Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor

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SUMMARY

Previous studies have shown that heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) mRNA is synthesized in the mouse uterine luminal epithelium, temporally, just prior to implantation, and spatially, only at the site of blastocyst apposition (Das, S. K., Wang, X. N., Paria, B. C., Damm, D., Abraham, J. A., Klagsbrun, M., Andrews, G. K. and Dey, S. K. (1994) Development 120, 1071-1083). HB-EGF is synthesized as a transmembrane protein (HB-EGFTM) that can be processed to release the soluble growth factor. An antibody that cross-reacts only with the transmembrane form detected HB-EGFTM in uterine luminal epithelium in a spatial manner similar to that of HB-EGF mRNA. HB-EGFTM is a juxtacrine growth factor that mediates cell-cell contact. To ascertain if HB-EGFTM could be an adhesion factor for blastocysts, a mouse cell line synthesizing human HB-EGFTM was co-cultured with mouse blastocysts. Cells synthesizing HB-EGFTM adhered to day-4 mouse blastocysts more extensively than parental cells or cells synthesizing a constitutively secreted form of HB-EGF.

Adhesion of cells synthesizing HB-EGFTM to blastocysts was inhibited by excess recombinant HB-EGF but less so by TGF-α. Adhesion was also inhibited by the synthetic peptide P21 corresponding to the HB-EGF heparin binding domain, and by incubating the blastocysts with heparinase. In addition, adhesion to delayed implanting dormant blastocysts, which lack EGF receptor (EGFR), was diminished relative to normal blastocysts. These results suggested that adhesion between blastocysts and cells synthesizing HB-EGFTM was mediated via interactions with both blastocyst EGFR and heparan sulfate proteoglycan (HSPG). It was concluded that HB-EGFTM, which is synthesized exclusively in the luminal epithelium at the site of blastocyst apposition, and which is a juxtacrine adhesion factor for blastocysts, could be one of the mediators of blastocyst adhesion to the uterus in the process of implantation.

Key words: juxtacrine growth factor, EGF receptor, heparan sulfate proteoglycan, TGF-α, mouse

INTRODUCTION

Implantation is a complex sequence of events that leads to an intimate association of the blastocyst with the uterine endometrium. Prior to these interactions, a growing embryo is largely independent of maternally derived factors, since a fertilized egg can grow in vitro in a simple medium and develop into a blastocyst (Adamson, 1993). However, further development is dependent on an exchange of materials between the mother and the embryo. In the mouse, the blastocyst is apposed to the uterine epithelium prior to implantation, which is initiated at 20-22 hours on day 4 of pregnancy. Implantation proceeds to the adhesion stage at which point the apical surfaces of both the uterine epithelium and trophectoderm are lined up in parallel and separated only by a small cleft of 20 nm (Schlafke and Enders, 1975). At this stage, it is difficult to separate both cell layers without injuring cell surfaces. In subsequent stages, the trophectoderm cells of the embryo displace the uterine epithelium at the site of implantation, attach and penetrate the underlying basement membrane and invade the uterine stroma in a controlled fashion (reviewed by Graham and Lala, 1988; Denker, 1993).

For successful implantation to occur in the mouse, differentiation of the uterus and blastocyst development are synchronized by the effects of the maternal hormones, progesterone and estrogen (Paria et al., 1993b). Secretion of estrogen on day 4 of pregnancy renders the uterus receptive to blastocyst implantation (Yoshinaga and Adams, 1966; Paria et al., 1993b). In the absence of estrogen, for example, by ovariectomy, no attachment reaction occurs and the blastocyst remains in a dormant phase known as delayed implantation (Yoshinaga and Adams, 1966; Paria et al., 1993b). A single injection of estrogen is sufficient to reactivate the implantation process in the progesterone primed uterus (Yoshinaga and...
Adams, 1966; McCormack and Greenwald, 1974). It is not clear, however, whether estrogen acts directly on the blastocyst or whether its effects are mediated via the uterus (Wu et al., 1992; Hou and Gorski, 1993; Paria et al., 1993b).

There has been some progress in delineating the molecular basis of uterine epithelium/blastocyst interactions. A number of cell surface molecules has been identified that are capable of promoting the attachment and implantation reactions, for example, heparan sulfate proteoglycan (HSPG). HSPG has been identified on trophectoderm cells and uterine epithelium (Sutherland et al., 1991; Raboudi et al., 1992; Carson et al., 1993). Attachment and outgrowth of blastocysts in vitro are inhibited by addition of soluble heparin or heparinase (Farach et al., 1987). Other cell surface molecules that might mediate attachment include carbohydrate-binding sites on trophoblast cells (Chavez, 1986), uterine glycosaminoglycans (Jacobs and Carson, 1991), H-type oligosaccharides (Kimber et al., 1994), and integrin receptors for laminin, fibronectin and vitronectin (Sutherland et al., 1988, 1993).

