Peripheral blood mononuclear cells in early pregnancy promote invasion of human choriocarcinoma cell line, BeWo cells

Haruto Egawa1,4, Hiroshi Fujiwara1,5, Takeshi Hirano2, Takahiro Nakayama1,4, Toshihiro Higuchi1, Keiji Tatsumi1, Takahide Mori3 and Shingo Fujii1

1Department of Gynaecology and Obstetrics, Faculty of Medicine, Kyoto University, Sakyo-ku, 2Daigo Watanabe Hospital, Fushimi-ku, Kyoto, 3Osaka National Hospital, Osaka, Japan

4Present address: Department of Obstetrics and Gynaecology, Japan Baptist Hospital, Sakyo-ku, Kyoto, Japan

5Correspondence to: Hiroshi Fujiwara, Department of Gynaecology and Obstetrics, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, 606-8397, Japan. E-mail: fuji@kuhp.kyoto-u.ac.jp

BACKGROUND: During the establishment of the maternal blood circulation around the implanting human embryo, maternal peripheral blood mononuclear cells (PBMC) directly contact trophoblasts. To determine the physiological significance of this interaction, the effects of PBMC obtained from pregnant women on the proliferative and invasive properties of a human choriocarcinoma cell line, BeWo cells, were examined. METHODS AND RESULTS: PBMC were obtained from women in early pregnancy and from women in the secretory phase of the menstrual cycle. PBMC from pregnant women significantly increased the number of invading BeWo cells in an invasion assay without affecting the proliferation of BeWo cells ($P < 0.05$). No significant changes were observed in the co-cultures with PBMC from non-pregnant women. The addition of conditioned medium, which was prepared by 2 days of incubation with PBMC from pregnant women, also enhanced BeWo cell invasion in a dose-dependent manner. Moreover, when PBMC obtained from non-pregnant women were incubated with recombinant HCG (0–10 IU/ml) for 2 days, significant augmentation of the effect on BeWo cell invasion was observed in the conditioned medium from HCG-treated PBMC ($P < 0.05$). CONCLUSION: This study indicated that soluble factor(s) secreted from PBMC promote BeWo cell invasion. It also showed the possible involvement of HCG in the regulation of BeWo cell invasion by PBMC. These findings suggest crosstalk between maternal PBMC and trophoblasts via soluble factor(s), which may play an important role in early embryo implantation.

Key words: BeWo cells/human chorionic gonadotrophin/invasion/peripheral blood mononuclear cells/trophoblast

Introduction

Embryo invasion is one of the most important steps during implantation (Bischof and Martelli, 1992; Bischof et al., 1995; Tabibzadeh and Babaknia, 1995; Burrows et al., 1996). Within 1 week after the initiation of implantation, the human embryo is buried in the endometrial stroma, and maternal blood enters the lacunar spaces in the differentiating trophoblast (Boyd and Hamilton, 1970; Gersell et al., 1987; Woodruff et al., 1988; Aplin, 1991). In this site, maternal peripheral blood mononuclear cells (PBMC) interact directly with the trophoblast and then return to the systemic circulation.

Recently, we reported that human luteal cells in the corpus luteum during pregnancy express several cell adhesion molecules for T-lymphocytes on their cell surfaces (Fujiwara et al., 1993; Hattori et al., 1995; Higuchi et al., 1999). PBMC derived from women in early pregnancy promoted progesterone production by luteal cells of the corpus luteum during pregnancy to as high a level as HCG in vitro, implying that the function of the corpus luteum is maintained not only by HCG, but also by PBMC (Hashii et al., 1998). We demonstrated that spleen cells derived from pregnant mice promote murine embryo implantation by regulating endometrial receptivity (Takabatake et al., 1997a,b). From these findings, we proposed a new hypothesis that peripheral immune cells receive some signals from the conceptus in the early stage of pregnancy, and consequently they regulate the function of both the corpus luteum and endometrium to support embryo implantation (Hattori et al., 1995; Takabatake et al., 1997b, Hashii et al., 1998). Although these PBMC are suggested to be influenced by the implanting embryo, the physiological role of the interaction between maternal PBMC and trophoblasts at the early implantation site is not yet thoroughly understood.
Our recent study showed that human PBMC derived from pregnant women promoted spreading and invasion of murine embryos in vitro. These promoting effects of PBMC were found to be induced by HCG stimulation, which is one of the most important embryonal signals (Nakayama et al., 2002). These results suggested the presence of crosstalk between maternal PBMC and the implanting embryo. In the present study, to examine whether PBMC had similar effects on human trophoblastic cells, we used a human choriocarcinoma cell line, BeWo cells, and found that PBMC derived from pregnant women enhanced the invasive properties of BeWo cells. The characteristics of soluble factor(s) from PBMC and the cell population responsible for the enhancing effects were further investigated. Since integrins have been reported to be involved in extravillous trophoblast invasion (Damsky et al., 1994), we also examined the involvement of integrin β1 in the stimulatory effect of PBMC on BeWo cell invasion.

