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The characteristics of integrins expression in decidualized human endometrial stromal cell induced by 8-Br-cAMP in *in vitro*

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Abbreviations: 8-Br-cAMP, 8-bromo-cAMP; extracellular matrix, ECM

Abstract

Integrins are heterodimeric glycoproteins that have been found to undergo dynamic temporal and spatial changes in the endometrium during the menstrual cycle and in early pregnancy. Specificity of integrins is known to be different in human endometrial stromal cells and decidual cells. These shifts of integrins suggested to play an important role in embryo implantation and can be modulated by progesterone, cAMP derivatives, and cytokines. The mechanisms of decidualization and its precise physiological role are still not clearly understood and *in vitro* systems could provide an alternative that overcomes limitations of studying such complex biological phenomena *in vivo* at the time of implantation. This study was undertaken to establish an *in vitro* model system for human decidualization using 8-bromo-cAMP and to investigate the characteristics of stromal integrin expression *in vitro* by 8-Br-cAMP. Endometrial stromal cells were isolated and cultured, and then were induced to decidualize by 0.5 mM 8-Br-cAMP for 15 days. Immunofluorescence staining and flow cytometric analyses of the integrin subunits (α1, α4, α5, α6, β1 and αvβ3) were performed at day 9. In the presence of 8-Br-cAMP, the staining intensity of αvβ3 was significantly higher than control and measurements for α1, α4, α5, α6, and β1 were similar. Immunofluorescent localization of the integrins reflected the differences obtained from the flow cytometric analyses described above. In summary, the expression of αvβ3 integrin increased in stromal cells *in vitro* decidualized by 8-Br-cAMP and this up-regulation of αvβ3 integrin expression during decidualization might influence on human implantation.

Keywords: decidualization, integrins, 8-bromo-cAMP, human endometrial stromal cells

Introduction

The successful implantation depends on the timely interaction of the embryo with a receptive secretory endometrium, which requires both the secretory changes in the glandular epithelium and the decidual transformation of the stroma cells (Weitlauf, 1988). Decidualization of stromal cells involves extensive cell differentiation characterized by the production of many secretory products such as prolactin and insulin-like growth factor-binding protein-1 (Maslar *et al*., 1979; Bell *et al*., 1991). Decidual cells are believed to play a role in implantation and in the maintenance of pregnancy through control of trophoblast invasion, nutrition of the embryo (Pijnenborg *et al*., 1980).

Integrins, a class of cell adhesion molecules, are known to be involved in cell-cell and cell-substrate adhesion, and suggested to play an important role in embryo attachment and trophoblast invasion (Albelda *et al*., 1983; Kearns *et al*., 1983; Merviel *et al*., 2001). Integrins display dynamic temporal and spatial patterns of expression in the epithelial cells during the menstrual cycle and in early pregnancy (Tabibzadeh, 1992; Lessey *et al*., 1994). The decidualized stromal cells also adopt these epithelial patterns of extracellular matrix and its receptor expression (Aplin *et al*., 1988). The pattern of integrin during early pregnancy and principal feature were an apparent shift from epithelial to predominantly stromal expression. These shifts raise a number of questions regarding the mechanisms that regulate integrin expression in human endometrium. The α1 expression seems to be progesterone dependent and transforming growth factor-β, granulocyte-monocyte colony stimulating factor, and interleukin-1β have all been shown to alter the expression of integrin molecules (Grossinkinsky *et al*., 1996). However, it is little known about the characteristics of stromal integrin expression by 8-Br-cAMP *in vitro*.

The mechanisms of decidualization and its precise physiological role are still not clearly understood. *In vitro* systems provide an alternative that overcomes limitations of studying on complex biological phenomena

in vivo at the time of implantation. *In vitro* decidualization can be induced by two pathways, one by progesterone-mediated and another cAMP-mediated (Irwin *et al*., 1989; Tang *et al*., 1994; Brar *et al*., 1997; Mizuno *et al*., 1999). This study was undertaken to establish an *in vitro* model system for human decidualization using 8-Br-cAMP and investigate the characteristic of stromal integrin expression. The six integrin subunits (α1, α4, α5, α6, β1, and $\alpha v\beta$3) that were largely expressed in the stromal cells or pregnant decidual cells (Bishop *et al*., 1993; Lessey *et al*., 1994; Ruck *et al*., 1994), were examined in this study.