Another class of molecules that are possible mediators of blastocyst/uterus interactions are peptide growth factors. A number of growth factors and receptors are synthesized by the embryo during early development (reviewed by Adamson, 1993). At the same time growth factors are also secreted by the uterine tissue, e.g. transforming growth factor (TGF)-α (Tamada et al., 1991), transforming growth factor-β (Tamada et al., 1990), and leukemia inhibitory factor (LIF) (Bhatt et al., 1991). Though a fertilized egg can grow in vitro to a hatched blastocyst in the absence of maternal derived proteins, adding certain growth factors can be beneficial to its development (reviewed by Paria and Dey, 1990; Adamson, 1993). For example TGF-α, EGF, insulin or colony-stimulation factor-I have been shown to increase protein synthesis, cell number and hatching rate of embryos grown in vitro. Thus far, only LIF has been proven to be essential for blastocyst implantation (Stewart et al., 1992).

There have been a number of studies analyzing the role of EGF family members and their receptors in blastocyst development. TGF-α, EGF and HB-EGF promote attachment and embryo outgrowth in vitro (Haimovich and Anderson, 1993; Das et al., 1994). EGFR is synthesized in the mouse trophectoderm (Paria and Dey, 1990; Dardik et al., 1992; Wiley et al., 1992) and is developmentally regulated. Functional EGFR is detectable in normal blastocysts but not in dormant blastocysts resulting from delayed implantation (Paria et al., 1993a). Reactivated blastocysts synthesize EGFR within a few hours after terminating delayed implantation (Paria et al., 1993a).

HB-EGF is a heparin-binding member of the EGF family purified initially from the conditioned medium (CM) of macrophage-like cells (Higashiyama et al., 1991, 1992). It is synthesized as an insoluble membrane-spanning precursor that can be processed to release the soluble mature form (Higashiyama et al., 1991; Raab et al., 1994). Mature HB-EGF is a potent mitogen for fibroblasts, smooth muscle cells (SMC; Higashiyama et al., 1993) and keratinocytes (Marikovsky et al., 1993). When applied to mouse blastocysts in culture, HB-EGF stimulates EGFR phosphorylation, the rate of zona hatching, and an increase in blastocyst cell number and trophoblast outgrowth (Das et al., 1994). The bioactivity of HB-EGF, at least for SMC, is enhanced by interactions with cell surface HSPG (Higashiyama et al., 1993; Thompson et al., 1994). The heparin-binding property of HB-EGF is due to the presence of a 21 amino acid heparin-binding domain that lies mostly upstream of, but overlaps the EGF-like domain (Thompson et al., 1994). Both a synthetic peptide corresponding to the heparin-binding domain and heparinase inhibit HB-EGF bioactivity (Higashiyama et al., 1993; Thompson et al., 1994).

The transmembrane form of HB-EGF (HB-EGF(tm)), although insoluble, is bioactive. It is a juxta- and an increase in blastocyst cell number and trophoblast outgrowth (Das et al., 1994). The bioactivity of HB-EGF, at least for SMC, is enhanced by interactions with cell surface HSPG (Higashiyama et al., 1993; Thompson et al., 1994).

The transmembrane form of HB-EGF (HB-EGF(tm)), although insoluble, is bioactive. It is a juxtamembrane growth factor for adjacent cells (Higashiyama et al., 1995) and serves as the receptor for diphtheria toxin (DT; Naglich et al., 1992). Transmembrane growth factors, such as proTGF-α, have been shown to promote cell-cell adhesion (Anklesaria et al., 1990). Our previous study has suggested that HB-EGF might have adhesive properties during blastocyst implantation in the mouse (Das et al., 1994). It was found using in situ hybridization that HB-EGF is synthesized by the uterine epithelium just prior to the attachment reaction and only at the site of blastocyst implantation. No HB-EGF gene expression was detected in pseudopregnant mice (Das et al., 1994). These results suggested that the blastocyst induces HB-EGF synthesis in the uterus. Since trophoblast cells synthesize EGFR and HSPG, we speculated that the uterine epithelium might synthesize a non-diffusible transmembrane form of HB-EGF, which could promote adhesion of the blastocyst via EGFR and HSPG (Das et al., 1994). In this report we demonstrate that the transmembrane form of HB-EGF is synthesized at the site of blastocyst implantation. Furthermore, we demonstrate in a model system that cells synthesizing HB-EGF(tm), but not parental cells or cells synthesizing soluble mature HB-EGF, adhere to mouse blastocysts via interactions with trophectoderm EGFR and HSPG.

