Basigin expression and hormonal regulation in the rat uterus during the peri-implantation period

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Basigin is essential for fertilization and implantation. The aim of this study was to determine the expression and hormonal regulation of the basigin gene in the rat uterus during the peri-implantation period. Basigin mRNA was localized strongly in the luminal epithelium on day 1 of pregnancy and gradually decreased to a basal concentration from day 3 to day 5 of pregnancy. Basigin mRNA and protein were expressed strongly in the implanting blastocyst and primary decidua on day 6 of pregnancy. A similar expression pattern was also induced in the uterus after delayed implantation was terminated by oestrogen treat-

ment and the embryo implanted, whereas expression was not detected during delayed implantation. Basigin expression was not detected on day 6 of pseudopregnancy. Basigin mRNA was expressed strongly in the decidua on days 7 and 8 of pregnancy. Furthermore, both basigin mRNA and protein were induced in the decidua during artificial decidualization. In addition, oestrogen stimulated strong expression of basigin mRNA in the uterine epithelium of ovariectomized rats. These findings indicate that basigin may play a role during implantation and decidualization in rats.

Introduction

Basigin, a highly glycosylated transmembrane protein, belongs to the immunoglobulin superfamily with two immunoglobulin domains (Miyachi *et al.*, 1990). Basigin has been identified independently by several groups and given different names: HT7, neurothelin and 5A11 in chickens; gp42 in mice; OX-47 and CE9 in rats; and M6 and EMMPRIN in humans (for a review, see Igakura *et al.*, 1998). Basigin expression in mouse endometrium was stronger at sites with embryos in the lumen than at sites without embryos (Kuno *et al.*, 1998). Basigin mRNA was expressed strongly in the inner cell mass and embryo on days 4.0–7.5 (Igakura *et al.*, 1998). Basigin (–/–) mouse embryos developed normally during preimplantation stages; however, most of these embryos died at about the time of implantation. Many null mutant embryos were lost because of deficiencies in implantation. Surviving male and female mice with basigin deficiency were sterile (Igakura *et al.*, 1998). The expression and regulation of the basigin gene in the uterus of other mammalian species during early pregnancy has not been studied.

The aim of the present study was to determine whether basigin is essential for embryo implantation in mammalian species other than mice. *In situ* hybridization and immunohistochemistry were used to examine the expression of the

basigin gene in the rat uterus during early pregnancy and pseudopregnancy, delayed implantation and artificial decidualization, and also its regulation by steroid hormones.

Materials and Methods

Animals and treatments

Mature Sprague–Dawley rats were maintained in cages in a controlled environment with a 14 h light:10 h dark photoperiod. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Northeast Agricultural University. The stage of the oestrous cycle was determined by inspection of vaginal smears. Adult females were mated with fertile males of the same strain to induce pregnancy. Pregnancy was confirmed by examining for the presence of spermatozoa in the vaginal smear (day 1 = day of vaginal smear positive for spermatozoa). On days 1–5, pregnancy was confirmed by flushing embryos from the reproductive tracts. On days 6–7, the implantation sites were identified by i.v. injection of 0.1 ml of 1% (w/v) trypan blue solution in 0.85% (w/v) NaCl. Pseudopregnancy was induced by caging adult females with vasectomized males and was confirmed by checking for a vaginal plug (either in the vagina or on the cage floor) the next morning. At least three rats were used in each stage or treatment in this study.

Hormonal treatments were initiated 2 weeks after mature female rats were ovariectomized. The ovariectomized rats were treated with an injection of oestradiol (250 ng per rat;

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Fig. 1. *In situ* hybridization (left panels) and immunohistochemistry (right panels) of basigin gene in rat uterus on (a,b) day 1, (c,d) day 5, (e,f) day 6 and (g,h) day 8 of pregnancy, respectively. (i) Basigin

Sigma Chemical Co., St Louis, MO), progesterone (4 mg per rat; Sigma), or a combination of the same doses of progesterone and oestradiol. All steroids were dissolved in sesame oil and injected s.c. Controls received vehicle only (0.2 ml per rat). Treated rats were killed 24 h later.

