Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation

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SUMMARY

Heparin-binding EGF-like growth factor (HB-EGF) is a newly discovered member of the EGF family of growth factors. HB-EGF can bind to two loci on cell surfaces, heparan sulphate proteoglycans and EGF-receptor (EGF-R), and either one or both of these interactions could play a role in cell-cell interactions. In the rodent, increased endometrial vascular permeability at the site of blastocyst apposition is considered to be an earliest discernible prerequisite event in the process of implantation and this event coincides with the initial attachment reaction between the blastocyst trophectoderm and uterine luminal epithelium. This investigation demonstrates that the HB-EGF gene is expressed in the mouse uterine luminal epithelium surrounding the blastocyst 6-7 hours before the attachment reaction that occurs at 2200-2300 hours on day 4 of pregnancy. It was further demonstrated that this gene is not expressed in the luminal epithelium at the site of the blastocyst apposition during the progesterone-maintained delayed implantation, but is readily induced in the luminal epithelium surrounding an activated blastocyst after termination of the delay by an estrogen injection. In vitro studies showed that HB-EGF induced blastocyst EGF-R autophosphorylation, and promoted blastocyst growth, zona-hatching and trophoblast outgrowth. These results suggest possible interactions between the uterine HB-EGF and blastocyst EGF-R very early in the process of implantation, earlier than any other embryo-uterine interactions defined to date at the molecular level.

Key words: HB-EGF, EGF-R, blastocyst, uterus, implantation, delayed implantation, mouse

INTRODUCTION

The synchronized development of the preimplantation embryo to the blastocyst stage, escape of the blastocyst from the zona pellucida, and differentiation of the uterus to the receptive state are all essential to the process of implantation (Psychoyos, 1973). The establishment of a differentiated uterine environment for supporting embryo development and implantation is primarily dependent on the coordinated effects of estrogen and progesterone (Psychoyos, 1973; Paria et al., 1993b). In the rodent, the first conspicuous sign that the implantation process has been initiated is an increased endometrial vascular permeability at the site of the blastocyst. This can be visualized as discrete blue bands along the uterus after an intravenous injection of a blue dye solution (Psychoyos, 1973). This increased localized vascular permeability coincides with the initial attachment reaction between the uterine luminal epithelium (LE) and trophectoderm (Enders and Schlafke, 1967), and is considered as one of the earliest prerequisite events in implantation (Psychoyos, 1973). In the mouse, the attachment reaction occurs in the evening (2200-2300 hours) of day 4 of pregnancy, and is preceded by uterine luminal closure which results in an intimate apposition of the blastocyst trophectoderm with the LE (Enders and Schlafke, 1967; Psychoyos, 1973; Enders, 1976). The attachment reaction is followed by localized decidualization of the stroma and apoptosis of the LE at the site of blastocyst implantation (Parr et al., 1987). This results in subsequent adherence and penetration by trophoblast cells through the underlying basement membrane (Schlafke and Enders, 1975). Invasion of trophoblast cells continues through the stroma in a regulated manner by the remodeling of the extracellular matrix.

Ovarectomy in the morning of day 4 of pregnancy prior to preimplantation ovarian estrogen secretion results in blastocyst dormancy and failure in attachment reaction, a condition termed delayed implantation. Delayed implantation can be maintained by continued progesterone (P4) treatment, but is terminated by an injection of estrogen with blastocyst activa-
tion and initiation of implantation (attachment reaction) (Yoshinaga and Adams, 1966; Huet and Dey, 1987). The luminal closure and apposition occur during the P4-treated delayed implantation, but the attachment reaction (uterine blue reaction) and the subsequent events do not occur unless estrogen treatment is provided (Psychoyos, 1973; Nilsson, 1974; Enders, 1976).

Since the process of implantation is restricted to the site of blastocyst apposition, it is considered that the attachment reaction culminates from an intimate ‘cross-talk’ between the trophectoderm epithelium of the active blastocyst and LE of the receptive uterus. However, the molecular basis of this ‘cross-talk’ for blastocyst attachment to the LE remains an enigma. The interactions between cell surface molecules of the trophectoderm and LE appear to be important for the attachment reaction (Enders and Schlaflke, 1974). Many basement membrane and extracellular matrix components, such as laminin, fibronectin, entactin, collagen-type IV, hyaluronic acid and heparan sulfate proteoglycan (HSPG), promote blastocyst attachment and outgrowth in vitro (reviewed by Arman, 1991; Carson et al., 1993). It is proposed that integrin receptors on trophoblast cells interact with extracellular matrix components in mediating these events. However, some of the attachment factors (laminin, fibronectin, and collagen) that can support ‘implantation’ in vitro are present in basement membranes, but are not displayed at the apical surface of the LE. Therefore, these components may be important for trophoblast penetration and invasion later in the process of implantation, but not for the initial attachment reaction between the trophectoderm and LE. On the other hand, the recent observation of expression of HSPG molecules on the trophectodermal cell surface and their beneficial role in blastocyst attachment in vitro (Carson et al., 1993) points towards an important function, as discussed below, for HSPG in embryo-uterine interaction during implantation.