**MATERIALS AND METHODS**

**Blastocysts**

CD-1 female mice (20-25 g, Charles River, Raleigh, NC) were mated with males of the same strain. Mouse blastocysts were recovered in Whitten’s medium (Whitten, 1971) at 1400 hours on day 4 (day 1 = vaginal plug) of pregnancy. They were freed of zona pellucidae, washed and cultured as previously described (Paria and Dey, 1990). Induction of blastocyst dormancy by ovariectomy, maintenance by progesterone and reactivation by estradiol-17β was carried out as previously described (Yoshinaga and Adams, 1966; Huet-Hudson and Dey, 1990; Paria et al., 1993b). Zona-free dormant and activated blastocysts were collected in Whitten’s medium 16 hours after the last steroid injections.

**Expression of transmembrane and mature HB-EGF in 3D cells**

HB-EGF constructs were expressed in an interleukin-3-dependent mouse cell line, 3D, obtained from Dr Jackie Pierce (NIH, Rockville, MD; Pierce et al., 1988). The 3D cells were grown in suspension in RPMI 1640 supplemented with 10% FCS, GPS, and 5% WEHI-3 conditioned medium (CM) as previously described (Blotnick et al., 1994).

**HB-EGF(tm) and mature HB-EGF**

In order to synthesize HB-EGF(tm), full length human HB-EGF cDNA encoding the entire 208 amino acid open reading frame (ORF) (Higashiyama et al., 1991) was amplified by polymerase chain reaction (PCR) using synthetic DNA oligonucleotide primers (P20: GCT CTA GAC CAT GAA GCT GCT GCC GTC G and P21: GCT CTA GAT CAG TGG GAA TTA GTC AT) and ligated into the XbaI-
site of the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA) (pRcHB-EGF TM). In order to synthesize the secreted form of HB-EGF, the cDNA encoding amino acids 1-149 and thereby lacking the transmembrane and cytoplasmic tail domains was amplified by PCR using synthetic DNA oligonucleotide primers P20 and P31 (P31: GCT GTA GAC TAT CTT AGG AGG CTC TCG CCA TGG). The resulting DNA fragment was digested with XbaI and ligated into the XbaI-site of pRc/CMV (pRcHB-EGF). For DNA transfections, about 3x10^6 32D cells were washed with serum-free medium, incubated on ice for 10 minutes and then electroporated with 20 μg of linearized plasmid DNA using an electroporator (BioRad, Hercules, CA). The transfected cells were kept on ice for 10 minutes, incubated for 48 hours in RPMI 1640/10% FCS/GPS/5% WEHI, and then transferred into selective medium containing 600 μg/ml G418-sulfate. Since human HB-EGF TM is the DT receptor, cells transfected with pRcHB-EGF TM were screened for DT sensitivity (Naglich et al., 1992; Raab et al., 1994). Cells transfected with pRcHB-EGF and pRcHB-EGF TM were screened for the production of HB-EGF mitogenic activity in the conditioned media.

HB-EGF-alkaline phosphatase fusion proteins

In order to visualize HB-EGF on the cell surface of 32D cells, epitope-tagged constructs were prepared encoding HB-EGF-alkaline phosphatase (AP) fusion proteins as described previously (Chen et al., 1995). Briefly, a 1.5 kb BglII-HpaI fragment of an alkaline phosphatase (AP) coding (tag-I) plasmid (Flanagan and Leder, 1990) was blunt-ended with Klenow polymerase and then ligated into the MscI-site of the human HB-EGF ORF to construct pHB-EGF TM-AP, pHBEFG-AP was constructed by replacing the 2.2 kb BsmI-fragment of pHBEFG-AP with a 2.1 kb BsmI-fragment derived from pRcHBEGF and thereby deleting the HB-EGF transmembrane and cytoplasmic domains. Cells transfected with pHB-EGF TM-AP were screened for cell-associated AP in a colorimetric enzyme assay as follows. Cells (0.5-1x10^5) were washed 2 times with PBS and then suspended in 200-400 μl 450 mM Tris-HCl, pH 9.7, 135 mM NaCl, 5 mM EDTA, 5 mM p-nitrophenol phosphate, 1 mM diethanolamine (AP buffer), and incubated at 37˚C until a yellow color developed. AP activity was quantitated by measuring the optical density at 410 nm. Released mature HB-EGF-AP was determined by mixing CM with the AP buffer. To localize HB-EGF-AP fusion proteins on the 32D cell membrane, the transfected cells were immunostained with monoclonal anti-AP antibody 1801 and FITC-conjugated secondary antibody and then examined by confocal laser microscopy using a confocal imaging system as previously described (Chen et al., 1995).

