Physiologic Role of Decidual \( \beta_1 \) Integrin and Focal Adhesion Kinase in Embryonic Implantation

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Abstract. Implantation refers to a series of interactions between embryo and endometrium including hatching, attachment, and outgrowth. We investigated the expression and function of \( \beta_1 \) integrin and focal adhesion kinase (FAK) in human decidual cells during implantation. Immunofluorescent staining localized \( \beta_1 \) integrin to surfaces of cultured decidual cells. Double staining for \( \beta_1 \) integrin and mediators of intracellular signaling involving \( \beta_1 \) integrin, such as FAK and vinculin, colocalized \( \beta_1 \) integrin with these substances, suggesting that human decidual cells express \( \beta_1 \) integrin in the focal adhesion region. We next investigated the actions of \( \beta_1 \) integrin and FAK in implantation by co-culturing mouse embryos and human decidual cells. Mouse blastocysts attached to cultured decidual cells after embryo hatching, usually within 24 h of culture initiation. Blastocysts attached to decidual cells exhibited extensive outgrowth at 48 h. Treatment of decidual cells with an antibody against \( \beta_1 \) integrin or with an antisense FAK oligonucleotide did not affect hatching or attachment of blastocysts, but either one could inhibit outgrowth. Thus, it was concluded that human decidual \( \beta_1 \) integrin and FAK participate in this final step of implantation.

Key words: \( \beta_1 \) integrin/Focal adhesion kinase, Focal adhesions, Decidual cell, Spreading, Implantation

INTERACTIONS of cells with their neighbors through extracellular matrix (ECM) proteins are observed in a variety of biologic processes including embryonic development, cell migration, growth control, and tissue repair [1]. These interactions are mediated largely by members of the integrin family, which are located on cell surfaces, and consist of \( \alpha \) and \( \beta \) subunits [2–5]. Each subunit is composed of a large extracellular and a short intracellular domain; an exception is the \( \beta_1 \) integrin subunit, which possesses an extremely long cytoplasmic tail. The \( \beta_1 \) subunit can be associated with at least 10 different \( \alpha \) subunits, constituting the largest subfamily among integrins. Integrins act as transmembrane receptors for ECM constituents. As such, integrins play a critical role in cell attachment through focal adhesion molecules. Focal adhesion kinase (FAK) is a key protein expressed at focal adhesion sites [6], which are aggregates of protein complexes including activated FAK that recruit other proteins including paxillin, p130Cas, vinculin, and talin [7]. These protein complexes anchor the actin cytoskeleton and maintain the structural integrity of cells [8]. Phosphorylation of focal adhesion proteins is important for turnover at focal adhesion sites, allowing for reorganization of the actin cytoskeleton and cellular movement [9].

In human embryonic implantation a large number of trophoblasts invade the decidua, extending as far as the third layer of the myometrium [10]. Earlier studies have demonstrated that mouse blastocysts at the pre-
implantation stage express β1 integrin, which interacts with fibronectin (FN) as well as laminin [11–13]. These ECM proteins, when individually precoated on tissue culture plates, promote in vitro attachment and outgrowth of mouse blastocysts in serum-free medium [14]. This finding suggests that ECM proteins produced by decidua provide a site for trophoblast attachment [15–17]. Human endometrial and decidual cells express many specific substances [18, 19] including members of the β1 integrin family on their surfaces in a dynamic process related to the menstrual cycle [20–23]. Expression of β1 integrins in human endometrium increases at the time of implantation and remains high in the decidua during early pregnancy [23–26]. In addition, decidual β1 integrin is involved in the implantation, as is trophoblastic β1 integrin [27]. Integrins themselves have no enzymatic activity and must rely upon interactions with accessory proteins for generation of cytoplasmic signals. FAK, considered important in integrin-mediated signal transduction, is associated with integrins both functionally and spatially. The aim of the present study was to elucidate the physiologic significance of decidual β1 integrin and FAK in implantation. We localized β1 integrin and FAK on human decidual cells by immunofluorescent staining, and also studied the effects of anti-β1 integrin antibody and antisense FAK oligonucleotide on mouse embryo hatching, attachment, and outgrowth in an experimental system using human decidual cells.

