CHAPTER-4

Embryonic regulation of $\alpha_v \beta_3$ integrin expression in rat endometrial epithelial cells
INTRODUCTION

The process of implantation turning into successful pregnancy is achieved only when the uterine receptivity is in synchronized state with the developmental stage of the embryo (Psychoyos, 1986; Harper, 1992). This is a process in which the two aliens having different genetic backgrounds influence each other by exchanging signals (Weitlauf, 1994). The presence of embryo in uterine lumen is assumed to play an important role in successful implantation (Carlone and Rider, 1993). Shiotani et al., (1993) illustrated the nature of interaction between the embryo and the endometrium by studying the biological effect of the former on the latter. According to them, the presence of embryo in the oviduct followed by its entry in the uterine cavity may promote a functional transformation by inducing receptivity in the endometrial epithelium.

Several lines of evidence substantiate the idea that the embryo-derived factors may influence, directly or indirectly, uterine receptivity and implantation. A study in the rhesus monkey has indicated that the endometrial physiology during mid-luteal phase, in the presence of the conceptus, is discernibly different from that in non-fecund mid-luteal phase (Ghosh and Sengupta, 1998). Another study in the baboon has shown that chorionic gonadotrophin (CG), when infused in a manner that mimics the transitional presence of blastocyst in uterus, had similar physiological effects in the three major cell types (i.e., luminal and glandular epithelium and stromal fibroblasts) of the endometrium (Fazleabas et al., 1999a).
It is believed that the embryo modulates uterine receptivity by regulating the expression of adhesion/anti-adhesion molecules. Amongst the adhesion molecules, more emphasis has been laid on $\alpha_\nu\beta_3$ integrin as it is alleged to be one of the most important markers of uterine receptivity in recent years. Most of the information on embryonic regulation of integrins in EEC has come from human (Simon et al., 1997a). In preceding chapters it has been shown that $\alpha_\nu\beta_3$ integrin acts as the marker of uterine receptivity in rat and is regulated by the influence of ovarian steroids. However, the adequate information in defining the role of embryo on the regulation of this integrin in rat EEC is still lacking. Accordingly, the primary objective of this study was to investigate the effect of rat embryo on the expression of $\alpha_\nu\beta_3$ integrin in EEC.
MATERIALS AND METHODS

Biochemicals

Dulbecco’s Modified Eagle’s Medium (DMEM) having L-Glutamine and 1000mg glucose/L without phenol red and NaHCO3, Phosphate buffered saline (PBS) without Ca2+ and Mg2+, Fetal Bovine Serum (FBS), Antibiotic/Antimycotic solution (100X), Trypsin solution (10X), Pancreatin, Deoxyribonuclease-I, Insulin, L-Glutamine, ECM gel (Engelbreth Holm Swarm Tumour Matrix), Cell dissociation solution were obtained from Sigma Chemical Company, St. Louis, U.S.A. Primary and secondary antibodies for β3 integrin subunit were obtained from Santa Cruz, California, U.S.A.

Animal experimentation

Adult female (150-200 g) Sprague-Dawley rats (Rattus norvegicus) of the institute’s animal colony maintained in air-conditioned quarters (temperature 21 ± 4°C) under a regulated photoperiod (14 hrs light and 10 hrs dark) were used. Pseudopregnancy was induced in these rats at estrous stage by injecting 3.0 ml of 10% sodium chloride subcutaneously (Psychoyos, 1959). The treated animals were not supplied drinking water for 10 hrs and then autopsied four days (fifth day) after the treatment. Pseudopregnancy was confirmed by inducing deciduoma on day 5 of pseudopregnancy.
Collection of rat blastocysts

Pregnant rats were autopsied on day 5 p.c. (10.00 hrs). Their uterine horns (collected in PBS) were cleared of mesenteries and then placed on a wet filter paper. The lumen of each horn was flushed with PBS (0.5 ml) and every drop of the flushing was collected in watch glass and examined under the microscope for the presence of blastocysts. The blastocysts were picked up under the inverted microscope (Zeiss, Germany) using a micro-pipex (United Germany). Minimum 4-5 blastocysts were used in each co-culture experiment.

Embryo co-culture

Round tissue culture petridishes (60 mm; Corning, USA) were coated with ECM gel (1/4 dilution in DMEM) and then placed in CO₂ incubator for at least 1 hr and then rinsed with PBS before seeding the cells. EEC, isolated from pseudopregnant rats on day 5 (day 1 being the day on which pseudopregnancy was induced), were cultured on matrix (ECM gel) coated tissue culture petridishes (60 mm) till confluence. Once the monolayers of EEC became confluent, they were divided into two groups. In one group, EEC were co-cultured with rat blastocysts; the co-culture was continued till the late blastocyst stage (24-48 hrs). The other group was comprised of EEC cultured without blastocysts. This served as a control.

Flow-cytometry

After the completion of co-culture, medium was removed from cultures of both the groups (EEC with and without blastocysts) and the EEC of respective
cultures were detached from the substratum by treatment with the 1X non-
enzymatic cell dissociation solution (Sigma, U.S.A.), washed in PBS, centrifuged
to form the cell pellets that were processed for flow-cytometry in separate tubes.
The expression of $\alpha_\nu\beta_3$ integrin in the EEC of both the groups was studied by a
quantitative flow-cytometric estimation of its $\beta_3$ subunit. The detailed procedure
for flow-cytometry has been described in Chapter-2. The results obtained were
analyzed by a paired t-test and the data are presented as mean ± SEM. Statistical
significance was considered at $p<0.05$. 
Fig. 1: a. EEC of day 5 pseudopregnant rats cultured in the absence of blastocyst. b. EEC of day 5 pseudopregnant rats cultured in the presence of blastocyst. Note the presence of an expanded bulged blastocyst over the monolayer. (Total Magnification 100X).
Fig. 2: Expression of α₄β₃ integrin in day 5 pseudopregnant rat EEC cultured without blastocysts (EEC) or co-cultured with the rat blastocysts (EEC + Blastocyst). Note the increase in the fluorescence intensity of EEC co-cultured with blastocysts as compared to EEC cultured without blastocysts. Negative control represents the background fluorescence.
Fig. 2