Heparin affinity chromatography and mitogenic activity of 32D cell HB-EGF

Samples of CM were analyzed on TSK-heparin columns using fast liquid performance chromatography (FPLC) and fractions were collected and analyzed for the ability to stimulate DNA synthesis in EP170.7 cells (Pierce et al., 1988) as previously described (Blotnick et al., 1994).

Adhesion of blastocytes to 32D cells

Adhesion

Parental 32D cells and stable transfectants synthesizing HB-EGF TM or mature HB-EGF were co-cultured (1x10^6 cells/well) with a single zona-free normal day-4 blastocyst, a progesterone-treated dormant blastocyst, or an estrogen-activated blastocyst in RPMI-1640 containing 10% FCS, 5% WEHI-conditioned medium and antibiotics, for 36 hours in 96-well plates. Co-cultures were carried out in a humidified incubator with 5% CO₂ and 95% air at 37˚C. After terminating the co-cultures, blastocysts were washed six times in Whitten’s medium to eliminate loosely adhering cells. The cells associated with the blastocyst surface were counted under an inverted microscope with Hoffman modulation contrast optics. For each experiment, the adhesion assays were carried out by one investigator and anywhere from 4 to 87 blastocysts were counted by two other investigators independently in a blind unbiased manner. The standard error of the mean (s.e.m.) was determined using ANOVA which was followed by the Newman-Keuls multiple comparison test. The numbers of adherent cells counted represent an underestimate, since only a portion of the 3-dimensional blastocyst can be seen in the culture well. After counting, blastocysts were placed onto glass slides and stained with hematoxylin prior to analysis by light microscopy. Adhesion of blastocysts to 32D cells was also examined by scanning electron microscopy as previously described (Enders et al., 1986). Briefly, blastocysts were fixed in 2.5% glutaraldehyde in PBS (pH 7.3) overnight. After rinsing in PBS, they were post-fixed in 1% osmium tetroxide/PBS for 30 minutes. The blastocysts were then dehydrated in ascending grades of alcohol (30-100%) in a small sack made from optical lens tissue. They were critical point-dried, mounted on aluminum stubs, sputter-coated with gold, and then examined and photographed using a Hitachi S-2700 scanning electron microscope.

Inhibition of adhesion

Day-4 mouse blastocysts were collected at 1300 hours and zona pel- lucidae were removed. After incubation for 1 hour in Whitten’s medium at 37˚C, blastocysts were incubated with 0.05 U/ml heparinase for 1 hour in the same medium. A second aliquot of heparinase was added to a final concentration of 0.1 U/ml and incubation was continued for another 2 hours at 37˚C. The control group was incubated with Whitten’s medium alone. Blastocysts were then co-incubated with 32D cells as described above and the co-culture was terminated after 36 hours. For competitive inhibition analysis, zona-free blastocysts were incubated with various concentrations of either HB-EGF, TGF-α, bFGF, or 25 μg/ml of the HB-EGF heparin-binding domain peptide P21 (Higashiyama et al., 1993) in Whitten’s medium for 1 hour at 37˚C before being placed in co-culture with 32D cells. Co-culture and harvesting of the blastocysts were performed as described above.

Immunohistochemistry

Immunohistochemical analysis of uterine sections during implantation was carried out as described previously (Das et al., 1994) except that an antibody (Ab 3100) directed against the 16 C-terminal amino acids of the 208 amino acid primary translation product was used (Ono et al., 1994). This antibody is specific in that it detects transmembrane but not soluble mature HB-EGF.

RESULTS

Mouse uterine epithelium synthesizes the transmembrane form of HB-EGF at the time of implantation

In a previous report, it was demonstrated that HB-EGF gene expression was induced by the blastocyst just prior to implantation (Das et al., 1994). In addition, anti-HB-EGF antibodies detected HB-EGF protein in the uterine luminal epithelium surrounding the blastocyst. However, the antibodies used in that study could not differentiate between the mature and transmembrane forms of HB-EGF. Since then, we have produced an antibody, Ab 3100, directed against the C-terminal portion of the transmembrane HB-EGF cytoplasmic tail, which is highly specific in recognizing transmembrane HB-EGF only (Ono et al., 1994). Ab 3100 detected HB-EGF TM in the uterine luminal epithelium surrounding the blastocyst on the morning of day 5 of pregnancy (Fig. 1). These results demonstrate that...
the transmembrane form of HB-EGF is present only in the luminal epithelium of the implantation chamber.