Materials and methods

Cells and culture conditions

BeWo cells, a continuous cell line established from a human choriocarcinoma (Patillo and Gey, 1968) were obtained from the Japanese Cancer Research Resources Bank and maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS) (Life Technologies), 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies). The cells were maintained as monolayers in 25 cm² flasks at 37°C in a humidified atmosphere of 5% CO₂ in air.

Antibody

The mouse anti-human CD29 (integrin β1) monoclonal antibody (mAb, clone P4C10, IgG1 class) (Carter et al., 1990) was purchased from Sigma Chemical Company (St Louis, MO, USA). A mouse monoclonal IgG1 antibody (clone DAK-G01; DAKO, Glostrup, Denmark) was used as a negative control antibody. An FITC-conjugated rabbit anti-mouse immunoglobulin polyclonal antibody and a rabbit anti-HCG polyclonal antibody were purchased from DAKO.

Preparation of PBMC

PBMC were prepared as described previously (Emi et al., 1991). Volunteers were recruited from healthy non-pregnant women (secretory phase, cycle day 18–24, n = 17) with a regular menstrual cycle, and from healthy pregnant women (5–10 weeks of gestation, n = 30) with a known duration of gestation. Menstrual cycle dating was determined from the initial day of menstruation. PBMC were isolated from 16 ml of venous blood using Ficol-Hypaque. After centrifugation, PBMC were collected from the interphase layer and washed four times with RPMI 1640. PBMC were suspended in RPMI 1640 supplemented with 10% of FCS at a concentration of 1×10⁶ cells/ml. PBMC were cultured as described above were added to the culture well. In each experiment, a pair of matched PBMC from non-pregnant and pregnant women was used for the invasion assay. After a 24-h culture period, non-invasive BeWo cells and Matrigel on the upper surface of the filter were thoroughly removed with cotton swabs. Cells remaining on the lower surface of the filter, which had migrated through the Matrigel and the filter, were fixed in methanol for 10 min at room temperature, and stained with haematoxylin. For quantification, the cells on the lower surface of the filter were counted under a microscope in five pre-determined fields at a magnification of ×200. The assay was performed in triplicate chambers. The results were expressed as the percentage of the number of invading BeWo cells in the assay in the absence of PBMC. The experiments were repeated 6 times.

Cell viability and proliferation

To examine the viability of BeWo cells, the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) reduction assay was performed in some experiments. The tetrazolium salt, MTT, is metabolized to a coloured formazan salt by mitochondrial enzyme activity in viable cells (Mosmann, 1983).

BeWo cells (2×10⁴) were plated into each well of a 24-well plate in 1 ml of RPMI 1640 with 10% FCS. A basket-type culture well unit (Intercell, 0.45 µm diameter pore; Kurabo, Tokyo, Japan) was put in each well of the culture plates. Then, PBMC (5×10⁵ in 500 µl) were inoculated into the Intercell and were co-cultured with BeWo cells. Direct interaction between BeWo cells and PBMC was prevented by the microper membrane located in the bottom of the Intercell, which could transmit soluble factors such as cytokines. The cells were cultured in triplicate.

After 24-h culture of the BeWo cells with PBMC derived from pregnant or non-pregnant women, the Interells containing PBMC were removed. After the remaining BeWo cells were washed with culture medium, they were incubated in RPMI 1640 with 10% FCS and 500 µg/ml MTT. After 4 h, 300 µl of 2-propanol containing 0.04 N HCl was added to each well. MTT reduction was determined using an automated enzyme-linked immunosorbent assay plate reader (Molecular Devices, Menlo Park, CA, USA) at an optical density of 570 nm. The results were expressed as a percentage of the value in the control assay (BeWo cell culture in the absence of PBMC). The experiments were repeated 5 times.