Materials and Methods

Isolation and culture of human endometrial stromal cells

Human endometrium was obtained at hysterectomy from normally cycling pre-menopausal women, aged 35 to 44 years, who underwent surgery for non-endometrial abnormalities. The ethical committee at Oxford University approved this study and informed consent was obtained from every patient.

A portion of each endometrial specimen obtained was examined histologically. Endometrial stromal cells were isolated as described previously (Shiokawa *et al*., 1996). Briefly, tissue samples were collected in Dulbeccos Modified Eagle Medium (DMEM) containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum. After cleaning and trimming to remove blood clots and mucus, the specimens were minced to fragments less than 1 mm in size under a laminar flow hood and then digested at 37°C for 60 min with 0.25% collagenase I (Sigma Chemical Co., Poole, U.K.) and deoxyribonuclease (Sigma Chemical Co.). The cell suspension was filtered first through a 250 μm and then through a 40 μm sieve. After enzymatic digestion, the most of the stromal cells were present as single cells or small aggregates and then purified. The purity of stromal cells obtained by this method was usually >90%. The purified stromal cells were washed and the number of viable cells were counted by dye exclusion using trypan blue and the viability of isolated cells were at least 90% in each experiment.

In vitro decidualization of human endometrial stromal cells

A total of $1x10^4$ viable cells were plated into each well of a 24-well plate containing glass coverslips for immunohistochemistry, and stromal cells were plated in 175 cm^2 Falcon flasks and cultured for flowcytometry. Cells were cultured in duplicate with phenol red-free Dulbecco's Modified Eagle Medium-Ham's F-12 mixture (DMEM/F12) containing 10% charcoal-treated bovine calf serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. After cells were grown to confluency, decidualization was induced by incubating the cells in media containing 0.5mM 8-Br-cAMP (Sigma Chemical Co.). In selected experiments, exposed and non-exposed cells to 8-Br-cAMP were cultured respectively for 15 days. The culture medium was changed every 3 days with continuous supplementation with 8-Br-cAMP. At the completion of the culture period, immunohistochemical staining and flowcytometry for integrins were performed. Phase contrast microscopy was used to verify morphological changes associated with differentiation *in vitro* in response to 8-Br-cAMP.

Prolactin assays

To confirm the decidualization of endometrial stromal cells by 8-Br-cAMP, prolactin levels in supernatant were measured by Immunoassay (Immulite^R, DPC, UK) using a commercial kit. The coefficient of variation within was 5.7-6.8%, and between assays were 6.4-9.6%. The detection limit of this assay was 0.5 ng/ml. DMEM/F12 supplemented with charcoal-stripped bovine calf serum did not have measurable prolactin concentrations. Prolactin levels were standardised on the basis of cell protein content of each culture wells at the end of each treatment period. Bovine serum albumin was used as standard. All experiments in this study were performed in duplicate and experiments were repeated three times.

Immunocytochemistry

Immunofluorescence staining was performed on stromal cells grown on glass coverslips for 9 days. Samples were fixed in 3% paraformaldehyde for 5 min and were treated with 1:100 diluted primary antibody to each integrin subunits (α1, α4, α5, α6, β1, and $\alpha v\beta$3; Serotec Co, Oxford, UK; Table 1) and then with 1:75 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody for 1 h. Samples were subsequently washed in phosphate buffered saline and mounted.

Flow cytometric analysis

After 9 days of culture, stromal cells were detached from

Table 1. Monoclonal antibodies used for the detection of integrin subunits

Integrin Subunits	Clone
α1	TS2/7
α4	44H6
α5	SAM-1
α6	4F10
β1	8E3
$\alpha v\beta$3	23C6

tissue culture flasks. Cells were treated with primary monoclonal antibodies, and then treated with 1:75 dilution of FITC-conjugated anti-mouse antibody each for 30 min. Dead cells were labelled by propidium iodide and gated out during analysis. Anti-Thy-1 and anti-mouse IgG were used as positive and negative controls respectively. Fluorescence from 10^4 stromal cells in each labelled sample was measured and calibrated, by comparison with Flow-Set™ (Coulter Corporation, Miami, USA) in a Coulter EPICS® ELITE ESP flow cytometer.

