

Insulin-like growth factor binding protein-1 induces decidualization of human endometrial stromal cells via $\alpha 5 \beta 1$ integrin

Hironori Matsumoto, Keiji Sakai¹ and Mitsutoshi Iwashita

Department of Obstetrics and Gynecology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan

¹Correspondence address. Tel: +81-422-47-5511; Fax: +81-422-47-3177; E-mail: sakaik@aol.com

Progesterone is known to induce decidualization of human endometrial stromal cells *in vitro*. Decidualized stromal cells produce insulin-like growth factor binding protein-1 (IGFBP-1) as well as prolactin (PRL). In this study, we tested the possibility that IGFBP-1 directly stimulates endometrial stromal cell decidualization. Endometrial stromal cells were obtained from normal menstruating patients with uterine myoma at hysterectomy. Stromal cells were cultured for up to 4 weeks with estradiol (E_2) and/or medroxy progesterone acetate (MPA) in the presence or the absence of IGFBP-1 and, LR³-IGF-I (an IGF-I analogue) that binds to the IGF-I receptor but has reduced affinity for IGFBPs. Decidualization of endometrial stromal cells was evaluated by morphological changes and PRL release into culture media. The binding of IGFBP-1 to endometrial cells was analysed using a biosensor. MPA and E_2 induced decidualization of stromal cells, while LR³-IGF-I inhibited decidualization by MPA and E_2 as well as PRL and IGFBP-1 secretion into medium. IGFBP-1 induced decidualization of stromal cells in the absence of MPA and E_2 in the medium. IGFBP-1-induced decidualization was not inhibited by the addition of LR³IGF-1 but was inhibited by the addition of an RGD peptide, however, the RGD peptide had no effect on decidualization when added alone. The binding analysis showed that IGFBP-1 bound to the surface of endometrial stromal cells and an anti- $\alpha 5 \beta 1$ integrin antibody inhibited its binding. These results suggest that IGFBP-1 produced by endometrium can mediate progesterone-induced decidualization possibly by interacting with $\alpha 5 \beta 1$ integrin on the surface of endometrial stromal cells.

Keywords: IGFBP-1; decidualization; endometrium; integrin; human stromal cells

Introduction

Endometrial stromal differentiation is essential for the implantation of developing blastocyst (Glasser *et al.*, 1993; Mehrotra *et al.*, 1995). Recently, an *in vitro* model of human decidualization has been developed by a number of investigators that has proven useful in investigating the regulation of endometrial stromal cell differentiation (Lockwood *et al.*, 1993; Brosens *et al.*, 1999; Mark *et al.*, 2002). In this model, human endometrial stromal cells are cultured in the presence of progesterone or medroxy progesterone acetate (MPA), with or without estradiol (E_2), and undergo differentiation into cells that morphologically resemble decidual cells. In addition, the decidualized stromal cells produce prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1), a decidual specific protein that is not expressed in undifferentiated stromal cells (Bell *et al.*, 1991). Estrogen stimulates production of IGF-I in human endometrium (Giudice *et al.*, 1994; Strowitzki *et al.*, 1996; Rutanen, 1998) suggesting that estrogen-induced proliferation of endometrium is regulated by local IGF-I. In contrast, decidualized endometrium produces IGFBP-1 that inhibits IGF-I action by limiting IGF-I binding to its receptor (Jones *et al.*, 1993a; Rajkumar *et al.*, 1996). Thus, IGF-I and IGFBP-1 have the potential to coordinately regulate endometrial physiology. Although physiological significance of IGFBP-1 produced in decidua is not fully understood, it has been presumed that decidual IGFBP-1 negatively controls the trophoblast

invasion into uterine myometrium (Lee *et al.*, 1997). Recently, it has been become apparent that growth factors are likely to play an important role in regulating the cell growth and differentiation in the female reproductive tract (Fowler *et al.*, 2000).