The effect of PBMC on the proliferation of BeWo cells was also examined. After a 48-h culture with PBMC derived from pregnant or non-pregnant women, the BeWo cells were dispersed with 0.05% trypsin (Difco Laboratories, Detroit, MI, USA) and 0.05% EDTA, and the number of cell nuclei was determined by the citric acid-crystal violet method (Patterson, 1979). The results were expressed as a percentage of the number of BeWo cells cultured in the absence of PBMC. The experiments were repeated 4 times.

Invasion assay using the conditioned media of PBMC

PBMC obtained from pregnant or non-pregnant women were cultured for 48 h at a concentration of 1×10⁶ cells/ml in RPMI 1640 supplemented with 10% FCS. After centrifuging the media from the cultures at 800 g, the supernatant fluids were collected as the conditioned media and subjected to the invasion assay. Part of the fresh culture medium in the lower well (800 µl in total) was replaced by the conditioned media (100, 200 or 400 µl from PBMC of pregnant...
women or 400 µl from PBMC of non-pregnant women). BeWo cells were inoculated on the upper chamber and cultured for 24 h. The number of invading BeWo cells was measured as described above. The results were expressed as a percentage of the number of invading BeWo cells in the assay without any conditioned media. The experiments were repeated 7 times.

In some experiments of the BeWo cell invasion assay, (i) half of the fresh culture medium in the upper chamber (200 µl in total) was replaced by the conditioned medium (100 µl), (ii) half of the culture medium in the lower well (800 µl in total) was replaced by conditioned medium (400 µl), or (iii) both the culture media in the upper chamber and the lower well were replaced by conditioned medium (100 and 400 µl respectively). As a control, the BeWo cell invasion assay was performed without conditioned medium. The PBMC-conditioned media derived from pregnant women were used in the above experiments (n = 6).

In other experiments, mouse anti-human integrin β1 mAb (0, 0.5, 1 or 10 µg/ml, clone P4C10) or control mouse mAb (10 µg/ml, clone DAK-GO-1) was added to the Cell Culture Inserts where BeWo cells had been inoculated. The fresh culture medium in the lower well was replaced by PBMC-conditioned media from pregnant women. The results were expressed as a percentage of the number of invading BeWo cells in the assays without any conditioned medium or mAbs. The experiments were repeated 6 times.

Effects of adherent or non-adherent PBMC on BeWo cell invasion

After isolating PBMC by the Ficoll-Hypaque method, the separated plasma was incubated in 24-well culture plates (Becton Dickinson Labware) for 30 min at 37°C and the plates were washed with PBS to prepare them for use in the following PBMC culture. The washed PBMC suspended in RPMI with 10% FCS were inoculated into six wells (1×10^6 cells/ml, 1 ml per well) of the pretreated 24-well culture plate for 48 h. The non-adherent PBMC were then recovered from each well by washing twice with PBS. All the collected non-adherent PBMC were sedimented and resuspended in fresh RPMI medium containing 10% FCS (4.8 ml). Half of these cells were inoculated into fresh plasma-pretreated wells (three wells, 800 µl per well) and the other half (800 µl/well) was added to three of the wells previously used for PBMC culture; these wells contained unremoved adherent PBMC, which were hereby combined again with non-adherent PBMC.

To the remaining three wells containing adherent PBMC, 800 µl of fresh RPMI medium containing 10% FCS was added per well. Then BeWo cells suspended in 200 µl of culture medium were inoculated into a Matrigel-coated chamber inserted in each well and the invasion assay was performed as described above. As a control, the BeWo cell invasion assay was performed without PBMC in the plasma-pretreated wells. The results were expressed as a percentage of the number of invading BeWo cells in the assays without PBMC. The experiments were repeated 6 times.

Flow cytometric analysis of integrin β1 expression in BeWo cells

BeWo cells were cultured with PBMC derived from pregnant or non-pregnant women for 24 h using Intercells as described above. After removing PBMC, BeWo cells were dispersed with 0.05% trypsin (Difco Laboratories) and 0.05% EDTA. The dispersed BeWo cells were washed with Hank’s balanced salt solution (HBSS) containing 0.1% NaN₃. The precipitated BeWo cells were incubated with anti-human integrin β1 mAb (P4C10) or control mAb (DAK-GO-1) for 30 min at 4°C. After they were washed 3 times, the BeWo cells were incubated with FITC-conjugated rabbit anti-mouse immunoglobulin polyclonal antibody (DAKO) for 30 min at 4°C in the dark. The expression of integrin β1 in the washed BeWo cells was analysed using FACScan flow cytometry (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). The experiments were repeated 3 times.