Statistical analysis

Unless stated otherwise, the numerical data presented are the mean ± SD of at least three experiments. For the comparison of two populations, the nonparametric Wilcoxon test was used. Differences were considered significant when p-values were less than 0.05.

Results

Morphological assessment

Endometrial stromal cells cultured without 8-Br-cAMP retained a fibroblast-like appearance throughout the culture period (Figure 1A). In the presence of 8-Br-cAMP, these spindle-shaped stromal cells were transformed into large polygonal cells (Figure 1B).

Measurement of prolactin

To confirm the decidualization of endometrial stromal cells by 8-Br-cAMP, prolactin levels in supernatant were measured as described above. The concentration of prolactin in supernatant treated with 8-Br-cAMP was increased and peaked at day 9 of culture (Figure 2). The cells cultured for 9-day period was used in the studies for flowcytometry and immunofluorecent staining.

Immunofluorecent staining for integrin subunits

Human stromal cells cultured for 9 days with and without 8-Br-cAMP showed an increase in immunofluorecent staining for integrin αvβ3 in the cells treated with 8-Br-cAMP (Figure 3). Immunostaining for α1 was peripheral, and the αvβ3 integrin was specifically localized to focal contacts of stromal cells *in vitro*. The expression of α4 integrin was very weak in the stromal cells from the midsecretory phase. The staining of α5, α6, and β1 integrins were similar (Figure 3).

Flow cytometric analysis of integrin subunits

Determination of median fluorescence intensity by flow cytometry showed that human stromal cells cultured for 9 days with 8-Br-cAMP enhanced expression of αvβ3 (Figures 4 and 5). Figure 4 shows the shifts in medial fluorescence intensity curves. But the expressions of α1, α4, α5, α6, and β1 are similar in the treated and non-treated cells (Figures 4 and 5).

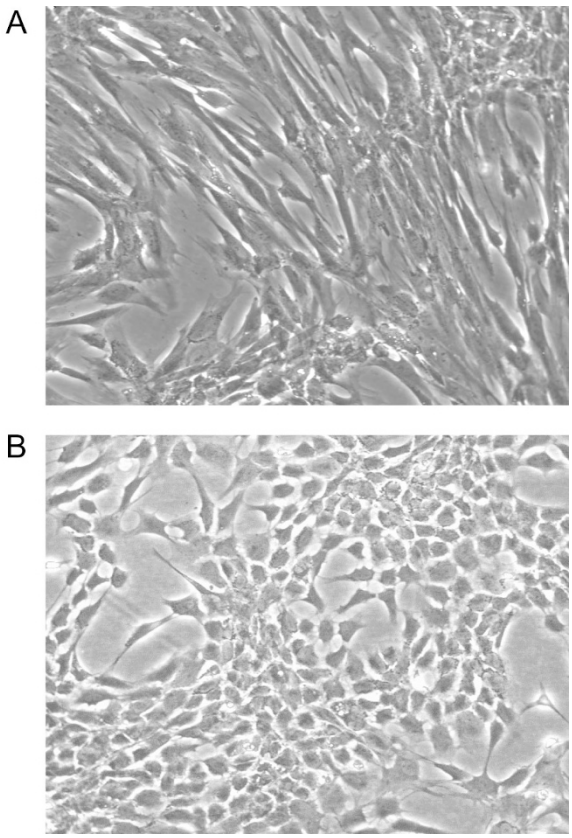

Figure 1. Endometrial stromal cells cultured without 8-Br-cAMP retained a fibroblast-like appearance throughout the culture period (A, x40). In the presence of 8-Br-cAMP, these spindle-shaped stromal cells started to change morphologically from day 3 of culture and completely transformed into large polygonal cells by day 6 of culture. Pictures were taken at day 6 of culture (B, x40).