**Characterization of 32D cells synthesizing transmembrane HB-EGF**

Since HB-EGF made in the implantation chamber is in its transmembrane form, we speculated that blastocyst EGFR and possibly HSPG could interact with uterine luminal epithelial HB-EGF\textsubscript{TM} in a juxtacrine fashion and thereby promote blastocyst-cell adhesion and signaling. In order to study HB-EGF\textsubscript{TM} independent of other luminal epithelial cell surface molecules, we transfected 32D cells with a cDNA encoding the entire HB-EGF 208 amino acid ORF. For comparison, 32D cells were also transfected with a construct encoding only the amino acids 1-149 of the HB-EGF ORF in order to produce constitutively released mature HB-EGF. Northern blot analysis indicated that these two transfectants synthesized equal levels of HB-EGF (data not shown). The CM of the transfectants were analyzed by heparin affinity chromatography (Fig. 2A). Cells transfected with the mature form of HB-EGF released a heparin-binding growth factor that eluted from a TSK heparin column with 1.1 M NaCl and that was mitogenic for EP170.7 cells, a cell line overexpressing the EGFR gene (Pierce et al., 1988). This mitogenic activity could be neutralized by anti-HB-EGF antibodies (data not shown) that were previously demonstrated to be highly specific for HB-EGF (Blotnick et al., 1994). However, neither parental cells nor cells synthesizing HB-EGF\textsubscript{TM} released HB-EGF into CM. These results suggested that HB-EGF\textsubscript{TM} was 32D cell-associated.

To show cell surface tethered HB-EGF\textsubscript{TM} directly, epitope-tagged HB-EGF fusion proteins were synthesized in 32D cells. To do so, the ORF of the human secreted alkaline phosphatase gene was inserted in frame into the ectodomain of either the transmembrane or the released mature form of HB-EGF. Cells synthesizing the fusion proteins were examined by confocal fluorescence microscopy after immunostaining with anti-AP antibody and FITC-conjugated secondary antibodies (Fig. 2B). All of the epitope-tagged HB-EGF\textsubscript{TM} appeared to be associated with the cell surface. In contrast, virtually no cell surface staining was detected in 32D cells synthesizing equivalent amounts of constitutively released mature HB-EGF-AP, thus demonstrating that the released growth factor is not retained on the cell surface. There was no immunostaining of parental 32D cells. These results suggest that 32D cells synthesizing HB-EGF\textsubscript{TM} would be useful for blastocyst adhesion studies.

**Adhesion of mouse blastocysts is specifically promoted by the transmembrane form of HB-EGF**

To test the idea that HB-EGF\textsubscript{TM} is an adhesion factor, blastocysts were co-cultured with cells synthesizing either the transmembrane or the secreted form of HB-EGF. After 36 hours, the blastocysts were examined by scanning electron microscopy. It appeared that cells synthesizing HB-EGF\textsubscript{TM} adhered to blastocysts (Fig. 3B) to a much greater extent than did parental 32D cells (Fig. 3A) or cells constitutively releasing mature HB-EGF (Fig. 3C). Attached cells were quantitated by using Hoffman modulation contrast optics and tested statistically by using ANOVA followed by the Newman-Keuls multiple comparison test (Table 1). On average, there were about 24 visible 32D cells synthesizing HB-EGF\textsubscript{TM} attached per blastocyst compared to 4 attached 32D cells synthesizing mature HB-EGF and 2 attached parental 32D cells (Table 1A). Using the comparison test, the differences in adhesion of cells expressing HB-EGF\textsubscript{TM} compared to cells expressing mature HB-EGF or parental cells were found to be statistically significant. However, there were no statistically significant differences between the latter two cell types. The ability of soluble growth factors to inhibit adhesion of blastocysts to 32D cells...
Blastocysts were harvested at 1400 hours on day 4 of pregnancy and preincubated in the absence or presence of various concentrations of recombinant mature HB-EGF, TGF-α and bFGF for 1 hour before co-culture with HB-EGF-transfected or parental 32D cells for 36 hours. Blastocysts were recovered, washed extensively to remove non-adherent 32D cells and adherent cells were counted under a microscope with Hoffman objectives. Statistical analysis was prepared using ANOVA followed by the Newman-Keuls multiple comparison test. (A) In the comparison test, there was a statistically significant difference (P<0.05) between blastocyst adhesion to cells expressing HB-EGF TM and cells expressing either mature HB-EGF or not expressing any HB-EGF, but no statistical difference between the latter two cell types. (B) There was a statistically significant (P<0.05) inhibition of blastocyst adhesion to cells expressing HB-EGF TM, (a) in the absence of added HB-EGF, when compared to addition of 0.1, 1.0 and 10.0 nM HB-EGF but no significant differences among these three HB-EGF concentrations themselves, and (b) with 10 nM but not 0.1 nM or 1.0 nM TGF-α. (c) There was no statistically significant inhibition of blastocyst adhesion to cells expressing HB-EGF TM by bFGF.