An emerging concept is that polypeptide growth factors could be involved in preimplantation embryo development and implantation. Indeed, the expression of several growth factors and their receptors in the uterus and/or the embryo suggests that these factors could be involved in these processes (Lingham et al., 1988; Murphy et al., 1987; Rappolee et al., 1988; Heyner et al., 1989; Huet-Hudson et al., 1990b; Paria and Dey, 1990; Tamada et al., 1990, 1991; Kapur et al., 1992; Das et al., 1992, 1994; Paria et al., 1992, 1993a; Wiley et al., 1992 and references therein). Recent observations particularly suggest the possibility of a ligand-receptor signaling with the EGF family of mitogens in the initiation of the implantation process: in the mouse, EGF receptor (EGF-R) is expressed in the blastocyst trophectoderm, and this expression is tightly regulated by the maternal steroid hormonal status at the time of implantation (Paria et al., 1993a). These results suggested a possible role for the EGF family members in luminal epithelial and trophectodermal cell-cell interactions in the implantation process, but the ligand involved in interaction with the blastocyst EGF-R had remained undefined. Among the EGF family of growth factors, EGF itself, transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor (HB-EGF) and amphiregulin, all mediate their effects apparently via binding to EGF-R (Massague and Pandiella, 1993). TGF-α is expressed in the periimplantation mouse uterus (Tamada et al., 1991; Paria et al., 1994), but a role for this growth factor in implantation has been questioned since implantation appears to be normal in TGF-α-deficient mice (Bruce-Mann et al., 1993; Luetteke et al., 1993). In addition, EGF mRNA could not be detected in the mouse uterus on day 4 of pregnancy prior to implantation (Huet-Hudson et al., 1990a). Whether amphiregulin is expressed in the uterus is yet to be determined.

We are particularly interested in a possible role of HB-EGF in embryo-uterine interaction. HB-EGF was identified as a secreted product of a macrophage-like cell line and can bind to the EGF-R (Higashiyama et al., 1991, 1992, 1993). HB-EGF is a potent mitogen for smooth muscle cells and fibroblasts, but not endothelial cells. HB-EGF is also mitogenic for keratinocytes (Marikovsky et al., 1993) and can trigger the migration of smooth muscle cells (Higashiyama et al., 1993). This growth factor is structurally similar to TGF-β in that it exists in two forms: a cell membrane-associated precursor and a soluble mature paracrine factor. Both forms can interact with the EGF-R receptor. The cell-associated precursor is also the receptor for the diphtheria toxin (Naglich et al., 1992). An important property of HB-EGF is that, unlike TGF-α and EGF, it binds tightly to immobilized heparin or to cell surface HSPG (Higashiyama et al., 1991, 1992, 1993; Thompson et al., 1994). The ability of HB-EGF to bind to cell surface HSPG via a 21 amino acid heparin-binding domain is, in part, responsible for its greatly enhanced potency for smooth muscle cell proliferation when compared to TGF-α or EGF (Higashiyama et al., 1993; Thompson et al., 1994). In addition to EGF-R (Paria et al., 1993a), HSPG is also associated with the trophectoderm cell surface and its presence is coordinated with the development of attachment competence of the mouse blastocyst (Carson et al., 1993). Since HB-EGF can bind to two loci on neighboring cells, HSPG and EGF-R (Higashiyama et al., 1993), either one of these interactions could play a role in mediating embryo-uterine attachment. If HB-EGF in the LE remained in a membrane-associated form, such interactions could be via a juxtacrine mechanism. Since HB-EGF is a potent mitogen and motility factor (Higashiyama et al., 1993), we postulate that HB-EGF could also directly modulate blastocyst growth and functions. Given these considerations, we examined the temporal and cell-specific expression of HB-EGF in the mouse uterus during the periimplantation period. We report here that 6-7 hours prior to the attachment reaction (blue reaction), HB-EGF is expressed in the LE exclusively surrounding the blastocyst. In addition, recombirant mature HB-EGF triggered phosphorylation of the blastocyst EGF-R, and stimulated blastocyst zona-hatching, development and outgrowth. Thus, our observations are consistent with embryo-uterine interactions being mediated at least in part at the molecular level by a growth factor very early in the process of implantation.

**MATERIALS AND METHODS**

**Animals**

Adult CD-1 females (Charles River Laboratories, Raleigh, NC) were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (day 1 = vaginal plug), respectively. Mice on days 1-3 were killed between 0830-0930 hours and pregnancy was confirmed by recovering embryos from oviducts. Mice
were also killed at various times on days 4 and 5 of pregnancy or pseudopregnancy. On days 6-8, mice were killed at 0900 hours. Implantation sites (increased uterine vascular permeability) on day 4 at 2300 hours through day 6 were visualized by intravenous injections (0.1 ml/mouse) of a Chicago Blue dye solution (1% in saline) and killing mice 5 minutes later. Implantation sites were demarcated by discrete blue bands (Huet and Dey, 1987).