Invasion assay using recombinant HCG-treated PBMC

PBMC derived from women in the secretory phase were cultured with recombinant HCG (rHCG; Rohto Pharmaceutical Co. Ltd., Osaka, Japan; 0, 1 or 10 IU/ml) in RPMI 1640 medium supplemented with 10% FCS for 48 h at 37°C in a humidified atmosphere of 5% CO₂ in air. To exclude the influence of possible contamination by unknown agents in the r-HCG, the solution of rHCG (10 IU/ml) was preadsorbed with anti-HCG polyclonal antibody (DAKO). Anti-HCG polyclonal antibody was conjugated to Affigel-10 (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Honda et al., 1995). The reagent containing r-HCG (10 IU/ml) was preincubated with anti-HCG polyclonal antibody-conjugated Affigel-10 for 2 h at 4°C. After it was sterilized by passage through a microfilter, this anti-HCG-adsorbed solution was added to PBMC cultures. After 2 days of culturing, all the cells, including the attached cells, were collected and washed with the medium 4 times. The cells were then suspended at a concentration of 1×10^6 cells/ml and subjected to the invasion assay. The results were expressed as a percentage of the number of invading BeWo cells in the assay without PBMC. The experiments were repeated 5 times.

Direct effects of rHCG on the viability and invasive property of BeWo cells

To examine the direct effect of HCG on BeWo cell viability, rHCG (0, 1, or 10 IU/ml) was added to BeWo cell culture. After 24 h of culturing, the MTT reduction assay was performed as described above.

In addition, rHCG (0, 1, or 10 IU/ml) was added to the BeWo cell invasion assay in the absence of PBMC. After 24 h, the numbers of invading BeWo cells were counted and compared among the three groups. These experiments were repeated 5 times.

Statistical analysis

The data are expressed as mean ± SEM, and were analysed by the paired t-test or one-way analysis of variance, followed by Scheffé’s F-test for multiple comparisons. Differences were regarded as significant at P < 0.05.

Results

Invasion assay of BeWo cells co-cultured with PBMC

The BeWo cell invasion assay was performed in the presence of PBMC derived from pregnant or non-pregnant women. PBMC derived from pregnant women significantly increased the number of invading BeWo cells as compared with PBMC from non-pregnant women and with the control (assay without PBMC) (P < 0.05, P < 0.01 respectively, n = 6). On the other hand, in the presence of PBMC derived from non-pregnant women, a slight, but not significant, increase in BeWo cell invasion was observed (Figure 1).

Effect of PBMC on BeWo cell viability and proliferation

The MTT reduction assay was performed to assess the effects of PBMC on BeWo cell viability after a 24 h co-culture (n = 5). No significant effect on BeWo cell viability was observed in co-cultures with either non-pregnant or pregnant PBMC (Table 1).

The proliferation of BeWo cells co-cultured with PBMC for 48 h was also assessed (n = 4). BeWo cell proliferation was
Effects of PBMC derived from non-pregnant and pregnant women on BeWo cell invasion. The invasion assay was carried out in the presence of PBMC from non-pregnant women. (B) Assayed with PBMC from pregnant women. The results are expressed as a percentage of the number of invading BeWo cells without PBMC (control assay), and are expressed as the mean ± SEM of six independent experiments. PBMC derived from women in early pregnancy promoted BeWo cell invasion as compared with PBMC from non-pregnant women. *Indicates P < 0.05.

Invasion assay using conditioned media of PBMC

BeWo cell invasion assay was carried out in the presence of conditioned media prepared by incubation with PBMC derived from pregnant or non-pregnant women (n = 5). The invading cell number was significantly increased by the PBMC-conditioned medium derived from pregnant women in a dose-dependent manner as compared with the control (assay without conditioned medium) when the culture medium in the lower well was partially replaced by conditioned medium (Figure 2). In contrast, the PBMC-conditioned medium derived from non-pregnant women had no significant effect on the BeWo cell invasion even when a large volume of it was used for supplementation.

