J. Mamm. Ova Res. Vol. 15, 96–102, 1998

An Enhancement of Uterine Epidermal Growth Factor Receptor Associated with Embryo Implantation in Rats

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Abstract: *The present study was designed to investigate the physiological significance of epidermal growth factor (EGF) receptors expressed on rat uterine epithelial cells during embryo implantation. The number of uterine EGF receptor sites, as determined with ^{125}I-labelled EGF as a ligand, progressively increased from the 3rd to the 7th day of pregnancy. Analysis of the EGF receptor by Scatchard transformation of EGF-binding data and affinity labelling with ^{125}I-labelled EGF indicated that EGF receptor proteins were noticeably expressed during postimplantation. Determination for EGF receptor sites was further carried out in the uterine tissues of rats, which were superovulating after transplantation of pituitary tissue into a capsule of kidney or subcutaneous injection of 50 IU pregnant mare serum gonadotropin (PMSG) and 50 IU human chorionic gonadotropin (hCG). The transplantation caused an increase in the number of implantation sites (24.5 ± 3.9, n=4) as compared to control rats (14.0 ± 0.6, n=7). Nevertheless, PMSG- and hCG-primed rats totally failed to initiate the implantation process, whereas the number of EGF receptor sites significantly increased during preimplantation (3rd day). In pituitary-transplanted rats on the 7th day, the number of EGF receptor sites was increased as observed in control rats (30.0 ± 0.5 fmol/mg proteins). In PMSG- and hCG-primed rats, however, the EGF receptor was noticeably reduced (7.3 ± 0.4 fmol/mg proteins). These results strongly suggest that functional EGF receptor proteins were noticeably expressed during postimplantation and that its drastic enhancement depends upon the interaction of blastocysts with uterine epithelial cells.*

Key words: *Epidermal growth factor receptor, Uterine, Embryo implantation, Rat.*

Received: January 16, 1998
Accepted: January 26, 1998
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Implantation in multiparous mammals such as rat and mouse is a complex process characterized by changes in vascularization and endometrial growth, generating a decidual reaction at the site of implantation. The lack of this reaction in the regions between the implantation sites implies that some regulatory factors are acting to limit decidual formation at the sites of implantation. Although ovarian steroids are believed to be involved the initiation of implantation, there is a growing body of evidence that several growth factors and their specific receptors are expressed in the embryos [1–4] and the uterus [5–10] prior to implantation, suggesting that at least some ovarian steroids are mediated via actions of growth factors during the processes of implantation [11–13]. Epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and their common receptor have been identified in the mouse uterus [11, 14–17]. Furthermore, it was recently reported that their mRNAs were detected in the embryos prior to implantation [18, 19]. The EGF receptor is present on all major uterine cell types [20].

EGF is effective in the improvement of mouse embryo development and hatching [14–17]. Synthesis of the uterine EGF receptor increases after estrogen treatment [12, 13, 21] and prior to implantation, especially at the sites of implantation [6]. Since the response of cell populations to EGF depends upon the receptor levels, alterations in EGF receptor levels may cause changes in the implantation, but the factors responsible for development of the uterine receptor during periimplantation remain to be fully understood. In a previous study [22], we reported that an intraluminal injection of EGF was effective in the implantation of rat blastocysts. In the present study, we extend our recent report to evaluate the physiological significance of EGF receptors expressed on rat uterine epithelial cells during embryo implantation. That report described how functional EGF

receptor proteins were noticeably expressed during postimplantation and that its drastic enhancement might depend upon the interaction of blastocysts with uterine epithelial cells.

Materials and Methods

Animals

Adult female rats (200 to 300 g) of the Sprague-Dawley strain that have an 4-day estrus cycle were obtained from the University Experimental Animal Laboratory and housed in an air-conditioned room maintained at 22–24°C with controlled illumination (lights on from 8:00 to 20:00). They were placed with male rats in the afternoon of the proestrus day. In the morning of the next day, mating was confirmed by detection of spermatozoa in the vaginal smear (day 1 of pregnancy).

Superovulation and determination of implantation sites

The rats had ovulation induced by subcutaneous injection of 20 IU or 50 IU per head of pregnant mare serum gonadotropin (PMSG, Teikokuzouki Mfg. Co., Tokyo) dissolved in physiological saline on the metaestrus day, followed by 20 IU or 50 IU per head of human chorionic gonadotropin (hCG, Mochida Pharmaceutical Co., Tokyo) after 48 h. In some rats on the metaestrus day, the pituitary gland obtained from male rats at 3–4 weeks of age was transplanted into a capsule of kidney under pentobarbital anesthesia. The gonadotropin-treated and pituitary-transplanted rats were allowed to mate as described above. On day 7 of pregnancy, implantation of embryos, which form a discrete blue band around the uterus, was estimated by intravenous injection of 1 ml of a 1% (w/v) Evans Blue solution dissolved in saline 10 min before the rats were killed. The number of implantation sites in each uterine horn was counted.