IGFBP-1 is present in extracellular fluid as several different phosphoisoforms. Phosphorylated IGFBP-1 usually has an inhibitory effect on IGF-I action (Lee *et al.*, 1997), whereas non-phosphorylated IGFBP-1 can stimulate IGF-I action (Elgin *et al.*, 1987; Jones *et al.*, 1991, 1993b; Rutanen *et al.*, 1998). Since decidual IGFBP-1 is mostly phosphorylated, it has been shown that decidual IGFBP-1 is likely to inhibit trophoblast invasion (Irwin and Giudice, 1998). Recently, IGFBP-1 has been shown to stimulate cell migration and adhesion (Jones *et al.*, 1993a). These actions of IGFBP-1 are IGF-independent and are mediated through its RGD sequence in the molecule which interacts with $\alpha 5 \beta 1$ integrin on the cell surface (Jones *et al.*, 1993a; Frost and Lang 1999).

We hypothesized that decidualization of endometrium by progesterone might be mediated in part by locally produced IGFBP-1 and thereby be coordinately regulated with the proliferative effect of E_2 that is mediated by local IGF-I in endometrium. To test the possibility, the effects of IGFBP-1 on decidualization were evaluated using cultured endometrial stromal cells. Furthermore, we investigated whether IGFBP-1 could bind to $\alpha 5 \beta 1$ integrin on cell surface and evaluated the effect of inhibiting binding to the $\alpha 5 \beta 1$ integrin on decidualization.

Materials and Methods

Materials

E₂, MPA, recombinant human IGFBP-1 expressed in CHO cells (phosphorylated IGFBP-1) and RGD peptide were purchased from Sigma Chemical Co. (St Louis, MO). Recombinant human IGF-I was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). Collagenase (type-1) and DNase-I were from Wako-Junyaku-Kogyo (Osaka, Japan). LR³-IGF-I (an IGF-I analog with an arginine for glutamate substitution at position 3 and a 13 amino acid extension peptide at the N-terminus) was from GroPep (Adelaide, Australia). The monoclonal antibody for $\alpha 5 \beta 1$ integrin (JBS5) was purchased from Chemicon (Temecula, CA). Cell culture media, antibiotics (streptomycin and penicillin) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). The water soluble cross-linker BS³ was from Pierce (Rockford, IL).

Specimens and cell culture

Mid-proliferative phase human endometrial tissues were obtained from normal menstruating patients who received no hormonal therapy within the prior 3 months and underwent hysterectomies for the treatment of uterine fibroids. A portion of each endometrial specimen was dated histologically according to the criteria of Noyes *et al.* (1950) and only histologically normal tissues were used for cell culture. Informed consent was obtained from all patients and ethical approval for this study was granted by the ethics committee of Kyorin University. The fragments of endometrial tissues were washed with Dulbecco's Modified Eagle's Medium (DMEM) containing antibiotics and 10% FBS. Selected tissues were cut into small pieces and incubated with 0.1% type-I collagenase and 0.01% DNase-I for 1 h at 37°C to disperse the cells. The cell suspension was filtered through nylon mesh with pore size of 10⁵ μ m (Millipore, Eschborn, Germany) to remove undigested tissue debris. Cells were collected from the filter by rinsing and centrifugation at 800g for 10 min. Then endometrial cells were rinsed three times and suspended in DMEM containing 10% charcoal-treated FBS and streptomycin (100 μ g/ml) and penicillin (100 U/ml) in 5% CO₂–95% air at 37°C. Media was replaced every 48 h and the cultures were continued for 7–10 days until they reached confluency.

Cells were detached using trypsin–EDTA solution (Sigma Chemical Co.) and incubating for 5 min at 37°C. Detached cells were diluted with DMEM containing 10% charcoal-treated FBS, and streptomycin (100 μ g/ml) and penicillin (100 U/ml), then seeded into 12-well plates (Costar, Cambridge, MA) at a concentration of 200 000 cells/well. Cells were grown to confluence in this growth medium (1 ml/well). The identity of endometrial stromal cells was established by immunohistochemical staining: positive for fibronectin and vimentin (Nasu *et al.*, 1998), and negative for cytokeratin and factor VIII (Zoumakis, *et al.*, 2000). The media were replaced with DMEM containing 5% charcoal-treated FBS and test substances as indicated in the figures and figure legends, when cells were grown to confluence. The cultures attained confluence within 14 days. Then, the media were replaced every 7 days and the cultures were continued for additional 14 days. At Day 28, morphological changes of endometrial stromal cells were characterized and conditioned media were collected to measure the concentrations of PRL and IGFBP-1. The population of endometrial stromal cells was routinely over 98% pure.

