Epidermal Growth Factor Enhances Invasive Activity of BeWo Choriocarcinoma Cells by Inducing $\alpha 2$ Integrin Expression

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Abstract. The trophoblast, an important component of the mammalian placenta, has several essential biological roles in the maintenance of pregnancy. First, trophoblast cells must attach to the uterine endometrium, and then they must invade to a depth at which the vascular network exists. Here, we investigated the effects of epidermal growth factor (EGF) on $\alpha 2$ integrin expression, adhesiveness to collagen, and invasive activity using human BeWo choriocarcinoma cells. EGF induced the expression of $\alpha 2$ integrin mRNA and protein, as shown by Northern blotting, Western blotting, and flow cytometry. Human chorionic gonadotropin (hCG) secretion was enhanced by the addition of EGF, which suggests that the BeWo cells functionally differentiated similarly to normal trophoblasts. EGF also dose-dependently stimulated the invasiveness of BeWo cells. Antibody against $\alpha 2$ integrin inhibited this effect, suggesting that it may be mediated by an increase of cell surface integrin. EGF had no effect on the adhesiveness of BeWo cells to collagen, whereas it stimulated the chemokinetic activity in a dose-dependent manner. The increase of chemokinetic activity was suppressed by antibody against $\alpha 2$ integrin. These results suggest that EGF may induce $\alpha 2$ integrin expression in trophoblast cells, thereby enhancing their invasiveness into the endometrium via an increase of their chemokinetic activity.

Key words: Trophoblast, Epidermal growth factor, $\alpha 2$ integrin, Invasion

(Endocrine Journal 50: 703–714, 2003)

FORMATION of the maternal-fetal interface, a consequence of trophoblast differentiation, is essential for the maintenance of pregnancy. During human placental development, trophoblast cells establish a firm adhesive base on the uterine wall, invade the superficial uterine vasculature and migrate along the endometrium [1, 2]. The invasion is initiated as soon as implantation occurs, and the invasive activity is greatly accelerated when the columnar epithelium (chorionic villi) is formed [3, 4]. The final destination of the trophoblast invasion is the uterine spiral arteries,

where the cells obtain an oxygen supply [5].

The interactions of trophoblast cells with various extracellular matrix (ECM) proteins play important roles in the maintenance of the adhesion and invasion into the uterus. Integrins, the major cell surface receptors for ECM proteins, are membrane glycoproteins that anchor extracellular adhesion proteins to cytoskeletal components [6, 7]. Integrins are heterodimers composed of different α and β subunits and include cell surface receptors for fibronectin, collagen, laminin and vitronectin [8, 9]. Many reports have shown that cell surface integrins are expressed in trophoblast cells [10–12]. For example, cytotrophoblast (CTB) and villi stem cells express $\alpha 6 \beta 4$ integrin. As differentiation proceeds, the CTB upregulates the expression of $\alpha 5 \beta 1$ integrin in cell columns and of $\alpha 1 \beta 1$ integrin in the uterine wall, while it downregulates the expression of

Received: December 3, 2002

Accepted: July 22, 2003

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$\alpha 6 \beta 4$ integrin. The switch from $\alpha 6 \beta 4$ to $\alpha 5 \beta 1$ integrin appears to be essential to the differentiation of the CTB [13].

In general, $\alpha 2 \beta 1$ integrin is a major receptor for collagen, and its expression is associated with reorganization of connective tissue in fibroblasts [14]. Recently, it was reported that $\alpha 2 \beta 1$ integrin is transiently expressed in the CTB during implantation [15]. These facts raise the possibility that cytokines or growth factors secreted around the maternal-fetal interface play a role in regulating the integrin expression profiles. We focused on epidermal growth factor (EGF) among such factors, because there have been several reports that EGF is produced in the uterus during the peri-implantation period [16–18]. In addition, the EGF receptor is expressed on the trophoectoderm [19] and EGF promotes mouse trophoblast outgrowth [20] and blastocoel expansion [21] as well as human cytotrophoblast invasion [22].

