RESEARCH ARTICLE

Integrins expressed on the surface of human endometrial stromal cells derived from a female patient experiencing spontaneous abortion

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Abstract

Here, as a basic study in revealing the correlation between extracellular matrix components and spontaneous abortion, we defined the types of integrins expressed on the surface of endometrial stromal (ES) cells retrieved from the uterus of a patient experiencing spontaneous abortion. For these, the types of integrin subunits in the ES cells retrieved from a woman with spontaneous abortion were identified at the transcriptional and translational levels, and functional assay was conducted for confirming the combinations of integrin α and β subunits. Among the genes encoding 25 integrin subunits, significantly high transcription was seen in integrins α_1, α_2, α_3, α_4, α_5, α_V, β_1, β_3, and β_5. Translation of integrins α_1, α_3, α_5, α_V, and β_1 on the cell surface was detected in almost all ES cells, whereas integrins α_2, α_4, β_3, and β_4 were expressed translationally only in some ES cells. Subsequently, ES cells showed significantly increased adhesion to collagen I, laminin, fibronectin, and vitronectin, and functional blocking of integrin α_1, α_3, α_5, and α_V significantly inhibited adhesion to these molecules. These results demonstrated that active heterodimers composed of integrins $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_1$ were co-localized on the surface of ES cells derived from a patient experiencing spontaneous abortion.

Keywords Human · Infertility · Spontaneous abortion · Endometrial stromal cell · Integrins

Introduction

Implantation, one of the most fundamental and important processes in pregnancy, comprises a series of molecular and cellular events that accompany the growth and differentiation of the uterus, adhesion and invasion of the blastocyst, and formation of the placenta [1, 2]. However, the molecular and biological mechanisms of implantation are not yet fully known. Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage, and a synchronized dialogue between maternal and embryonic tissues [3, 4]. A number of growth factors and cytokines, as well as the extracellular matrix (ECM) and adhesion molecules, are known to be involved in the interaction between embryos and the endometrium, which governs the success of implantation

Jie Ohn Sohn and Hye Jin Park contributed equally to this work and are considered to be co-first authors.

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[4, 5]. Identification of the molecular factors involved in each stage of implantation is essential to understand the process.

Among implantation related molecular factors, the ECM is a macromolecular network containing a variety of dissolvable ECM proteins, polysaccharides, and glycoproteins [6, 7]. These contribute to a physical scaffold alleviating mechanical stress in the cytoplasm of cells [8, 9], and to signals inducing a variety of cellular responses [10, 11]. Specifically, for successful completion of the implantation process essential for embryogenesis, the endometrial ECM plays an important role as an inducer of cellular physiological events, including cell proliferation, migration, attachment, spreading, differentiation, survival, homeostasis, apoptosis, and morphogenesis [12–14]. At this time, the transduction of signals derived from each ECM component is mediated through integrin molecules [15], a group of cell adhesion molecules that exist as heterodimers with various combinations of α and β subunits [16]. Whether integrin heterodimers interacting with the specific ECM components are present or absent on the surface of endometrial cells determines the function and utility of ECM components.

Spontaneous abortion is defined as the loss of pregnancy prior to 20 weeks of gestation without medical treatment. Three or more consecutive spontaneous abortions is defined as recurrent spontaneous abortion (RSA), or habitual abortion. Fetal chromosome and endocrine abnormalities, immunological factors, and a variety of unknown factors are understood to be the major causes of spontaneous abortion [17, 18]. It has been reported that abnormalities in the localization of ECM components in the endometrium are associated with RSA [19, 20]. These abnormalities may play a pivotal role in the occurrence of spontaneous abortion. Unfortunately, there have been no studies to date on the correlation between ECM components and spontaneous abortion in the endometrium. Therefore, information on integrins contributing directly to the transmission of multiple signals derived from ECM components in the endometrium of a spontaneous abortion triggered uterus is required for determining the origin of spontaneous abortion.

Here, we examined the types of integrins expressed on the surface of endometrial stromal (ES) cells retrieved from the uterus of a infertile woman experiencing spontaneous abortion. The types of integrin subunits expressed in these ES cells were identified at the transcriptional and translational levels, and functional assays were conducted to confirm the combinations of integrin α and β subunits.

Materials and methods

Retrieval of human endometrial tissues

During this study, only one reproductive age fertile woman with experiences of spontaneous abortion was observed.

The patient was 33 years old female and attempted ART (artificial reproductive technology) program twice due to the male factor, but both were aborted spontaneously. BMI (body mass index) and AMH (anti mullerian hormone) levels were 23.5 kg/m^2 and 5.41 ng/ml, respectively, and the basal follicular stimulating hormone (FSH) level was 3.65 mIU/ml. Also, the patient was confirmed as having normal chromosomal karyotypes and has been never exposed to toxic or severe radiation in living or working environment.