Measurement of PRL and IGFBP-1 in conditioned media

The concentration of PRL in conditioned media from hormone-treated endometrial stromal cells was measured using a specific homologous enzyme immunoassay. hPRL standards and antibodies were obtained from Tosoh Corporation (Tokyo, Japan). Both intra-assay and inter-assay coefficients were <10%, and sensitivity of this assay was 1 ng/ml. IGFBP-1 concentrations were measured by ELISA kits from Diagnostic System Laboratories, Inc. (Webster, TX) according to the manufacturer's instruction. The sensitivity of this assay was 0.8 ng/ml, and both intra-assay and inter-assay coefficients were <5%.

Analysis of endometrial stromal cells surface and IGFBP-1 binding by biosensor

IASys plus (Affinity Sensors, Cambridge, UK) was used to analyse the binding interaction between the endometrial stromal cells and IGFBP-1 at 25°C using

planar aminosilane surface (Baker *et al.*, 2002). This instrument has a dual-well stirred cuvette and monitors the interactions between the ligand and ligate in the cuvette.

The cells were grown to 80% confluency and a non-enzymatic cell dissociation solution (Sigma, Cat# c-5914) was used to remove the cells from the tissue culture dishes. The cells were washed with PBS three times and incubated with 50 nM of anti- $\alpha 5 \beta 1$ monoclonal antibody (B: test cells) or mouse IgG (A: control cells) for 1 h at 4°C to block the binding of IGFBP-1 to $\alpha 5 \beta 1$ integrins. The water soluble cross-linker BS³ (2 mM) was added for 30 min at 4°C to form covalent linkages, and the mixture washed three times with PBS. Endometrial stromal cells were chemically immobilized to free amino groups on the surface of an aminosilane coated cuvette using BS³ (Mochizuki *et al.*, 2006). BS³ was added to the cuvette and allowed to bind to the cuvette for 5 min. The cuvette was washed with PBS to remove unbound BS³. Twenty thousand cells were then immobilized on the cuvette surface. The remaining sites on the activated surface of cuvette were blocked by PBS containing 2% BSA solution and cuvette was washed with PBS three times after stable baseline was obtained, 1 μ M IGFBP-1 that was dissolved in PBS was applied to the cuvette and the resonant angle response was recorded. Dissociation was initiated by adding PBS and 0.05% Tween-20 to the cuvette and recording resonant angle response for 5 min. Results were analysed with Fastfit software from IAsys.

Statistical analysis

Experiments were carried out in triplicate at three times. Mean $\pm$ SEM values from three separate experiments were described in figures and table. Statistical analysis was carried out using Stat View 5.0 (SAS Institute Inc., Cary, NC) and a paired Student's *t*-test was used to compare difference between the control and the test groups.

Results

During the process of decidualization, stromal cells change from a fibroblastic spindle shape to a spherical appearance. Endometrial stromal cells cultured in media with no addition remained fibroblastic in appearance throughout the incubation period (Fig. 1A). Addition of E₂ and MPA to the media altered the endometrial stromal cells from fibroblastic shape to a spherical shape (Fig. 1B). Exposure to LR³-IGF-I in the presence of E₂ and MPA, however, inhibited morphological changes in the endometrial stromal cells (Fig. 1C). Addition of IGFBP-1 in culture media also altered the appearance of stromal cells from spindle shape to spherical shape (Fig. 1D). Co-incubation with LR³-IGF-I did not alter the morphological changes induced by IGFBP-1 (Fig. 1E). At the end of the experiments, cell numbers were counted after trypsinization, and the difference between the control and the test groups compared by alternate Welch's *t*-test and the Mann–Whitney *U*-test. There were no differences in cell numbers between the control group and test groups (data not shown).

Decidualized endometrial stromal cells also show secretion of specific proteins (e.g. PRL and IGFBP-1). Therefore, secretion of PRL and IGFBP-1 into culture media from endometrial stromal cells was analysed to confirm the presence of decidualization biochemically. Addition of E₂ and MPA significantly increased secretion of PRL and IGFBP-1 from endometrial stromal cells into the medium, 13.6- and 41.4-fold above control, respectively (*P* < 0.0001) (Table I). On the other hand, IGF-I or LR³-IGF-I reduced PRL and IGFBP-1 secretion that have been stimulated to nearly control levels (Table I). These results demonstrated that co-incubation with E₂ and MPA potentiated decidualization of endometrial stromal cells, and that IGF-I inhibited decidualization of these cells that had been induced by E₂ and MPA.