In this study, we investigated the effect of EGF on the integrin expression profile and the invasive activity of human BeWo choriocarcinoma cells. BeWo cells have several characteristics of the normal trophoblast, including the secretion of human chorionic gonadotropin (hCG) and the expression of high affinity receptors to EGF [23], and provide a system for elucidating biological mechanisms in trophoblast cells. We show here that EGF induces the invasiveness of BeWo cells via an increase of their chemokinetic activity and that the expression of $\alpha 2$ integrin is involved in the induction.

Materials and Methods

Antibodies

The anti-human $\alpha 2$ integrin antibodies used in this study were as follows: for immunoblot experiments, rabbit polyclonal antibody (Cat #AB1936, Chemicon) was used; for invasion assays and Boyden chamber assays, mouse monoclonal antibody P1E6 (Cat #MAB1950Z, Chemicon) was used. Anti-GSK3 α antibody (H-75) was purchased from Santa Cruz Biotechnology Inc.

The antibodies used in flow cytometry analysis, invasion assays, adhesion assays, and Boyden chamber assays were as follows: anti- $\alpha 1$: HP2B6, $\alpha 2$: P1E6, $\alpha 3$: P1B5, $\alpha 4$: SG/73, $\alpha 5$: KH/72, $\alpha 6$: GoH3, $\beta 1$: SG/

19, respectively. SG73, KH/72, and SG/19 were kind gifts from Dr. Kensuke Miyake (Institute of Medical Science, University of Tokyo). GoH3 and HP2B6 were purchased from Chemicon.

Cell culture

The BeWo human choriocarcinoma cell line was maintained in HAM's F-12K (Sigma) supplemented with 15% FBS (JRH Biosciences Nichirei Co.), 100 IU/ml penicillin, and 100 μ g/ml streptomycin. The cells were maintained as monolayers in 250-ml flasks (Falcon Becton Dickinson) at 37°C in a humidified atmosphere of 5% CO₂ in air. Human recombinant EGF (236-EG), LIF (250-L), and TGF- β (100-B) were purchased from R & D Systems, Inc.

Determination of hCG secretion profile

The BeWo cells (5×10^5) were plated in 24-well plates in 1 ml/well of HAM's F-12K supplemented with 15% FBS. The next day, the medium was replaced with HAM's F-12K containing 1% FBS with or without 10 nM EGF. After 12 h, 24 h, or 48 h, the culture media were collected and the concentration of hCG in the culture media was measured using an enzyme immunoassay analyzer (Nissui Seiyaku Corporation). To normalize for the cell growth effects, the number of cells was counted by staining the cells with Trypan Blue and examining them under a microscope at each time indicated.

Flow cytometry

The BeWo cells were treated with HAM's F-12K medium containing 1% FBS with or without 10 nM EGF for 24 h. Then the cells were trypsinized and resuspended in suspension medium (MEM with 0.03% NaN₃, 1% FBS). The suspended cells were transferred to a 96-well cell culture plate at a density of 2×10^5 cells/well and incubated for 30 min at 4°C with 10 μ l of anti- $\alpha 2$ integrin mouse monoclonal antibody (100 μ g/ml). The cells were washed twice with the suspension medium and incubated with 1.5 μ l of FITC-conjugated rabbit anti-mouse immunoglobulin for 30 min at 4°C. The cells were then washed twice and resuspended in the suspension medium. Cell surface integrin expression was analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

RNA extraction

Total cellular RNA was extracted from BeWo cells cultured in HAM's F-12K medium supplemented with 1% FBS for 24 h and harvested immediately (0 time) or further cultured for 3, 6, 12, or 24 h in the same medium in the presence of 10 nM EGF and then harvested. Total RNA was extracted using Trizol Reagent (Gibco BRL) following the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260 nm.

