

# Human peripheral blood mononuclear cells (PBMC) in early pregnancy promote embryo invasion *in vitro*: HCG enhances the effects of PBMC

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**BACKGROUND:** The aim of this study was to investigate the role of peripheral blood mononuclear cells (PBMC) in embryo invasion at the implantation site and to estimate the effect on PBMC function of human chorionic gonadotrophin (HCG) that is secreted from the human embryo. **METHODS AND RESULTS:** The effect of PBMC on the invasiveness of murine embryos was examined using an invasion assay. PBMC obtained from women in early pregnancy (5–9 weeks gestation) significantly enhanced both spreading of murine embryos on Matrigel and invasion beneath the gel. These effects were greater than those of PBMC obtained from non-pregnant women in the secretory phase (cycle day 16–24) and the control (in the absence of PBMC). When PBMC obtained from non-pregnant women were incubated with recombinant HCG (10 IU/ml) for 2 days and were subjected to invasion assay using murine embryos, PBMC treated with HCG significantly promoted both spreading and invasion of murine embryos as compared with the non-treated PBMC. On the other hand, embryo outgrowth was not affected by HCG in the absence of PBMC, showing no direct effect of HCG on embryo invasion. **CONCLUSION:** This study indicated that PBMC from pregnant women promoted murine embryo invasion *in vitro* and this effect of PBMC was enhanced by HCG. These findings suggest that PBMC at the implantation site are activated by HCG secreted from the embryo, following which PBMC regulate embryo invasion.

*Key words:* early pregnancy/embryo invasion/HCG/PBMC

## 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 differentiating trophoblasts of the human embryo destroy the surrounding capillaries in the endometrial stroma, and maternal blood enters the lacunar spaces in the trophoblasts (Boyd and Hamilton, 1970; Gersell *et al.*, 1987; Woodruff *et al.*, 1988; Aplin, 1991). At this site, maternal peripheral blood mononuclear cells (PBMC) directly interact with trophoblasts and then they return to the systemic circulation. These PBMC are probably influenced by several signals from the implanting embryo, but the physiological role of the interaction between trophoblasts and PBMC at the early implantation site has not yet been reported.

Recently, we have reported that spleen cells derived from pregnant mice promote murine embryo implantation by regulating endometrial receptivity (Takabatake *et al.*, 1997a,b). In humans, 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 the early stage of pregnancy promoted progesterone production by luteal cells of the corpus luteum during pregnancy as much as did human chorionic gonadotrophin (HCG) *in vitro*, implying that the function of the corpus luteum of pregnancy is maintained not only by HCG, but also by PBMC (Hashii *et al.*, 1998). From these findings, we proposed the new hypothesis that peripheral immune cells receive signals from the conceptus in the early stage of pregnancy, then regulate the differentiation of both corpus luteum and endometrium to support embryo implantation (Hattori *et al.*, 1995; Takabatake *et al.*, 1997b, Hashii *et al.*, 1998).

In this study, to elucidate the physiological roles of PBMC in the embryo at the implantation site, we examined the effects of PBMC obtained from women in the early stage of pregnancy on embryo invasion by invasion assay using murine embryos. In addition, to examine how PBMC are activated by the embryo, we investigated the effect of HCG on PBMC, which is an embryonal signal.

## Materials and methods

### 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 16 to 24,  $n = 10$ ) with a regular menstrual cycle and from healthy pregnant women (5 to 9 weeks of gestation,  $n = 10$ ) whose duration of gestation was known. PBMC were isolated from 16 ml of venous blood using Ficoll-Hypaque. After centrifugation, PBMC were collected from the interface and washed with RPMI 1640 medium. After washing four times, the supernatant of PBMC from pregnant and non-pregnant women was combined to make a common suspension medium. All PBMC were resuspended using this common suspension medium and recentrifuged. By using this procedure, even if very potent soluble factors were still present in the suspension medium from pregnant PBMC, these factors would be equally divided into each group, preventing the effects of contaminated soluble factors (Hashii *et al.*, 1998). PBMC were suspended at a concentration of  $1 \times 10^6$  cells/ml with RPMI 1640 supplemented with 10% of fetal calf serum (FCS) (Dainippon Pharmaceutical Co., Osaka, Japan), 100 U/ml penicillin and 100 µg/ml of streptomycin, and were subjected to invasion assay, as described below.