Materials and Methods

Reagents

Mouse anti-human integrin β1 subunit monoclonal antibody was purchased from Chemicon International (Temecula, CA, USA). Rabbit anti-human FAK polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphorylated (phospho)-FAK rabbit polyclonal IgG, from which antibodies to nonphosphorylated-FAK had been immunosorbed was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Vinculin monoclonal anti-mouse IgG1 was purchased from Sigma Chemical (St. Louis, MO, USA). Fluorescently labeled secondary antibodies including Cy2-labeled donkey anti-rabbit IgG and Cy3-labeled donkey anti-mouse IgG, were purchased from Jackson Immuno Research (West Grove, PA, USA). We purchased 4,6-diamidine-2-phenylindole hydrochloride (DAPI) from Boehringer (Mannheim, Germany), and fibronectin (FN) from Iwaki Glass (Chiba).

Decidual cell culture

Specimens of decidua were obtained from 20 women undergoing therapeutic abortion at 7 to 11 weeks of gestation. All women gave informed consent for collection of the tissues, and the study was approved by the Ethics Committee at Kyorin University School of Medicine. Blood clots were removed, and the decidual tissue was rinsed extensively in ice-cold medium 199 (Invitrogen, Carlsbad, CA, USA). Tissue was trimmed and cut into small pieces using a small pair of scissors. A portion of tissue was stained with hematoxylin and eosin for histologic examination. The remaining tissue was treated enzymatically to disperse cells. Isolation of the decidual cells was performed by the methods described previously [28, 29], with minor modifications. The tissue was treated with 0.1% collagenase (Type IA; Sigma) and 0.1% hyaluronidase (Type IS; Sigma) in Ca2+-free phosphate-buffered saline (PBS) at 37°C for 1 h. After digestion and dispersion, the cell suspension was filtered through a nylon mesh (pore size, 105 μm) to remove undigested tissue debris. The cells were collected from the filtrate by centrifugation at 800 × G for 10 min, and the pellet was resuspended in medium 199 containing 10% fetal calf serum (FCS; Invitrogen). The cell suspension was filtered through a 38-μm stainless steel mesh (Spectrum, Los Angeles, CA, USA) to retain glandular elements as previously described [28, 30]. Stromal cells then were collected by centrifugation. These decidual cells were washed three times in medium 199 supplemented with 10% FCS and 1% antibiotic-antimycotic mixture. Aliquots of decidual cell suspension were counted using dye exclusion (0.4% trypan blue by volume in PBS) to assure viability. More than 90% of cells were shown to be alive. The stromal cells were plated at 5 × 10^6 cells/ml in a plastic Petri dish (35 × 10 mm, Falcon #3001; Beckton-Dickinson, Billerica, MA, USA). The culture medium was changed every 48 h, and the cultures were maintained at 37°C for 10 days in a humidified mixture of 95% air/5% CO₂.
Table 1. Oligonucleotide sequences for focal adhesion kinase (FAK)

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>FAK-AS (20-mer)</td>
<td>ATATCCAGCTTGAACCAAG</td>
</tr>
<tr>
<td>FAK-MS (20-mer)</td>
<td>ATATTCAGCTTCAACCAAG</td>
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AS, antisense; MS, missense.