Northern blotting

Total RNA (10 μ g) was separated by formaldehyde-agarose gel electrophoresis, transferred to Nytran membranes (Hybond-N, Amersham-Pharmacia) and analyzed by Northern blot hybridization using a 32 P-labeled cDNA probe. Loading of equal amounts of RNA in each lane was verified by staining 28S and 18S ribosomal RNAs on the membranes with methylene blue and assessment by visual inspection. The radiolabeled probes for human $\alpha 2$ integrin or human GAPDH were synthesized by random priming of full-length cDNA using a Megaprime labeling kit (Amersham-Pharmacia) according to standard methods. The final post-hybridization washing consisted of two rinses in $2 \times$ SSC at 42°C for 10 min each, followed by a rinse in $0.5 \times$ SSC, 0.1% SDS at 65°C for 10 min. The radioactivity of each band was quantified using a BAS2000 imaging system (Fuji Film, Tokyo, JAPAN).

Western blotting

The BeWo cells were washed twice with phosphate-buffered saline and lysed in ice-cold buffer consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl_2 , 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM PMSF. The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent (Bio-Rad). The extracts (150 μ g of protein) were then subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. After a blocking reaction (5% nonfat dry milk in Tris-buffered saline, pH 7.4, 0.05% Tween-20) for 1 h, membranes were incubated in the blocking buffer supplemented

with anti- $\alpha 2$ integrin or anti-GSK-3 α rabbit polyclonal antibody overnight at 4°C. After incubation with horseradish peroxidase-linked anti-rabbit IgG, the membranes were developed by using the ECL system (Amersham-Pharmacia) according to the manufacturer's instructions. The signal intensity of each band was quantified using a FluorChem 8000 (Alpha Innotech Corporation Inc., San Leandro, CA, USA).

Invasion assay

BeWo cells (2×10^5) were trypsinized and resuspended in HAM's F-12K medium containing 0.1% BSA (Sigma). Then the cells were seeded into the upper compartments of transwell chambers containing polycarbonate membranes with 12- μ m-diameter pores, which were placed in the wells of a 12-well tissue culture plate (Corning Costar Corporation) [24, 25]. The upper surface of the membranes was coated with cold Matrigel (100 μ g/ml) and the filters were air-dried for 24 h at room temperature. Prior to use, the upper chambers were rehydrated with 100 μ l of HAM's F-12K medium for 1 h. The lower chambers were loaded with 1.5 ml of HAM's F-12K medium containing 0.1% BSA in the absence or presence of EGF at the indicated concentrations for 24 h. After 24 h of culturing, non-invading cells in the upper chambers were removed with a cotton swab. Cells on the underside of the membranes were fixed with 10% formaldehyde for 30 min at room temperature, and stained with hematoxylin and eosin. For quantification, the cells invading to the lower surface were counted under a microscope in pre-determined fields at a magnification of $\times 200$. For blocking assays, anti- $\alpha 1$ or - $\alpha 2$ integrin antibody or control mouse IgG1 was added to the medium in the upper chamber at a final concentration of 10 μ g/ml immediately after plating the cells.

Cell adhesion assay

The BeWo cells were treated with HAM's F-12K medium containing 1% FBS in the absence or presence of EGF at the indicated concentrations for 24 h. For adhesion assays [26, 27], 96-well tissue culture plates were coated with type IV collagen or fibronectin for 2 h at 37°C. Nonspecific binding sites were blocked by preincubation with 3% BSA for 1 h at 37°C. The blocking buffer was then removed by suctioning. The

cells were trypsinized and resuspended in HAM's F-12K medium containing 0.1% BSA. The dispersed cells were seeded (2×10^5 cells/well) in the culture plates and allowed to adhere for 1 h at 37°C. Then the medium was removed by suctioning and 0.04% crystal violet was added to each well. The cells were stained for 10 min at room temperature and washed three times with PBS, and then 30 μ l of DMSO was added to each well. After incubation for 10 min at room temperature, 70 μ l of double-distilled water was added to each well. The absorbance was measured at 590 nm.

