The potential role of stem cell factor and its receptor c-kit in the mouse blastocyst implantation

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Embryo implantation is a complex process that requires the interaction of embryo and endometrium. Several growth factors and cytokines appear to be involved in this process. Stem cell factor (SCF) and its receptor c-kit regulate the proliferation and survival of germ cells and play an important role in follicular development. However, little information is available on the role of SCF and c-kit in the process of blastocyst implantation. In the present study, we examined the expression of SCF and c-kit mRNA in mouse embryos and in the stromal and epithelial cells of the uterine endometrium by reverse transcription–polymerase chain reaction (RT–PCR). SCF mRNA was expressed in the spreading blastocysts and endometrial cells, with especially strong expression occurring in the stromal cells. Expression of c-kit mRNA was detected in the blastocyst and spreading blastocysts, as well as in the endometrial cells. By immunocytochemical studies, staining for c-kit protein was observed in the in-vitro spreading trophoblasts. We found that 50–100 ng/ml SCF significantly promoted the expansion of the surface area of the spreading blastocysts. We also assessed the effect of SCF on trophoblast outgrowth through its receptor c-kit.

Key words: c-kit /embryo development /implantation /stem cell factor/trophoblast spreading

Introduction

Mutations at either the mouse W (white spotting) or Sl (steel) loci cause defects in germ cell development, melanogenesis, and haematopoiesis. It has been reported that the W locus encodes the c-kit proto-oncogene, which is a receptor molecule with tyrosine kinase activity, and the Sl locus encodes its corresponding ligand stem cell factor (SCF) (Chabot \textit{et al.}, 1988; Geissler \textit{et al.}, 1988; Nocka \textit{et al.}, 1989; Yarden \textit{et al.}, 1989; Zsebo \textit{et al.}, 1990). These genes regulate signal transduction mechanisms in several types of cells.

During embryonic development, SCF and c-kit are essential for the survival and proliferation of the germ cell and migration toward the gonad. c-kit mRNA is expressed in the primordial germ cells, while the SCF transcript is expressed along their migratory pathway toward the genital ridge (Geissler \textit{et al.}, 1981; Kuroda \textit{et al.}, 1988; Copeland \textit{et al.}, 1990; Matsui \textit{et al.}, 1990). In the post-natal ovary, SCF and c-kit are important for follicular development. The function-blocking antibody for c-kit disturbs the onset of primordial follicle development, primary follicle growth, follicular fluid formation of preantral follicles, and penultimate-stage ovarian follicle maturation before ovulation (Yoshida \textit{et al.}, 1997). During the peri-ovulatory period, c-kit is expressed in theca cells and oocytes, while SCF is expressed in granulosa cells (Horie \textit{et al.}, 1991; Arceci \textit{et al.}, 1992; Motro and Bernstein, 1993; Packer \textit{et al.}, 1994; Laitinen \textit{et al.}, 1995; Ismail \textit{et al.}, 1996; Tanikawa \textit{et al.}, 1998). After ovulation, c-kit mRNA in oocytes decreases rapidly to a point where expression cannot be detected. It is then re-expressed from the late 2-cell stage to the expanded and hatched blastocyst, just prior to attachment to the endometrium (Arceci \textit{et al.}, 1992). On the other hand, the SCF transcript is detected in the oviduct and uterus (Arceci \textit{et al.}, 1992). A recent study demonstrated that SCF is expressed in human endometrium, and that c-kit is expressed in placental tissues during pregnancy (Kauma \textit{et al.}, 1996). The expression of SCF increases in decidual tissue compared with non-pregnant endometrium (Kauma \textit{et al.}, 1996). The expression and localization of SCF and c-kit at the feto–maternal interface suggest that SCF has an important role in embryo development as well as in trophoblast and placental growth. However, no information is available about the role of SCF and c-kit during the implantation process.