To induce and maintain delayed implantation, mice were ovarioctomized in the morning (0800-0900 hours) of day 4 of pregnancy and received daily injections of progesterone (P₄, 2 mg/mouse) from days 5-7 (Yoshinaga and Adams, 1966; Paria et al., 1993a,b). To terminate delayed implantation and to induce blastocyst activation, the P₄-primed delayed pregnant mice were given an injection of estradiol-17β (E₂, 25 ng/mouse) on the 3rd day of the delay (day 7). Mice were killed at 2, 6, 12, and 24 hours after E₂ injection. Under these conditions, the first visually detectable implantation sites after blue dye injection became evident 18-24 hours after an E₂ injection. All steroids were dissolved in sesame oil and injected subcutaneously.

Hybridization probes
A rat HB-EGF cDNA fragment, derived from cRNA (7-6 (Abraham et al., 1993), of approximately 1.5 kb insert was subcloned into a pGEM7ZF(+) vector at the EcoRI site. For northern hybridization, an antisense 32P-labeled cRNA probe was generated using SP6 polymerase. For in situ hybridization, sense and antisense 35S-labeled cRNA probes were generated using T7 and SP6 polymerases, respectively. A cDNA clone of human fibroblast cystolinic β-actin (Ponte et al., 1984) was subcloned into a pGEM vector containing a promoter for SP6 polymerase and used as a template for synthesis of a 32P-labeled cRNA probe (Das et al., 1994). Probes had specific activities of about 2×10⁶ disintegrants per minute/µg.

Northern blot hybridization
Total RNA was extracted from whole uteri by a modified guanidine thiocyanate procedure (Han et al. 1987; Das et al., 1992, 1994). Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose column chromatography (Sambrook et al., 1989). Poly(A)⁺ RNA (2.0 µg) was denatured, separated by formaldehyde-agarose gel electrophoresis, transferred and cross-linked to the membranes by UV irradiation (Sambrook, Spectronics Corp., Westbury, NY). Northern blots were prehybridized, hybridized and washed as described previously (Das et al., 1992, 1994). Hybridization was carried out for 20 hours at 68°C in 3x SET (1x SET = 150 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl, pH 8.0), 20 mM phosphate buffer (pH 7.2), 250 µCi/ml tRNA, 10% dextran sulphate and 1x10⁶ cts/minute of labeled HB-EGF sense or antisense cRNA probes for 4 hours at 68°C. After hybridization and washing, the slides were incubated with RNase A (20 µg/ml) at 37°C for 15 minutes. RNase A resistant hybrids were detected after 1-3 weeks of autoradiography using Kodak NTB-2 liquid emulsion. The slides were poststained with hematoxylin and eosin.

Anti-HB-EGF antibodies
Antibody no. 197 (Ab 197) was raised in a goat by Triple J Farms (Redmond, WA) by intramuscular injection of recombinant human HB-EGF (met-HB-EGF), see below). Purified IgG was obtained from the goat plasma by octanoic acid precipitation. Antibody no. 850 (Ab 850) was raised in a chicken at East Acres Biologicals (Southbridge, MA) by subcutaneous injection of rat HB-EGF that had been recombinantly expressed in, and secreted by CHO-K1 cells. Purified immunoglobulin was obtained from egg yolks, using the method of Polson et al. (1985). Antibody no. 1001.1 (Ab 1001.1) is a monoclonal antibody derived from a mouse that was initially immunized with a peptide representing the carboxy-terminal region of secreted human HB-EGF (ICHPGYHGERCHGLSLP); prior to fusion of the spleen cells, the mouse was boosted with a peptide representing the carboxyl terminus of rat HB-EGF (HCLPGYHGQRCGLTL). Ab 1001.1 was purified on Protein G Sepharose.

Recombinant HB-EGF
Human HB-EGF protein (met-HB-EGF), made recombinantly in E. coli, consisted of a translation-initiating methionine residue followed by amino acids 73 through 149 of the 208-residue HB-EGF primary translation product.

Immunohistochemistry
Immunohistochemical localization of HB-EGF was performed as described previously (Das et al., 1992; Slayden et al., 1993). Uterine pieces were subjected to microwave fixation for 7 seconds in Hank’s balanced salt solution (HBSS) followed by incubation in ice-cold 10% sucrose solution in PBS for 20 minutes. Tissues were then flash frozen in liquid freon and stored at −70°C until used. Frozen sections (12 µm) were mounted onto poly-L-lysine coated glass slides, microwaved again for 2 seconds followed by fixation in a mixture of 0.2% picric acid and 2% paraformaldehyde solution in PBS for 10 minutes. Sections were rinsed in cold (4°C) PBS containing 1.5% polyvinyl pyrrolidone (PVP), 1.5% PVP plus 0.37% glycine, and 1.5% PVP plus 0.1% gelatin in succession. Immunostaining was achieved as described previously (Das et al., 1992). Each blot was first hybridized to the HB-EGF probe, and then to a β-actin probe to confirm integrity, equal loading and blotting of RNA samples.