Boyden chamber assay

Chemotaxis of BeWo cells was quantitated using a modified 48-well Boyden chamber (Neuroprobe, MD) [26–28] and polycarbonate membranes with a pore diameter of 12 μ m (Neuro Probe Inc., Gaithersburg, MD) coated on both sides with collagen by immersion in a 10 μ g/ml collagen solution. The BeWo cells were treated with HAM's F-12K medium containing 1% FBS in the absence or presence of EGF at the indicated concentrations for 24 h. Then the cells were seeded at a concentration of 1×10^6 /ml in HAM's F-12K medium containing 0.1% BSA in the top wells, and HAM's F-12K medium containing 0.1% BSA and various concentrations of EGF was placed in the bottom wells. Cells were then allowed to migrate for 3 h at 37°C in a humidified atmosphere of 5% CO₂ in air. Migrating cells on the lower surface of the membranes were fixed with formaldehyde and stained with hematoxylin. For quantitative assessment of migrating cells, the cells in four different fields from each well were counted. Next, blocking assays were performed in the same way, except that the cells were pretreated with anti- α 1 or α 2 integrin antibody or mouse IgG1 for 1 h at 4°C before they were added to the top wells.

Statistical analysis

The paired *t* test was used to assess whether there were significant differences in hCG secretion between EGF-treated and untreated cells at each time point after EGF treatment. The same test was performed to examine the statistical significance of differences in the invasive activity or chemokinesis activity between the control and EGF-treated cells at each EGF concentration. Two-way ANOVA was used to deter-

mine whether there were significant differences in the invasive activity or chemokinesis activity among treatments with different antibodies. $P < 0.05$ was considered statistically significant. The analyses were performed with a statistics software package (Statview 4.02, Abacus Concepts Inc., Berkeley, CA).

Results

Effect of EGF on functional differentiation of BeWo choriocarcinoma cells

Previous studies utilizing isolated trophoblast cells as well as several choriocarcinoma cell lines [29, 30] indicated that hCG secretion was increased by addition of EGF in a time-dependent manner. We first examined the responsiveness of BeWo choriocarcinoma cells to EGF by monitoring hCG secretion in order to confirm that the characteristics of the BeWo cells were appropriate for subsequent analysis. Fig. 1 shows a representative hCG secretion profile after 12 h, 24 h,

Fig. 1. Effect of EGF on hCG secretion from BeWo choriocarcinoma cells

BeWo choriocarcinoma cells were cultured in HAM's F-12K medium supplemented with 1% FBS in the absence or presence of different concentrations of EGF for 12, 24, or 48 h. The culture medium was collected and subjected to quantification of the hCG concentration using an automated enzyme immunoassay system. The effect of cell number was taken into account by normalizing the level of hCG relative to the number of cells in each well. Data are shown as the mean $\pm$ S.D. ($n = 4$). The paired *t* test was used to assess whether there were significant differences in hCG secretion levels between control and EGF-treated conditions at each treatment time point. Statistically significant differences are indicated by asterisks (**, $P < 0.01$).

and 48 h in the presence or absence of various concentrations of EGF. The hCG concentration increased with time in a dose-dependent manner when the cells were treated with EGF, whereas the hCG concentration remained unchanged in the absence of EGF. These results show that the BeWo cells we used in the following experiments possess important biological

characteristics of CTBs.

Expression of $\alpha 2$ integrin in response to EGF

We next investigated the effects of EGF on the expression of integrins on the surface of BeWo cells. Fig. 2A shows representative flow cytometry analyses

Fig. 2. Effect of EGF on the expression of various integrins on the surface of BeWo cells

BeWo cells were cultured for 24 h under the same conditions described in the legend for Fig. 1. The cells were stripped from the culture flasks, incubated with mouse anti-integrin antibodies, and then washed extensively with chilled PBS. The washed cells were then incubated with FITC-conjugated anti-mouse IgG antibody and analyzed using a FACScan flow cytometer. The effects of 10 nM EGF on (A) $\alpha 1$ - $\alpha 6$ integrins and (B) $\beta 1$ integrin, and the effect of 0.1 ng/ml LIF and 10 ng/ml TGF- β on $\alpha 2$ integrin expression (C) are shown. The *ordinate* shows cell numbers (Counts), and the *abscissa* shows the relative fluorescence intensity on an arbitrary scale (FL1-H). All experiments were repeated at least three times with consistent results.