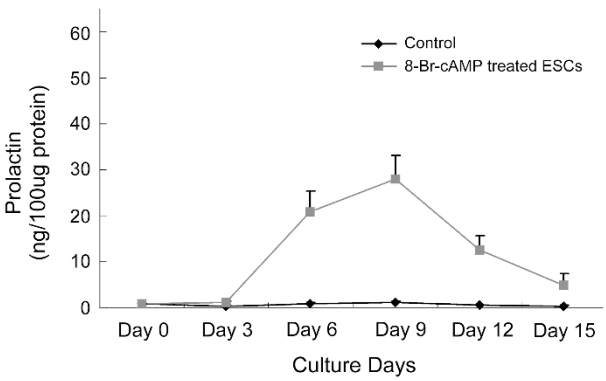

Figure 2. The concentration of prolactin in supernatant treated with 8-Br-cAMP was increased from day 3 of culture and peaked at day 9. Stromal cells at the day 9 was harvested and used to perform immunohistochemical staining and flow cytometry for integrin expression. Data represents mean ± SD of three separate experiments.

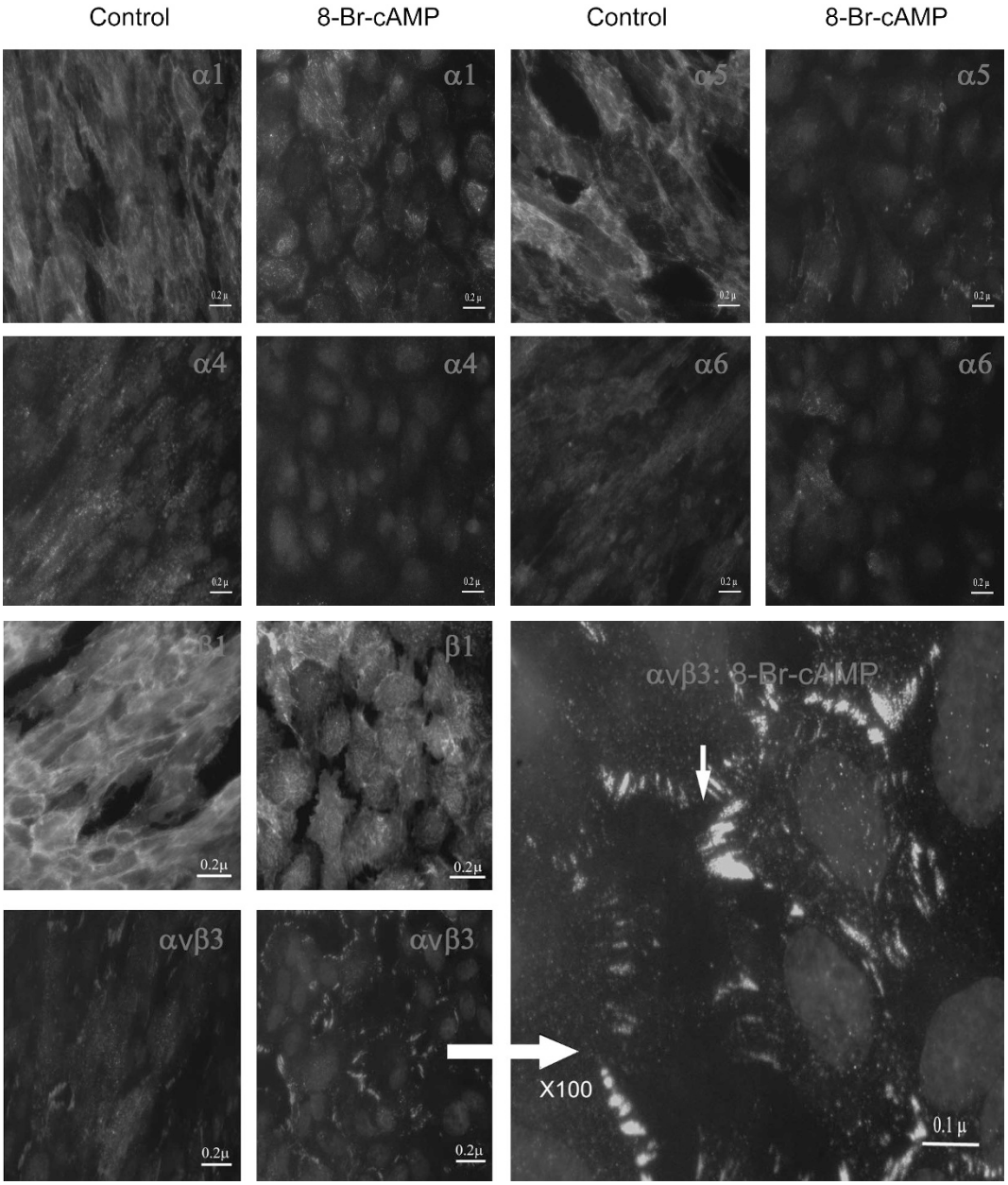

Figure 3. Immunostaining of integrin subunits. In presence of 8-Br-cAMP, αvβ3 was significantly higher, and other subunits (α1, α4, α5, α6, and β1) were similar. Scale bar indicates 0.1 μm (x100), and 0.2 μm (x40), respectively. White arrow indicates focal deposits of integrin αvβ3 on the cell membrane of human endometrial stromal cells.

Discussion

Decidualization begins in the human endometrium during the secretory phase of menstrual cycles, under the influence of estradiol and progesterone, even in the absence of conception. The decidual transformation of stromal cells *in vivo* is not only associated with characteristic morpholgical patterns, but is also accompanied by prolactin secretion (Maslar *et al.*, 1979). Because the mechanisms of decidualization and its physiological role are very complex and not clearly defined *in vivo*, *in vitro* systems may provide an alternative that overcomes some of the inherent limitations of studying. Our study was undertaken to establish an *in vitro* model system for human decidualization using not progestins but 8-Br-cAMP. We have shown here that human endometrial stromal cells also undergo such changes *in vitro* in response to 8-Br-cAMP stimulation as shown by progesterone.

The production of prolactin by the human endometrium has been shown to correlate with the degree of histological decidualization (Maslar *et al.*, 1979; Irwin *et*