In situ hybridization
In situ hybridization was performed as described previously (De et al., 1989; McMaster et al., 1992). On specific days and times of pregnancy, pseudopregnancy or delayed implantation, each uterine horn was excised, cut into halves, or separated into implantation and interimplantation sites. Frozen sections (10 µm) were mounted onto poly-L-lysine coated slides and stored at −70°C until used. When required, frozen sections were cut serially to detect the sites of blastocysts. After removal from −70°C, the slides with the uterine sections were placed on a slide warmer (37°C) for 2 minutes and then fixed in 4% paraformaldehyde in PBS for 15 minutes at 4°C. Following prehybridization, uterine sections were hybridized to 35S-labeled HB-EGF sense or antisense cRNA probes for 4 hours at 45°C. After hybridization and washing, the slides were incubated with RNase A (20 µg/ml) at 37°C for 15 minutes. RNase A resistant hybrids were detected after 1-3 weeks of autoradiography using Kodak NTB-2 liquid emulsion. The slides were poststained with hematoxylin and eosin.

**Fig. 1.** Northern blot hybridization of HB-EGF mRNA in the mouse uterus. The mRNA levels were detected in samples obtained from the whole uterus on days 1-7 of pregnancy, or from the separated deciduum (Dec) and myometrium (Myo) on day 8 as indicated. RNAs isolated from lung (Lu) or liver (Lv) tissues were used as positive and negative controls, respectively. Poly(A)⁺ RNA (2 µg) samples were separated by formaldehyde-agarose gel electrophoresis, transferred and UV cross-linked to nylon membranes and hybridized sequentially to 32P-labeled HB-EGF and β-actin probes. Autoradiographic exposures were for 2 hours for HB-EGF and 0.5 hour for β-actin. The two species of β-actin transcripts present in whole uterine RNA samples reflect the abundance of muscle-specific actin mRNA contributed by the myometrium.
performed using a goat (Ab 197) or a chicken (Ab 850) antibody (2 µg/ml) utilizing a Zymed-Histostain-SP kit (Zymed Laboratories, San Francisco, CA). Red deposits indicated the sites of immunostaining. Immunoneutralization experiments were performed by incubating sections first with a 10-fold molar excess of a 21 amino acid peptide encompassing the heparin-binding domain of HB-EGF (Thompson et al., 1994; Higashiyama et al., 1993) followed by incubation with pre-neutralized primary antibody with a 10-fold molar excess of the antigen.

Western blot analysis
Proteins were extracted from uterine LE by sonication in 0.01 M sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and proteinase inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 µg/ml pepstatin and 1 µg/ml leupeptin). Triton X-100 (1%) was added to the homogenate, which was incubated for 40 minutes at 4°C. The homogenate was centrifuged at 14,000 g for 5 minutes and the supernatant was loaded onto a heparin-affinity column (Pharmacia LKB, Piscataway, NJ). The column was equilibrated with 0.01 M sodium phosphate buffer. After loading, the sample was eluted first with 0.2 M NaCl and then with 2 M NaCl in equilibration buffer. The fractions were collected, dialyzed in distilled water for 4 hours and then in 0.01 M sodium phosphate buffer overnight and lyophilized. These fractions (80 µg protein/lane) were subjected to a 15% SDS-PAGE under reducing conditions. Recombinant human HB-EGF (met-HB-EGF77, 30 ng) was run on the same gel as a positive control. Proteins were transferred to a nitrocellulose membrane. The membrane was incubated in 20% Carnation milk in Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 (TBST) for 2 hours. After rinsing, the blots were incubated with the chicken anti-HB-EGF IgG (Ab 850, 2 µg/ml), goat anti-HB-EGF IgG (Ab 197, 2 µg/ml) or mouse monoclonal anti-HB-EGF (Ab 1001.1, 3 µg/ml) in TBST with 3% BSA for 2 hours. Species-specific secondary antibodies conjugated with alkaline phosphatase (Sigma) were used (1:5000 dilution in 3% BSA-TBST) for recognizing the primary antibodies, followed by development of color reaction (Paria et al., 1994).

