Role of αVβ3 integrin in embryo implantation in the mouse

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Abstract Integrin, a heterodimeric adhesive molecule composed of α and β subunits, can regulate cell adhesion and trafficking. Recent data have documented that, at the "implantation window" stage, αVβ3 integrin participates in the maternal-fetal interaction and becomes a potential marker of uterine receptivity. Furthermore, it can affect invasiveness of embryo. This work made a further study about its action mechanism. Results of indirect immunofluorescence and laser scanning confocal microscopy showed that αVβ3 integrin was clearly expressed in the mouse blastocyst. Injection of αVβ3 integrin antiserum into a uterine horn of a pregnant mouse on day 3 markedly decreased the number of embryos implanted ($P < 0.001$). In a co-culture model, αVβ3 integrin antisera at 1 : 100 and 1 : 200 dilutions significantly depressed the attachment and outgrowth reactions of blastocysts on monolayer of uterine epithelial cells. Analysis of correlation manifested that the inhibitory effect of αVβ3 integrin antiserum was dosage/dilution-dependent. Thus, αVβ3 integrin is an essential factor in the uterine endometrium for embryo implantation in the mouse. This integrin distinctly expressed in the mouse blastocyst at "implantation" stage affected the process of embryo implantation by route of mediating both the attachment and the outgrowth processes of blastocyst on uterine epithelial cells.

Keywords: αVβ3 integrin, co-culture, embryo implantation, mouse.

Since the mid-1980s, there has been considerable excitement regarding the discovery and characterization of a new family of cell adhesion molecules (CAMs) known as integrin[1]. These heterodimeric glycoprotein receptors consist of a 120—180 ku α-subunit and a 90—110 ku β-subunit, which combine variously to form over 21 receptors with distinct ligand-binding specificity[2]. It is well established that, by way of interaction with extracellular matrix components such as fibronectin and

vitronectin, integrin plays an important role in cell adhesion and trafficking and contributes to diverse functions of cell migration, organization of the cytoskeleton, and transduction of differentiation signals[3]. Successful embryo implantation into uterine endometrium depends upon the synchronized development of both the invasiveness of blastocyst and the receptivity of uterine endometrium, as well as on their intimate co-ordination. This process was elaborately orchestrated by a series of cellular or molecular events[4]. αVβ3 integrin plays an important role in embryo implantation, and has been widely regarded as an epithelial marker of the opening of the window of implantation[1]. The effect of αVβ3 integrin was reported in the mouse[5] and human[6]. Till now, however, the defined pathway in which αVβ3 integrin functions during embryo implantation has not been fully known. Therefore, by *in vivo* (injection into uterine horn and indirect immunofluorescence) and *in vitro* (co-culture) methods, effect of αVβ3 integrin on trophoblast invasiveness in the mouse was further studied, and its action machinery was also elucidated in this note.

1 Materials and methods

(i) Reagents. Polyclonal rabbit antiserum against αVβ3 integrin, Ham's F_{12} medium, fetal calf serum (FCS) and trypsin were purchased from Gibco BRL; FITC-conjugated anti-rabbit secondary antibody from the goat, paraformaldehyde (PFA), bovine serum albumin (BSA) and propidium iodide (PI) were from Sigma.

(ii) Animals. Adult mice of the outbred Kunming white strain (5—6 weeks old, 25—30 g), were purchased from the Experimental Animal Center of the Institute of Heredity Science, the Chinese Academy of Sciences. The animals were raised at room temperature (about 25℃) and with a constant photoperiod (light : dark cycle, 12L:12D). Food and water were provided *ad libitum*.

(iii) Indirect immunofluorescence and confocal microscopy. Blastocysts of D4 mice, free of zona pellucida hatched or removed naturally by Tyrode's buffer, were fixed 30—60 min at 4℃ in freshly prepared 4% PFA containing 0.2% Triton X-100. After being rinsed 3 times each for 5 min in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4), the blastocysts were blocked in the non-specific antibodies for 45 min by 5%—10% BSA at room temperature, then incubated in primary antiserum against αVβ3 integrin diluted in PBS 4℃ overnight. After being rinsed in PBS as above, the blastocysts were incubated for 60 min at 37℃ in FITC-conjugated secondary antibody, then rinsed as above, stained in 0.01 mg/mL PI for 15 min. Finally, the blastocysts were rinsed in PBS, mounted on dishes and viewed under a confocal laser scanning microscope (Leica).