The purpose of this study was to investigate the action of SCF and c-kit during blastocyst implantation. We examined the expression of SCF and c-kit mRNA in embryos and in epithelial and stromal cells of the uterine endometrium of mice by reverse transcription–polymerase chain reaction (RT–PCR). We detected the localization of c-kit protein in spreading blastocysts. We also assessed the effect of SCF on trophoblast outgrowth \textit{in vitro}. 

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Materials and methods

Animals

Ovulation was stimulated in B6C3F1 female mice aged 5–7 weeks (Shimizu Experimental Animals Laboratory Co Ltd, Kyoto, Japan) by i.p. injection of 7.5 IU pregnant mare’s serum gonadotrophin (PMSG; Sankyo Co Ltd, Tokyo, Japan), followed 48 h later by the injection of 7.5 IU human chorionic gonadotrophin (HCG; Mochida Co Ltd, Tokyo, Japan). Immediately thereafter, the mice were mated overnight with males. All experiments were performed in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Culture media

The blcMEM-F0 medium described by Tachi was used in this study (Tachi, 1992; Taniguchi et al., 1998). Powdered Eagle’s minimal essential medium (MEM) was prepared without bicarbonate, glutamine, antibiotics, and Phenol Red by dissolving a powdered mixture (Nissui Co Ltd, Tokyo, Japan) in distilled water. All non-essential amino acids were added at a concentration of 10 ml/l (×100; Gibco Oriental Co Ltd, Tokyo, Japan). The other components were added as follows: sodium bicarbonate (6.8 g/l), t-glutamine (292.0 mg/l), cysteine HCl–H2O (200.0 mg/l), co-carboxylase (1.0 mg/l), thymidine (5.0 mg/l), uridine (5.0 mg/l), penicillin G (10 000 IU/l), and streptomycin sulphate (50.0 mg/l). The osmolarity of the culture medium was adjusted to 285–295 mOsmol, then sterilized with a Millex 0.22 µm filter unit, and stored at 4°C for up to 4 weeks until use, and then added to this medium at the concentration of 5% (vol/vol).

SFM-101 medium ‘Nissui’ (Nissui Co Ltd, Tokyo, Japan) was also used in this study to exclude the influence of serum. SFM-101 medium was supplemented with 0.5% bovine serum albumin (BSA fraction V; Sigma), then sterilized with a Millex 0.22 µm filter unit.

Reagents

Recombinant murine stem cell factor was purchased from Pepro Tech EC Ltd. (London, UK). Rat anti-mouse monoclonal antibody to the extracellular domain of the c-kit receptor, ACK2, was kindly provided by Professor Shin-Ichi Nishikawa, Kyoto University, Kyoto, Japan. Detailed characteristics of this antibody have been described previously (Nishikawa et al., 1991; Yoshida et al., 1993).

Collection and culture of blastocysts

Blastocysts were flushed and collected from the resected uteri at 96 h after the HCG injection, using modified human tubal fluid medium with or without 100 ng/ml SCF. These microdrops were preincubated in the blcMEM-F0 medium with 100 µg/ml ACK2 for 1 h, then cultured singly in microdrops (30 µl) of the blcMEM-F0 medium with or without 100 ng/ml SCF. These microdrops were placed for 72 h under mineral oil on 35 mm tissue culture dishes coated with human fibronectin (Becton Dickinson Labware, Bedford, MA, USA) at 37°C in a humidified atmosphere of 95% O2/5% CO2 (Taniguchi et al., 1998). In another study, the SFM-101 serum-free medium, instead of the blcMEM-F0 medium, was used, and blastocysts were incubated for 96 h under the same conditions.