### HCG pretreatment of PBMC

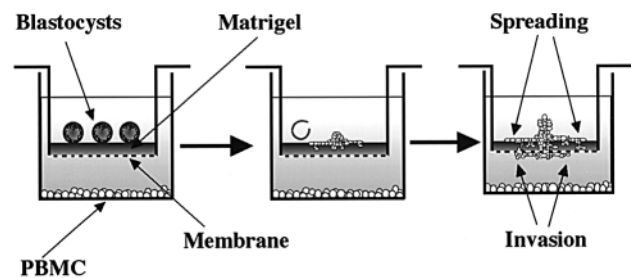
PBMC derived from women in the secretory phase ( $n = 6$ , cycle day 16–24) were cultured with recombinant HCG (rHCG; Rohto Pharmaceutical Co. Ltd., Osaka, Japan; 0 and 10 IU/ml) in RPMI 1640 medium supplemented with 10% FCS for 48 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After culture, the cells were collected and washed ( $\times 4$ ) with the same medium as described above. PBMC were then suspended at a concentration of  $1 \times 10^6$  cells/ml and were used for invasion assays.

### Preparation of mouse blastocysts

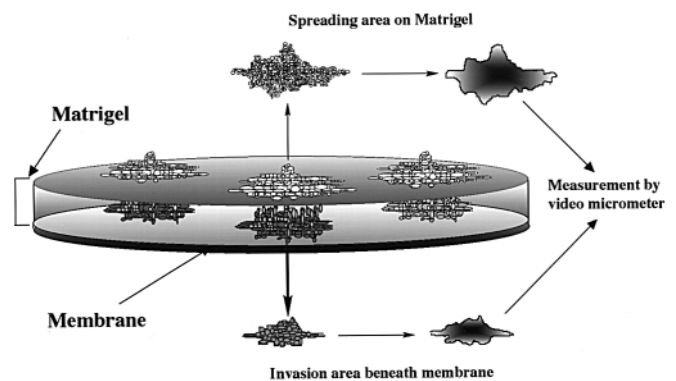
Mouse blastocysts were prepared as previously reported (Takabatake *et al.*, 1997a). Briefly, 4-week-old female and 3-month-old male ICR mice were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed under controlled lighting (14 h light, 10 h dark) and given water and food *ad libitum*. Blastocysts were recovered from donor ICR mice (pregnancy day 4), which were 5 weeks old and had been mated after stimulation by pregnant mares serum gonadotrophin (PMSG) (5 IU; Teikokuzoki Co., Tokyo, Japan) and HCG (5 IU; Teikokuzoki Co.).

### Invasion assay

The invasion assay was carried out as previously described, with slight modifications (Katsuragawa *et al.*, 1997). As shown in Figure 1, blastocysts were cultured on cell culture inserts (three blastocysts per culture insert, 6.4 mm in diameter, Becton Dickinson Labware, Bedford, MA, USA) containing polyethylene terephthalate membranes with 8 µm diameter pores, and these culture inserts were placed in each well of a 24-well tissue culture plate (Corning Costar Co., Cambridge, MA, USA). The upper surface of the filters was coated with cold Matrigel (15 µg/filter, Collaborative Research Co., Bedford, MA, USA) and air-dried aseptically. Prior to use, the inserts were rehydrated with 100 µl of warm RPMI 1640 for 2 h. Three blastocysts suspended in 500 µl of RPMI 1640 with 10% FCS were added to the filter coated with Matrigel (three embryos in each insert). Under the Matrigel, PBMC ( $1.2 \times 10^6$  cells in 1200 µl of RPMI 1640 with 10% FCS), which were prepared from non-pregnant women or pregnant women as described above, or culture medium only (control) were added to each culture well. In each experiment, one pair of



**Figure 1.** An invasion assay. The cell culture insert containing polyethylene terephthalate membrane with 8 µm diameter pores and Matrigel were placed in each well of a 24-well tissue culture plate. Three murine blastocysts were located on the Matrigel. Under the Matrigel, PBMC ( $1.2 \times 10^6$  cells), prepared from non-pregnant or pregnant women, or culture medium only (control) were added to each culture well. The invasion assay was performed under 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub> for 7 days.