Preparation of uterine membrane homogenates

The rat uteri on day 3 to day 7 of pregnancy were placed on a chilled surface, the adhering connective and adipose tissue removed, and the uteri separated at the vaginal and oviductal junctions, then pooled, homogenized in 3 ml of phosphate-buffered saline (PBS) with a teflon-glass homogenizer under ice cooling, and centrifuged at 400 g for 5 min. Protein content in the supernatant fluids was determined with BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) and the samples were stored at −96°C until use.

Determination of EGF receptors

EGF binding studies with ^{125}I-labelled EGF iodinated by the lactoperoxidase method [23] were performed principally by the method of Carpenter & Cohen [24]. The EGF receptor content of tissue homogenates was measured by quantifying the amounts of ^{125}I-EGF bound to the homogenates. The homogenates (200 μg proteins) were incubated in 0.5 ml assay buffer, PBS containing 0.1% bovine serum albumin, with the tracer (3×10^5 counts/min, 1 ng) for 60 min at 25°C, reaching the equilibrium state. The membrane particle bound and free radioactive ligands were separated by rapid filtration on 0.45-μm pore nitrocellulose filters (Millipore Co.). Then the filters were washed three times with 1 ml assay buffer, and the receptor bound radioactivity remaining on the filters was quantified with a gamma-radiation scintillation counter. Scatchard analyses were performed in the presence of increasing amounts of ^{125}I-EGF (0.1–2 ng/tube), and the maximum number of EGF receptor sites and the affinity dissociation constant (Kd) were calculated [25]. The nonspecific binding was determined in the presence of excess unlabeled EGF (200 ng/tube). The specifically bound EGF is expressed as fmol bound EGF per mg proteins.

Affinity labelling

The uterus homogenates (1 mg proteins) prepared from rats on days 3 and 7 of pregnancy were suspended in 0.5 ml assay buffer and incubated with ^{125}I-EGF (20 ng) for 60 min at 25°C. Thereafter, they were washed with assay buffer and suspended in 0.5 ml assay buffer. To cross-link the bound ^{125}I-EGF, disuccinimidyl suberate (Pierce, Rockford, IL), freshly prepared in dimethyl sulfoxide, was added to a final concentration of 0.5 mmol/l and incubated for 20 min at 4°C. The reaction was terminated with 1 ml of 20 mmol tris(hydroxymethyl)aminomethane (Tris)/l-HCl (pH 7.4) containing 0.15 mol NaCl/l and centrifuged at 1,500 g for 15 min. The pellets were the solubilized in 20 mmol Tris/l-HCl (pH 7.4) containing 1% (w/v) Triton X-100, 2 mmol p-amidinophenylmethylsulfonyl fluoride/l (Wako, Osaka, Japan), 5 mmol EDTA/l, and 5 mmol iodoacetate/l. The suspension was stirred gently for 30 min at 4°C and centrifuged at 1,500 g and 4°C for 15 min. The supernatants were mixed with 0.6 volumes of threefold concentrated Laemmli electrophoresis sample buffer [26] and boiled for 2 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the affinity-labeled samples was carried out with 7.5% (w/v) gels. After SDS-PAGE was performed, the gels were fixed, stained with 0.25% (w/v) Coomassie brilliant blue R-250, destained, dried and exposed to Kodak XAR film at −80°C for 2 weeks.

Statistical analysis

The data are expressed as the means ± SE, and differences between them were analyzed by Student's t-test, with $p<0.05$ or $p<0.01$ considered to be statistically significant [27].

Results

Changes in EGF receptor levels in uterine tissues of pregnant rats

Figure 1 shows the specific binding of ^{125}I-EGF to uterine membrane particles prepared from pregnant rats at day 3 to day 7. The amount of EGF receptors at day 3 was relatively low (about 9.17 fmol EGF bound per mg proteins), thereafter, EGF receptor levels increased in a time-dependent manner. A 2-fold increase in EGF receptors was observed at day 5 of pregnancy ($p<0.01$), at which implantation is initiated. Then, at day 7, EGF receptor levels were further augmented, with a 3-fold increase over day 3 ($p<0.01$).