### Table 1. Co-culture of blastocysts with 32D cells synthesizing constitutively released mature HB-EGF or transmembrane HB-EGF

<table>
<thead>
<tr>
<th>Type of blastocyst</th>
<th>No. of cells adhered/blastocyst</th>
<th>No. of blastocysts assayed</th>
</tr>
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<tbody>
<tr>
<td>A. 32D cells synthesizing HB-EGF TM</td>
<td>24.0±1.5</td>
<td>87</td>
</tr>
<tr>
<td>Mature HB-EGF</td>
<td>4.3±0.6</td>
<td>35</td>
</tr>
<tr>
<td>No HB-EGF</td>
<td>1.8±0.6</td>
<td>20</td>
</tr>
</tbody>
</table>

**B. Competition of cells synthesizing HB-EGF TM with:**

- (a) HB-EGF
  - 0.0 | 24.0±1.5 | 87 |
  - 0.1 nM | 5.8±1.3 | 20 |
  - 1.0 nM | 10.0±4.1 | 6 |
  - 10.0 nM | 2.8±0.7 | 20 |
- (b) TGF-α
  - 0.1 nM | 15.3±4.5 | 4 |
  - 1.0 nM | 15.7±2.5 | 7 |
  - 10.0 nM | 7.2±5.2 | 6 |
- (c) bFGF
  - 10.0 nM | 21.1±3.7 | 13 |

No HB-EGF 1.8±0.6 20
Mature HB-EGF 4.3±0.6 35
No HB-EGF 1.8±0.6 20

Blastocysts bind to transmembrane HB-EGF via EGFR

Besides inhibition by TGF-α, the participation of blastocyst EGFR in HB-EGF TM-mediated adhesion was also shown using normal, progesterone-treated dormant, and estrogen-reactivated blastocysts. Mice were ovarioctomized on day 4 of pregnancy and injected with progesterone on days 5 and 6 to produce dormant blastocysts that lack EGFR (Paria et al., 1993a). Administration of estrogen reactivates the dormant blastocysts and EGFR synthesis occurs within 12 hours (Paria et al., 1993a). Adhesion of blastocysts to 32D cells was monitored by light microscopy (Fig. 4). Normal blastocysts (Fig. 4B) and estrogen-reactivated blastocysts (Fig. 4D) adhered to 32D cells synthesizing HB-EGF TM, but dormant blastocysts (Fig. 4C) adhered to a much lesser extent. Quantitatively, there were about 4 attached cells/dormant blastocyst compared to about 24 attached cells/normal blastocyst and 16 attached cells/reactivated blastocyst (Table 2). Using the comparison test, the adhesion of normal and reactivated blastocysts was statistically significant compared to dormant blastocysts. Taken together, these results indicate that blastocyst adhesion to cells is mediated in part via the blastocyst EGFR interacting with cell surface HB-EGF TM.

Trophoblast cell surface HSPG contributes to HB-EGF TM-mediated blastocyst adhesion

In Table 1, it was shown that excess TGF-α did not inhibit adhesion mediated by HB-EGF TM as efficiently as excess HB-EGF. One possible explanation is that HB-EGF TM interacts with high affinity EGFR and not expressing any HB-EGF, but no statistical difference between the latter two cell types. (B) There was a statistically significant (P<0.05) inhibition of blastocyst adhesion to cells expressing HB-EGF TM, (a) in the absence of added HB-EGF, when compared to addition of 0.1, 1.0 and 10.0 nM HB-EGF but no significant differences among these three HB-EGF concentrations themselves, and (b) with 10 nM but not 0.1 nM or 1.0 nM TGF-α. (c) There was no statistically significant inhibition of blastocyst adhesion to cells expressing HB-EGF TM by bFGF.