Exposure of 100 nM IGFBP-1 to endometrial stromal cells significantly increased PRL secretion into the medium (5.3 ± 0.2 ng/ml) to a level that was similar to that induced by E₂ and MPA (6.8 ± 0.8 ng/ml).

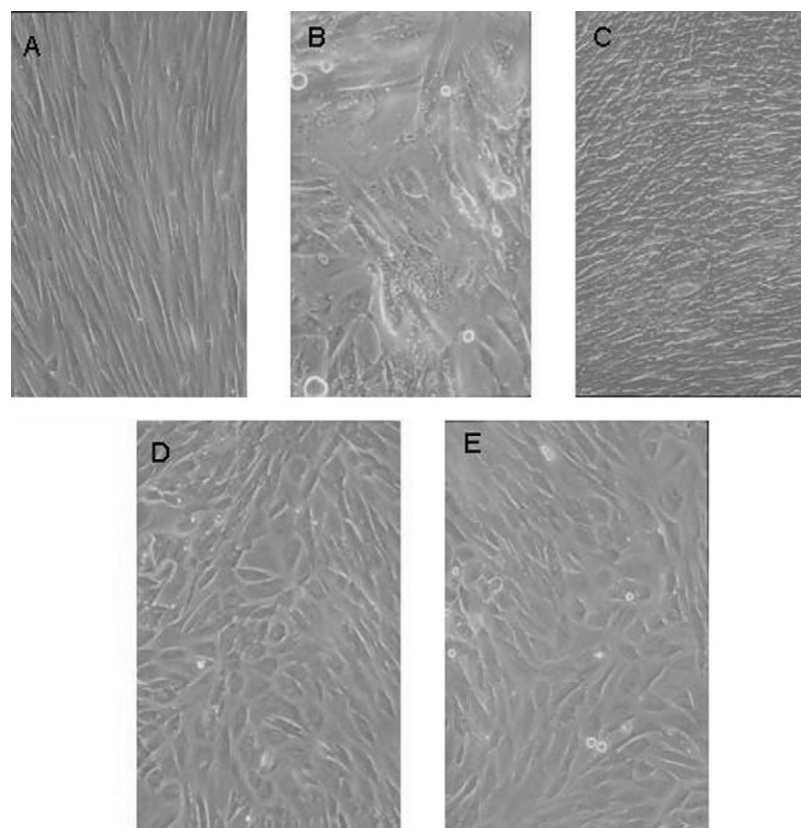


Figure 1: Decidualization of endometrial stromal cells by placental steroid hormones, IGF and IGFBP-1.

Endometrial stromal cells were grown to confluence then media were replaced with DMEM containing 5% charcoal-treated FBS and test substances: (A) no addition, (B) E_2 (10^{-8} M) and MPA (10^{-6} M), (C) E_2 (10^{-8} M), MPA (10^{-6} M) and LR³-IGF-I (10^{-7} M), (D) IGFBP-1 (10^{-7} M), (E) IGFBP-1 (10^{-7} M) and LR³-IGF-I (10^{-7} M). The media were changed every 7 days including the test substances and the cultures were continued for 28 days. At Day 28, morphological changes of endometrial stromal cells were shown.

PRL production stimulated by IGFBP-1 was not inhibited by the addition of LR³-IGF-I, while IGFBP-1 stimulated PRL release was inhibited by the addition of RGD peptide by 1.1 ± 0.2 ng/ml, whereas RGD peptide alone had no effect on PRL release (Fig. 2).

The addition of IGFBP-1 in the culture media also altered the appearance of stromal cells to spherical shape, and co-incubation with the RGD peptide had no effect on the morphological changes induced by IGFBP-1 (data not shown).