of cells incubated with or without 10 nM EGF for 24 h. With regard to α integrins, it was demonstrated that several molecules, including $\alpha 1$, $\alpha 2$, $\alpha 5$, and $\alpha 6$ integrins, were constitutively expressed, while the expression of $\alpha 3$ and $\alpha 4$ integrins was weak. Treatment of the cells with EGF resulted in enhanced expression of $\alpha 2$ integrin alone. Flow cytometry at various points, including 6 h and 12 h, after the addition of 10 nM EGF showed that EGF enhanced the expression of $\alpha 2$ integrin at those times, but not as markedly as at 24 h after the addition of EGF (data not shown). Because $\alpha 2$ integrin forms a heterodimer with $\beta 1$ integrin, we also examined the effect of EGF on $\beta 1$ integrin expression by performing flow cytometry analyses under the same conditions as for $\alpha 2$ integrin. We found that $\beta 1$ integrin was abundantly expressed on BeWo cells. However, EGF had no apparent effect on $\beta 1$ integrin expression (Fig. 2B). To test whether the augmentation of $\alpha 2$ integrin expression occurs with cytokines other than EGF, the cells were also treated with 0.1 ng/ml of leukemia inhibitory factor (LIF) or 10 ng/ml transforming growth factor β (TGF- β), both of which are cytokines known to induce

differentiation of trophoblast cells [31, 32]. These two cytokines did not alter the expression profile of $\alpha 2$ integrin (Fig. 2C).

Enhancement of $\alpha 2$ integrin mRNA and protein expression by EGF

To examine the mechanism by which the cell surface $\alpha 2$ integrin protein was induced by EGF, we next investigated the effect of EGF on $\alpha 2$ integrin expression at the level of mRNA expression. For this purpose, we performed Northern blot analysis with total RNA samples prepared from BeWo cells after stimulation with 10 nM EGF. As shown in Fig. 3A, $\alpha 2$ integrin mRNA expression was maximally augmented after 3 h of EGF treatment (image scanning showed a 8.5-fold increase compared to the basal level; data not shown). Moreover, a time-course study showed that the amount of $\alpha 2$ integrin mRNA gradually decreased thereafter through 24 h after stimulation. These results suggest that the expression of $\alpha 2$ integrin is induced by EGF at the level of mRNA expression. Next, we examined the time-course of

Fig. 3. Induction of $\alpha 2$ integrin mRNA and protein by EGF

A, BeWo cells were cultured for the periods indicated in the panel in the presence of 10 nM EGF under the same culture conditions as described in the legend for Fig. 1. The RNA samples were electrophoresed on a 1.2% agarose gel and then blotted onto a Nytran membrane. The blot was hybridized with ^{32}P -labeled $\alpha 2$ integrin cDNA. The same membrane was rehybridized with ^{32}P -labeled GAPDH cDNA and the radioactivity in each band was quantitated as described in Materials and Methods. B, (Left) Total cell lysates from BeWo cells treated under the same conditions as in A were prepared as described in Materials and Methods. Each lysate was subjected to SDS-PAGE and the separated proteins were transferred to a nitrocellulose membrane. Immunoblotting was performed with rabbit anti-human $\alpha 2$ -integrin antibody (top) or anti-GSK3 α antibody (bottom) and immunoreactive proteins were visualized using the ECL system (Amersham-Pharmacia). $\alpha 2$: $\alpha 2$ integrin, GSK3: glycogen synthase kinase 3 alpha. (Right) Densitometric analysis was carried out to quantify $\alpha 2$ integrin protein expression in the absence (white column) or the presence (black columns) of EGF. The density of each $\alpha 2$ integrin band was normalized by the density of the GSK3 protein band in the same lane.

expression of $\alpha 2$ integrin protein by Western blot analysis. EGF augmented $\alpha 2$ integrin protein expression; however, the induction was observed much later than that of mRNA. The level of $\alpha 2$ integrin protein gradually increased starting 6 h after the addition of EGF, and it reached a plateau from 12 h through 24 h, while the amount of GSK-3 α protein remained constant (Fig. 3B).