### Table 2. Co-culture of dormant and activated blastocysts with 32D cells synthesizing HB-EGF TM

<table>
<thead>
<tr>
<th>Type of blastocyst</th>
<th>No. of cells adhered/blastocyst</th>
<th>No. of blastocysts assayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>24.0±1.5</td>
<td>87</td>
</tr>
<tr>
<td>Dormant</td>
<td>4.2±0.4</td>
<td>53</td>
</tr>
<tr>
<td>Reactivated</td>
<td>15.8±1.3</td>
<td>31</td>
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Mice were ovarioctomized at 0830-0900 hours on day 4 of pregnancy and blastocyst dormancy was maintained with progesterone. To induce implantation and blastocyst reactivation, mice were given an injection of estradiol-17β on day 6. Dormant and activated blastocysts were removed 16 hours after the last steroid injections. Normal blastocysts were collected at 1400 hours on day 4 of pregnancy. Blastocysts were co-cultured with 32D cells synthesizing HB-EGF TM, and adherent cells were counted and analyzed statistically as described in Table 1. In the comparison test, there was a statistically significant difference (P<0.05) between normal or reactivated blastocysts compared to dormant blastocysts in their adhesion to cells expressing HB-EGF TM.
TGF-α, non-heparin binding growth factors were not affected by these treatments. Accordingly, we tested the possibility that trophectodermal cell HSPG contributed to HB-EGF\textsubscript{TM}/blastocyst interactions (Table 3) by counting adherent cells and carrying out statistical analysis. When blastocysts were pre-treated with heparinase (Ibex, Montreal, Canada) to remove cell surface HS, the number of attached 32D cells synthesizing HB-EGF\textsubscript{TM} was reduced by nearly 50%, from 23.0 to 11.6 cells. Incubation with P21 reduced the number of attached cells by 63%, to 8.6 cells. The loss of adhesion by both these treatments was statistically significant compared to the untreated control. Thus, the ability of HB-EGF\textsubscript{TM} to interact with HSPG, an interaction that does not occur with TGF-α, appears to contribute to the binding of HB-EGF\textsubscript{TM} to blastocysts.

**DISCUSSION**

In this report, we have demonstrated (i) that the transmembrane form of HB-EGF is synthesized in the uterus at the site of blastocyst implantation, and (ii) that in a model system transmembrane HB-EGF alone is sufficient to mediate the adhesion of cells to blastocysts by interactions with blastocyst EGFR and HSPG. In a previous study using in situ hybridization, we demonstrated that HB-EGF synthesis in the mouse uterus during early pregnancy is tightly regulated both in a temporal and spatial manner (Das et al., 1994). HB-EGF mRNA accumulation occurred in the mouse uterine epithelium 6-7 hours prior to blastocyst attachment reaction and was limited to the luminal epithelium immediately adjacent to the blastocyst. HB-EGF protein was also detected in the uterus on day 5 of pregnancy, but the antibody used could not distinguish between mature HB-EGF and HB-EGF\textsubscript{TM}. Recently, we have produced an antibody directed against a synthetic peptide corresponding to the C-terminal 16 amino acids of the HB-EGF ORF. This antibody (Ab 3100) can detect the transmembrane, but not the mature form of this growth factor. For example, Ab 3100 detects HB-EGF\textsubscript{TM} on the surface of muscle cells upon differentiation (Chen et al., 1995). In the present study, we observed that Ab 3100 detected HB-EGF\textsubscript{TM} in the luminal epithelium on the morning of day 5 after implantation had been initiated and only at the site of implantation. Thus, these results showing spatial synthesis of HB-EGF\textsubscript{TM} are consistent with the in situ hybridization data. Whether any mature HB-EGF is released from its precursor is difficult to ascertain since antibodies directed against the mature HB-EGF domain will detect both soluble and transmembrane forms. However, previous studies have shown that breast, kidney and prostate epithelial cells do not spontaneously release detectable amounts of mature HB-EGF (Raab et al., 1994, Goishi et al., 1995, Freeman and Klagsbrun, unpublished observations).

Having established that the transmembrane form of HB-EGF is present on the apical surface of the uterine epithelium at the site of blastocyst attachment, we speculated that it could act as an insoluble membrane-anchored adhesion molecule promoting cell-cell interactions. A number of transmembrane growth factors, including TGF-α, have been shown to mediate juxtacrine activities, such as phosphorylation of EGFR, cell proliferation, Ca\textsuperscript{2+} uptake and adhesion to adjacent cells (Brachmann et al., 1989; Wong et al., 1989; Anklesaria et al., 1990). We developed a model system to analyze the adhesion properties of HB-EGF\textsubscript{TM} for mouse blastocysts since trophectodermal cells possess abundant EGFR (Dardik et al., 1992) and HSPG (Carson et al., 1993). In this model, 32D cell lines synthesizing either transmembrane or released mature HB-EGF were generated and then incubated with blastocysts. The 32D cells were chosen since they do not synthesize HB-EGF, EGFR or HSPG, and they do not process the transmembrane form of HB-EGF to the soluble form, even when exposed to phorbol ester (data not shown) which induces processing in carcinoma cells (Raab et al., 1994). In addition, their relatively small size and round shape readily distinguishes them from blastocysts. Using scanning electron microscopy and counting of adherent cells, it was found that 32D cells synthesizing HB-EGF\textsubscript{TM} adhered to mouse blastocysts in co-culture. The number of adherent cells was, in a statistically significant
HB-EGF with EGFR is consistent with previous results to dormant blastocysts is more pronounced than to blastocysts. Blastocyst/cell adhesion via HB-EGF is a novel demonstration of a juxtacrine interaction occurring at a multicellular level. Although, it would be of interest to use uterine luminal epithelial cells for these adhesion studies, it would be difficult to carry out the necessary control experiments ruling out non-HB-EGF interactions with blastocysts. Furthermore, these cells in culture might not synthesize HB-EGF since it appears that uterine HB-EGF gene expression is temporally and spatially regulated by the blastocyst itself (Das et al., 1994).

