_5 \beta_1 \)-integrin. Furthermore, immunofluorescent staining exhibited the co-localization of \( \alpha_5 \beta_1 \)-integrin with Rab5a in EVT cells, which implies its intracellular transportation (Bucci et al., 1992). Rab5 is exclusively localized to the plasma membrane, early endosomes, and plasma membrane-derived clathrin-coated vesicles (Chavrier et al., 1990; Bucci et al., 1992). Using green fluorescent protein (GFP) in living cells, \( \alpha_5 \)-integrin has been seen to remain on the substrate behind the migrating cells or appeared in the vesicles that move toward the cell body where they are either degraded or recycled to the cell front for incorporation into the new adhesions (Paleyek et al., 1998; Pierini et al., 2000; Laukaitis et al., 2001). The fact that prevention of integrin internalization by a calcineurin inhibitor that inhibits the cell mobility provides the best evidence to date that integrin recycling is an essential aspect of cell motility (Lawson and Maxfield, 1995). On the other hand, a biphasic relationship exists between the adhesive strength of \( \beta_1 \)-integrins and their ability to mediate the cell movement. Cell movement increases progressively with adhesive strength, but is reduced beyond a certain point of optimal interaction (Hangan-Steinmann et al., 1999). This is why changing in the expression of \( \alpha_5 \beta_1 \)-integrin in some tumours correlates with their invasiveness (Fujita et al., 1995). Thus, we propose that the increased internalization of \( \alpha_5 \beta_1 \)-integrin following IGF-I stimulation may promote migration of EVT cells either via modulating the adhesive strength of \( \alpha_5 \beta_1 \)-integrin or though up-regulation of adhesion turnover and formation of new adhesions. The importance of the increased internalization of \( \alpha_5 \beta_1 \)-integrin in IGF-I-induced migration of EVT cells needs further study.

Considering the fact that IGF-I can stimulate the formation of the FA in several cell types (Leventhal et al., 1997; Kabir-Salmani et al., 2003), the possibility of the co-localization of \( \alpha_5 \beta_1 \)-integrin with the components of FA as integrin-based structures was studied next. Results obtained from immunofluorescent double-staining demonstrated that \( \alpha_5 \beta_1 \)-integrin was not co-localized with \( \alpha_5 \beta_1 \)-integrin, pFAK, paxillin or vinculin at FA of EVT cells. This is in agreement with previous reports showing that \( \alpha_5 \beta_1 \)-integrin was not detected in FA of fibroblast cells in two-dimensional culture systems (Geiger et al., 2001). Likewise, using green fluorescent protein in living migrating cells, the co-localization of \( \alpha_5 \)-integrin with paxillin or \( \alpha \)-actinin was not observed (Laukaitis et al., 2001). This is in contrast to the previous report that employed a three-dimensional culture system for fibroblastic cells and observed the co-localization of \( \alpha_5 \beta_1 \)-integrin with paxillin and FAK (Cukierman et al., 2001). More studies are required to determine the possibility of the co-existence of \( \alpha_5 \beta_1 \)-integrin with components of the EVT cells and its interaction with \( \alpha_5 \beta_1 \)-integrin on EVT cell surface in three-dimensional culture system and in vivo. Emerging evidence shows that interactions between different integrins on the cell surface can strongly influence the adhesive function of individual receptors (Lishko et al., 2003; Ly et al., 2003). Considering the fact that \( \alpha_5 \beta_1 \)-integrin has a pivotal role in the migratory function of several cell types (Jones et al., 1996; Kabir-Salmani et al., 2003) and that \( \alpha_5 \beta_1 \) regulates the \( \alpha_5 \beta_1 \)-mediated adhesion and migration (Ly et al., 2003), it is tempting to suggest that IGF-I might affect the migratory function of EVT cells though ‘crosstalk’ and activation of both \( \alpha_5 \beta_1 \) and \( \alpha_5 \beta_1 \)-integrins.

In conclusion, our findings indicate that the binding of \( \alpha_5 \beta_1 \)-integrin to the RGD domain of the FN substrate is essential to the migratory event of EVT cells. We speculate that IGF-I stimulates the migration of these cells, at least in part, via modulation of \( \alpha_5 \beta_1 \)-integrin recycling by regulating its internalization.

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