For induction of delayed implantation, pregnant rats were treated with progesterone (5 mg per rat, s.c.) on day 4 of pregnancy and ovariectomized at 08:30–09:00 h. Progesterone (5 mg per rat) was injected to maintain delayed implantation from day 5 to day 7. Delayed implantation in the progesterone-primed rats was terminated by administration of oestrogen (250 ng per rat). The rats were killed by stunning and cervical dislocation at 24 h after oestrogen treatment and uteri were collected. The implantation sites were identified by i.v. injection of trypan blue solution into the tail.

Artificial decidualization

Mature female rats were ovariectomized and were sensitized for the decidual cell reaction 5 days later according to the procedures described by Kennedy and Ross (1997). Some of the rats were administered with a bilateral intrauterine injection of 100 µl sesame oil at about 12:00 h on the equivalent of day 5 of pseudopregnancy. The animals that did not receive the intrauterine injection served as non-stimulated controls (Orlando-Mathur *et al.*, 1996). Treated rats were killed 6 days after the intrauterine injection of oil.

Immunohistochemistry

Uteri were fixed immediately in Bouin's solution for 24 h and embedded in paraffin wax. After the sections (7 µm) were cut, the paraffin wax was removed and the sections were rehydrated. Antigen retrieval was performed by incubating the sections in 0.01 mol citrate buffer l⁻¹ (pH 6.0) at 95°C for 10 min and by cooling at room temperature for 20 min. Non-specific binding was blocked with 10% (v/v) normal horse serum in PBS for 1 h. The sections were incubated with rabbit anti-mouse basigin IgG in 10% (v/v) horse serum (1:100) for 1 h. The anti-basigin antibodies had been raised against a glutathione-S-transferase–basigin fusion protein containing the whole extracellular domain of mouse basigin (Fan *et al.*, 1998). The sections were then incubated with biotinylated secondary antibody followed by an avidin–alkaline phosphatase complex and Vector Red, according to the manufacturer's protocol (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA). Endogenous alkaline phosphatase activity was inhibited by supplementing 1 mmol levamisole l⁻¹ (Sigma) into Vector Red substrate solution. Moreover, rabbit anti-mouse basigin IgG was replaced with the same concentration of normal rabbit IgG as a negative control. The sections were counter-

stained with haematoxylin and mounted. The degree of staining was assessed subjectively by blind examination of the slides by two investigators.

In situ hybridization

A 553 bp rat basigin cDNA fragment was reverse transcribed and amplified with the total RNA from rat uterus using forward primer 5'-CGGAATTCCGGAACACGCCA-GTGAGG and reverse primer 5'-GCGGATCCACAGGAGT-GGAGGCAGACG, designed according to the rat basigin cDNA sequence (556–1108 bp, Genbank accession number X54640). Within these primers, an *Eco*RI site was added at the 5' end of the forward primer and a *Bam*H1 site at the 5' end of the reverse primer. The PCR fragment for rat basigin was recovered from the agarose gel and cloned into pGEM-3Zf (+) plasmid through *Eco*RI and *Bam*H1 sites, respectively. The cloned rat basigin fragment was verified further by sequencing. These plasmids were linearized with enzymes for labelling. Digoxigenin-labelled antisense or sense cRNA probes were transcribed *in vitro* using a digoxigenin RNA labelling kit (T7 for sense, SP6 for antisense; Boehringer Mannheim, Mannheim).