Immunofluorescent staining of decidual cells

Decidual cells were cultured for 48 h in medium 199 containing 10% FCS on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA). To prevent cells from slipping off from slides following treatment, slides were coated with FN. The cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed three times with PBS. The cells were then permeabilized with 0.5% Triton X-100 for 10 min and incubated at 37°C for 60 min with one of two sets of antibodies: (1) rabbit anti-human phospho-FAK polyclonal antibody (1:50) mixed with mouse anti-vinculin monoclonal antibody (1:50); or (2) rabbit anti-human phospho-FAK polyclonal antibody (1:50) mixed with mouse anti-human integrin β1 subunit monoclonal antibody (1:50). The cells were rinsed extensively in PBS and then stained with a mixture of Cy2-labeled donkey anti-rabbit IgG, Cy3-labeled donkey anti-mouse IgG, and DAPI for 60 min at 37°C. Slides were washed in PBS, rinsed in deionized water, and coverslipped with glycerol mounting medium. Cells were observed by confocal laser scanning microscopy (Olympus Optical, Tokyo). All double stainings was examined to ensure that no undesired cross-reactivity existed between primary and secondary antibodies.

FAK antisense oligonucleotides

Phosphorothioated oligonucleotides directed against the portion of the FAK gene encoding amino acids 262 to 268 were used as FAK antisense oligonucleotides (FAK-AS) [31]. A sequence containing a five-nucleotide base mismatch of FAK-AS was used as a control (FAK-MS). Oligonucleotides were purchased from Sawaday Technology (Tokyo). For an FAK attenuation study, decidual cells were plated at a density of 2 × 10^5/well in 6-well plates or 10^4 per 100-mm tissue culture plate and incubated for 10 days at 37°C. Cells were then treated with FAK-AS or -MS [31, 32]. Oligonucleotides were added at 0.15 μM with 5 μg/μl lipofectin reagent (Invitrogen Corp., Carlsbad, CA, USA) and incubated for 24 h at 37°C. The treated decidual cells were immunostained with rabbit anti-human FAK polyclonal antibody. Immunostaining intensity was quantified by using the NIH Image program (National Institutes of Health, Bethesda, MD USA).

Protein extraction and Western blotting

Decidual cells treated with oligonucleotides were lysed in NP40 lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 5 mM EDTA, 1 mM Na3VO4, and 10 μg/ml each of aprotinin and leupeptin]. Protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL). Portions of each sample (50 μg) were subjected to 7.5% sodium dodecyl sulfate (SDS-PAGE) and Western blotting, followed by incubation with rabbit anti-human FAK polyclonal antibody.

Mouse embryo attachment and spreading assay

Female ICR mice (10 weeks old; Clea Japan Tokyo) were superovulated with an injection of 5 IU of eCG (Teikoku-Zoki, Tokyo) followed after 48 h by an injection of 2.5 IU of human chorionic gonadotrophin (hCG; Teikoku-Zoki). Treated female mice then were caged with male mice overnight. Embryos at the late morula stage or early blastocyst stage (96 h after administration of hCG) were obtained by flushing the uterine horns and rinsed in medium 199 supplemented with 0.4% bovine serum albumin (BSA). After decidual cells had been cultured in medium 199 supplemented with 10% FCS for 10 days, the cells were incubated with a monoclonal antibody against the β1 subunit at concentrations of 1 to 100 ng/ml. Five to eight embryos were then placed in dishes prepared with a subconfluent monolayer culture of decidual cells for coculture up to 96 h. In control cultures, decidual cells were incubated with a purified mouse monoclonal IgG1 antibody at a concentration of 100 ng/ml before addition of the embryos. To determine embryo attachment, a small amount of medium was gently flushed over each embryo using a fine-bore glass pipette. While being observed under an inverted phase-contrast microscope (Olympus) embryos that
showed no movement were considered to have attached. Spread of embryos was defined as migration of individual cells or monolayers of trophoblasts from the ectoplacental cone rudiment. To determine the extent of spread embryos were photographed at a magnification of 200x; each negative was printed at the same enlargement. The area of outgrowth was measured using a color image analyzing system (SP500; Olympus) by a method described by Imamura et al. [33]. Area of spread of each embryo was calculated from the average of three tracings. The result of each treatment represents the mean of measurements for at least 35 embryos. Determinations of embryo attachment and spread were made at 24 and 48 h of incubation, respectively. The area of embryo outgrowth was determined at 48 to 96 h incubation.