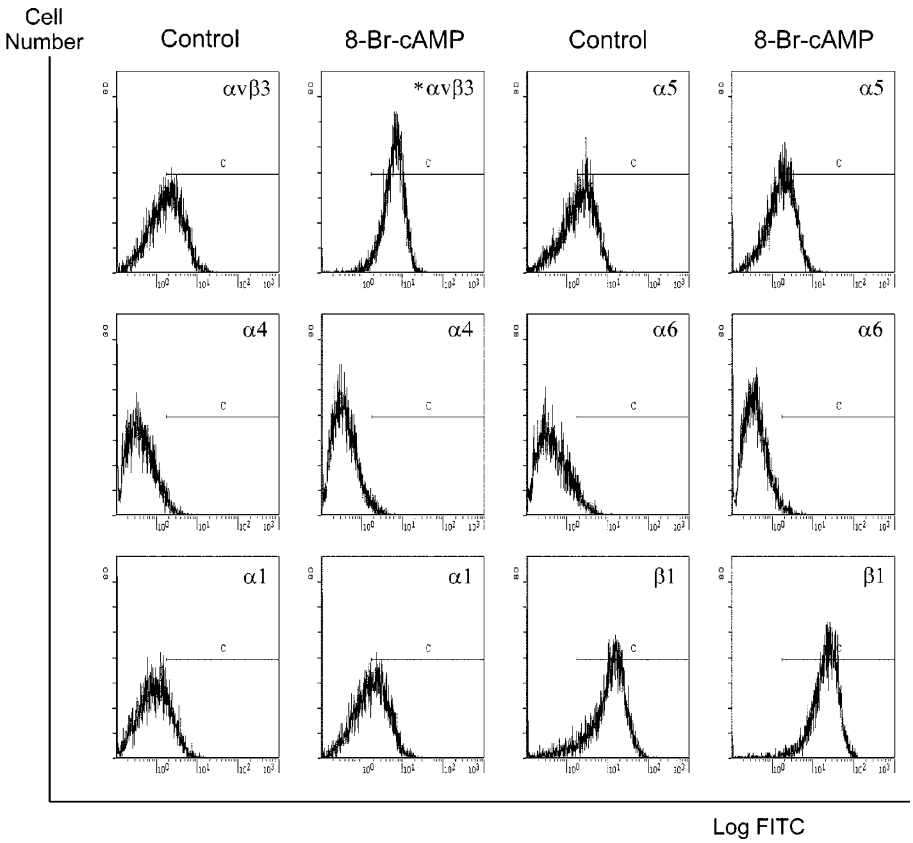

Figure 4. Quantitation by flow cytometry of integrin subunits. Intensity of fluorescence (log FITC) and number of labelled cells are represented on the X and Y axes, respectively. As described in Materials and Methods, the stromal cells or decidualized cells were specifically labelled with monoclonal antibodies against several integrin subunits (α1, α4, α5, α6, β1, and αvβ3), and the number of cells stained was quantitated using flow cytometry. The significant enhancement of αvβ3 expression was noticed in *in vitro* decidualized stromal cells induced by 8-Br-cAMP, this shows the shifts in medial fluorescence intensity curves. But the expressions of other integrins (α1, α4, α5, α6, and β1) are not different between the stromal cells and the decidualized cells (*: p<0.05).

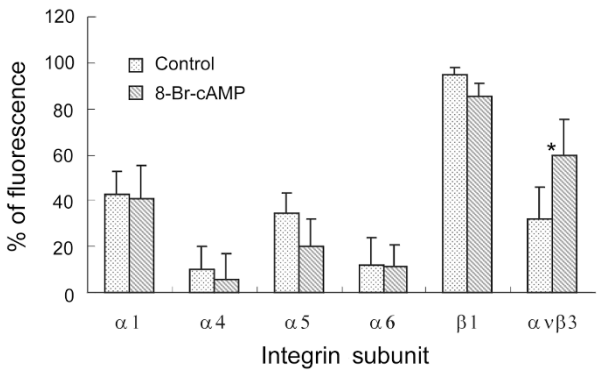

Figure 5. The quantitative flow cytometric analysis of integrin expression. The significant enhancement of αvβ3 expression was noticed in the cells treated with 8-Br-cAMP, however, α1, α4, α5, α6, and β1 integrins are similar in the treated and non-treated cells with 8-Br-cAMP. Data represents mean ± SD of three separate experiments (*:p<0.05).

al., 1989). We found that serially subcultured stromal cells produce prolactin in response to 8-Br-cAMP stimulation. Our findings did confirm and extended earlier observations of prolactin production with progestins and cAMP derivatives by primary stromal cell cultures (Irwin *et al.*, 1989; Mizuno *et al.*, 1999). Stromal cells released detectable amounts of prolactin from day 3 of culture and peaked at day 9 of culture (Figure 2).

Integrins are a large family of heterodimeric (α- and β-chain) cell-surface receptors, which anchor cells to the extracellular matrix (ECM) by their interaction with extracellular matrix proteins, such as fibronectins, laminins, collagens and tenascin, at focal contacts and in hemidesmosomes. Intracellularly, integrins are connected via complex molecular interaction to the cytoskeleton, thereby influencing cytoskeletal organization and signalling (Hynes *et al.*, 1992; Dedhar *et al.*, 1996). The regulated integrin-mediated interaction of cells with the extracellular matrix or adjacent cells such as trophoblasts is important for fundamental biologic processes during cellular differentiation, proliferation and cell mobility during implantations (Hynes *et al.*, 1992).