Involvement of $\alpha 2$ integrin expression in EGF-induced invasive activity of BeWo cells

To assess the biological effects of EGF-induced $\alpha 2$ integrin expression, we examined the invasive activity of BeWo cells in the presence or absence of EGF. Invasion assays showed that treatment with EGF for 24 h increased the invasive activity of BeWo cells in a dose-dependent manner (Fig. 4A). Because EGF induced $\alpha 2$ integrin expression in the cells, we next examined the effect of antibody against $\alpha 2$ integrin on the invasive activity to address whether $\alpha 2$ integrin is involved in this effect. As shown in Fig. 4B, the invasive activity of the EGF-treated cells was markedly

decreased by the addition of the anti- $\alpha 2$ integrin antibody compared to the activity of cells treated with control mouse IgG1 or anti- $\alpha 1$ integrin antibody. These data indicate that $\alpha 2$ integrin plays a major role in the EGF-induced invasive activity of BeWo cells.

Effect of EGF on adhesion of BeWo cells to collagen

Because the invasion process involves adhesion and chemokinesis of the cells, we investigated the effect of EGF on the adhesion of BeWo cells. Fig. 5 shows the results of adhesion assays using a type IV collagen matrix. EGF had no effect on the adhesion of BeWo cells at a concentration of 1 nM or 10 nM. When the matrix was changed to fibronectin, another ligand for $\alpha 2$ integrin, EGF did not alter the adhesion of BeWo cells either under the same experimental conditions (data not shown).

Enhancement of chemokinesis of BeWo cells by EGF via induction of $\alpha 2$ integrin

We next performed Boyden chamber assays to

Fig. 4. Effect of EGF on, and involvement of $\alpha 2$ integrin in, the invasive activity of BeWo cells

A, BeWo cells (2×10^5) were pre-incubated in the absence (control) (white column) or presence (black columns) of different concentrations of human EGF for 24 h, collected from the culture flasks and resuspended in HAM's F-12K medium. The cells were then placed in transwell chambers on membranes coated with 100 μ g/ml Matrigel. Twelve hours later, the membrane was removed from each chamber and stained with hematoxylin to visualize the cells that had reached the bottom surface of the membrane. The cell numbers are shown on the graph. Regression analysis indicated a significant positive relationship between the EGF concentration and the number of invading cells. The paired *t* test was used to assess whether there were significant differences in the number of invading cells between the control and each EGF-treated condition. Statistically significant differences are indicated by asterisks (**, $P < 0.01$).

B, BeWo cells (2×10^5) were pre-incubated with (right set of columns) or without (control) (left set of columns) 10 nM human EGF for 24 h in the presence of mouse anti- $\alpha 2$ integrin antibody (black columns), control mouse IgG1 (white columns), or anti- $\alpha 1$ integrin antibody (shaded columns). Results are expressed as the number of invading cells. Data are shown as the mean $\pm$ S.D. ($n = 4$). Two-way ANOVA was used to assess whether there were significant differences in the number of invading cells within the same EGF concentration with different antibody treatments. Statistically significant differences are indicated by asterisks (**, $P < 0.01$).

Fig. 5. Effect of EGF on the adhesion of BeWo cells to a collagen matrix

BeWo cells were cultured in the absence (control) (white column) or presence (black columns) of different concentrations of human EGF for 24 h. The cells were trypsinized and resuspended in HAM's F-12K medium. The dispersed cells were seeded (2×10^5 cells/well) in 96-well collagen-coated tissue culture plates coated with a 10 $\mu\text{g/ml}$ collagen solution. One hour later, the culture medium was removed by aspiration and the cells remaining on the culture plate surface were stained with 0.04% crystal violet solution and the A595 was measured to quantify the ability to adhere to collagen and is shown on the ordinate (Fluorescence units). Data are shown as the mean $\pm$ S.D. ($n = 4$).

quantify the chemokinetic activity of the cells. EGF enhanced the chemokinetic activity of BeWo cells in a dose-dependent manner (Fig. 6A). We also investigated the effect of anti- $\alpha 2$ integrin antibody on the EGF-induced chemokinetic activity. Fig. 6B shows that the chemokinetic activity of BeWo cells was suppressed by the addition of the same antibody that caused inhibition in the invasion assay compared to the chemokinetic activity of cells treated with control mouse IgG1 or anti- $\alpha 1$ integrin antibody. These results indicate that the increase of $\alpha 2$ integrin is involved in the induction of the chemokinetic activity of BeWo cells by EGF.