**Figure 2.** Measurement of spreading and invasion. Every 24 h during the culture, spreading areas on the Matrigel were measured by video micrometer. After 7 days of culture, cells on the lower surface of the membrane, which had migrated (invaded) through the Matrigel and the filter membrane, were fixed in methanol and stained with haematoxylin. The invaded areas were also measured by video micrometer.

blood samples from pregnant and non-pregnant women was used. The invasion assay was performed under the hypo-oxygenic conditions (5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>) to prevent embryos from oxygenic toxicity (Nakayama *et al.*, 1998, 1999). Every 24 h, spreading areas were measured by video micrometer (VM-50; Olympus, Tokyo, Japan) (Figure 2). After 7 days of culture, the upper surface of the filter was 'scrubbed' three times with a cotton swab. 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. The invaded areas were also measured by video micrometer (Figure 2). From each well, the median values for spreading and invasion areas for three embryos were obtained. The assay was performed in triplicate chambers. The means of the three wells were defined as the value for each group for one experiment.

In some experiments, blastocysts were inoculated into the culture chamber inserts in the presence or absence of HCG (10 IU/ml) without PBMC. The spreading and invasion areas were measured as described above during a 7 day culture.

### Statistical analysis

The data were expressed as means  $\pm$  SEM, and were analysed by repeated measures ANOVA, followed by Scheffé's *F*-test for post-hoc multiple comparisons. To assess the direct effect of HCG on

**Table I.** Spreading areas of murine embryos in invasion assay co-cultured with PBMC

Culture day	2	3	4	5	6	7
Control (without PBMC)	4.5 ± 0.4	9.2 ± 0.5	14.6 ± 1.1	22.9 ± 2.8	33.1 ± 1.8	52.9 ± 3.2
PBMC from non-pregnant women	4.7 ± 0.4	9.7 ± 0.5	16.3 ± 0.7	34.9 ± 1.7	63.3 ± 3.8 <sup>b</sup>	100.8 ± 7.2 <sup>b</sup>
PBMC from pregnant women	4.8 ± 0.5	10.5 ± 0.5	18.2 ± 0.7 <sup>a</sup>	38.0 ± 2.0 <sup>b</sup>	82.4 ± 6.5 <sup>bc</sup>	154.0 ± 11.1 <sup>bd</sup>

Values are expressed as means ± SEM of the spreading areas from 10 experiments. Significant differences as compared with the control are denoted by <sup>a</sup>*P* < 0.05 and <sup>b</sup>*P* < 0.01 respectively. Significant differences as compared with PBMC from non-pregnant women are denoted by <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 respectively.

embryo invasion, paired *t*-tests were used to compare the HCG treated group with the control group. Differences were regarded as significant at the 5% level.

## Results

### *Invasion assay of murine embryos co-cultured with PBMC*

PBMC derived from pregnant women significantly increased the spreading areas of murine embryos from day 4 to day 7 (Table I). PBMC derived from non-pregnant women also promoted spreading areas, but this effect was significantly less than that from pregnant women. These spreading embryos are shown in Figure 3.

In the same invasion assay, the extra-embryonic cells that invaded beneath the filter membrane were fixed, stained with haematoxylin and measured by video micrometer to assess the invasion areas after a 7 day culture.

Similarly to embryo spreading, the invasion areas of murine embryos beneath the filter were significantly enhanced by co-culture with PBMC derived from pregnant women (Figure 4). PBMC derived from non-pregnant women also promoted murine embryo invasion, but this effect was significantly lower than in that from pregnant women. These cells are shown in Figure 5.

### *Invasion assay of murine embryo co-cultured with rHCG-treated PBMC*

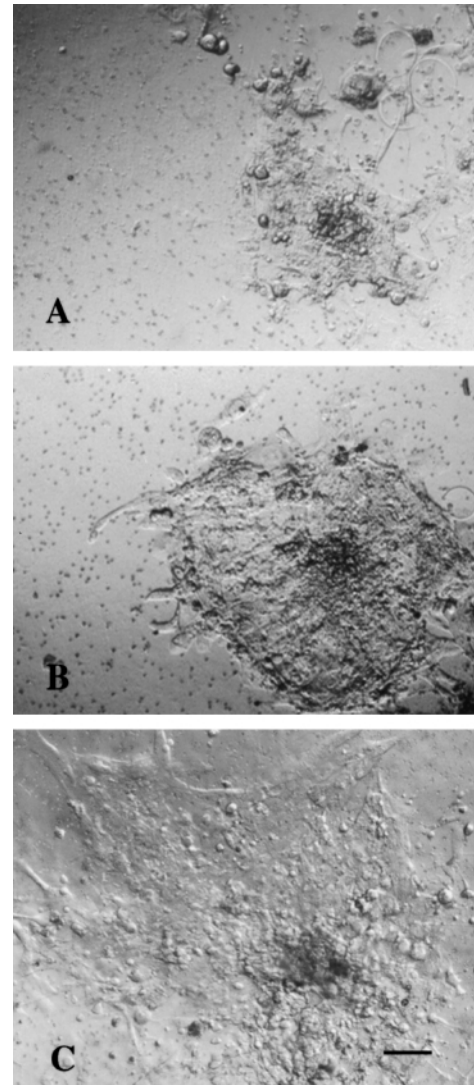
PBMC that were pretreated with HCG showed significantly increased spreading areas of murine embryos as compared with HCG non-treated PBMC (Table II). HCG treated PBMC also enlarged the invasion areas of murine embryos (Figure 6).