Autophosphorylation of blastocyst EGF-R
Day-4 blastocysts collected in groups of 43 were placed in microfuge tubes containing 10 µl of 10 mM Tris-HCl (pH 7.4) plus 0.25 M sucrose, 10 µg/ml leupeptin and 20 µg/ml PMSF, and sonicated. The homogenate (approx. 0.86 µg protein) was suspended in 50 µl reaction buffer (50 mM Pipes, pH 7.0, 1 mM MnCl2 and 0.1 mM sodium vanadate) and incubated for 10 minutes at 4°C in the presence (11 nM) or absence of HB-EGF. The phosphorylation reaction was carried...
out as described previously (Das et al., 1994). After 1 hour of incubation on ice, the reaction mixtures were centrifuged. The precipitates were washed with a mixture of diethyl ether and ethanol (1:1) and resuspended in an equal volume of 50 µl of Tris buffer (50 mM, pH 7.5) and protein A Sepharose/an EGF-R monoclonal antibody complex. The mixture was then incubated for 90 minutes at 4°C. The protein A complex was precipitated by centrifugation and washed (Das et al., 1994). The pellets were dissolved in 1X SDS sample buffer and subjected to 7.5% SDS-PAGE. The gels were dried and exposed for autoradiography.

**Blastocest growth and hatching in vitro**
To study effects of HB-EGF on blastocyst growth and hatching in vitro, 8-cell embryos recovered on day 3 (1000-1030 hours) were cultured singly for 72 hours in 25 µl of Whitten’s medium (Whitten, 1971) in the presence or absence of recombinant human met-HB-EGFβ7 (11-1100 pM) (Paria and Dey, 1990). Embryonic development was monitored every 24 hours. The number of blastocysts showing complete hatching or considerable protrusion from zona-pellucidae was recorded at the end of the culture period. Cell number per hatched blastocyst was determined (Paria and Dey, 1990).

**Blastocyst attachment and trophoblast outgrowth in culture**
The details of the procedure have been described previously (Armant et al., 1986). In brief, blastocysts were collected at 1500 hours on day 4 and cultured on Falcon Petri dishes (no. 1008) in groups of 10-15 in 25 µl CMRL-1066 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mg/ml sodium lactate, 60 µg/ml sodium pyruvate, 4 mg/ml BSA, 20 µg/ml penicillin and 20 µg/ml streptomycin under silicon oil at 37°C in an environment of 5% CO2/95% air for 48 hours to allow complete zona hatching. Blastocysts were then cultured singly for 24 hours in 10 µl of the same culture medium on the laminin-coated Petri dishes in the presence or absence of recombinant human met-HB-EGFβ7. Coating of dishes with laminin was achieved as described previously (Armant et al., 1986). After 24 hours, the number of blastocysts attached to the dish was scored, and the surface area of trophoblast outgrowth was measured using a computerized image processing system (Analytical Imaging Concepts, Irvine, CA). Outgrowth was defined as the visible appearance of trophoblast monolayer around the embryo.

**RESULTS**

**Northern blot hybridization of HB-EGF mRNA in the perimplantation uterus**

Steady state levels of HB-EGF mRNA in the uterus on days 1-8 of pregnancy were examined by northern blot hybridization using a 32P-labeled cRNA probe (Fig. 1). Mouse liver (Lv) and lung (Lu) RNAs served as negative and positive controls, respectively (Abraham et al., 1993). A 2.5 kb transcript of HB-EGF mRNA, similar to that identified in other tissues (Higashiyama et al., 1991; Abraham et al., 1993), was detected in the uterus and lung, but not in the liver (Fig. 1). Levels of HB-EGF mRNA in whole uterine poly (A)+ RNA samples were higher on day 1 of pregnancy, but remained virtually unaltered during the rest of the perimplantation period. Integrity and loading of RNA samples were examined by rehybridizing the same blots to a β-actin probe (Fig. 1). The presence of two species of β-actin transcripts in RNA samples from the whole uterus reflects the abundance of muscle-specific actin mRNA contributed by the myometrium (Das et al., 1994).