Using biopsy catheter (Rampipella, RI.MOS, Italy), endometrial biopsy was conducted from a reproductive age fertile woman with normal menstrual cycles and twice experiences of spontaneous abortion. Samples of human endometrial tissues in proliferative phase (Day 12 of the menstrual cycle) were retrieved from uterus of a woman who received no hormonal therapy within 30 days before biopsy. All of the human tissue sampling, handling, and experimental procedures were approved by the institutional review board (IRB) of the Seoul Women's Hospital (IRB approval No. SWH-IC-A_2016001). Informed consent was obtained from all individual participants included in the study.

Isolation of ES cells from human endometrial tissues

The retrieved human endometrial tissues were washed with Dulbecco's Phosphate Buffered Saline (DPBS; Welgene, Gyeongsan, Korea) supplemented with 1% (v/v) antibiotic antimycotic solution (Invitrogen, Waltham, MA, USA) and then mechanically dissected into small pieces < 1 mm^3 using surgical blades. Subsequently, the fragmented tissues were digested using 0.25% trypsin EDTA (Invitrogen, Waltham, MA, USA) at 37 °C for 10 min on a shaker and filtered through a sterile 40 μm nylon strainer (Falcon, New York, NY, USA). Then, the remaining tissues on the cell strainer were retrieved and washed with DPBS supplemented with 1% (v/v) antibiotic antimycotic solution. Enzymatic digestion of the strained tissues into a single cell suspension was sequentially and separately conducted using 1 mg/ml Collagenase from Clostridium histolyticum type IV (Sigma Aldrich, St. Louis, MO, USA) in DPBS at 37 °C for 20 min on a shaker and 10 mg/ml Dispase® II (Roche, Basel, Switzerland) in DPBS at 37 °C for 10 min on a shaker. After washing twice with DPBS supplemented with 1% (v/v) antibiotic antimycotic solution, human ES cells were collected by centrifuging at 1500 rpm for 5 min and enumerated using a hemocytometer.

Culture of human ES cells

Primary human ES cells were cultured in Dulbecco's modified Eagle's medium: nutrient mixture F12 (DMEM/F12; Invitrogen, Waltham, MA, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; HyClone,

Logan, UT, USA), 1% (v/v) non essential amino acids solution (NEAA; Invitrogen, Waltham, MA, USA), and 1% (v/v) antibiotic antimycotic solution (hereafter referred to as ES cell culture medium) at 37 °C under an atmosphere of 5% CO_2 in air until 90% confluency was reached. Subsequently, subculturing was conducted at 5 days intervals and the fresh ES cell culture medium was changed every third day.

Real time polymerase chain reaction

According to the manufacturer's instructions, the Dynabeads mRNA Direct Kit (Ambion, Austin, TX, USA) was used for extracting total mRNA from the cells and cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo, Osaka, Japan). Then, the quantification of the specific gene expression was conducted using a Prime Q Matermix (GeNet Bio, Daejeon, Korea) under the qTOWER^3 Real Time PCR Thermal Cycler (Analytik Jena AG, Jena, Germany), and melting curve date was analyzed for identifying PCR specificity. The mRNA level was presented as $2^{-\Delta Ct}$, where Ct = threshold cycle for target amplification, $\Delta Ct = Ct_{\text{target gene}}$ (specific genes for each sample) − Ct$_{\text{internal reference}}$ (β-*actin* for each sample). Design of primer sequences by Primer3 software (Whitehead Institute/MIT Center for Genome Research) was performed with information of cDNA sequences obtained from GenBank for Human, and Supplementary Table 1 shows general information and sequences of primers.

Flow cytometry

Cells were fixed with 4% (v/v) paraformaldehyde (Junsei Chemical Co., Ltd., Chuo ku, Japan) for 10 min and washed with DPBS. The fixed cells were stained for 16 h at 4 °C with fluorescence conjugated anti integrin primary antibodies diluted in DPBS supplemented with 2% (v/v) FBS. Supplementary Table 2 describes the detailed information and dilution rate of the used primary antibodies. The stained cells were washed twice with DPBS and sorted using a FACS Calibur (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The BD CellQuest Pro software (Becton, Dickinson and Co.) were used for data analysis.