On the other hand, BeWo cell invasion tended to be suppressed when half of the fresh culture medium in the upper chamber was replaced with PBMC-conditioned media derived from pregnant women, although the suppression was not significant as compared with the control. No significant effect on BeWo cell invasion was observed when the fresh media in both the upper chamber and lower well were simultaneously replaced by the conditioned medium (Figure 3).

Effects of adherent or non-adherent PBMC on BeWo cell invasion

BeWo cell invasion was significantly promoted when the BeWo cells were co-cultured with adherent PBMC, whereas no increase was observed when BeWo cells were co-cultured with non-adherent PBMC (Figure 4). There was also no significant difference in the effect on BeWo cell invasion between adherent PBMC alone and combined adherent and non-adherent PBMC.
PBMC promote trophoblast invasion

Figure 4. Effects of adherent or non-adherent PBMC on BeWo cell invasion. PBMC were separated into adherent and non-adherent PBMC as described in Materials and methods. BeWo cell invasion was significantly enhanced when the BeWo cells were co-cultured with adherent PBMC (A), whereas no increase was observed when BeWo cells were co-cultured with non-adherent PBMC (B). There was also no significant difference in the effect on BeWo cell invasion between adherent PBMC alone (A) and combined adherent and non-adherent PBMC (C). ** Indicates P < 0.01. # Shows a significant difference as compared with the assay without PBMC.

Figure 5. Effect of anti-integrin β1 antibody on BeWo cell invasion. The BeWo cell invasion assay was performed in the presence of anti-integrin β1 mAb (P4C10, 0, 0.5, 1 or 10 µg/ml) or control mAb (DAK-GO-1, 10 µg/ml). Open bar: in the absence of conditioned medium. Closed bar: in the presence of of medium conditioned by 2 days of incubation with pregnant PBMC. The results are expressed as a percentage of the number of invading cells in the assay without conditioned medium or antibodies (control assay). Each experiment was repeated 6 times and the results were expressed as the mean ± SEM. Anti-integrin β1 mAb (P4C10, 10 µg/ml) significantly decreased BeWo cell invasion to 70.8% in the absence of conditioned medium and to 52.2% in the presence of conditioned medium as compared with the negative control mAb (DAK-GO-1, 10 µg/ml). * ** Indicates P < 0.05 and P < 0.01 respectively. # Shows a significant difference as compared with the assay without the conditioned medium or antibodies.

Effect of anti-integrin β1 mAb on BeWo cell invasion in the presence of PBMC-derived conditioned media

In the absence of the conditioned medium, anti-integrin β1 mAb (10 µg/ml) decreased BeWo cell invasion to 70.8% of the control (negative control mAb, DAK-GO-1, 10 µg/ml) (P < 0.01, n = 6) (Figure 5).

In the presence of the conditioned medium (400 µl), BeWo invasion was significantly increased as described above. Under these conditions, anti-integrin β1 mAb (10 µg/ml) significantly decreased BeWo cell invasion to 52.2% of the control (negative control mAb, DAK-GO-1, 10 µg/ml) (P < 0.05, n = 6) (Figure 5).

Effect of PBMC on the expression of integrin β1 on the BeWo cells

Flow cytometric analysis of integrin β1 expression on BeWo cells was performed after co-culturing with PBMC (n = 3). No significant alteration in integrin β1 expression was detected. Typical histograms are shown in Figure 6.

Invasion assay of BeWo cells co-cultured with r-HCG-treated PBMC

When BeWo cells were co-cultured with r-HCG-treated PBMC, the PBMC that had been pretreated with r-HCG at a concentration of 10 IU/ml significantly increased the number of invading BeWo cells compared with PBMC without rHCG pretreatment (P < 0.05, n = 5) (Figure 7). On the other hand, PBMC treated with anti-HCG-adsorbed solution did not show a promoting effect on BeWo cell invasion.

Direct effects of r-HCG on the viability and invasive activity of BeWo cells

In the absence of PBMC, no significant effects of rHCG were observed in either the MTT reduction assay or the invasion assay (data not shown).