Decidual stromal cells, like endometrial glands and endothelium, express integrins that bind basement components. These integrins represent the basis for the formation of the pericellular basement membrane of

these cells and also bind ECM that support outgrowth and attachment of the trophoblast in vitro (Damsky *et al*., 1993; Ruck *et al*., 1994). After implantation and penetration of the endometrial basement membrane have occurred, the decidual stroma represents the largest physical barrier confronting the invading trophoblast. Because of the particular pattern of organization of the ECM in the decidual stroma, the expression of ECM receptors by decidual stromal cells is of considerable interest (Aplin *et al*., 1988; Ruck *et al*., 1994). Immunostaining for the integrin subunits α1, α3, α5, α6, αv, β1 and β3 was identified on stromal cells and the integrins bind the following ECM proteins: laminin (α1β1, α3β1, and α6β1), fibronectin (α3β1, α5β1, αvβ1 and αvβ3), vitronectin (αvβ1 and αvβ3), and collagen types I and IV (α1β1). The expression of receptors for the basement membrane components laminin, fibronectin, and collagen type IV at the cell-substratum interface characterizes the decidual stromal cells (Aplin *et al*., 1988; Ruck *et al*., 1994). The function of the basement membrane surrounding the stromal cells has not yet been determined, but it is possible that it restricts the motility of the stromal cells and provides a supporting framework, thus contributing to the structural stability of the decidualized endometrium (Aplin *et al*., 1988).

Integrin expression in endometrial stroma has been well studied *in vivo* (Bischof *et al*., 1993; Tabibzadeh, 1993; Lessey *et al*., 1994; Ruck *et al*., 1994). During menstrual cycle, the fibronectin receptor α5β1 is constitutively expressed. α1β1 is weakly expressed in nonpregnant stroma, but its levels increase greatly in early pregnancy. Similarly, decidualized stroma expresses increased α3β1, α6β1, and αvβ3 (Lessey *et al*, 1994; Ruck *et al*., 1994). In addition, α5β1 has been noted to increase and human deciduas expressed similar levels of α5 and increased levels of α1, α3, α6, and αvβ3 compared with cycling endometrial stroma (Bischof *et al*., 1993; Lessey *et al*., 1994; Ruck *et al*., 1994; Grosskinsky *et al*., 1996). We found that stromal cells express α1, α5, β1, and αvβ3, with low levels of α4 integrin subunits. In decidualized stromal cells *in vitro*, α1 slightly increased not significantly. However, αvβ3 integrin significantly increased after treatment of 8-Br-cAMP. The control and functional significance of these shifts during decidualization are not fully understood.

The pattern of integrin expression during early pregnancy or *in vitro* decidualization was changed and its principle feature was an apparent shift from epithelial to predominantly stromal expression (Aplin *et al*., 1988; Lessey *et al*., 1994). These shifts in the integrin pattern raise a number of questions regarding the mechanisms that regulate integrin expression in human endometrium and need further studies. Why is αvβ3 increased during decidualization and pregnancy? What controls the up-regulation of the various integrin expressions in decidualized stroma?

The ligands for the αvβ3 integrin include vitronectin, fibronectin, fibrinogen, Von Willebrands factor and thrombospondin. The αvβ3 integrin is present not only on endometrial stromal and grandular cells but on the surface of human trophoblastic cells and embryo (Damsky *et al*., 1993; Lessey *et al*., 1994). Therefore, it is likely that αvβ3 and its ligands participate in a synchronous and bilateral trophoblast-endometrial interaction. In this study, the αvβ3 integrin was specifically localized to focal contacts of stromal cells *in vitro* (Figure 3). This pattern suggests a function in attachment to the substrate and signifies a stationary cell type. Focal contacts link the extracellular matrix to the cytoplasmic matrix and enhance trophoblast invasion to the endometrium (Damsky *et al*., 1993; Grosskinsky *et al*., 1996). The suboptimal expression of integrins αvβ3 has been reported in the epithelial cells of certain groups of women suffering from reproductive failure such as unexplained infertility, endometriosis or luteal phase defect (Lessey *et al*., 1992; Lessey *et al*., 1994; Lessey *et al*., 1995). However, the functional significances of the stromal αvβ3 integrins are currently not understood. In summary, we have employed an *in vitro* system of human stromal cells from proliferative endometrium to explore integrin expression in this cell type as 8-Br-cAMP model of decidualization. The αvβ3 integrins increased in decidualized endometrial stromal cells by 8-Br-cAMP, but the functional significance of these changes was not clear. Further study will need an understanding of the role of αvβ3 integrins in decidualization and implantatioin, and of the regulatory events in the trophoblast-decidual interaction.