Scatchard analysis of EGF receptors in uterine membrane particles

Scatchard analysis of ^{125}I-EGF binding was performed with uterine membrane particles prepared from pregnant rats at day 3 and day 7. Scatchard transformation of ^{125}I-EGF binding isotherm data indicated that the number of EGF receptor sites was significantly higher at day 7 than at day 3 of pregnancy (9.8 fmol/mg proteins at day 3; 66.8 at day 7). A significant alteration in their binding affinities was also observed (Kd = 1.00 pM at day 3; 3.11 pM at day 7).

Analysis of uterine EGF receptor by ligand-affinity cross-linking

In order to identify the EGF receptor augmented during pregnancy, the EGF receptor was analyzed by affinity labelling with ^{125}I-EGF in uterine membrane particles by using disuccinimidyl suberate. Figure 3 shows an autoradiogram obtained from SDS-PAGE of the membrane particles cross-linked with ^{125}I-EGF. Labeled bands with a molecular mass of approximately 170-180 kDa were detected, and these bands were abolished by an excess of cold EGF, indicating the EGF receptor with a molecular mass of 170 kDa [28]. The intensity of the autoradiographic appearance of the specific EGF binder showed a higher level of bound ^{125}I-EGF at day 7 of pregnancy, and migration of the EGF receptor cross-linked with ^{125}I-EGF was identical in both groups, thus indicating increases in intact EGF receptor proteins during the progress of pregnancy.

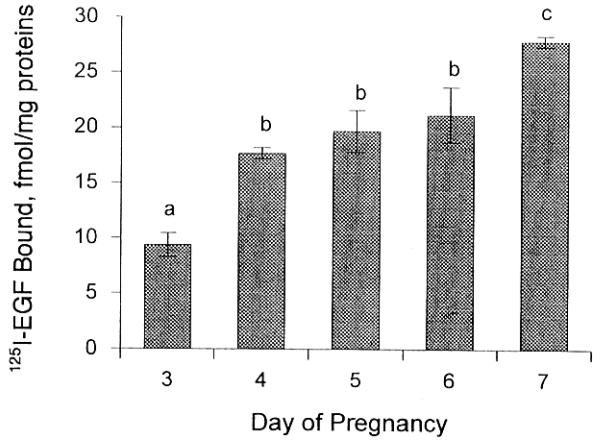

Fig. 1. Changes in ^{125}I-EGF binding sites in uterine tissues of pregnant rats. The rat uteri on day 3 to day 7 of pregnancy were removed, pooled and homogenized as described in Materials and Methods. The homogenates (200 μg proteins) were analysed for EGF receptor content. The specifically bound EGF is expressed as fmol bound EGF per mg proteins. Data are the means ± S.E. of triplicate determinations and values with different letters are significantly different ($p<0.01$).

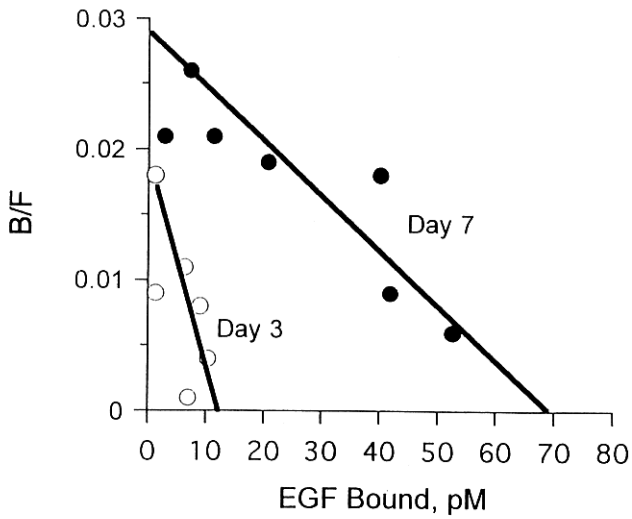

Fig. 2. Scatchard analyses of EGF receptors in rat uterine tissues. The uteri were prepared from pregnant rats on the 3rd day (open circle) and the 7th day (closed circle) of pregnancy. The homogenates were incubated with increasing concentrations (0.1–2 ng/tube) of ^{125}I-labelled EGF as described in Materials and Methods. Data are the means of duplicate determinations.