Attachment assay

96-well tissue culture plates were coated with the following concentration of purified extracellular matrix (ECM) proteins overnight (minimum 18 h) at 4 °C: 0, 5, 10 and 15 μg/ml collagen I (Sigma Aldrich, St. Louis, MO, USA); 0, 100, 200 and 300 μg/ml laminin (Sigma Aldrich, St. Louis, MO, USA); 0, 20, 40 and 60 μg/ml fibronectin (Millipore, Billerica, MA, USA); and 0, 5, 10 and 15 μg/ml vitronectin (Invitrogen, Waltham, MA, USA). Subsequently, for inhibiting

non specific binding of cells, each well was blocked with 1% (w/v) bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA) at 4 °C for 1 h and then the wells were washed three times with DPBS. Two times 10^4 cells resuspended in human ES cell culture medium were plated to each blocked well. After incubating at 37 °C for 2 h, the removal of non adherent cells were conducted by washing sufficiently each well and adherent cells were fixed in 4% (v/v) paraformaldehyde at room temperature for 10 min. Then, the fixed adherent cells were stained with 0.1% (w/v) crystal violet (Sigma Aldrich, St. Louis, MO, USA) in 20% (v/v) methanol (Sigma Aldrich, St. Louis, MO, USA) for 5 min and washed twice with DPBS. The amount of adherent cells were quantified at 570 nm using a microplate reader (Epoch Microplate Spectrophotometer; BioTek Instruments Inc., Winooski, VT, USA) after adding 50 μl of 0.2% (v/v) triton X 100 (Biopure, Cambridge, MA, USA) diluted with distilled water.

Peptide and antibody inhibition assay

Each well of 96well tissue culture plate was coated with 5 μg/ml collagen I, 100 μg/ml laminin, 20 μg/ml fibronectin or 10 μg/ml vitronectin overnight at 4 °C, and the coated wells was blocked with 1% (w/v) BSA for 1 h at 4 °C. Subsequently, function of integrin was inhibited by incubating 2×10^4 cells in human ES cell culture medium including integrin α_1 blocking peptides and anti integrin α_3 (MTSV1-7), anti-integrin α_4 (9C10), anti integrin α_5 (NKI-SAM-1) or anti integrin α_V (NKI-M9) blocking antibody for 2 h at 37 °C, and the detailed information regarding the used peptides and antibodies is described in Supplementary Table 2. Next, the functionally blocked cells was plated on the each well and incubated at 37 °C for 8 h. The non-adherent cells were removed by washing extensively with DPBS, the adherent cells were fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature and the fixed adherent cells were stained with 0.1% (w/v) crystal violet in 20% (v/v) methanol for 5 min. Finally, the wells washed twice with DPBS were supplemented with 50 μl 0.2% (v/v) triton X 100 diluted with distilled water and the amount of dye was measured at 570 nm using a microplate reader.

Statistical analysis

The statistical analysis system (SAS) program was used for analyzing statistically all the numerical data shown in each experiment. Comparison among treatment groups was performed by the least square or DUNCAN method, when significance of the main effects through variance (ANOVA) analysis was detected in the SAS package. Moreover, significant differences among treatments were determined when p value was less than 0.05.

Results

Determination of integrin subunits expressed on the membranes of ES cells derived from human uterine endometrium experiencing spontaneous abortion

To gain insights into the types of integrin heterodimers expressed on the membranes of ES cells in the uterine tissue derived from a infertile woman experiencing spontaneous abortion, we examined the expression of integrin subunits at the transcription and translation levels. In the transcriptional analyses of genes encoding 17 α and 8 β integrin subunits, significantly higher levels of expression were observed for integrins α_1, α_2, α_3, α_4, α_5, and α_V (Fig. 1a), and integrin β_1, β_3, and β_5 (Fig. 1b) subunit genes. Integrins α_6, α_7, α_8, α_9, α_{10}, α_{11}, α_L, and α_X (Fig. 1a), and integrin β_2, β_4, and β_8 (Fig. 1b) subunit genes, showed minimal transcriptional expression. No transcriptional expression was detected in integrins α_M, α_D, or α_E (Fig. 1a), or in integrin β_6 or β_7 subunit genes (Fig. 1b). Subsequently, we investigated the translational regulation of integrins α_1, α_2, α_3, α_4, α_5, and α_V (Fig. 1a) and integrin β_1, β_3, and β_5 (Fig. 1b) subunit genes showing increased transcription. Among the six integrin α subunit genes, almost all human ES cells showed translational expression of the integrin α_1 ($93.92 \pm 4.74\%$), α_3 ($96.61 \pm 1.52\%$),

α_5 ($99.97 \pm 0.03\%$), and α_V ($99.39 \pm 0.43\%$) subunit genes on the cell surface. However, the percentage of human ES cells showing translational expression of the integrin α_2 ($14.45 \pm 5.89\%$) or α_4 ($54.46 \pm 14.36\%$) subunit genes was significantly lower compared to the other integrin subunit genes (Fig. 2a). In the case of the integrin β subunits (Fig. 2b), translational expression of the integrin β_1 subunit gene on the cell surface was detected in almost all human ES cells ($99.95 \pm 0.05\%$). However, a significantly low percentage of human ES cells showed translational expression of integrin β_3 ($5.01 \pm 2.28\%$) and β_5 ($1.63 \pm 0.60\%$) subunit genes. These results indicate that integrins α_1, α_3, α_5, and α_V, and integrin β_1 subunits, are expressed on the surface of almost all ES cells derived from human uterine endometrium experiencing spontaneous abortion, but integrin α_2, α_4, β_3, and β_5 subunits are not.