Negative control

EEC

EEC + Blastocyst
Fig. 3: Histogram showing the expression of $\alpha_v\beta_3$ integrin in day 5 pseudopregnant rat EEC cultured without blastocysts (EEC) or co-cultured with the rat blastocysts (EEC + Blastocyst). Values represent mean ± SEM of five individual observations. a-p<0.01 vs. EEC. Note the high expression of $\alpha_v\beta_3$ integrin in day 5 pseudopregnant EEC co-cultured with blastocysts as compared to EEC cultured without blastocysts.
RESULTS

To investigate the embryonic regulation of αvβ3 integrin in EEC, the expression of its β3 subunit was quantitated in pseudopregnant rat EEC co-cultured with blastocysts and its expression was compared with the same population of EEC cultured in the absence of blastocysts. Fig. 1a and 1b shows formation of monolayers of sensitized EEC (sensitized by inducing pseudopregnancy) cultured alone or in the presence of blastocysts respectively. At 24 hrs of culture the EEC formed characteristic features of cuboidal contours with cytoplasmic extensions on the sides to form the link with neighbouring cells and then the formation of monolayer occurred. In co-culture (Fig. 1b), the blastocyst was fully expanded and was floating over the surface of EEC monolayer. In this culture it was noticed that though most of the EEC remained cuboidal, some of them in the periphery of blastocyst changed their contour characterized by formation of long cytoplasmic extension on the either side of the cells.

The expression of αvβ3 integrin in EEC cultured with and without blastocysts was quantitated by flow-cytometry. The results of the flow-cytometric study revealed a high expression of αvβ3 integrin in EEC that were co-cultured with blastocysts (Fig. 2). Where as the EEC that were maintained nascent in culture (without blastocysts) showed relatively low expression (Fig. 2). The expression of αvβ3 in EEC co-cultured with blastocysts was significant (p<0.01) when compared to its expression in EEC cultured without blastocysts (Fig. 3).
DISCUSSION

The purpose of this study was to gain knowledge about the extent of embryonic ability in modulating uterine receptivity manifested by the expression of \( \alpha_\text{v}\beta_3 \) integrin in this species. This crucial concept has been demonstrated in human (Simon et al., 1997a) as well as in animals such as mouse (Shiotani et al., 1993), rabbit (Harper et al., 1989) and sheep (Godkin et al., 1984, Valle et al., 1987). The role of embryo in regulating the uterine receptivity using integrin expression as a marker has never been investigated in rat.

The results of the flow-cytometric study have clearly shown an up-regulation of \( \alpha_\text{v}\beta_3 \) integrin in EEC when maintained in co-culture with blastocysts. However, the expression of \( \alpha_\text{v}\beta_3 \) in EEC, when maintained nascent in culture i.e., without blastocysts, remains significantly low as compared to EEC in co-culture with the blastocysts. This denotes the involvement of blastocyst-endometrial epithelial interactions in the regulation of uterine receptivity, and the importance of blastocyst that plays a putative role in regulating the expression of \( \alpha_\text{v}\beta_3 \) in EEC. These quantitative \((in\ vitro)\) results have distinctly demonstrated that the blastocysts indeed up-regulate the expression of \( \alpha_\text{v}\beta_3 \) in rat EEC.

Based on earlier studies (Simon et al., 1993a, 1993b, 1994a, 1994b; De Los Santos et al., 1996), the notion has been well established that the embryo secretes complete Interleukin-1 (IL-1) system (IL-1\( \alpha \), IL-1\( \beta \)/IL-1ra) in response to some unknown factors secreted by the EEC. In this context the up-regulation of
αvβ3 integrin in EEC (as demonstrated by flow-cytometry) by the blastocysts is being possibly triggered by the binding and activation of embryonic IL-1α + IL-1β to the endometrial epithelial IL-1R type1. The embryonic IL-1 system acts as the paracrine factor in regulating the expression of αvβ3 integrin in EEC during the receptivity period. This paracrine effect of the IL-1 system is further mediated through Leptin (Gonzalez et al., 2003). Leptin is a 16 kDa polypeptide, which plays a key role in the development of preimplantation embryo and the implantation process (Kawamura et al., 2002; Malik et al., 2001). Leptin and its receptor (Ob-R) are expressed in the endometrium (Alfer et al., 2000; Gonzalez et al., 2000; Kitawaki et al., 2000; Wu et al., 2002) and their expression is modulated directly by the embryonic IL-1β (Gonzalez and Leavis, 2001). IL-1β stimulates leptin secretion and Ob-R expression in EEC. Leptin secreted in the EEC act in an autocrine or paracrine manner and mediates uterine receptivity by up-regulating the expression of αvβ3 integrin. Leptin acts as a mediator molecule for the actions of IL-1β.

In conclusion, it may be stated that the rat blastocysts when co-cultured with sensitized EEC, modulate uterine receptivity by up-regulating the expression of αvβ3 integrin in rat EEC. This statement holds true by the results of the present study that has successfully established an in vitro system to demonstrate the potential influence of embryo on the regulation of αvβ3 integrin in EEC whose expression reflects the onset of uterine receptivity required for the successful implantation of blastocyst.