Uteri were cut into pieces of 4–6 mm and flash frozen in liquid nitrogen. Frozen sections (10 µm) were mounted on slides coated with 3-aminopropyltriethoxy-silane (Sigma) and fixed in 4% (w/v) paraformaldehyde solution in PBS. The sections were treated in 0.2 mol HCl l⁻¹ for 10 min after two washes in PBS, digested with 0.1 µg proteinase K ml⁻¹ at room temperature for 10–15 min and incubated (2 × 5 min) in 0.2% (w/v) glycine in PBS. The sections were post-fixed in 4% (w/v) paraformaldehyde for 2 min. After acetylation in freshly prepared 0.25% (w/v) acetic anhydride in 0.1 mol triethanolamine l⁻¹ (pH 8.0) for 10 min, the sections were washed (2 × 5 min) in 2 × SSC (1 × SSC: 0.15 mol sodium chloride l⁻¹, 0.015 mol sodium citrate l⁻¹), dehydrated in serial dilutions of ethanol and air dried. After prehybridization in hybridization buffer (4 × SSC, 50% (v/v) formamide, 5% (w/v) dextran sulphate, 1 × Denhardt's solution, 0.5 mg denatured salmon sperm DNA ml⁻¹, 0.25 mg yeast tRNA ml⁻¹) at 25°C for 2–3 h, the sections were hybridized in hybridization buffer with 1–5 µg ml⁻¹ of digoxigenin-labelled antisense or sense RNA probe for rat basigin at 49°C for 16 h. After hybridization, the sections were washed in 4 × SSC at room temperature for 10 min and digested in 10 µg RNase A ml⁻¹ (Boehringer Mannheim) in 0.01 mol Tris–HCl l⁻¹ and 0.5 mol NaCl l⁻¹ (pH 8.0) at 37°C for 30 min. The sections were washed in 4 × SSC, 1 × SSC and 0.5 × SSC (2 × 10 min each) at 42°C. After non-specific binding was blocked with 0.5% (w/v) block mix (Boehringer Mannheim), the sections were incubated in sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:200; Boehringer Mannheim). The signal

mRNA signal was not observed in the rat uterus on day 8 of pregnancy when basigin sense cRNA probe was used. (j) Basigin immunostaining was not observed in the rat uterus on day 8 of pregnancy when rabbit anti-basigin antibody was replaced with normal rabbit IgG. Black arrow: implanting blastocyst; white arrow: luminal epithelium. Scale bars represent 25 µm.

Fig. 2. *In situ* hybridization of basigin mRNA in rat uterus. (a) Basigin mRNA was detected strongly in the luminal epithelium on day 1 of pseudopregnancy, but (b) expression was very low in the luminal and glandular epithelia on day 6 of pseudopregnancy. (c) Basal expression of basigin mRNA was observed in the luminal epithelium during delayed implantation. (d) When delayed implantation was terminated by oestrogen treatment and embryos implanted, basigin expression was induced significantly in the implanting blastocyst (arrow) and in the luminal and glandular epithelia at the implantation site. Scale bars represent 25 μm .

was visualized with 0.4 mmol 5-bromo-4-chloro-3-indolyl phosphate I^{-1} and 0.4 mmol nitroblue tetrazolium I^{-1} in the buffer containing 100 mmol Tris-HCl I^{-1} (pH 9.5), 100 mmol NaCl I^{-1} and 50 mmol MgCl_2 I^{-1} . Endogenous alkaline phosphatase activity was inhibited with 1 mmol levamisole I^{-1} (Sigma). All of the sections were counter-stained with 1% (w/v) methyl green in 0.12 mol glacial acetic acid I^{-1} and 0.08 mol sodium acetate I^{-1} for 30 min.

Results

Basigin expression during early pregnancy

Basigin mRNA was localized mainly in the luminal epithelium on day 1 of pregnancy (Fig. 1a). The amount of basigin mRNA in the luminal epithelium gradually decreased from day 2 of pregnancy and was low on days 3–5 of pregnancy (Fig. 1c). However, an intermediate amount of basigin mRNA was first detected in the glandular epithelium on day 3 of pregnancy and was maintained throughout days 4 and 5 of pregnancy. On day 6, basigin mRNA was

detected mainly in the implanting blastocyst and in the luminal epithelium surrounding the blastocyst (Fig. 1e). From day 7 to day 8 of pregnancy, basigin mRNA signal gradually increased and was detected strongly in the embryos and decidualized cells (Fig. 1g). When digoxigenin-labelled basigin antisense cRNA was replaced by digoxigenin-labelled basigin sense cRNA as a probe, no basigin mRNA signal was observed in the uterus on day 8 of pregnancy (Fig. 1i).