Functional identification of integrin heterodimers expressed on the plasma membrane of ES cells derived from human uterine endometrium experiencing spontaneous abortion

Based on the results regarding the integrin α and β subunits expressed on the surface of ES cells derived from human uterine endometrium experiencing spontaneous abortion, we speculated that these cells may possess the active integrin heterodimers $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_1$, as described
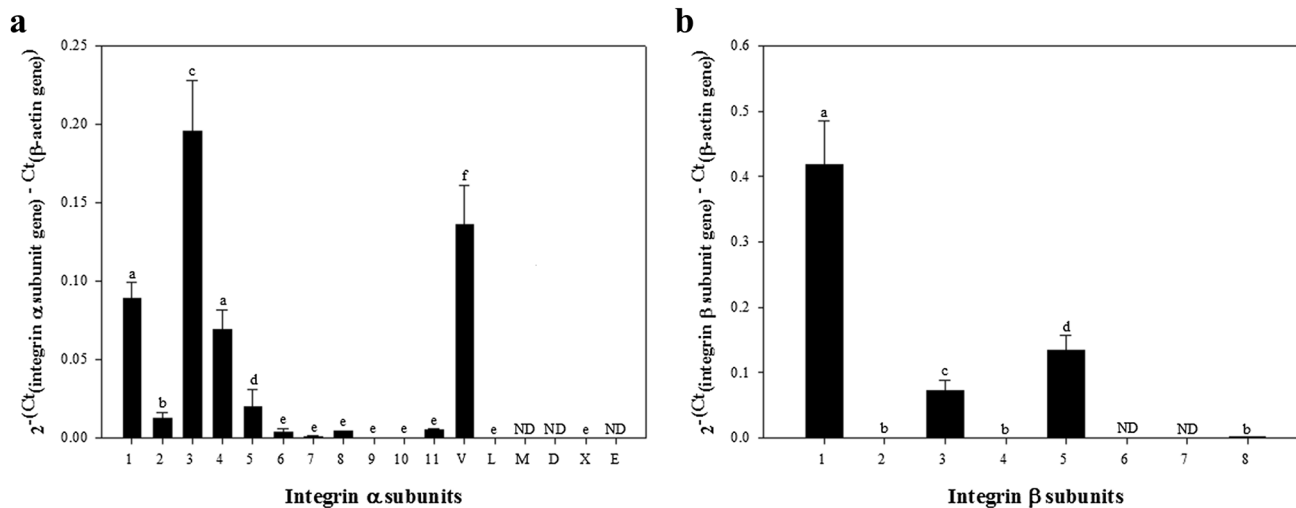
Fig. 1 Transcriptional levels of α and β integrin subunit genes in the endometrial stromal (ES) cells of uterine tissue derived from a infertile woman experiencing spontaneous abortion. Enzymatic retrieval of ES cells from uterine tissue of a infertile woman experiencing spontaneous abortion was conducted, and the retrieved human ES cells were cultured in ES cell culture medium. Transcriptional levels of α and β integrin subunit genes in the human ES cells were quantified through real time polymerase chain reaction. Among a total of 17 α and 8 β integrin subunit genes, 6 α (α_1, α_2, α_3, α_4, α_5 and α_V) (**a**) and 3 β (β_1, β_3, and β_5) (**b**) subunits in human ES cells showed significantly stronger transcription than 8 α (α_6, α_7, α_8, α_9, α_{10}, α_{11}, α_L, and α_X) (**a**) and 3 β (β_2, β_4, and β_8) (**b**) subunits showing extremely low transcription. Moreover, no transcription