**In situ hybridization of HB-EGF mRNA in the perimplantation uterus**
The above observation suggested that, if HB-EGF expression is important for implantation at around days 4-6 of pregnancy, it would be through localized cell type-specific expression in the uterus. Thus we examined the distribution of HB-EGF mRNA in the uterus by in situ hybridization. On day 1 of pregnancy, autoradiographic signals showed heterogeneous distribution in the luminal and glandular epithelia. The signals virtually disappeared on days 2 and 3 of pregnancy (data not shown). These results correlated with the pattern of northern blot hybridization. The expression of HB-EGF on day 1 is probably the result of stimulation of epithelial cells by preovulatory estrogen secretion (Huet-Hudson et al., 1989). However, HB-EGF mRNA was again detected in a specific cell-type and at a specific time on day 4 of pregnancy. Autoradiographic signals of HB-EGF hybrids were detected in the LE surrounding the blastocyst at 1600 hours (Fig. 2A-D), but not at 0900 hours (Fig. 2A′,B′) or 1400 hours (Fig. 2C′,D′). Furthermore, no autoradiographic signals could be detected in LE in between sites of blastocysts (Fig. 2A,B), or in uterine cells of pseudopregnant mice at 1600 h on day 4 (Fig. 2E,F), as well as in the morning and afternoon of day 5 (data not shown). With the initiation of attachment reaction (blue reaction) at 2300 hours on day 4 of pregnancy, the distribution of the mRNA extended along the LE above the implantation chamber towards the mesometrial pole of the uterus (Fig. 3A,B). A similar pattern was noted on day 5 of pregnancy at 0900 hours (data not shown). With the progression of the implantation process in the afternoon of day 5, the intensity of the autoradiographic signals remained high in the LE, and were also present in proliferating stromal cells (Huet-Hudson et al., 1989), particularly cells at the mesometrial pole of the implantation site (Fig. 3C,D). A similar distribution pattern was evident on the morning of day 6 of pregnancy (data not shown). However, levels of HB-EGF mRNA were very low at interimplantation sites (data not shown). With the further progression of the decidualization process on days 7 (data not shown) and 8 of pregnancy (Fig. 3E,F), secondary decidual cells at the antimesometrial region showed accumulation of HB-EGF mRNA. Implanting blastocysts on day 8 also exhibited accumulation of HB-EGF mRNA (Fig. 3E,F). The in situ hybridization signals shown in Figs 2 and 3 appeared to be specific for HB-EGF mRNA, because the sense probe exhibited no specific signals (data not shown), and because hybridization with TGF-β receptors type II and III probes showed different temporal and spatial distribution in uterine sections obtained from the same mice as were used for HB-EGF mRNA localization (Das et al., unpublished data). Furthermore, hybridization of uterine sections to a metallothionein-I mRNA probe showed a different distribution pattern (McMaster et al., 1993).

**In situ hybridization of HB-EGF mRNA in the delayed implanting uterus before and after initiation of implantation**
To examine further whether the expression of the HB-EGF gene in the LE requires the presence of an active blastocyst, in situ hybridization was performed on uterine sections obtained from P4-treated delayed implanting mice, or after the initiation
of blastocyst activation and implantation by an E₂ injection (Fig. 4). No hybridization signals were detected in luminal epithelial cells surrounding dormant blastocysts that had been in close apposition with luminal epithelial cells for 3 days during P₄ treatment (Fig. 4A,B). In contrast, with the E₂-induced termination of delayed implantation and onset of blastocyst activation, temporal and cell type-specific expression of uterine HB-EGF mRNA again became evident. Although no hybridization signal was detected 2 hours after an E₂ injection, a weak signal was observed in the LE surrounding a few blastocysts 6 hours after an E₂ injection (data not shown). The intensity of the signal increased at the site of each blastocyst at 12 hours (Fig. 4C,D), followed by a further increase at 24 hours (Fig. 4E-H) after an E₂ injection. Attachment reaction (blue reaction) occurs between 18 to 20 hours after estrogen injection of P₄-treated delayed implanting mice. Therefore, the
temporal and spatial pattern of expression of the HB-EGF gene, observed in these experiments, matched that observed during normal implantation as described above (Figs 2, 3). On many occasions, luminal epithelia outside the implantation chamber, but in close proximity to the embryo, exhibited hybridization signals (Fig. 4E,F).

Immunostaining of HB-EGF in the uterus
To examine whether HB-EGF mRNA is translated in the mouse uterus, cellular distribution of HB-EGF protein was examined by immunohistochemistry using a goat or chicken polyclonal antibody raised against recombinant HB-EGF. A similar distribution pattern of HB-EGF was observed with both of the antibodies. The results obtained with the goat antibody are presented in Fig. 5. Red deposits indicate the sites of immunostaining. On day 1 of pregnancy, immunoreactive HB-EGF was present in the luminal and glandular epithelium (data not shown). On the morning of day 5 (0900 hours), HB-EGF was detected in the LE surrounding the blastocyst and along the implantation chamber (Fig. 5A). No immunostaining was noted in the blastocyst (Fig. 5A). Preneutralization of the antibody with an excess of antigen resulted in loss of specific staining in similar day-5 uterine sections (Fig. 5B). The pattern of HB-EGF immunostaining was consistent with the distribution of HB-EGF mRNA as described above. Very little or no immunostaining was observed in the interimplantation sites (data not shown).

Western blot analysis of luminal epithelial HB-EGF
Immunohistological studies were complemented by immunoblot analysis in separated luminal epithelial cell extracts obtained from pregnant uteri on the morning of day 5.
Effects of HB-EGF on blastocyst zona-hatching and growth in vitro

HB-EGF is a member of the EGF family of growth factors, known for their role in cell proliferation, differentiation, and survival. In this section, we discuss the effects of HB-EGF on blastocyst zona-hatching and growth in vitro, focusing on how this growth factor influences cell development and behavior.