(iv) Intra-uterus injection. Pregnant mice on day 3 were injected with 3μL antiserum against αVβ3 integrin in the lumen of the left uterine horn while the right horn was injected with normal rabbit serum of the sdame mass as control. On day 8, the treated animals (n=17) were killed to count the number of embryos implanted.

(v) Co-culture. The co-culture protocol was referred to Zeng et al.[7], which was described briefly as follows. Female mice were injected intraperitoneally with 10 IU of pregnant mare serum gonadotropin (PMSG) and 48 h later with 10 IU of human chorionic gonadotropin (hCG). Following hCG injection, each female was caged with a male mouse overnight. Pregnancy was confirmed by the presence of a vaginal plug. Preimplantation blastocysts were flushed from the uterus of day 4 pregnant mice with Hank's solution. Uteri from day 4 pregnant mice were split longitudinally to expose the epithelial cell surface and digested with 0.6% trypsin solution at 4℃ for 2 h. Tissues were gently shaken to dislodge the epithelium from the endometrial bed. The epithelial cells and fragments were collected by centrifugation at 600 × g for 10 min. Cells were washed three times by centrifugation as above, with Ham's-F_{12} containing 2.2 mmol/L calcium lactate, 2.05 mmol/L glutamine, 12.5 mmol/L $NaHCO_3$, 400 IU/mL gentamycin sulfate. Finally, the cells were resuspended in the same medium with 10% FCS. A cell suspension adjusted to 1×10^6 cells/mL was put on 24-well Falcon plates, and incubated at 37℃ in 5% CO_2 at atmosphere in a humidified chamber. After monolayer of mouse uterine epithelial cells attached the plate (about 24 h), the blastocysts were transferred in wells (about 35 blastocysts per well) and the same experiment was repeated at least three times. The medium in control group (C) was F_{12}-medium containing 0.4% BSA, and 2.2 mmol/L calcium lactate, 2.05

mmol/L glutamine, 12.5 mmol/L $NaHCO_3$, 400 IU/mL gentamycin sulfate as above. The media in the treatment groups were F_{12}-medium complemented with the following dilutions of antisera against αVβ3 integrin: 1 : 400 (T_1), 1 : 200 (T_2), 1 : 100 (T_3). After 24, 48 and 72 h of co-culture, the attachment or outgrowth of blastocysts was observed and defined under a phase-contrast microscope (Olympus) according to the following criteria. After shaking the plate gently and briefly, we could find the blastocyst at the same place, which was designated as "attachment". If primary giant trophoblast cells were visible around the attachment site, we designated the blastocyst as "outgrowth"[8, 9]. Ratios of attachment and outgrowth were counted under the microscope as above, and the outgrowth areas were also measured through a grid.

(vi) Statistical analysis. All the results are shown as mean ± standard error (SE). Data were analyzed by *t*-test, and a value of $P < 0.05$ was considered to be significant.

2 Results

(i) Expression of αVβ3 protein. In this study, we used a double-staining technique: the nucleus was stained by PI, which showed red under a confocal microscope. The FITC-conjugated secondary antibody bound specifically with the primary antibody (αVβ3 integrin antiserum) showed green, which manifested the location of the detected antigen. As Plate I shows, αVβ3 integrin is obviously expressed in trophoblast in the mouse.

(ii) Intra-uterine injection. Fig. 1 summarizes the result of injection of αVβ3 integrin antiserum into a uterine horn of a pregnant mouse on day 3. The number of embryos implanted in the treatment horn was dramatically decreased compared with that in the control horn ($P < 0.001$), which suggested that αVβ3 integrin promoted embryo implantation in the mouse.

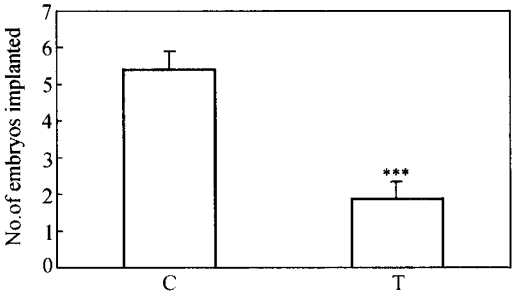

Fig. 1. Effect of intra-uterine injection of αVβ3 integrin antiserum on embryo implantation in the mouse. C indicates the control horn injected with normal rabbit serum while T indicates the treatment horn injected with αVβ3 integrin antiserum. *** indicates a significant difference from the control ($P < 0.001$).