Discussion

In the presence of PBMC derived from pregnant women, the number of invading BeWo cells was significantly increased. Since PBMC did not affect the proliferation of BeWo cells, we concluded that PBMC derived from pregnant women enhanced the invasive activity of BeWo cells. In this co-culture system, PBMC and BeWo cells were separated by an inserted chamber that inhibited their direct contact. Therefore, we speculated that some soluble factor(s) secreted from PBMC are involved in the promotion of BeWo cell invasion. To confirm this, the BeWo cell invasion assay was performed in the presence of conditioned medium that had been cultured with PBMC derived from pregnant and non-pregnant women. The addition of medium conditioned by the PBMC from pregnant women stimulated BeWo cell invasion in a dose-dependent manner. This indicates that some soluble factor(s) secreted from PBMC enhanced the invasive activity of BeWo cells.

Next, the characteristics of the soluble factors that affect BeWo cell invasion were investigated. In contrast to the effect of adding the PBMC-conditioned medium to the lower well, BeWo cell invasion was rather suppressed when the conditioned medium was added to the upper chamber in the invasion assay. Since no significant effect was observed when this conditioned medium was added to both the upper chamber and lower well, the soluble factors in the conditioned medium may affect BeWo cell function as chemoattractants for BeWo cell migration rather than as stimulators of BeWo cell invasion.
Integrin $\beta_1$ expression on BeWo cells detected by flow cytometry. BeWo cells were cultured for 24 h with PBMC obtained from non-pregnant and pregnant women, and the level of integrin $\beta_1$ expression in BeWo cells was analysed by flow cytometry. A representative histogram of the flow cytometry is shown. A, control using a negative control antibody (DAK-GO-1, CTR). B-D, integrin $\beta_1$ expression detected by immunostaining with anti-human integrin $\beta_1$ mAb (P4C10). (A), (B) Cultured without PBMC. (C) Cultured with PBMC from non-pregnant women. (D) Cultured with PBMC from pregnant women. No notable difference was observed between the culture with PBMC from non-pregnant and pregnant women.

Effect of PBMC treated with r-HCG on BeWo cell invasion. PBMC from non-pregnant women were incubated with r-HCG for 48 h and their effect on the BeWo cell invasion assay was tested. (A) PBMC that had been incubated without HCG for 48 h. (B) PBMC incubated with HCG (1 IU/ml). (C) PBMC incubated with HCG (10 IU/ml). (D) PBMC incubated with anti-HCG-adsorbed solution. The results are expressed as a percentage of the number of invading cells in the assay without PBMC (control assay), and shown as the mean ± SEM of five independent experiments. The promoting effect of PBMC on BeWo cell invasion was significantly enhanced by HCG treatment in a dose-dependent manner. * Indicates $P < 0.05$. # Shows a significant difference as compared with the control assay without PBMC ($P < 0.01$).

The promoting effect of PBMC derived from non-pregnant women was lower than that of PBMC derived from pregnant women and not significant, suggesting that there are functional differences between the PBMC of pregnant and non-pregnant women. Similarly, functional differences were observed in the stimulatory effects of PBMC on progesterone production of luteal cells derived from the corpus luteum of early pregnancy (Hashii et al., 1998). When luteal cells were co-cultured with PBMC, the cytokines produced differed depending on whether PBMC from pregnant women or from non-pregnant women were used (Hashii et al., 1998). Thus, the biological activity of PBMC is considered to be changed by the presence of a conceptus during early pregnancy. During implantation, the human embryo invades the maternal capillaries in the endometrial stroma. At this site, maternal PBMC can directly interact with the trophoblast. This local interaction between PBMC and the embryo may induce the alteration in PBMC function.

To investigate the signalling pathway from the embryo to PBMC, we examined the effect of HCG, which is a major embryonal signal, on PBMC function. Using the invasion assay, we found that HCG-treated PBMC enhanced the invasive activity of BeWo cells, indicating that HCG can induce PBMC to become activators of BeWo cell invasion. Yagel et al. reported that high concentrations of HCG directly attenuated the invasion of trophoblasts during the first trimester by decreasing urokinase plasminogen activator and collagenase activities (Yagel et al., 1993). On the other hand, Zygmunt et al. demonstrated that HCG increased the invasive property and collagenase activity of JEG-3 cells (Zygmunt et al., 1998). In the present study, HCG had no direct effect on BeWo cell viability or invasion in the absence of PBMC. Although our findings are not in agreement with those of the above reports,
we can say at least that the promoting effect of HCG observed in this study is exerted via PBMC, not directly on BeWo cells.