Discussion

Integrins provide physical linkage between the cytoskeleton and ECM and they transduce signals initiated by extracellular interactions [33]. The α -integrin subunits have diverse amino acid sequences and distinct functions [33]. $\alpha 2$ integrin is expressed on many cell types, including breast, colon, fibroblast and endothelial cells, and forms a heterodimer with $\beta 1$ integrin [34]. In skin and mucosa, $\alpha 2$ integrin is located

Fig. 6. Effect of EGF on, and the involvement of $\alpha 2$ integrin in, the chemokinetic activity of BeWo cells

A, Nitrocellulose membranes with 12- μm pores were coated with 10 $\mu\text{g/ml}$ collagen solution and placed in Boyden chambers. BeWo cells (1×10^6) pre-incubated in the absence (control) (white column) or presence (black columns) of different concentrations of EGF for 24 h were placed into each chamber well and cultured for 3 h. The cells that reached the bottom surface of the membrane were stained with hematoxylin and the numbers of cells were counted. Regression analysis indicated a significant positive relationship between the EGF concentration and the number of migrating cells. Statistically significant differences are indicated by asterisks (**, $P < 0.01$).

B, BeWo cells were pre-incubated with (right columns) or without (left columns) 10 nM human EGF for 24 h in the presence of the same anti- $\alpha 2$ integrin antibody described in the legend for Fig. 4 (black columns), control mouse IgG1 (white columns), or anti- $\alpha 1$ integrin antibody (shaded columns). The antibody or control IgG1 was added to the cells immediately after the cells were plated in the Boyden chambers. Results are expressed as the number of migrating cells. Data are shown as the mean $\pm$ S.D. ($n = 5$). Two-way ANOVA was used to assess whether there were significant differences in the number of migrating cells within the same EGF concentration among each antibody treatment. Statistically significant differences are indicated by asterisks (**, $P < 0.01$).

between keratinocytes, suggesting its participation in cell-cell adhesion [35]. $\alpha 2$ integrin also plays an important role in cell migration and in the reorganization of the collagenous matrix of fibroblasts [36] and osteogenic cells [37]. In the present study we demonstrated a positive effect of EGF on $\alpha 2$ integrin expression in human BeWo choriocarcinoma cells. Previous reports indicated that EGF increases the level of $\alpha 2$ integrin expression in various cells such as squamous carcinoma cells [38] and a colon carcinoma cell line [39]. Furthermore, EGF was shown to induce cell migration activity via upregulation of $\alpha 2$ integrin in gastric cancer cells [26]. However, little information is available about how EGF regulates $\alpha 2$ integrin expression and affects the invasive activity of trophoblast cells. Therefore, our findings should provide new insights into the possible functions of $\alpha 2$ integrin in human trophoblast cells.

EGF increased the cell surface expression of $\alpha 2$ integrin alone among several α subunits in BeWo cells. These results are consistent with reports showing that EGF upregulated the expression of $\alpha 2$ integrin but not that of $\alpha 3$ or $\alpha 5$ integrins in human squamous carcinoma cells [40]. Moreover, it is notable that the responses of $\alpha 2$ and $\beta 1$ integrins to EGF were different. Other studies have also demonstrated such differences between the responses of these two integrins. For example, increased surface expression of $\alpha 2\beta 1$ integrin was reported to be a consequence of increased steady-state levels of $\alpha 2$ integrin, while $\beta 1$ mRNA was not altered during megakaryocytic differentiation [40]. Glucocorticoid suppressed $\alpha 2$ integrin expression markedly in the CTB, but had a weaker effect on $\beta 1$ integrin [41]. In addition, EGF did not stimulate the biosynthesis of $\beta 1$ integrin in 3T3-L1 cells [42]. These data suggest the possibility that $\alpha 2\beta 1$ integrin expression is mainly affected by the regulation of $\alpha 2$ integrin, and that signaling events specific for individual integrin heterodimers are triggered by the α -subunit.