To determine whether IGFBP-1 bound to the $\alpha 5\beta 1$ integrin, endometrial stromal cells which were incubated with mouse IgG (A) or $\alpha 5\beta 1$ integrin monoclonal antibody (B), were immobilized on the aminosilane cuvette using standard amine coupling procedures, and these cells were utilized for the binding analysis by biosensor. The sensorgram depicted in Fig. 3 demonstrates the binding of IGFBP-1 to endometrial stromal cell surface. After IGFBP-1 bound to endometrial stromal cells (association phase), the cuvette was exposed to PBS and 0.05% Tween-20 in PBS to remove the unbound IGFBP-1 (dissociation phase). The response curve (A) shows a significant increase above baseline compared with response curve (B).

Table I. Biochemical properties of decidualized endometrial stromal cells.

	PRL (ng/ml)	IGFBP-1 (ng/ml)
Control	0.5 ± 0.1	3.2 ± 0.2
E_2 + MPA	6.8 ± 0.8	132.4 ± 5.8
IGF -I	0.8 ± 0.1	2.0 ± 0.5
E_2 + MPA + IGF-I	1.0 ± 0.2	5.8 ± 0.8
LR ³ IGF-I	0.8 ± 0.1	3.3 ± 0.6

It reveals that IGFBP-1 bound to mouse IgG immobilized cells but did not bind to $\alpha 5\beta 1$ integrin antibody immobilized cells, and demonstrates that one of the binding sites of endometrial stromal cells for IGFBP-1 is $\alpha 5\beta 1$ integrin.

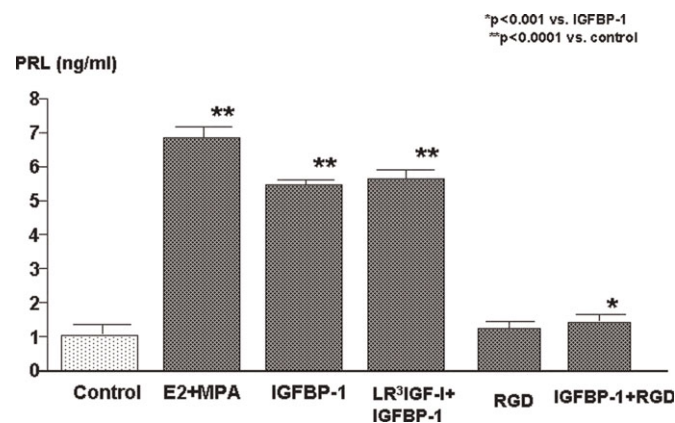


Figure 2: Effects of IGFBP-1 and RGD peptide on PRL secretion from endometrial stromal cells.

Endometrial stromal cells were grown to confluence in 24-well plates, then the media were replaced with DMEM containing 5% charcoal-treated FBS and test substances after cells were grown to confluence: E_2 (10^{-8} M) and MPA (10^{-6} M), IGFBP-1 (10^{-7} M), IGFBP-1 (10^{-7} M), LR³-IGF-I (10^{-7} M), RGD peptide (3×10^{-5} M) and IGFBP-1 (10^{-7} M) and RGD peptide (3×10^{-5} M). Media were replaced every 7 days including test substances and the cultures were continued for additional 14 days. At Day 28, and conditioned media were collected and the concentrations of PRL were measured by EIA. Each value is the mean $\pm$ SEM. of triplicate determinations.