At a concentration of 1 IU/ml, HCG-stimulated PBMC showed a slight promoting effect on BeWo invasiveness, but it was not significant. A significant effect of HCG was clearly observed at the higher concentration of 10 IU/ml. From the early stages of embryo implantation, such a high concentration of HCG can be achieved locally at the implantation site. Since a relatively high dose of HCG was used, we needed to exclude the possible effect of unknown contaminating substances in the recombinant HCG to correctly assess our experimental results. Therefore, by adsorption to an anti-HCG antibody-conjugated gel, HCG was specifically removed from the solution, which was prepared as HCG reagent. The anti-HCG-adsorbed solution had diminished ability to induce PBMC to promote BeWo cell invasion, indicating a specific effect of HCG on PBMC.

Our recent study showed that human PBMC derived from pregnant women promoted spreading and invasion of murine embryo cells in vitro and that HCG could increase the promoting effect of PBMC (Nakayama et al., 2002). The results of the present study are compatible with the findings with murine embryo cells. In this study, we also demonstrated that the enhancing effects on BeWo cells were mainly exerted by adherent PBMC. When peripheral blood immune cells migrate into various tissues, they must attach to endothelial cells via adhesion molecules. The adherent PBMC probably contain the immune cell populations that are thought to be able to migrate into extravascular spaces. Several cytokines such as interleukin-1β, transforming growth factor-β, tumour necrosis factor-α, interleukin-10 and so on had been reported to regulate trophoblast invasion (Librach et al., 1994; Irving and LaLa, 1995; Todt et al., 1996; Meisser et al., 1999; Roth and Fisher, 1999). On the other hand, there is recruitment from the vascular spaces of immune cells, including large granular cells, T-lymphocytes and monocytes, during embryo implantation and placentation in the decidual tissue (Bulmer, 1996). Since these immune cells are thought to secrete various cytokines, it is possible that the peripheral blood immune cells are activated initially at the implantation site, then move to the endometrial vessels and migrate into the endometrial stroma, influencing the invasion of extravillous trophoblasts in the decidual tissue.

Although BeWo cells have several characteristics of normal trophoblasts, they were originally derived from a human choriocarcinoma. When choriocarcinoma cells invade the surrounding tissues, they interact with immune cells. Thus, the results of this study also indicate the possibility that the invasive activity of choriocarcinoma cells can be affected by immune cells that are activated to produce enhancing factors via the stimulation by HCG at the interaction site, because choriocarcinomas usually produce HCG hormone.

It has been reported that β1-related integrins, especially integrin α5β1, are involved in trophoblast invasion, and the switching of integrin expression on extravillous trophoblasts has been proposed to regulate normal trophoblast invasion (Damsky et al., 1992; Damsky et al., 1994). Recently, we found that extravillous trophoblasts express the CD9 molecule on the cell surface in association with integrin α5β1 (Hirano et al., 1999a). Using the same invasion assay, anti-integrin β1 antibody (P4C10) was demonstrated to inhibit BeWo cell invasion in a dose-dependent manner (Hirano et al., 1999b). In the present study, we examined the inhibitory effect of P4C10 against BeWo cell invasion that was stimulated by conditioned medium derived from PBMC cultures. In the absence of the conditioned medium, P4C10 attenuated BeWo cell invasion to 70% of the control level. In this study, we also demonstrated that the enhancing effects on BeWo cells were mainly exerted by co-culturing with PBMC. P4C10 further inhibited BeWo cell invasion to nearly 50% of the control level in the presence of PBMC-conditioned medium. These findings indicate that integrin β1 is involved in the stimulatory mechanism of PBMC on BeWo cell invasion.

In conclusion, this study demonstrated that some soluble factor(s) secreted by PBMC derived from pregnant women promoted BeWo cell invasion in vitro.

The major factor(s) responsible for the promoting effect were found to be chemotactants for BeWo cell migration rather than stimulators. The population responsible for this promoting effect was adherent PBMC. Although BeWo cells are derived from choriocarcinoma, not from normal trophoblast cells, these findings suggest that there is crosstalk between the embryo and PBMC at the implantation site, where the embryo secretes HCG that acts on PBMC, and that consequently the stimulated PBMC change their function so that they positively regulate trophoblast invasion. Since the interaction between PBMC in maternal blood and the invading embryo is an inevitable event in the early stage of embryo implantation, its physiological significance should be further clarified.

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