The expression of $\alpha 2$ integrin was enhanced by EGF at the level of mRNA expression. The analysis of the 5'-flanking region of the $\alpha 2$ integrin gene revealed that several transcription factor binding sites, including AP-1, Sp1 and nuclear factor- κ B (NF- κ B) sites, are present in the promoter region [43], and in fact the regulation of $\alpha 2$ integrin has been shown to involve NF- κ B [44]. Furthermore, EGF has been shown to induce NF- κ B in breast cancer cell lines

[45] and A431 carcinoma cells [34]. Both $\alpha 2$ and $\beta 1$ integrin promoters contain AP-1 and Sp1 sites. Therefore, NF- κ B is a potential candidate for the mediator of EGF induction of $\alpha 2$ integrin mRNA by EGF.

By culturing mouse embryos in culture vessels coated with ECM proteins, it was shown that at least three alpha integrins ($\alpha 2$, $\alpha 6$, and $\alpha 7$) are expressed when embryos attach to the matrix [46]. With regard to EGF action, direct effects of EGF and transforming growth factor alpha (TGF- α) on trophoblast outgrowth of the mouse blastocyst *in vitro* was demonstrated [20]. Based on these facts, we hypothesized that $\alpha 2$ integrin plays a significant role during the initial stage of trophoblast invasion into the endometrium. We have provided several lines of evidence here that an increase of $\alpha 2$ integrin is involved in the EGF-induced invasiveness and chemokinesis of BeWo cells. In normal human pregnancy, the invasion of the uterus by the CTB is accompanied by marked changes in the expression of cell adhesion molecules [47]. There have been previous reports showing that $\alpha 1$ or $\alpha 5$ integrins are involved in the invasive activity of the trophoblast cells [48]. However, it was hitherto unknown whether $\alpha 2$ integrin affects the invasiveness of these cells. Therefore, it is noteworthy that we have identified $\alpha 2$ integrin as an important player in the invasive activity of BeWo cells. EGF and the EGF receptor are expressed by normal placental trophoblast cells from as early as the fourth to fifth week of gestation, and their expression then continues until term [49]. Thus, EGF may be a key factor in the biological function of the trophoblast via the regulation of $\alpha 2\beta 1$ integrin expression throughout pregnancy. The fact that $\alpha 2$ integrin is involved in growth factor-mediated cell invasion gives us new insights into the biology of the trophoblast. Because both human placental cells and choriocarcinoma cells contain EGF receptors, ligand (s) for the EGF receptor, such as EGF, TGF- α , and heparin-binding EGF, may play important roles in normal as well as abnormal placental cell growth and differentiation [47].

Invasion through the uterine epithelium is presumably promoted by proteases on the trophoblast cell surface. Integrins facilitate invasion by recruiting ECM-degrading proteinases such as matrix metalloproteinases (MMPs) [50]. Recent studies have revealed that two collagen receptors, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, regulate distinct functions in cells, although

both integrins are expressed by many cell types. $\alpha 2\beta 1$ integrin mediates activation of p38 mitogen-activated protein kinase (p38MAPK) [51] and regulates MMP-1 and MMP-13 [52], whereas $\alpha 1\beta 1$ integrin activates the Ras-extracellular signal-related kinase (Ras-ERK) signal transduction pathway [53, 54]. The distinct functions of individual α -subunits sharing a common $\beta 1$ -integrin may be due to their activation of different signaling processes. Further studies of the $\alpha 2\beta 1$ integrin signaling cascade will lead to a better understanding of the biological activity of trophoblast cells.

Acknowledgments

The authors thank Dr. Kensuke Miyake (Institute of Medical Science, University of Tokyo) for providing anti- $\alpha 4$, - $\alpha 5$ and - $\beta 1$ integrin antibodies, Dr. Dai Imamura (Nippon Organon K.K.) for valuable assistance with the statistical analysis, Dr. Masahiro Sakata and Dr. Elizabeth Nakajima for critical reading of the manuscript, and Ms. Ayako Okamura and Ms. Tomoko Iwaki for technical and secretarial assistance.

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