### *The direct effect of rHCG on embryo invasion*

To examine the direct effect of HCG on murine embryo invasion, we subjected murine embryos to invasion assay in the presence or absence of HCG, and the spreading and invasion areas were analysed. These experiments were repeated five times. There were no significant differences in spreading or invasion areas between the HCG treated and non-treated embryos, showing no direct effect of HCG on murine embryo invasion (Table III).

## Discussion

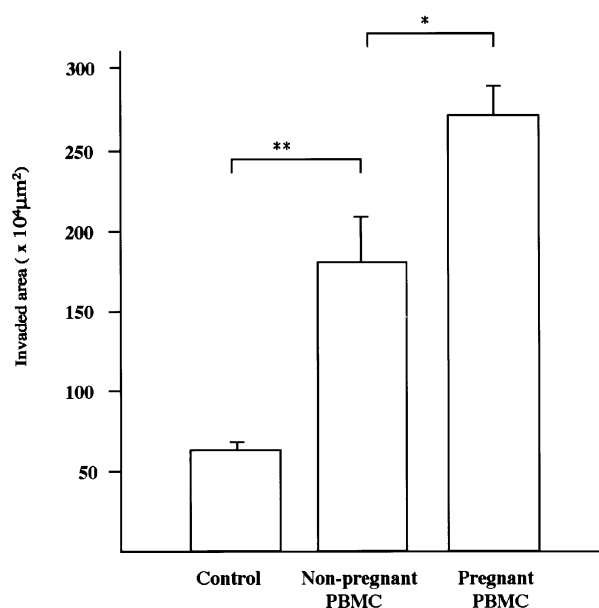
After the embryo attaches to the endometrial epithelial cells, it invades the endometrial stroma by degrading maternal surrounding tissue and destroying the endometrial vessels.



**Figure 3.** Spreading murine embryos on Matrigel. Spreading embryos on day 6 were observed by inverted microscope. (A) Control (without PBMC). (B) Cultured with PBMC obtained from non-pregnant women. (C) Cultured with PBMC from pregnant women. Bar = 100 µm.

During this process, the circulatory system of maternal blood around the embryo becomes established. Although the interaction between PBMC in the maternal blood and the invading embryo is an inevitable event in the early stage of embryo implantation, the physiological significance of this is not yet fully understood. In this study, human PBMC were shown to have promoting effects on spreading and invasion of murine