was observed in three α (α_M, α_D, and α_E) (**a**) and two β (β_6 and β_7) (**b**) integrin subunit genes. All data are the mean $\pm$ standard deviation of three independent experiments. [a–e]$p < 0.05$. *ND* not detected
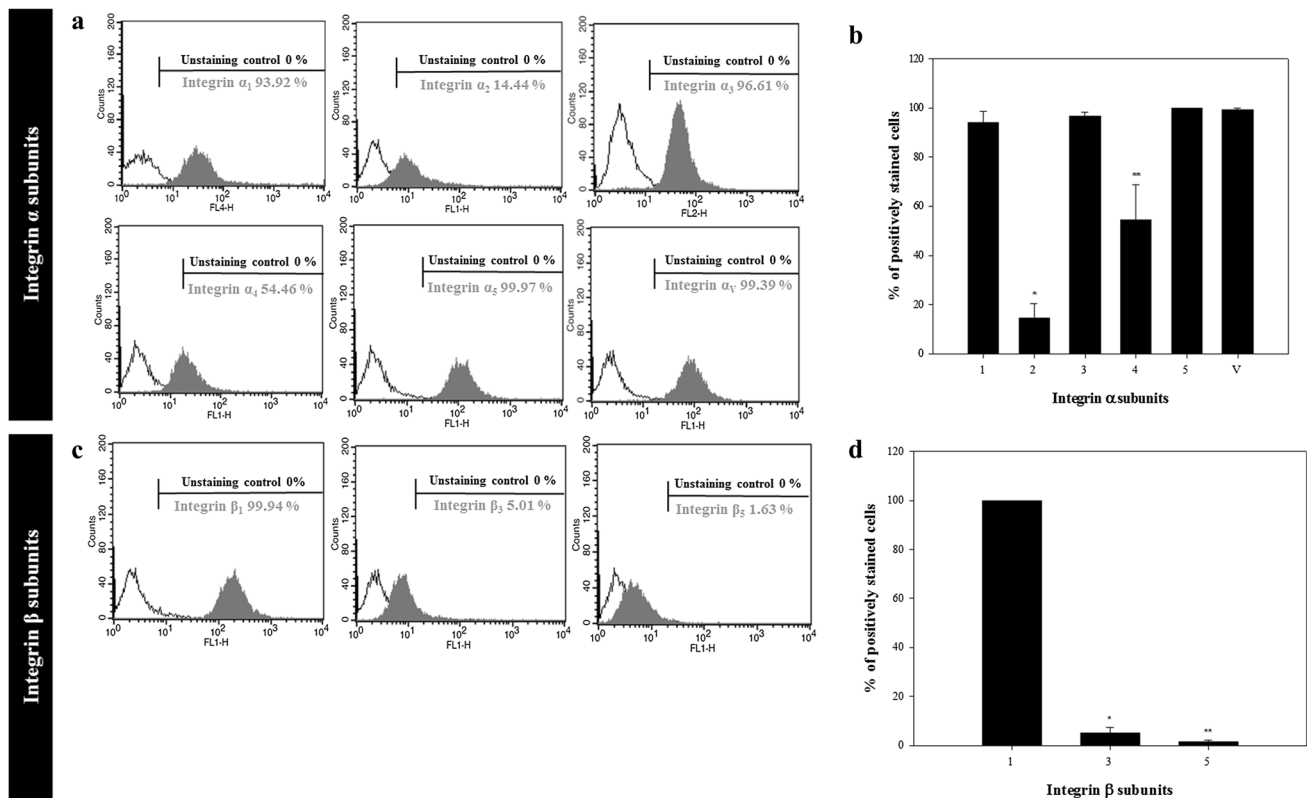
Fig. 2 Translational levels of α and β integrin subunit genes highly expressed in ES cells derived from the uterine tissue of a infertile woman experiencing spontaneous abortion. The ES cells retrieved enzymatically from uterine tissues derived from a infertile woman undergoing spontaneous abortion were cultured in ES cell culture medium. Translational levels of α and β integrin subunit genes in the human ES cells were quantitatively analyzed by flow cytometry. Among six α and three β integrin subunits, four α (α_1, α_3, α_5, and α_V) and one β (β_1) integrin subunit were detected on the surface of almost all human ES cells. However, the expression of two α (α_2 and α_4) and two β (β_3 and β_5) integrin subunits was observed only in some of the human ES cells. **a, c** are representative fluorescence activated cell sorting (FACS) analyses of the percentages of cells positively stained with antibodies to integrin α or β subunit proteins, and **b, d** are composite averages (mean ± standard deviation) of the percentage of cells positively stained with antibodies to integrin α or β subunit proteins from three independent experiments. *,**$p < 0.05$