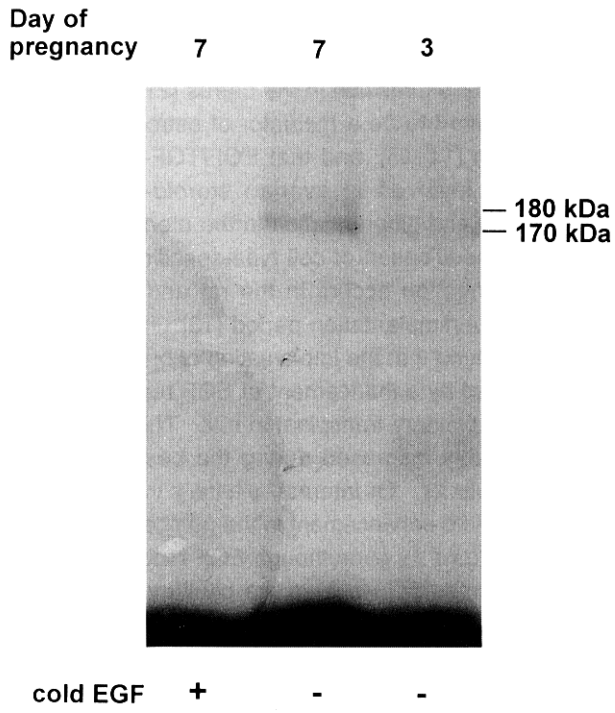

Fig. 3. Analysis of uterine EGF receptor by ligand-affinity cross-linking. Affinity cross-linking of ^{125}I-EGF to the uteri homogenates (1 mg proteins) was performed as described in Materials and Methods. *Left*: Incubation with homogenates on the 7th day of pregnancy was carried out in the presence of excess unlabelled EGF. Sizes of ^{125}I-EGF-receptor complexes are indicated on the right. *Middle*: 7th day of pregnancy. *Right*: 3rd day of pregnancy.

Embryo implantation and uterine EGF receptor level under hormonal stimulation

To further evaluate the role of uterine membrane EGF receptor during the periimplantation periods, we examined its changes in pre- and post-implantation of embryos ovulated by PMSG-hCG-primed or pituitary-transplanted rats. Subcutaneous injection of PMSG (20 IU or 50 IU) was carried out on the metaestrus day, and thereafter hCG (20 IU or 50 IU) was subcutaneously injected. Transplantation of pituitary into a capsule of kidney was carried out on the metaestrus day. As shown Table 1, there were no significant differences between control and PMSG-hCG-primed (each 20 IU) rats in the number of implantation sites. The number of implantation sites was increased 2-fold by transplantation of pituitary. In contrast, treatment with higher doses of PMSG and hCG (each 50 IU) significantly decreased the numbers of implantation sites ($p<0.01$).

The amounts of uterine EGF receptors were determined with the membrane particles prepared from PMSG-hCG-primed (each 50 IU) and pituitary-transplanted rat uteri on days 3 and 7 of pregnancy. There were no significant differences between pituitary transplantation and treatment with PMSG plus hCG in EGF receptor levels in day 3 pregnant uteri. In day 7 pregnant uteri, EGF receptor levels were noticeably augmented in pituitary-transplanted rats, as observed in control rats showing regular ovulation. In contrast to control and pituitary-transplanted rats, treatment with higher doses of PMSG and hCG decreased EGF receptor levels in day 7 pregnant uteri as well as the number of implantation sites.

Table 1. Implantation of embryos in pituitary-transplanted and PMSG-hCG-primed rats

Treatment	No. of rats used	No. of implantations (%)	No. of implantation sites
Control	7	7/7 (100.0)	14.0 ± 0.6^a
Pituitary transplantation	4	4/4 (100.0)	24.5 ± 3.9^b
PMSG and hCG			
20 IU + 20 IU	8	7/8 (87.5)	13.4 ± 1.3^a
50 IU + 50 IU	4	1/4 (25.0)	3.0

Mature rats were induced to ovulate by pituitary transplantation or hormonal treatment. Transplantation of the pituitary from male rats (3–4 weeks old) into a capsule of the kidney was carried out on the metaestrus day. Hormonal treatment was carried out by subcutaneous injection of PMSG (20 IU or 50 IU) on the metaestrus day, followed by hCG (20 IU or 50 IU) after 48 h. The treated females were allowed to mate. Data are the mean ± S.E. and values with different letters are significantly different ($p<0.01$).