Basigin protein was barely detectable in the uterus on day 1 (Fig. 1b). A small amount of basigin protein appeared in the luminal epithelium on day 3 and the amount increased gradually from day 4 to day 5. On day 5, basigin protein was detected strongly in the glandular epithelium, luminal epithelium and underlying stromal cells (Fig. 1d). On day 6, basigin mRNA signal was observed mainly in the implanting blastocyst and sub-luminal stromal cells at the implantation site (Fig. 1f). From day 7 to day 8, basigin protein was detected strongly in the embryos and the luminal epithelium at the implantation site (Fig. 1h). However, basigin immunostaining was not observed in the rat

Fig. 3. Basigin expression in the rat uterus during artificial decidualization. (a) Low expression of basigin was observed in only the luminal epithelium in the uterus of control rats, whereas (b) basigin mRNA was detected strongly in the decidualized cells (*) and luminal epithelium (arrow) during artificial decidualization. (c) Basigin protein was barely detectable in the luminal epithelium in the uterus of control rats, but (d) was induced strongly in the decidualized cells (*) and luminal epithelium (arrow) during artificial decidualization. Scale bars represent 25 μm .

uterus on day 8 of pregnancy when rabbit anti-mouse basigin antibody was replaced with normal rabbit IgG (Fig. 1j).

Basigin expression during pseudopregnancy

During pseudopregnancy, basigin mRNA was localized strongly in the luminal epithelium on day 1, decreased from day 2 and reached a low concentration that was maintained from day 3 to day 8 (Fig. 2a,b). Basigin mRNA was not observed in the glandular epithelium on day 1, but a basal amount was present in the glandular epithelium from day 2 to day 8 of pseudopregnancy (Fig. 2a,b).

Basigin expression during delayed implantation

During delayed implantation, a basal amount of basigin mRNA was present in the uterus (Fig. 2c). After delayed implantation was terminated by oestrogen treatment and embryos implanted, basigin mRNA signal was induced strongly in the implanting blastocyst and outlining luminal epithelium, and glandular epithelium. A small amount of basigin mRNA was also detected in the sub-luminal stroma (Fig. 2d).

Basigin expression during artificial decidualization

A small amount of basigin mRNA was detected in the luminal epithelium in only the control uninjected uterine horn (Fig. 3a), whereas basigin mRNA was detected strongly in the luminal epithelium and decidualized stromal cells during artificial decidualization (Fig. 3b).

When decidualization was induced in the uterine horn by injecting sesame oil into the uterine lumen, basigin protein was detected strongly in the decidualized cells (Fig. 3d), whereas only a basal amount of basigin protein was observed in the control horn (Fig. 3c).

Hormonal regulation of basigin expression

A hybridization signal was not observed in the uterus of ovariectomized rats (Fig. 4a). A small amount of basigin mRNA was detected after progesterone treatment (Fig. 4b). Compared with progesterone treatment, oestrogen induced a significant increase in basigin mRNA signal in the luminal epithelium and glandular epithelium (Fig. 4c). A combination of oestradiol and progesterone stimulated a similar expression pattern as that induced by oestrogen treatment.

Fig. 4. Hormonal regulation of (a) basigin expression in the uterus of ovariectomized rats. (a) Basigin mRNA signal was not observed in the uterus of ovariectomized rats. (b) Basal expression of basigin mRNA was observed in the glandular epithelium after progesterone treatment. (c) Expression of basigin was induced strongly in the luminal and glandular epithelia after oestrogen treatment. Scale bars represent 25 μm .