Separation of epithelial and stromal cells from endometrial cells

Tissue was obtained from resected uteri at the same time as the blastocysts were collected. Stromal cells were collected from the mouse endometrial tissue as previously described (Osteen et al., 1989; Taniguchi et al., 1998). Briefly, the endometrial tissues were minced in Hank’s balanced salt solution and then digested for 60 min at 37°C with 0.5% collagenase in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12, 1:1, vol/vol). The resultant dispersed cells were then filtered through a 70 µm nylon mesh to remove the undigested tissue pieces containing the glandular epithelium. The filtered fraction was next separated further from the epithelial cell clumps by differential sedimentation at unity gravity. Finally, the stromal cells obtained from the second sedimentation were filtered through a 40 µm nylon mesh. Final purification was achieved by allowing the stromal cells, which attach rapidly to plates, to adhere selectively to the culture dishes for 30 min at 37°C in 95% O2/5% CO2. Non-adhering epithelial cells were removed. The stromal cells were cultured in DMEM/F-12 supplemented with penicillin G (100 IU/ml), streptomycin sulphate (50 mg/ml), amphotericine B (2.5 µg/ml), and 10% FBS (vol/vol) at 37°C in 95% O2/5% CO2. In the present experiments, we used epithelial and stromal cells in a primary monolayer culture without passage.

Immunocytochemical analysis of the isolated endometrial stromal cells was performed using cytokeratin (Dako, Kyoto, Japan) as a marker for the epithelial cells, vimentin (Dako) as a marker for the stromal cells, and factor VIII (Dako) as a marker for the endothelial cells. The purity of epithelial and stromal cells were found to be 95 and 98% respectively. However, there may still be the possibility of contamination by smooth muscle cells in endometrial stromal cell cultures.

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted from the blastocysts, in-vitro spreading blastocysts, and cultured endometrial cells by the guaniendioxyanate method (Chomczynski and Sacchi, 1987). The NIH/3T3 cells and the F9 cells were used as a positive control for SCF and c-kit respectively. Reverse transcription of RNA into cDNA and PCR amplification were performed using a Gene Amp RNA PCR Core Kit (Perkin Elmer, NJ, USA) (Tanikawa et al., 1998). RNAs (2 µg) extracted from each sample were used. PCR amplification was performed for 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 60°C), and synthesis (90 s at 72°C). Specific primers for SCF, synthesized to generate extracellular domain including transmembrane domain, were 5′-AAGTGGATTACACTTGACAT-3′ (sense), and 5′-CAATGCAAGGTGATCCGATAG-3′ (antisense); these primers generated a product of 646 bp. Specific primers for c-kit, synthesized as described (Arceci et al., 1992), were 5′-CTGCTTGGCGCATGCGACG-3′ (sense), and 5′-CCGGCATCCCT-GGTTAAGG-3′ (antisense), generating an amplified product of 655 bp. Specific primers for β-actin, synthesized as described (Tanikawa et al., 1998), were 5′-TGGTGGTGCTTCTAGGAC-3′ (sense), and 5′-TGGCCATTGGTGTACGGAATG-3′ (antisense), generating an amplified product of 243 bp. The specificity of the PCR product was confirmed by Southern blot analysis. The bands generated by PCR were transferred to a nylon membrane (Sarton; Sartorius, Göttingen, Germany) using a vacuum blotter with 0.4 mol/l NaOH and 1 mol/l NaCl. The DNA on the membrane was fixed with a UV-crosslinker and hybridized with the biotinylated oligonucleotide internal probes (5′-TACCCCTGTTTATGC-3′ for SCF, 5′-TATAT-GGACATGAAGCCT-3′ for c-kit). The membrane was treated with streptavidin–alkaline phosphatase, followed by chemiluminescence
incubated in methanol containing 2% H2O2 to inactivate endogenous

Figure 1. Expression of stem cell factor (SCF) mRNA in the embryos and endometrium. The specificity of each polymerase chain reaction (PCR) product was confirmed by Southern blot analysis (upper panel). Ethidium bromide staining of the reverse transcription (RT)–PCR products of the β-actin was presented (lower panel). Data are representative of three independent experiments with samples derived from different animals in each experiment. Lane 1, blastocysts; Lane 2, in-vitro spreading blastocysts; Lane 3, endometrial epithelial cells; Lane 4, endometrial stromal cells; Lane 5, NIH/3T3 cells; Lane 6, no reverse transcriptase.