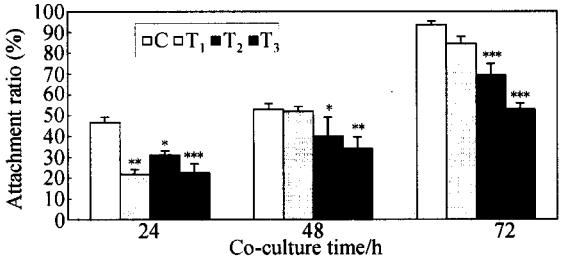

Fig. 2. Effect of αVβ3 integrin antisera on attachment ratios of blastocysts on monolayer of uterine epithelial cells in the mouse. *, ** and *** indicate significant differences from the control ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively).

(iii) Co-culture. At 24, 48 and 72 h of co-culture, the attachment ratios in treatment groups were greatly decreased by addition of αVβ3 integrin antiserum at dilutions of 1 : 100 and 1 : 200. However, at dilution of 1 : 400, only at 24 h of co-culture could an inhibitory effect occur ($P < 0.01$, fig. 2). Moreover, at 48 and 72 h of co-culture, there was a negative correlation between attachment ratios and dosages/dilutions of αVβ3 integrin antiserum ($df = 6$, $r = -0.95$, $P < 0.01$; $df = 6$, $r = -0.99$, $P < 0.01$).

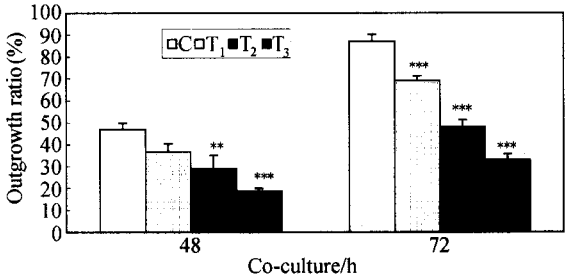

Fig. 3. Effect of αVβ3 integrin antisera on outgrowth ratios of blastocysts on monolayer of uterine epithelial cells in the mouse. ** and *** indicate significant differences from the control ($P < 0.01$ and $P < 0.001$, respectively).

As fig. 3 shows, at 48 and 72 h of co-culture, αVβ3 antisera at dilutions of 1 : 100 and 1 : 200 inhibited the outgrowth ratios ($P <$

0.01 and $P < 0.001$). However, at dilution of 1 : 400, only at 72 h could an inhibitory effect come out ($P < 0.001$, fig. 3). Moreover, the inhibitory effect was also dosage/dilution- depedent ($df = 6$, $r = -0.95$, $P < 0.01$; $df = 6$, $r = -0.99$, $P < 0.01$).

3 Discussion

Embryo implantation, a biological paradox, is mediated by a specialized population of cells, the trophoblast cells, which arise from the trophectoderm, the outer layer of epithelial cells that enclose the blastocoel cavity and the inner cell mass of the preimplantation blastocyst[5]. In the mouse, the first sign that the trophoblasts have developed the phenotype appropriate for implantation appears about 10—15 h after the embryo hatches from zona pellucida[4]. The developing trophoblasts simultaneously exhibit two distinct properties: i) they become competent to show cell-cell and cell-matrix binding; ii) they are consistent with invasive behavior and exhibit the ability to interpose themselves among other cells by separating them and degrading the components of the extracellular matrix[4]. Such phenotype of trophoblast is associated with cell differentiation, which is mainly displayed by expression of some specific proteins, such as CAMs.

Some researchers argued that blastocyst could be regarded as "a malign tumor", since the process that trophoblast invades uterine endometrium is much similar to that of tumor invasion and migration, so is their biological development[10]. This is exemplified by the action of integrin. As a common receptor of ECM components, integrin can bind specifically with collagen of type Ⅰ, fibronectin, vitronectin, von Willebrand factor, thrombospondin, osteopontin, and human compliment C_3 via RGD (arginine-glycine-aspartic acid) peptide sequence, which is involved in cell-cell and cell-ECM interaction. Consequently, integrin becomes a novel kind of CAMs. Integrin is not a simple adhesion site on cell surfaces, but can be viewed as two-way signaling molecule by way of "inside-to-outside" and "outside-to-inside" signaling pathways. These include the regulation of a Na^+/H^+ antiporter, Ca^{2+} influx, stimulation of inositol lipid synthesis and protein tyrosine phosphorylation of a group of cytoplasmic proteins of 100—130 ku[11].