previously [21–23]. To test for the presence of these integrin heterodimers, levels of adherent human ES cells to purified ECM proteins that interact selectively with each integrin heterodimer and adherent levels post culture of human ES cells treated with peptides or antibodies specifically blocking each integrin function on each purified ECM protein were estimated. Compared to those cultured on purified ECM protein free culture plates, the human ES cells incubated on collagen I (Fig. 3a), laminin (Fig. 3b), fibronectin (Fig. 3c), and vitronectin coated (Fig. 3d) culture plates showed significantly improved adhesion. These results suggest the presence of the collagen I interacting integrin $\alpha_1\beta_1$, laminin interacting integrin $\alpha_3\beta_1$, fibronectin interacting integrin $\alpha_5\beta_1$, and vitronectin interacting integrin $\alpha_V\beta_1$ on the plasma membrane of human ES cells. Subsequently, integrin $\alpha_1\beta_1$-, $\alpha_3\beta_1$-, or $\alpha_5\beta_1$-blocked human ES cells were incubated in 5 µg/ml collagen I, 100 µg/ml laminin, or 20 µg/ml fibronectin, as these were the minimum concentrations in the ECM associated with significantly improved adhesion of human ES cells.

In addition, integrin $\alpha_V\beta_1$-blocked human ES cells were incubated in 10 µg/ml vitronectin, as this concentration was associated with the strongest adhesion of human ES cells. As shown in Fig. 4, significantly weakened adhesion was detected in human ES cells with blockade of integrin $\alpha_1\beta_1$ (Fig. 4a), $\alpha_3\beta_1$ (Fig. 4b), $\alpha_5\beta_1$ (Fig. 4c), or $\alpha_V\beta_1$ (Fig. 4d) compared to cells without blockade of these integrin heterodimers. From these results, we confirmed that human ES cells derived from uterine endometrium experiencing spontaneous abortion simultaneously show functional expression of integrins $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_1$ on the plasma membrane.

Discussion

As communicators of external signals derived from the active sites of ECM proteins, integrin heterodimers localized on the surface of cells have been widely studied, and cell
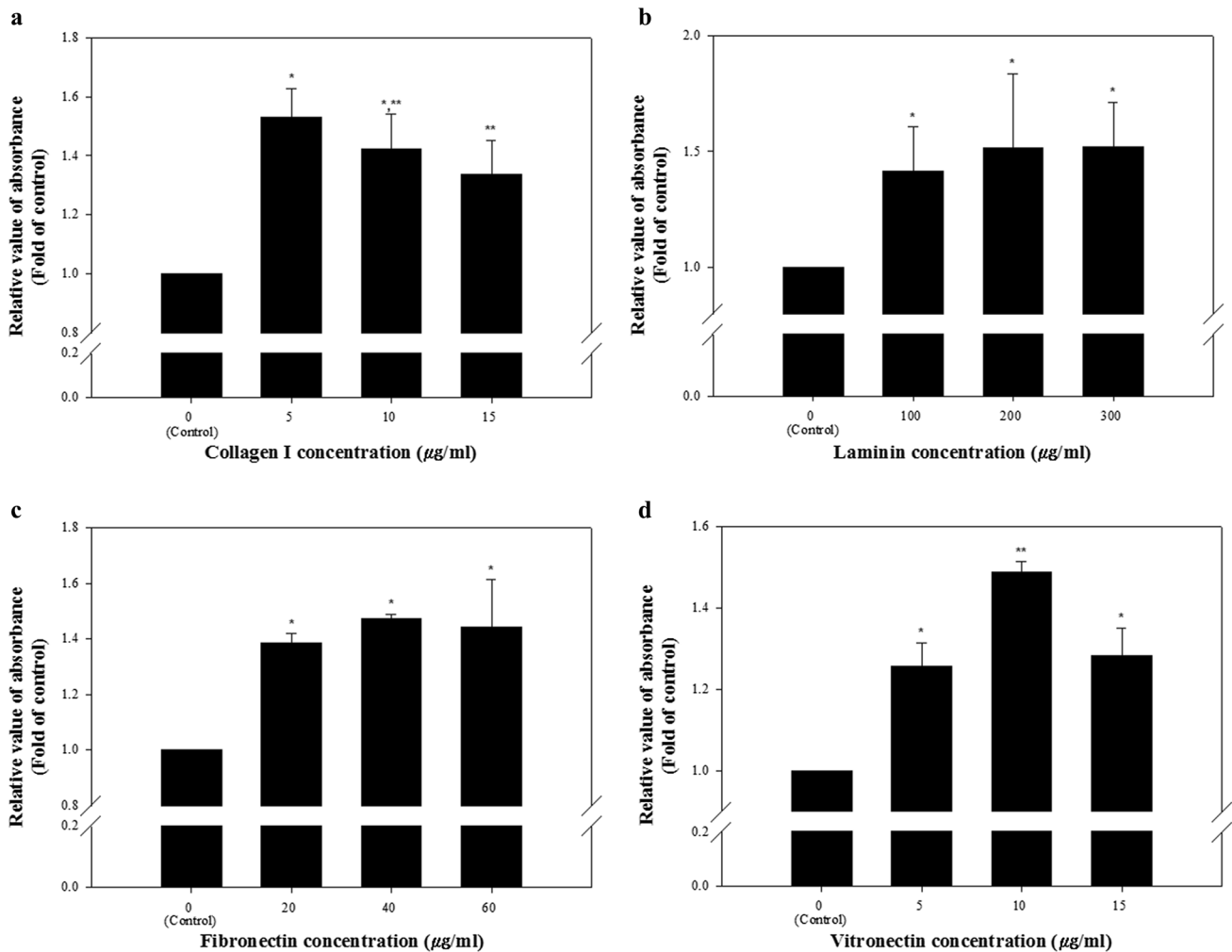
Fig. 3 Identification of integrin heterodimers interacting with collagen I, laminin, fibronectin, and vitronectin on the surface of ES cells derived from the uterine tissue of a infertile woman experiencing spontaneous abortion. Human ES cells resuspended in ES culture medium were plated in wells coated with collagen I, laminin, fibronectin, or vitronectin. Subsequently, the adherent cells stained with crystal violet were quantified using a microplate reader. The percentage of maximum adhesion is represented as the optical density of cells plated on extracellular matrix (ECM) protein free plates. The human ES cells cultured on collagen I (**a**), laminin (**B**), fibronectin (**c**) and vitronectin coated (**d**) culture plates showed significantly higher adhesion levels than those on ECM free culture plates. All data are the mean ± standard deviation of three independent experiments. *,**$p < 0.05$