Autophosphorylation of blastocyst EGF-R by HB-EGF

The present finding that HB-EGF is expressed in the LE surrounding the blastocyst around the time of implantation, coupled with the observation that EGF-R is expressed in the blastocyst (Paria and Dey, 1990; Wiley et al., 1992; Paria et al., 1991) but not in the uterine epithelium (Das et al., 1994), led us to speculate that LE-derived HB-EGF may interact with blastocyst EGF-R. Thus we studied HB-EGF-induced autophosphorylation of blastocyst EGF-R in vitro (Fig. 7). The result showed that HB-EGF could indeed trigger phosphorylation of the 170kDa EGF-R protein in day-4 blastocysts (Lane 2). This reaction was specific, since the intensity of the phosphorylated 170kDa EGF-R protein was severely compromised in the absence of HB-EGF (Lane 1). It was assumed that the content of membrane protein associated with the 43 blastocysts used was exceedingly low, since total protein content of each day-4 mouse blastocyst is about 20 ng (Brinster, 1967). This suggests that blastocyst EGF-R is very active in response to HB-EGF signaling.

Effects of HB-EGF on trophoblast outgrowths in vitro

Outgrowth of mouse blastocyst-derived trophoblast cells has been used by many investigators as an in vitro model for ‘implantation’ (reviewed by Sherman and Wudl, 1976; Armant et al., 1986). We used this model to examine effects of HB-EGF on trophoblast outgrowths in vitro. As reported previously (Armant et al., 1986), about 95% of zona-free blastocysts attached and outgrew on laminin-coated surface in a serum-free medium within 24 hours of culture both in the absence and

Fig. 6. Western blot analysis of HB-EGF in the mouse uterine LE on day 5 of pregnancy. An extract of luminal epithelia isolated from uteri at 0900 hours on day 5 of pregnancy was fractionated by heparin-affinity column chromatography. Eluates obtained using salt concentrations of 0.2 M (lane 3) or 2 M (lane 2) were immunoblotted along with recombinant HB-EGF (lane 1) using a polyclonal goat antibody. The recombinant HB-EGF (approx. 9×10^3 M_r) and two species of proteins (approx. 18×10^3 and 37×10^3 M_r) are evident in lanes 1 and 2, respectively.

Fig. 7. HB-EGF induced EGF-R autophosphorylation in day-4 (1100 hours) mouse blastocysts. EGF-R autophosphorylation was determined in blastocyst homogenates after preincubation with (lane 2) or without (lane 1) HB-EGF (11 nM). The labeling reaction was initiated by the addition of [γ-32P]ATP. After labeling, immunoprecipitations were performed with antibodies to EGF-R, the precipitates were fractionated by SDS-PAGE, and phosphorylated proteins were detected by autoradiography for 24 hours. Molecular size markers are shown as Std. Phosphorylation of a 170×10^3 M_r band specific to EGF-R is indicated.
were repeated several times. Blastocysts used for determining cell numbers. These experiments embryos. Numbers within parentheses indicate number of hatched per total numbers of blastocysts developed from 8-cell EGF. Numbers inside the bars indicate number of blastocysts test) compared with singly cultured embryos in the absence of HB-

tocyst with a hormonally prepared uterus. In this respect, recent expression of the amphiregulin gene in the uterus has not yet been deter-

4 of pregnancy (Huet-Hudson et al., 1990a), and the expression of specific blastocyst functions in vitro. The lack of expression of the HB-EGF gene in the regions of the LE not adjacent to the blastocyst prior to or after the initiation of the implantation process, and its absence in the pseudopregnant uterus asserts that the blastocyst is the major inducer of this gene. Furthermore, the induction of the HB-EGF gene by the blastocyst even before its zona dissolution, i.e., at 1600 hours on day 4 of pregnancy, suggests that cell-cell contact (trophectoderm epithelium-LE) is not the inducer of this growth factor gene in the LE. This suggestion is consistent with the observation of lack of expression of the HB-EGF gene in the LE closely apposed to the zona-free dormant blastocyst in the P4-treated delayed implanting mice. In contrast, expression of this gene after termination of the delay, by estrogen, suggests that this expression requires the interaction of an active blastocyst with a hormonally prepared uterus. In this respect, recent

observations document that the activated state of the blastocyst should match the receptive state of the uterus for successful implantation in the mouse (Paria et al., 1993b).

Increased uterine vascular permeability (blue reaction) at the site of the blastocyst has always been considered to be the earliest discernible prerequisite condition for successful implantation (Psychoyos, 1973). However, the results of the present investigation point towards an even earlier embryo-uterine interaction occurring 6-7 hours prior to the attachment reaction involving an important growth factor at the molecular level. The proposal that a blastocyst-derived diffusible molecule(s) participates in the induction of HB-EGF expression in the LE is consistent with the observation of induction of this gene in the LE outside the implantation chamber but very close to the blastocyst (Fig. 4, arrows in E and F). The failure of neighboring stromal or glandular epithelial cells to accumulate HB-EGF mRNA suggests that the blastocyst-derived molecule(s) primarily interacts with the LE. Alternatively, this mRNA could be rapidly degraded in the stromal and glandular epithelial cells. A major challenge will be to identify the chemical nature of the blastocyst-derived molecule(s).