Results

Expression of SCF and c-kit mRNA in embryo and endometrium

We examined the expressions of the SCF and c-kit mRNA in mouse blastocysts, the in-vitro spreading blastocysts, and the stromal and epithelial cells of the uterus endometrium using RT–PCR. SCF mRNA was expressed in the epithelial and stromal cells. In particular, we detected strong expression of SCF in the stromal cells (Figure 1). SCF mRNA was also expressed in the in-vitro spreading blastocysts, but not in blastocysts. This up-regulation in SCF expression may be the influence of in-vitro culture of blastocysts up to outgrowth stage. The shorter band, generated by alternative splicing (Flanagan et al., 1991), was observed in the stromal cells and the in-vitro spreading blastocysts. Although the possibility of stromal cell contamination in epithelial cell culture is not excluded, alternative spliced form (short form) of SCF mRNA was observed only in endometrial stromal cells, but not in epithelial cells, suggesting that the level of contamination may be negligible. Expression of c-kit mRNA was detected in the blastocysts and in-vitro spreading blastocysts (Figure 2). c-kit mRNA was also expressed in the epithelial and stromal cells of the endometrium.

Expression of c-kit protein in implanting embryos

Immunocytochemical staining for c-kit protein was performed on in-vitro spreading blastocysts. In-vitro spreading trophoblast giant cells were stained for c-kit (Figure 3A). Control sections were incubated with normal mouse IgG in place of the primary antibody (Figure 3B).
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Effects of SCF on the rates of blastocyst attachment and spreading

We did not observe any difference in the rate of blastocyst attachment with the addition of SCF to the blcMEM-F0 medium (data not shown). The addition of 100 ng/ml SCF significantly \((P < 0.05)\) enhanced the rate of blastocyst spreading (Figure 4). This effect of SCF was abolished by preincubation with 10 µg/ml ACK2 (Figure 4).

Effect of SCF on the trophoblast outgrowth

The mean surface area of the trophoblast spreading embryos was \(2.9 \times 10^{-2} \text{ mm}^2\) after 72 h of incubation with the blcMEM-F0 medium. By the addition of SCF in a concentration of 50 and 100 ng/ml, the areas significantly increased to \(4.4 \times 10^{-2} \text{ mm}^2\) and \(4.9 \times 10^{-2} \text{ mm}^2\), respectively (Figure 5). The mean surface area of the trophoblast spreading embryo was \(3.2 \times 10^{-2} \text{ mm}^2\) after 96 h of incubation with the SFM-101 serum-free medium. By adding 50 and 100 ng/ml SCF, the surface area significantly increased to \(4.3 \times 10^{-2} \text{ mm}^2\) and \(5.2 \times 10^{-2} \text{ mm}^2\), respectively (Figure 5). These effects of SCF were abolished by ACK2 (Figure 5). Abrogation with specific antibody to c-kit indicates that this promoting effect was specific for SCF.

Figure 3. Immunostaining for c-kit protein in the in-vitro spreading blastocysts. (A) Spreading blastocysts stained for c-kit. (B) Control sections incubated with normal mouse immunoglobulin G (IgG) in place of the primary antibody. Scale bar = 100 µm.

Figure 4. Effect of stem cell factor (SCF) on the rate of blastocyst spreading. Blastocysts were cultured for 72 h in the blcMEM-F0 medium supplemented with SCF (0–100 ng/ml). Pooled data from five separate experiments using at least 50 blastocysts/experiment. The effect of SCF was reduced by the anti-c-kit antibody ACK2 (10 µg/ml). *\(P < 0.05\) versus 0 ng/ml.
The role of colony-stimulating factor-1 (CSF-1)/c-kit during preimplantation development has been studied extensively. Mutations at the W locus are able to implant in the uterine endometrium, indicating that SCF and c-kit are involved in follicular development and tissue remodelling during the human menstrual cycle (Mori et al., 1997). However, no information is available about the role of SCF and c-kit in the implantation process.