It was suggested that integrin could regulate the cell-cell and cell-extracellular matrix interaction between the trophoblast and the uterus, which subsequently modulated the implantation process. In this work, αVβ3 integrin was clearly expressed in the mouse blastocyst using indirect immunofluorescence and confocal microscopy, which implicated that αVβ3 integrin possibly affected invasiveness of trophoblast. In the mouse, the "implantation window" was consumed to be on D4, when the attachment reaction between trophectoderm and uterine epithelium occurred at 22:00—23:00[12]. At this specific period, expression of αVβ3 integrin was necessary for the invasiveness of blastocyst, which suggested that blastocyst on D4 alerady developed maturation and gained the capacity of attaching and penetrating into uterine endometrium. This idea was supported by our result of intra-uterine injection; the number of embryos implanted was significantly decreased in the right uterine horn by treatment of integrin antiserum. This implicated that αVβ3 integrin played an important role in embryo implantation. Compared with other *in vivo* methods, injection into uterine horn has many advantages. First, it can reveal the local regulation on embryo implantation, which can hardly be proven with other *in vivo* methods, such as intra-muscular or subcutaneous injections. Second, the dose applied may be closer to its physiological level.

Lessey et al.[13, 14] argued that integrin was helpful for the switch of uterine endometrium from a non-adhesive state into an adhesive state. Consequently, integrin becomes a "receptive marker" of uterine endometrium during "implantation window". Other data manifested that trophoblast invasiveness at implantation stage was regulated by expression or distribution of integrin[5]. It was reported that differentiating/invading human cytotrophoblastic cells (CTBs) could express αVβ3 integrin, an integrin characteristic of endothelial cells during angiogenesis[6]. Functional studies showed that αVβ3 and α1β1 integrins, as well as VE-cadherin, enhanced CTB-invasiveness. Clinical evidence manifested that in preeclampsia (PE), a disease of pregnancy in which endovascular invasion is abrogated, CTBs fail to properly express integrin, caherin and immunoglobin superfamily proteins and cannot adopt a vascular adhesion phenotype. The consequences may affect negatively CTB endovascular and uterine invasion, thereby compromising blood flows to the maternal-fetal interface[1].

Thus, besides modulating receptivity of uterine endometrium, αVβ3 integrin determines invasive capacity of trophoblast cells.

In order to demonstrate the above hypothesis, a co-culture model was utilized to emulate the process of embryo implantation in the mouse. The results showed that antisera against αVβ3 integrin at dilution of 1 : 100 and 1 : 200 significantly inhibited the attachment reaction of blastocyst on monolayer of uterine epithelial cells with dosage-dependent relationship. This supported our presumption mentioned above. Some argue that attachment and outgrowth reactions were different processes during embryo implantation, which was mediated in different molecular and cellular events. In this study, antisera against αVβ3 integrin inhibited not only the attachment, but also the outgrowth, which indicated that this integrin antiserum had an inhibitory effect on both attachment and outgrowth of blastocyst. It was much similar to our previous result of murine ectoplacental cone outgrowth *in vitro*[15].

Insofar as the action mechanism of integrin on embryo implantation, Lessey et al.[11] postulated the suggestion as in the following: αVβ3 integrin binds and activates matrix metalloproteinase (MMP) and plasiminogen activators (PAs), and serves to position these proteases. The position of αVβ3 integrin on the apical portion of the epithelium on the luminal surface of the endometrium and on the outer surface of embryos raises the possibility that this integrin serves to activate and position proteinases during implantation. Proteolytic fragments that arise from MMP degradation of basement membrane serve as a signal for cell migration and might stimulate trophoblast invasion.

To be concluded, αVβ3 integrin plays an important role in embryo implantation in the mouse. Its expression coincides with the invasive behavior of trophoblast, which further regulates both the attachment and outgrowth processes of blastocyst on uterine epithelial cells.

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