Among the members of the EGF family, blastocyst-mediated expression of HB-EGF in the uterus appears to be specific and novel. Although TGF-α is expressed in the LE on day 4 of pregnancy, this expression is not limited to the site of the blastocyst apposition and at a specific time, very early in the process of implantation, and (ii) that this growth factor can influence specific blastocyst functions in vitro. The lack of expression of the HB-EGF gene in the absence of HB-EGF. Numbers within parentheses indicate number of hatched blastocysts used for determining cell numbers. These experiments were repeated several times.

The highlights of the present investigation are (i) that the expression of HB-EGF is induced in the LE by the blastocyst at the site of its apposition and at a specific time, very early in the process of implantation, and (ii) that this growth factor can influence specific blastocyst functions in vitro. The lack of expression of the HB-EGF gene in the absence of HB-EGF. Numbers within parentheses indicate number of hatched blastocysts per total numbers of blastocysts developed from 8-cell embryos. Numbers within parentheses indicate number of hatched blastocysts used for determining cell numbers. These experiments were repeated several times.

**DISCUSSION**

**Fig. 8.** Effects of HB-EGF on blastocyst zona-hatching and growth in vitro following culture of 8-cell mouse embryos. Embryos were cultured singly in the presence or absence of HB-EGF for 72 hours. After recording zona-hatching, cell number per blastocyst was determined. Results are mean ± s.e.m.*, *P<0.001 (ANOVA and t-test) compared with singly cultured embryos in the absence of HB-EGF. Numbers inside the bars indicate number of blastocysts hatched per total numbers of blastocysts developed from 8-cell embryos. Numbers within parentheses indicate number of hatched blastocysts used for determining cell numbers. These experiments were repeated several times.

**Fig. 9.** Effect of HB-EGF on trophoblast outgrowth in vitro. Zona-

free mouse blastocysts were cultured for 24 hours in the presence or absence of HB-EGF. After termination of the cultures, surface area of trophoblast cell monolayers were measured using an image analysis system. Results are mean ± s.e.m.*, *P<0.001 (ANOVA and t-test) compared with singly cultured blastocysts in the absence of HB-EGF. Numbers within parentheses indicate number of blastocysts used in three experiments.
membrane-associated forms on luminal epithelial cell surfaces, the process could also be influenced. If HB-EGF is displayed as certain blastocyst functions associated with the implantation stimulated trophoblast outgrowth of zona-free blastocysts in zona-free dormant blastocysts after an estrogen injection and more than one mechanism operates in this process.

Although it is not yet clear whether effects of uterine HB-EGF are mediated in paracrine and/or juxtacrine manner, the tightly regulated expression of HB-EGF in the LE as shown here and of EGF-R in the blastocyst around the time of implantation (Paria et al., 1993a) suggests an important function of this ligand-receptor signaling in embryo-uterine interactions during implantation. HB-EGF-induced autophosphorylation of blastocyst EGF-R is consistent with a potential role of this growth factor in receptor mediated blastocyst functions associated with implantation. An essential prerequisite to the implantation process is the escape of the blastocyst from its zona-pellucida prior to attachment to the LE. The majority of the blastocysts in our CD-1 mice become zona-free between 1800-2000 hours on day 4 of pregnancy. The observations that HB-EGF is expressed in the LE surrounding the blastocyst before its zona dissolution, and that a higher rate of HB-EGF induced zona-hatching occurs in vitro, suggest a paracrine role for diffusible HB-EGF in this process. Since HB-EGF can bind to HS PG, the expression of HS PG on trophectoderm cell surfaces at the time of acquisition of attachment competence of blastocysts (Carson et al., 1993) may provide an efficient or a sustained mechanism for delivery of uterine HB-EGF for further embryo-uterine signaling in the implantation process. Detailed characterization of uterine HB-EGF will be essential to address this issue. Nonetheless, the observation that homozgyous mouse blastocysts carrying mutated EGF-R genes die around the time of implantation (Thredgil and Magnuson, personal communication) suggests that ligand-receptor signaling with EGF-related growth factors could be important in implantation. HB-EGF is not likely to interact with LE cells, since these cells in the mouse lack EGF-R (Das et al., 1994). However, EGF-R is expressed in stromal and decidual cells (Das et al., 1994), and thus HB-EGF could play a role in stromal cell proliferation and decidualization.

In summary, the restricted expression of HB-EGF at a critical time of embryo-uterine interaction, as well as HB-EGF’s ability to induce blastocyst EGF-R autophosphorylation and promote blastocyst growth and zona-hatching in vitro point toward an important role for this signaling molecule during early pregnancy. However, the establishment of HB-EGF’s essential role in embryo-uterine interactions during the periimplantation period will require the creation of mice homozgyous for a disrupted HB-EGF gene.

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