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References

Albelda SM, Buck CA. Integrins and other cell adhesion molecules. FASEB J 1990;4:2868-80

Alpin JD, Charlton AK, Ayad S. An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. Cell Tissue Res 1988;253:231-40

Bell SC, Jackson A, Ashmore J, Zhu HH, Tseng L. Regulation of insulin-like growth factor-binding protein-1 synthesis and secretion by progestin and relaxin in long term cultures of human endometrial stromal cells. J Clin Endocrinol Metab 1991;72(5):1014-24

Bischof P, Redard M, Gindre P, Vassilakos P, Campana A. Localization of alpha 2, alpha 5, and alpha 6 integrin subunits

in human endometrium, decidua and trophoblast. Eur J Obstet Gynecol Reprod Biol. 1993;51:217-26

Brar AK, Frank GR, Kessler CA, Cedars MI, Handwerger S. Progesterone-dependent decidualization of the human endometrium is mediated by cAMP. Endocrine. 1997;6(3):301-7

Damsky CH, Sutherland AE, Fisher S. Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. FASEB 1993;7:320-1329

Dedhar S, Hannigan GE. Integrin cytoplasmic interactions and bidirectional transmembrane signalling. Curr Opin Cell Biol 1996;8:657-69

Grosskinsky CM, Yowell CW, Sun J, Parise LV, Lessey BA. Modulation of integrin expression in endometrial stromal cells in vitro. J Clin Endocrinol Metab 1996:81;2047-54

Hynes, R.O. Integrins : versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11-25

Irwin JC, Kirk D, King RJB, Quigley MM, Gwatkin RBL. Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization. Fertile Steril 1989;52:761-68

Kearns M, Lala PK. Life history of decidual cells: a review. Am J Reprod Immunol Microbiol 1983;3:78

Lessey BA, Catstelbaum AJ, Buck CA, Lei Y, Yowell CW, Sun J. Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. Fertil Steril 1994;62:497-506

Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W, Strom AL. Aberrant integrin expression in the endometrium of women with endometriosis. J Clin endocrinol Metab 1994;79:643-49

Lessey BA, Castelbaum AJ, Sawin SW, Sun J. Integrins as marker of uterine receptivity in women with primarily unexplained infertility. Fertil Steril 1995;63:535-42

Lessey BA, Damjanovich L, Coutufaris C, Castelbaum A, Alberda SM, Buck CA. Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. J Clin Invest. 1992;90:188-95

Maslar IA, Riddick DH: Prolactin production by human endometrium during the normal menstral cycle. Am J Obstet Gynecol 1979;135:751

Merviel P, Challier JC, Carbillon L, foidart JM, Uzan S. The role of integrins in human embryo implantation. Fetal Diagn Ther 2001;16(6):364-71

Mizuno K, Tanaka T, Umesaki N, Ogita S. Inhibition of cAMP-mediated decidualization in human endometrial stromal cells by IL-1beta and laminin. Horm Metab Res 1999;31(5):307-10

Pijnenborg R, Dixon G, Rovertson WB, Brosens I. Trophoblastic invasion of human deciduas from 8 to 18 weeks of pregnancy. Placenta 1950;1:3

Ruck P, Marzusch K, Kaiserling E, Horny HP, Dietl J, Geiselhart A, Handgretinger R, Redman CW. Distribution of cell adhesion molecules in decidua of early human pregnancy: an immunohistochemical study. Lab Invest 1994;71:94-101

Shiokawa S, Yoshimura Y, Nagamatsu S, Sawa H, Hanashi H, et al. Expression of β1 integrins in human endometrial stromal and decidual cells. J Clin Endocrinol Metab 1996;81:1533-40

Tabibzadeh S. Patterns of expression of integrin molecules in human endometrium throughout the menstrual cycle. Hum Reprod 1992;7:876-82

Weitlauf HM. Biology of implantation. In The Physiology of Reproduction, Edited by E Knobil, J Neill, LL Ewing, GS Greenwald, CL Markert, DW Pfaff. New York, Raven Press Ltd 1988;231-23