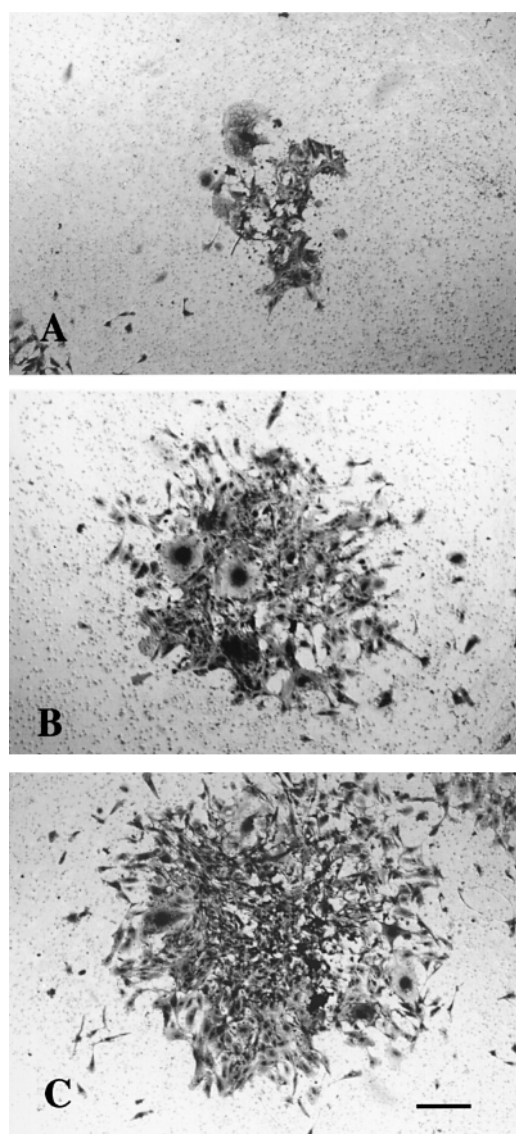


**Figure 4.** Invasion areas of murine embryos in invasion assay co-cultured with PBMC. Values are expressed as means  $\pm$  SEM of the invaded areas in 10 experiments. \* and \*\* indicate  $P < 0.05$  and  $P < 0.01$  respectively.

embryo. This suggests that the initial interaction of the invading embryo with PBMC at the implantation site is involved in the regulation of early embryo invasion.

The promoting effect of PBMC derived from women in early pregnancy was significantly greater than that of PBMC obtained from non-pregnant women, indicating that there are some functional differences in PBMC between pregnant and non-pregnant women. Using human luteal cell culture from the corpus luteum of early pregnancy, similar functional alterations of PBMC to assist embryo implantation have been observed (Hashii *et al.*, 1998). PBMC obtained from pregnant women more effectively stimulated progesterone production by luteal cells, which is advantageous in that it maintains endometrial receptivity for the embryo. Based on this and several related findings, we proposed that PBMC in early pregnancy receive information on the presence of the embryo at the implantation site. They then transmit this information into the ovary via blood circulation and regulate the function of the corpus luteum during pregnancy to indirectly facilitate embryo implantation in cooperation with HCG (Fujiwara *et al.*, 1993; Hattori *et al.*, 1995; Hashii *et al.*, 1998). Since PBMC from pregnant women were demonstrated to promote embryo invasion, it is also suggested that PBMC directly effect embryo outgrowth in the endometrium. This leads us to the concept that PBMC play an important role in establishing embryo implantation by indirectly regulating ovarian function and by directly promoting embryo invasion.

The next item for consideration is how the effects of PBMC on embryo invasion are enhanced. It is theoretically reasonable that PBMC are activated by the embryo at the implantation site. If so, what factors from the embryo serve as stimulators? At the implantation site, HCG is well known to be secreted from the embryo into the maternal blood and is transmitted to the ovary via blood circulation to support the function of the



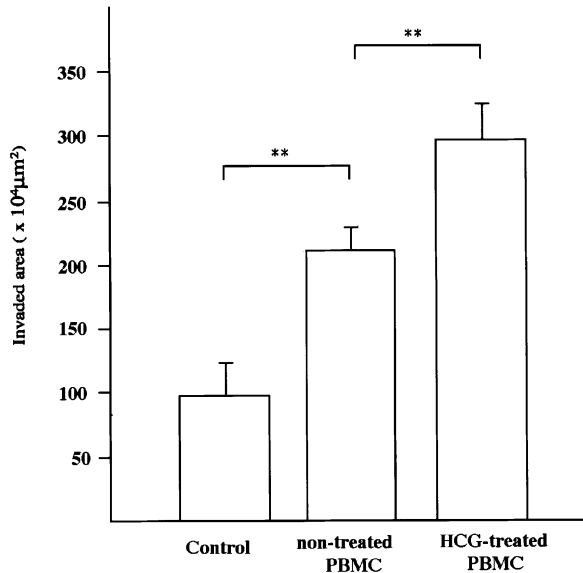
**Figure 5.** Invaded extra-embryonic cells beneath membranes were fixed in methanol on day 7 and stained by haematoxylin. (A) Control (without PBMC). (B) Cultured with PBMC obtained from non-pregnant women. (C) Cultured with PBMC from pregnant women. Bar = 200  $\mu$ m.