functions are dependent on types of these transmembrane proteins. Accordingly, information concerning the localization of integrin heterodimers on the surface of ES cells in a infertile woman experiencing spontaneous abortion is more valuable for understanding the mechanism underlying spontaneous abortion than information concerning ECM proteins. Here, we report the types of integrin heterodimers expressed on the surface of ES cells derived from a infertile woman experiencing spontaneous abortion. Transcriptional analysis of 17 α and 8 β integrin subunits, followed by confirmation of their expression at the translational level, attachment to ECM proteins, and inhibition with blocking antibodies revealed the presence of the integrin heterodimers $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_1$ on the plasma membrane of ES cells.

These results suggest that the collagen I interacting integrin $\alpha_1\beta_1$, laminin interacting integrin $\alpha_3\beta_1$, fibronectin interacting integrin $\alpha_5\beta_1$, and vitronectin interacting integrin $\alpha_V\beta_1$ in ES cells may be important for the transmission of extracellular signals that organize endometrial tissue. Moreover, collagen I, fibronectin, laminin, and vitronectin analogs will be important for the construction of artificial niches designed to promote the organization of endometrial tissue.

Previous studies have demonstrated that the expression of integrin subunits in the endometrium was extensively altered during the human menstrual cycle [24, 25]. Nevertheless, the uniform expression of integrin α_2 and α_4 subunits was observed throughout the menstrual cycle in human endometrium without any abortion experience [26, 27]. However,
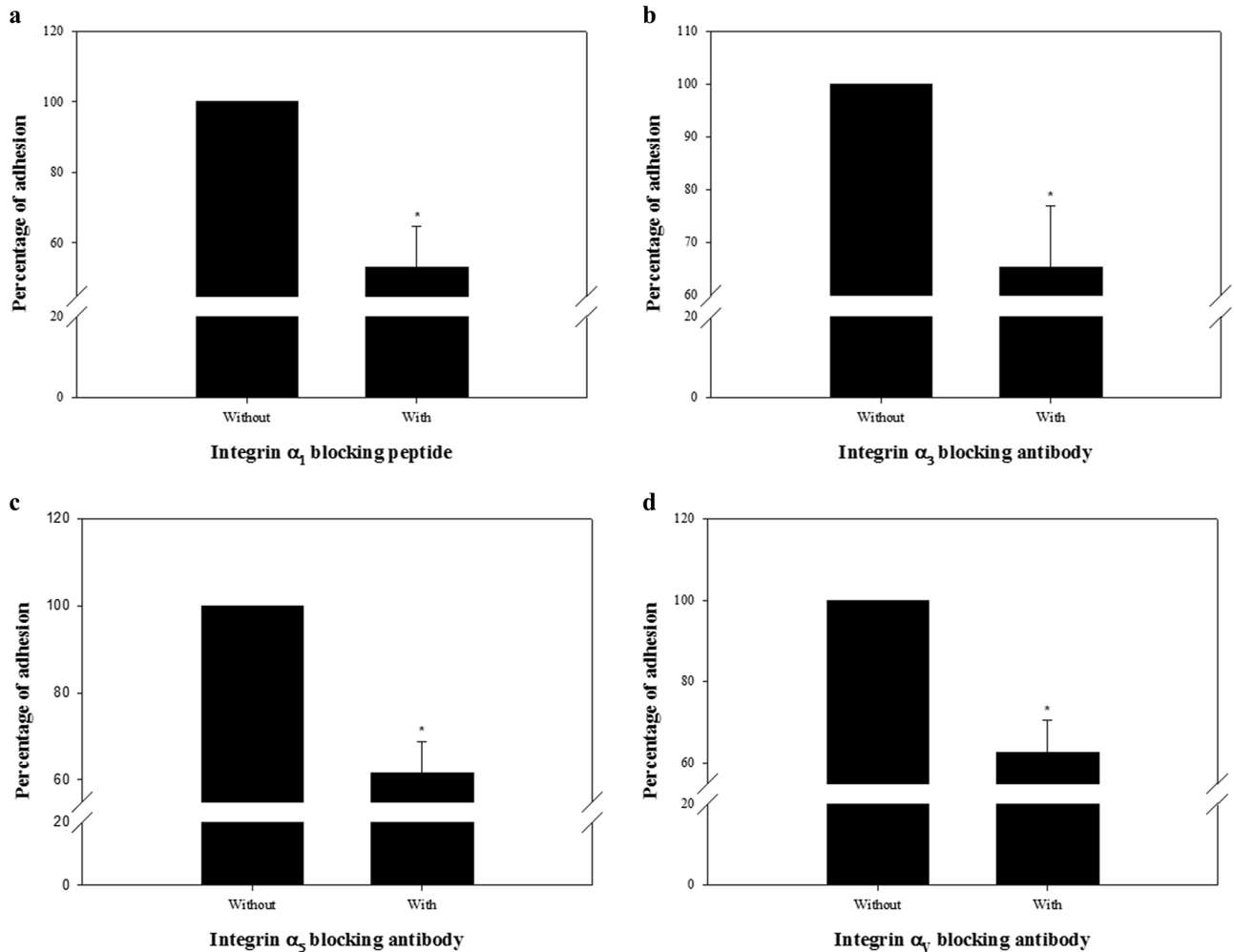
Fig. 4 Functional analysis of integrin heterodimers presumed to be expressed on the surface of ES cells derived from the uterine tissue of a infertile woman experiencing spontaneous abortion. Human ES cells were incubated in the absence or presence of integrin α_1 blocking peptides or anti integrin α_3, anti integrin α_4, anti integrin α_5, or anti integrin α_V blocking antibodies, and human ES cells with integrin blockade were plated on 5 µg/ml collagen I (integrin α_1 blockade), 100 µg/ml laminin (integrin α_3 blockade), 20 µg/ml fibronectin (integrin α_5 blockade), or 10 µg/ml vitronectin coated (integrin α_V blockade) wells. After incubation for 2 h at 37 °C, the adherent cells were stained with crystal violet and the adhesion level was quantified using a microplate reader. The percentage of maximum adhesion, represented by the optical density of cells plated on each ECM protein coated well in the absence of blocking peptides or antibodies, was elucidated. The human ES cells treated with integrin α_1 blocking peptides (**a**) or integrin α_3 (**b**), α_5 (**c**), or α_V (**d**) blocking antibodies showed significantly lower levels of adhesion than those without blocking peptides or antibodies. All data are the mean $\pm$ standard deviation of three independent experiments. *$p < 0.05$