The W/W mutant mice which lack c-kit function are born with deficiencies in the production of germ cells, melanocytes, and haematopoietic cells. Therefore, blastocysts with mutations at the W locus are able to implant in the uterine endometrium, suggesting that the role of SCF/c-kit during preimplantation development and implantation may not be essential. However, it has been shown that colony-stimulating factor-1 (CSF-1)/c-fms and SCF/c-kit may act in a compensatory or synergistic manner during preimplantation development (Arceci et al., 1992). The c-fms may compensate for the c-kit function of blastocysts mutated at the W locus during the peri-implantation period.

In the present study, we investigated the role of SCF and its receptor c-kit during mouse blastocyst implantation. We observed that embryos (during the implantation stage) expressed both SCF and c-kit mRNAs, and c-kit protein. In addition, the endometrial epithelial and stromal cells expressed both SCF and c-kit mRNAs. In particular, we observed a strong expression of SCF mRNA in the stromal cells. Adding SCF to the culture medium significantly increased the surface area of trophoblast outgrowth. These results suggest that SCF derived from embryo during implantation stage and endometrial cells promotes outgrowth of blastocyst through an autocrine and/or paracrine action. Thus, our study shows for the first time that SCF and c-kit may play a role in blastocyst implantation.

During the early implantation process, the polar trophectoderm of the blastocyst, overlying the inner cell mass, continues to proliferate after the initial implantation. It grows inward to form the extraembryonic ectoderm and outward to form the ectoplacental cone (EPC). Mouse embryo EPC consists of a core of adhesive and proliferating trophoblastic cells that transform into trophoblastic giant cells (TGC). TGC are localized at the fetomaternal interface, suggesting that TGC play an important role during the implantation process. In the present study, immunocytochemical analysis clearly illustrated that c-kit is expressed in both EPC and TGC of the trophoblast outgrowth. TGC grow out from the blastocyst and spread out. The surface area of the trophoblast outgrowth was predominantly occupied by TGC. Therefore, SCF may be involved in the differentiation of TGC rather than simply stimulating their growth.

We observed gene expression of SCF and c-kit in the uterine endometrium obtained when blastocysts were collected. A previous study showed that cytokines and growth factors, such as epidermal growth factor (EGF), bFGF, and platelet-derived growth factor (PDGF), play an integral role in endometrial proliferation and decidualization (Irwin et al., 1991). Strong expression of both SCF and c-kit mRNAs in the stromal cells was observed in the present study. Autocrine action of SCF in endometrial cell function may be an interesting issue for the future investigation.

It has been demonstrated that a primer for SCF synthesized to amplify an extracellular domain, including a transmembrane domain, generated an alternative spliced form (Flanagan et al., 1991). This form encoded a polypeptide missing 28 amino acid just outside the transmembrane domain (Flanagan et al., 1991). However, it has been reported that, in the testis, membrane-bound SCF is more potent form and has some activity that cannot be replaced by soluble SCF (Tajima et al., 1991; Allard et al., 1996). In the present study, the alternative spliced membrane bound SCF was expressed
in the stromal cells and embryos during implantation stage, and may act directly as a cell to cell contact mechanism. The regulation of production of these two forms of SCF by different cells may have important implications for control of the implantation process.

In summary, the localization of SCF and c-kit at the feto-maternal interface, and the stimulatory effect of SCF on trophoblast outgrowth suggest that SCF and c-kit may have a significant role during embryo implantation.

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References


Copeland, N.G., Gilbert, D.J., Cho, B.C. et al. (1990) Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell*, 63, 175–183.


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