corpus luteum during pregnancy. As an embryonal signal, we therefore examined the effects of HCG on PBMC function. After PBMC were incubated with rHCG for 2 days, embryo invasion assays were performed under co-culture with rHCG-treated PBMC. The spreading and invasion areas of murine embryos were significantly increased in the co-culture with rHCG-treated PBMC compared with those with non-treated PBMC. These findings suggest that the promoting effect of PBMC on embryo invasion is enhanced by an important embryonal signal, namely HCG. Although the effect of HCG on the residual immune cells in the endometrium was not examined in this study, it is also possible that HCG secreted by the embryo can activate endometrial immune cells to support its own invasion. A recent study reported that HCG directly stimulated the invasion of human cytotrophoblastic JEG-3 cells *in vitro* (Zygmunt *et al.*, 1998). On the contrary, the spreading and invasion of embryos were not affected by

**Table II.** Spreading areas of murine embryos in the invasion assay co-cultured with rHCG-treated PBMC

Culture day	2	3	4	5	6	7
Control (without PBMC)	5.6 ± 0.3	10.0 ± 0.3	13.4 ± 0.3	19.9 ± 1.0	29.6 ± 1.5	49.9 ± 3.1
Non-treated PBMC	5.5 ± 0.4	10.4 ± 0.6	14.6 ± 0.8	25.5 ± 1.8	64.3 ± 5.1 <sup>b</sup>	108.1 ± 10.3 <sup>b</sup>
HCG-treated PBMC	5.7 ± 0.4	10.5 ± 0.6	16.2 ± 0.9 <sup>a</sup>	31.1 ± 2.0 <sup>b</sup>	79.9 ± 3.6 <sup>bc</sup>	151.7 ± 11.2 <sup>bd</sup>

Values are expressed as means ± SEM from six experiments. Significant differences as compared with the control are denoted by <sup>a</sup> $P < 0.05$  and <sup>b</sup> $P < 0.01$  respectively. Significant differences as compared with non-treated PBMC are denoted by <sup>c</sup> $P < 0.05$  and <sup>d</sup> $P < 0.01$  respectively.

**Figure 6.** Invaded areas of murine embryos in the invasion assay co-cultured with rHCG treated PBMC. Values are expressed as means ± SEM from six experiments. \*\* indicates  $P < 0.01$ .**Table III.** The direct effect of rHCG on the spreading and invaded areas of murine embryos in the invasion assay

	Control	HCG-added	P value
Spreading area			
Culture day 2	4.5 ± 0.2	4.4 ± 0.2	NS
3	9.3 ± 0.3	8.8 ± 0.4	NS
4	13.9 ± 0.5	13.7 ± 0.5	NS
5	18.9 ± 1.0	18.6 ± 0.9	NS
6	30.1 ± 1.6	28.2 ± 1.4	NS
7	47.4 ± 2.7	48.4 ± 2.9	NS
Invading area	93.8 ± 4.8	87.9 ± 4.5	NS

The murine embryo invasion assay was performed in the presence or absence of rHCG (10 IU/ml). Values are expressed as means ± SEM from five experiments. NS = not significant.

HCG in the absence of PBMC in this study, showing that HCG has little direct effect on murine embryo outgrowth. This discrepancy may be because there is no production of chorionic gonadotrophin at the implantation site in mice.

Recently, attention has been focused on the patients who fail to achieve successful implantation in spite of repeated intrauterine transfers of morphologically good embryos (Edwards, 1995). We have previously proposed that local administration of autologous PBMC into the endometrium is a possible approach for patients suffering from implantation

failure (Takabatake *et al.*, 1997b; Fujita *et al.*, 1998). However, the effects of patients' PBMC on embryo implantation are estimated to be slight since the patients are not pregnant. In this regard, this study provides important evidence that HCG is a potent candidate for enhancing the promoting effect of patients' PBMC *in vitro* for clinical application. Lymphocytes from pregnant women were shown to express HCG receptor genes (Lin *et al.*, 1995). In addition, HCG was reported to modulate cytokine production by PBMC (Schäfer *et al.*, 1992). Further investigation should be performed to clarify what factors are produced from PBMC when pregnant and stimulated by HCG.

In conclusion, this study has demonstrated that PBMC promoted murine embryo invasion. This effect is prominent in PBMC derived from women in the early stage of pregnancy and was enhanced by HCG. These findings suggest a positive feedback loop in which PBMC in the implantation site is activated by HCG that is secreted from the embryo, and these PBMC then facilitate embryo implantation. In addition, results from this study raise the possibility that HCG activated patients' PBMC might be used for therapy in patients suffering from implantation failure.

### Acknowledgement

This work was supported in part by Grants-in-Aid for Scientific Research (no. 09671673, 09671674, 09671676).

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Received on April 27, 2001; accepted on August 21, 2001