Discussion

In the present study, both basigin mRNA and protein were highly expressed in the implanting blastocyst, the luminal epithelium and underlying stroma at the implantation site on day 6 of pregnancy in rats. This pattern of expression was very similar to that in the mouse uterus. Basigin mRNA expression in the mouse uterus increases transiently during early pregnancy and a peak is observed at about day 4.5

after mating, when implantation takes place. Basigin expression in the endometrium is much stronger at implantation sites than in the inter-implantation area (Kuno *et al.*, 1998). In the present study, basigin mRNA was also detected strongly in the implanting blastocyst, in the luminal epithelium and underlying stroma, and in the glandular epithelium after delayed implantation was terminated by oestrogen treatment and the embryos implanted, whereas only a basal amount of basigin mRNA was observed in the luminal epithelium during delayed implantation. In addition, although basigin expression in the rat uterus was very similar on days 1 and 2 between early pregnancy and pseudopregnancy, only basal expression of basigin was observed in the luminal and glandular epithelium on day 6 of pseudopregnancy. The strong expression of basigin in the implantation site and decidual cells indicates that basigin might play an important role during implantation and decidualization in rats. Igakura *et al.* (1998) reported that most basigin null mouse embryos died at about the time of implantation, although basigin-deficient mouse embryos developed normally during preimplantation stages.

In ovariectomized rats in the present study, basigin expression was not detected in the uterus. Oestrogen significantly induced basigin expression in the luminal and glandular epithelium, whereas progesterone induced only low expression of basigin in the glandular epithelium. These findings indicate that basigin expression was also upregulated by maternal oestrogen. In mice, the pattern of basigin expression during the preimplantation period and its regulation by maternal oestrogen were similar to those for leukaemia inhibitory factor (LIF) (Kuno *et al.*, 1998). Both LIF mRNA and protein were expressed strongly in the mouse uterus at oestrus and on day 4 of pregnancy, which just precedes blastocyst implantation (Bhatt *et al.*, 1991; Shen and Leder, 1992; Yang *et al.*, 1995). LIF expression was also upregulated by oestrogen in ovariectomized mice (Stewart *et al.*, 1992; Yang *et al.*, 1996). LIF is essential for mouse implantation in the LIF mutation model (Stewart *et al.*, 1992). Whether there is an interaction between LIF and basigin remains to be determined.

Although basigin was expressed specifically in the rat uterus during embryo implantation and decidualization, whether basigin acts through binding to its receptors or to other molecules is unclear. Basigin can form homo-oligomers in a *cis*-dependent manner on the plasma membrane. If the disulphide bond of the more N-terminally located Ig-like domain was destroyed by mutation, basigin could not form oligomers (Yoshida *et al.*, 2000). Thus it seems likely that the N-terminal Ig-like domain is necessary and sufficient for oligomer formation by basigin on the plasma membrane. Anti-basigin antibody 8G6 and recombinant basigin-Fc fusion protein markedly inhibit not only homophilic interaction but also the production of secreted matrix metalloproteinase 2 (MMP-2) by breast cancer cell line MDA-435 and the MMP-2-dependent invasion of MDA-435 cells through reconstituted basement membrane Matrigel (Sun and Hemler, 2001). Basigin on the surface of

trophectoderm cells may interact with basigin on the surface of the uterine endometrium via homophilic interaction to induce the production of MMPs and to initiate embryo implantation. In addition, basigin can bind integrins ($\alpha 3 \beta 1$ and $\alpha 6 \beta 1$) to form basigin–integrin complexes in the plasma membrane of the fibrosarcoma cell line (Berditchevski *et al.*, 1997). $\alpha v \beta 3$ integrin is expressed in mouse endometrium and blastocysts at the time of implantation (Illera *et al.*, 2000). Whether basigin in the mouse embryo and uterus is associated with embryonic and endometrial $\alpha v \beta 3$ integrin remains to be determined.

In the present study, the localization of basigin mRNA in rat embryos and decidualized cells on days 6–8 of pregnancy was consistent with a study in mice (Igakura *et al.*, 1998). Although basigin protein was detected in the primary decidual zone on day 6 of pregnancy, basigin protein was barely detectable in the decidua on days 7 and 8 of pregnancy, which differed from the expression of basigin mRNA in the decidua. The difference between the expression of basigin mRNA and protein was also apparent on day 1 of pregnancy. The reason for this difference is unknown; however, one possible explanation is post-transcriptional regulation of basigin expression, as basigin mRNA signal was expressed more strongly than basigin protein. However, basigin immunostaining and mRNA were both induced significantly in decidualized cells during artificial decidualization. These findings indicate that basigin may play a role during decidualization and placentation in rats.

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