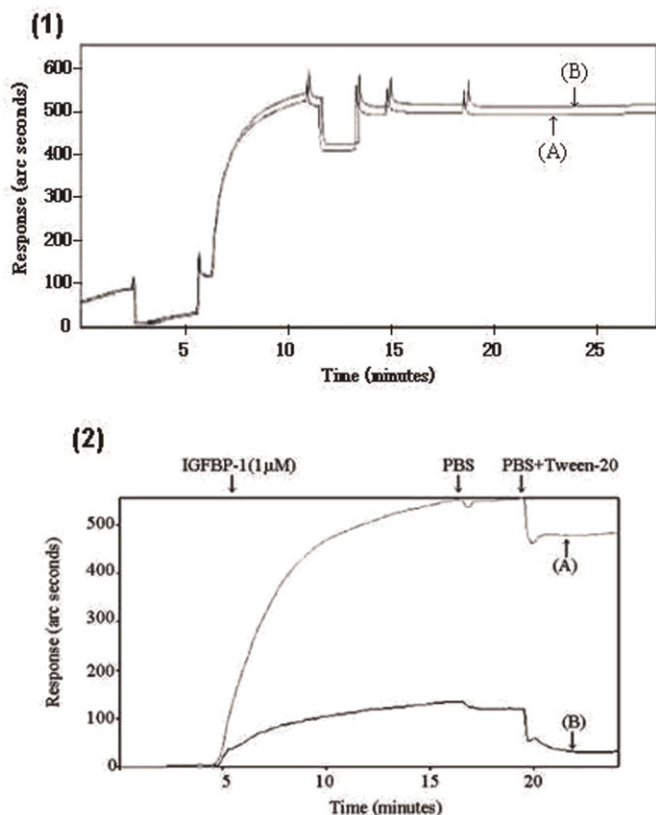


Figure 3: Analysis of IGFBP-1/endometrial stromal cell surface association using an IAsys plus.

The endometrial stromal cells were incubated with anti- $\alpha 5 \beta 1$ integrin monoclonal antibody (B: test cells) or mouse IgG (A: control cells) for 1 h at 4°C then BS³ added for 30 min at 4°C, and the cells were immobilized on the surface of an aminosilane cuvette (1). Association and dissociation curve for IGFBP-1 binding to endometrial stromal cell surface were determined using an IAsys plus Biosensor. IGFBP-1 (10^{-6} M) was passed over the immobilized endometrial control cell cuvette (A) or immobilized test cell cuvette (B) before switching to washing buffer to remove non-specific binding (2). The figure is a representative result from three separate experiments that gave similar results.

Discussion

It is well known that the initiation of pregnancy requires endometrial development and blastocyst implantation but the precise mechanisms that mediate the implantation process are not well understood. One of the basic requirements for the implantation and maintenance of pregnancy is the decidualization of uterine endometrium. Decidualization is defined as the differentiation of the fibroblast-like cells to polygonal cells which are morphologically distinct. Also decidualized cells are distinct from endometrial cells biochemically, and produce specific proteins such as PRL and IGFBP-1. IGFBP-1 is abundant in decidualized endometrial stromal cells and has been demonstrated to bind to IGF in extracellular fluids and to inhibit IGF actions *in vivo* and *in vitro* (Fowler *et al.*, 2000). IGFBPs have been demonstrated to have physiological and biological activities that are IGF independent, and the first report of an IGF-independent action was the effect of IGFBP-1 on cell migration. IGFBP-1 has been shown to have IGF-independent effects via binding to the $\alpha 5 \beta 1$ integrin through its RGD sequence (Jones *et al.*, 1993a). In the human endometrium, mRNAs for IGF-I, IGF-II and IGFBPs are expressed during the menstrual cycle. IGF-I mRNA is substantially higher during the proliferative phase than the secretory phase, whereas the converse is true for IGF-II (Zhou *et al.*, 1994; Gao *et al.*, 1995). IGFBP-1 mRNA is localized to predecidualized stromal cells in late secretory

phase of endometrium and to decidual cells in pregnant endometrium (Julkunen *et al.*, 1990; Rutanen *et al.*, 1991).

Stromal cells cultured in medium containing E₂ and MPA alter their shape from fibroblastic spindles to a spherical appearance, and secrete IGFBP-1. On the other hand, exposure to LR³IGF-I in the presence of E₂ and MPA inhibits the morphological changes of endometrial stromal cells, because LR³IGF-I inhibited secretion of IGFBP-1. It is reasonable to assume that locally produced IGFBP-1 might regulate in endometrial stromal decidualization.

In the current study, exposure to LR³IGF-I in the presence of exogenous IGFBP-1 did not inhibit decidualization of endometrial stromal cells, whereas LR³IGF-I did inhibit decidualization that was stimulated by E₂ and MPA because LR³IGF-I has reduced affinity for IGFBP-1. These results suggest that decidualization of endometrium by E₂ and MPA might be mediated through IGFBP-1 that is produced by the predecidual stromal cells in response to E₂ and MPA, since LR³IGF-I binds to IGF-I receptor and inhibits IGFBP-1 secretion (Fig. 4).