Statistical analysis

The percentage of embryos that hatched, attached, and spread, and the area of embryo spread are expressed as the mean ± SEM of triplicate cultures. To obtain a normal distribution, the percentages of embryo attachment and spreading were subjected to an arcsine transformation. The results represent duplicate measurements made in six separate experiments and are expressed as the mean ± SEM. The terms “increase” and “decrease” are applied to results only when were statistically significant. Statistical analysis was carried out using analysis of variance (ANOVA) and Scheffe’s test. A p value of less than 0.05 was considered to represent statistical significance.

Results

Immunofluorescent staining

To investigate localization of β1 integrin and adhesion molecules that mediate integrin signals in human decidual cells on FN-coated plates, double immunofluorescent staining was performed at 48 h after plating. The decidual cells had spread completely at this time. Cells were studied for the subcellular localization of β1 integrin and phospho-FAK (Fig. 1A, B). Phospho-FAK staining showed a pattern similar to that of vinculin (Fig. 1D, E), which is well known as a marker of focal adhesion. Combined images revealed identical expression sites; in these, nuclei were stained using DAPI (Fig. 1C, F).

Mouse embryo attachment and spreading assay

Blastocysts became attached to the cultured decidual cells in the presence of a purified mouse monoclonal IgG1 antibody after the embryos hatched from the zona pellucida. Immediately after hatching, the blastocysts were nonadherent to the cultured decidual cells, and their surface remained quiescent for 5 to 8 h. The trophoblasts of the embryos started to attach to the cultured decidual cells 10 to 12 h after initiation of culture with decidual cells, and exhibited extensive outgrowth after 48 h. Trophoblasts from attached blastocysts grew in a flat monolayer originating from the ectoplacental cone rudiment (Fig. 2A). Addition of anti-β1 subunit antibody to cultured decidual cells did not affect the hatching ratio. Attachment of the hatched blastocysts was slightly, but not significantly, reduced in cultures treated with the anti-β1 subunit antibody as compared to control cultures (Fig. 3A). Outgrowth of trophoblasts from attached blastocysts was observed at 48 to 96 h of coculture with decidual cells when incubation was carried out with a mouse monoclonal IgG1 antibody at 100 ng/ml as previously described [34]. The area of trophoblastic outgrowth from the attached blastocyst was significantly decreased by addition of anti-β1 subunit antibody at 100 ng/ml (Fig. 2). Furthermore, exposure of decidual cells to anti-β1 subunit antibody decreased the area of trophoblasts outgrowth in a dose-dependent manner as measured at 72 h of coculture (Fig. 3B). In the presence of anti-β1 subunit antibody, blastocysts remained attached to decidual cells for 48 h, but often appeared to have collapsed. Many of these embryos became detached from the decidual cell surface by 96 h.

FAK-AS reduced the mean immunofluorescence of FAK on cultured decidual cells (Figs. 4B, 5), while FAK-MS did not (Figs. 4C, 5). We confirmed by Western blot analysis, that FAK expression was diminished by treatment of decidual cells with FAK-AS (Fig. 6). Addition of FAK-AS or FAK-MS to cultured decidual cells did not affect the hatching ratio. Attachment of hatched blastocysts was slightly, but not significantly, reduced in cultures treated with FAK-AS compared with those treated with FAK-MS (Fig. 8A). Spread of trophoblasts from attached blastocysts was observed 48 to 96 h after coculture with decidual cells. The area of trophoblastic outgrowth from attached
Fig. 1. Double staining immunofluorescent detection of focal adhesion kinase (FAK) in human decidual cells 48 h after culture on fibronectin (FN)-coated coverslips. Staining for integrin β1 subunit (A), phosphorylated (phospho)-FAK (B) and a combined image including DAPI (C), are shown, as are staining for vinculin (D), phospho-FAK (E) and a combined image including DAPI (F). Immunofluorescent staining was carried out on permeabilized cells. Note staining at focal contacts indicated by arrows. Bar = 10 μm.