Fig. 2 in this study shows that almost all ES cells derived from the endometrium of a infertile woman experiencing spontaneous abortion did not express integrin α_2 or α_4 subunit proteins on the cell surface. In other words, the integrin α_2 subunit positive proportion of ES cells was very low, and the proportion of integrin α_4 subunit positive ES cells was intermediate. Therefore, the presence of integrin $\alpha_2\beta_1$- and/or $\alpha_4\beta_1$-free ES cells in the endometrium may be the cause of spontaneous abortion. This is supported by the fact that inappropriate localization of integrin heterodimers in endometrial tissue cells weakens the major endometrial functions [28, 29]. Simultaneously, transcriptional or translational level integrin α_2 and/or α_4 subunit expression in endometrial tissues may become a marker for estimating the likelihood of spontaneous abortion in fertile women.

In previous reports, localization of integrin α_M based heterodimers on the surface of neutrophils essential for innate immunity was identified in ES cells derived from animals [30, 31]. However, no transcription of integrin α_M subunit genes was detected in the ES cells derived from the endometrial tissue of a infertile woman experiencing spontaneous abortion (Fig. 1), resulting in an absence of integrin α_M based heterodimers. Accordingly, immunological disorders caused by the breakdown of innate immunity

can be considered as another factor involved in spontaneous abortion.

In conclusion, the surface of ES cells derived from a infertile woman experiencing spontaneous abortion clearly showed co localization of the active integrins $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_V\beta_1$ on the plasma membrane; simultaneously, integrin α_2, α_4, β_3, and β_5 subunits were identified in a subset of these integrins. These results will greatly contribute to research on the causes of miscarriage, as well as to new diagnostic tools, through comparative analysis with data on the integrin subunits and heterodimers expressed on the surface of ES cells derived from women with normal pregnancies.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional review board of Seoul Women's Hospital as well as the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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