Discussion

It is well known that EGF/TGF-α as well as its specific receptor are present in the rodent uterus and embryo prior to implantation [6, 13, 20, 21], suggesting the importance of their roles in the embryo-uterine interaction during implantation. The EGF receptor has been identified in the uterus of several species, especially in glandular epithelial cells and stromal cells from porcine endometrium [29]. Although the factors responsible for the development of specialized regions of the uterus involved in implantation are not fully understood, high expression of the EGF receptor gene in rat and mouse decidua is probably associated with its extensive proliferation and differentiation during this stage [17, 18]. Our present data for characterization of uterine EGF receptors as revealed by quantitative binding of iodinated EGF and analysis of ligand-affinity cross-linked receptors also support this view.

In the present study, we determined EGF receptors in the uterus of pregnant rats during the periimplantation period. Specific binding of iodinated EGF to uterine membrane particles was altered with the stage of implantation; a significant increase in EGF receptor was observed on day 5 (the day of implantation), and especially a marked enhancement of EGF receptors was noted on day 7 (Fig. 1). Scatchard analysis of EGF receptor binding and properties of ligand-affinity cross-linked receptors on SDS-PAGE indicate that intact EGF receptor proteins with a molecular mass of 170 kDa are noticeably synthesized during the postimplantation period (Figs. 2, 3). The rising EGF receptor levels in periimplantation may involve embryonic and uterine development.

Estrogen and progesterone are critical in regulating EGF receptor expression in the uterus [21]. EGF/TGF-α is considered to be a mediator of estrogen action in implantation [11–13], and that EGF/TGF-α and its receptor are involved in ovarian steroid-induced cell proliferation and differentiation in the uterus. A steroid hormonal modification of cell type-specific proliferation and differentiation occurs in the rat and mouse uteri during the periimplantation period [13]. In the present study, we found that the implantation of blastocysts was accompanied by enhancement of EGF receptors in the control and pituitary-transplanted rats. The EGF receptors noticeably increased during the postimplantation period (Table 2). Of interest, pituitary transplantation caused a 2-fold enhancement in the number of implantation sites (Table 1), even though EGF receptor levels in the uterine membranes from the pituitary transplanted group were not significantly increased as compared with the control group (Table 2), suggesting that the receptors on day 7 of pregnancy might not be always associated with the number of implanted embryos (Table 1).

Since the uterine receptivity for blastocyst implantation depends on an appropriate combination of progesterone and estrogen [30], our data indicate that continuous secretion of gonadotropins from transplanted pituitary might stimulate ovaries to produce steroid hormones and in turn to improve uterine receptivity. In contrast, treatment with higher doses of PMSG and hCG did not mimic the effect of pituitary transplantation (Table 1). Although treatment with PMSG plus hCG increased ovarian weight over the control, there was no significant difference between both groups in uterine weight (data not shown). We cannot yet exactly explain the decrease in implantation sites in PMSG- and hCG-primed rats, but it is noted that the decreased uterine receptivity is closely related to a drastic reduction in EGF receptor levels on day 7 (Fig. 4). These findings strongly suggest that some factors originating in blastocysts promote the EGF receptor during the postimplantation period.

Growing embryos have been reported to produce growth factors such as TGF-α, fibroblast growth factors and insulin-like growth factor-II [31, 32]. Studies on the promoter of the EGF receptor gene support that the receptor expression is regulated by multiple hormones and growth factors, including EGF/TGF-α [33–35]. Consequently, growth-promoting factors originating in the embryo are involved in an autocrine regulation of embryonic growth and differentiation, and they may also promote uterine EGF receptor for postimplantation embryonic and uterine development. Further investigation

Table 2. Changes in EGF receptor levels in uterine membranes from pituitary-transplanted and PMSG-hCG-primed rats

Treatment	^{125}I-EGF binding sites	
	day 3-pregnancy	day 7-pregnancy
	fmol/mg proteins	
Control	9.4 ± 1.1	27.8 ± 0.5
Pituitary transplantation	12.1 ± 0.4	30.0 ± 0.5
PMSG-hCG-priming	15.9 ± 0.2	7.3 ± 0.4

Mature rats were induced to ovulate by pituitary transplantation or hormonal treatment as described in Table 1. Hormonal treatment was carried out by subcutaneous injection of 50 IU PMSG on the metestrus day, followed by 50 IU hCG after 48 h. The treated females were allowed to mate. Data are the mean ± S.E. and values with different letters are significantly different.

for EGF receptor-promoting factors possibly originating in blastocysts postimplantation is necessary.

The results presented here provide evidence that functional EGF receptor proteins are noticeably expressed during the postimplantation period and that its drastic enhancement might depend upon interaction of blastocyst with uterine epithelial cells.

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