Fig. 2. Trophoblast spread of mouse blastocyst on human decidual cells treated with a monoclonal antibody against the integrin β1 subunit at various concentrations (A, 0 ng/ml; B, 1 ng/ml; C, 10 ng/ml; and D, 100 ng/ml). Spread of embryos was determined 72 h after beginning coculture with decidual cells. Trophoblast spread was inhibited by addition of the anti-integrin β1 subunit antibody to decidual cells. Bar = 100 μm.
blastocysts was decreased significantly by addition of FAK-AS, while addition of FAK-MS did not alter the area of trophoblastic outgrowth (Figs. 7, 8B).

**Discussion**

In this study, double staining for FAK, vinculin, and β1 integrin showed clear colocalization. The distribution of FAK in human decidual cells was prominent in the focal adhesion regions (adhesion plaques or focal contacts) where cells interact with ECM constituents [7]. We also found that the integrin β1 subunit in decidual cells was located in peripheral focal adhesions. These areas are enriched in integrins and in cytoskeletal and signaling proteins including talin, vinculin, paxillin, and FAK. Focal adhesions are thought to function as connections between the cytoskeleton and the ECM that confer structural integrity as well as acting as signaling organelles. These findings suggest that the focal adhesion on decidual cells may be the key region of interaction between maternal and embryonic cells.

To investigate the functional role of integrin β1 and FAK on implantation, we use a coculture system with mouse blastocyst and human decidual cells. Mouse blastocysts attach to and spread over human decidual cells. After a period of quiescence, hatched blastocysts are capable of attaching to decidual cells and forming extensive trophoblastic outgrowth. Because embryos grown in vitro displayed blastocyst attachment and outgrowth of trophoblasts, this system was chosen for studies of factors regulating trophoblast differentiation. Attachment of blastocysts to cultured decidual cells appeared to be a necessary prerequisite for further outgrowth of trophoblasts. In this study, outgrowth, but not attachment, of embryos on decidual cells was inhibited dose-dependently by a monoclonal antibody recognizing the integrin β1 subunit, suggesting that β1 integrins have an important role in blastocyst development and differentiation after attachment. The mechanism by which β1 integrins promote trophoblast outgrowth may reflect both their known adhesive action and their roles in cell attachment, detachment, and migration of cells [4, 35, 36]. ECM proteins are expressed during early embryogenesis; for example, laminin B1 and B2 chains appear at the four-cell stage [12, 13, 37] and fibronectin and collagen type IV are first detected at the blastocyst stage [9, 10]. Recently, isolated trophoblasts in primary culture have been shown to synthesize and secrete fibronectin molecules bearing a unique glycopeptide domain, defined as the oncofetal fibronectin class within the type-III connecting segment, this domain may mediate implantation and placental-uterine attachment throughout gestation [38]. Thus, blastocyst invasion may be mediated by β1 integrins on decidual cells.

In this study we showed localization of integrin β1 on decidual cells at focal adhesions. Focal adhesions are integrin-based structures that mediate strong cell-
Fig. 4. Inhibition of focal adhesion kinase (FAK) in decidual cells by antisense (AS) oligonucleotide. Decidual cells were cultured on fibronectin (FN)-coated coverslips for 48 h (A). The cells were treated with FAK-AS (B) or FAK-missense (MS) (C). FAK-AS treated decidual cells shows a marked decrease in FAK. Bar = 100 μm.

Fig. 5. Immunostaining intensity was quantified using the NIH Image program. Decidual cells was treated with focal adhesion kinase (FAK)-antisense (AS) (・), FAK-missense (MS) (○) or no oligonucleotide (□). *P<0.05, **P<0.01 vs. FAK-AS.