Trophoblast invasion is mainly caused by increasing cell migration (Irving *et al.*, 1995; Hamilton *et al.*, 1998). Trophoblast invasion of the human uterus is stringently controlled by the microenvironment. Invasion of extravillous trophoblast cells, *in situ* as well as in culture, is mediated through cell surface integrins. Since cell migration is a necessary step in the invasion cascade, specific integrins or invasion-regulatory molecules, i.e. TGF- β , IGF-II, and IGFBP-1 that are expressed at the feto-maternal interface, may have a functional role on trophoblast cell migration. IGFBP-1 stimulated cell migration and it was demonstrated that the migration of trophoblast cells *in vitro* requires the expression of $\alpha 5$ and $\beta 1$ integrin subunits (Irving and Lala, 1995). These biological actions of IGFBP-1 are achieved by IGFBP-1 binding to integrin $\alpha 5 \beta 1$ and these effects of IGFBP-1 are independent of IGF-I binding. Our results indicate that it is likely that IGFBP-1 caused decidualization of endometrial stromal cells via integrin $\alpha 5 \beta 1$ since the RGD peptide inhibited decidualization and IGFBP-1 bound to integrin $\alpha 5 \beta 1$ on these stromal cells. Other studies have suggested the possibility that IGFBP-1 binds not only to $\alpha 5 \beta 1$ but also to other proteins on the cell surface or in the extracellular matrix. The $\beta 1$ integrin has been shown to associate with tissue transglutaminase on cell surface, and it has been estimated that 5–40% of $\beta 1$ integrin on the cell surface is present in a complex with tissue transglutaminase.

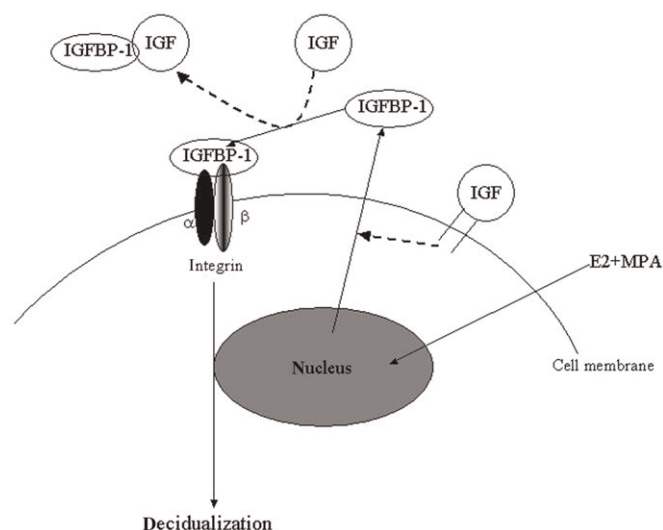


Figure 4: A model proposing role of IGFBP-1 and decidualization of endometrial stromal cells. Solid lines indicate stimulation of decidualization, and dotted lines indicate inhibition of decidualization.

Tissue transglutaminase on cell surface has been shown to increase cell adhesion and spreading on fibronectin (Akimov *et al.*, 2000). IGFBP-1 is a substrate for tissue transglutaminase (Sakai *et al.*, 2001). Ligand occupancy of $\alpha 5 \beta 1$ integrin by IGFBP-1 has been shown to induce focal adhesion kinase and PI-3 kinase. Therefore, it is assumed that IGFBP-1 could potentially modulate the $\alpha 5 \beta 1$ integrin-linked signaling, and the association of IGFBP-1 with tissue transglutaminase might contribute to the decidualization of endometrial stromal cells.

It has been demonstrated that estrogen increases endometrial IGF-I mRNA expression in the proliferative phase, and potentiates endometrial proliferation (Giudice *et al.*, 1993). In the current study, E_2 and MPA stimulated the production of IGFBP-1 and induced decidualization of endometrial stromal cells via $\alpha 5 \beta 1$ integrins (Table I, Fig. 4). These results suggest that menstrual cyclic changes of uterine endometrium by ovarian steroid hormones are regulated by the local IGF-I-IGFBP-1 axis.

In summary, the current studies demonstrated that IGFBP-1 can bind to $\alpha 5 \beta 1$ integrin on the endometrial stromal cell surface and suggest that IGFBP-1 might have biological role on endometrial stromal cell decidualization that is independent of its binding to IGF-I.

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