Fig. 6. Western blot analysis of focal adhesion kinase (FAK) expression in human decidual cells treated with FAK-antisense (AS) (line 1), FAK-missense (MS) (line 2) or no oligonucleotide (line 3). Expression of FAK was diminished by following treatment of decidual cells with FAK-AS.

substrate adhesion and also transmit information in a bidirectional manner between the ECM and the cytoplasm. Schmidt et al. reported that integrin-cytoskeleton linkages influence cell migration [39, 40]. In human implantation, numerous trophoblasts infiltrate through the decidua and extend as far as the third layer of myometrium [41]. We demonstrated that β1 integrins are involved in the spread of mouse embryos over decidual cells [27, 32]. Previous findings as well as the present results suggest that β1 integrin-cytoskeleton linkage in focal adhesions on human decidual cells may be important in mediating implantation.

Integrins themselves have no enzymatic activity and therefore must rely upon interactions with accessory proteins for generation of cytoplasmic signals. Focal
Fig. 7. Trophoblast spread of mouse blastocysts on human decidual cells treated without (A) or with focal adhesion kinase (FAK)-antisense (AS) (B) or FAK-missense (MS) (C). Spread of embryos was determined 72 h after beginning coculture with decidual cells. Trophoblast spread was inhibited by the addition of FAK-AS to decidual cells. Bar = 10 μm.

Fig. 8. Effects of focal adhesion kinase (FAK)-antisense (AS) or FAK-missense (MS). A. Hatching (□) and attachment (■) of blastocysts. Hatching and attachment were assessed at 12 h and 24 h after beginning coculture with decidual cells. B. Measurements of area of spreading (μm²) of embryos cultured without oligonucleotide (● ▲ ●) or with FAK-AS (● ● ●) or FAK-MS (■ ■ ■). The area of spread was determined at 48 h to 96 h of coculture with decidual cells. *P<0.1, **P<0.01, ***P<0.001 vs. control.

Adhesions are thought to be important not only as structural links between the ECM and the cytoskeleton, but also as sites of signal transduction from the ECM [42]. Furthermore, Maruyama et al. reported that tyrosine phosphorylation of decidual FAK may be regulated mainly by decidualization-associated growth factors [43]. We presently demonstrated that attenuation of FAK expression on decidual cells reduced embryo outgrowth on decidual cells. In a previous study we found that the outgrowth of embryos over decidual cells, but not the attachment, to be inhibited by a tyrosine kinase inhibitor, herbinicin A [44]. This suggests that tyrosine phosphorylation on decidual cells by tyrosin kinases, including FAK, may be important in development and differentiation following attachment. However, we cannot rule out the possibility that herbinicin A affects other tyrosine kinases. In the present study, we treated the decidual cells with antisense oli-
gonucleotide, which reduced the expression of FAK on decidual cells. This treatment also reduced embryo outgrowth on decidual cells. The function of FAK also has been investigated by gene manipulation [45]. FAK-deficient mice die at embryonic day 8 from multiple mesodermal defects. Mesodermal cells cultured from the embryos show decreased spread and motility. Although antisense FAK could possibly affect the trophoblast directly, the results suggest that FAK is involved in mediating spread and motility. These data along with previous findings underscore the importance of FAK on decidual cells in outgrowth of embryos on these cells.

In conclusion, β3 integrin and its accessory protein, FAK, are localized, in human decidual cells at regions known as focal adhesions. A β3 integrin-cytoskeletal linkage at focal contacts on human decidual cells may be an important mediator of implantation. Mouse blastocysts attached to and spread over human decidual cells. Outgrowth of embryos on decidual cells, but not attachment, was inhibited by FAK anti-sense oligonucleotide, suggesting that FAK on decidual cells may be important in development and differentiation following attachment. Activation of FAK may represent one step on the complex, multistep process of cytoskeletal assembly [46–48]. Further studies are needed to explore how other focal adhesion molecules participate in the process of implantation.

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References