Having demonstrated the adhesion properties of HB-EGF in mouse blastocysts, the next question was to determine which receptor types on the blastocysts were involved in these interactions. Based on the accumulated evidence, our conclusion is that HB-EGF-mediated adhesion depends on interactions with both blastocyst EGF receptors and blastocyst HSPG. A role for EGFR in mediating HB-EGF adhesion to blastocysts is demonstrated in that the adhesion is inhibited by soluble TGF-α, a growth factor known to act on cells via EGFR (Lee et al., 1985). In addition, cells expressing HB-EGF do not adhere to delayed-implanting dormant blastocysts that do not possess detectable EGFR (Paria et al., 1993a). These cells do adhere to blastocysts reactivated by estrogen, a treatment which results in reappearance of blastocyst EGFR (Paria et al., 1993a). However, these results might not be strictly EGFR-dependent since dormant blastocysts might undergo other changes besides loss of EGFR. For example, the levels of perlecan protein, an HSPG molecule, are low in dormant mouse blastocysts, but increase in estrogen-reactivated blastocysts (Smith et al., 1995). However, a partial role for EGFR is suggested in that the lack of HB-EGF adhesion to dormant blastocysts is more pronounced than to blastocysts treated with heparinase and P21. Interaction of cell-associated HB-EGF with EGFR is consistent with previous results showing that cells expressing HB-EGF stimulate juxtacrine proliferation of 32D cells engineered to express EGFR but not of parental 32D cells (Higashiyama et al., 1995). Although blastocyst EGFR appears to be a target for soluble HB-EGF (Das et al., 1994) as well as for HB-EGF, it is possible that blastocysts express other EGFR-related receptors that bind HB-EGF. Besides the classical EGFR (HER-1) first purified from A431 cells (Carpenter and Wahl, 1990), three other related gene products have been identified including HER-2/erbB2 (Hynes and Stern, 1994), HER-3/erbB3 (Kraus et al., 1989) and HER-4/erbB4 (Plowman et al., 1993).

A role for HSPG in mediating HB-EGF adhesion activity is also evident. HSPG synthesized by trophodermal cells is involved in attachment and trophoblast outgrowth in vitro on extracellular matrix molecules, e.g. laminin or fibronectin (Farach et al., 1987; Carson et al., 1994). HB-EGF is a heparin-binding growth factor and both mature and transmembrane forms bind to immobilized heparin with equal affinity (Ono et al., 1994). Pretreatment of blastocysts with heparinase or P21, a synthetic peptide corresponding to the heparin-binding domain of HB-EGF (Higashiyama et al., 1993) inhibits adhesion of 32D cells synthesizing HB-EGF to blastocysts by 51% and 63%, respectively. These results indicate that HB-EGF-mediated adhesion is HSPG-dependent. HSPG-dependence would explain why soluble HB-EGF is far more effective than soluble TGF-α in inhibiting the adhesion of 32D cells synthesizing HB-EGF to blastocysts. TGF-α does not bind to heparin (Besner et al., 1990) or HSPG (Higashiyama et al., 1993) and its activity is unaffected by treatments that destroy, or desulfate HSPG (Higashiyama et al., 1993). In analyzing EGFR ‘knock out’ mice it has been demonstrated that in one strain of mice, blastocysts homozygous for mutated EGFR exhibited perimplantation failure (Threadgill et al., 1995). However, the developmental failure was dependent on the genetic background of the mice and did not occur in all strains of mice examined (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). These results suggest that in these mice other members of the EGFR family or HSPG molecules might be able to compensate for the loss of EGFR during implantation. However, another possibility is that maternal EGFR mRNA is present during implantation (Threadgill et al., 1995).

Our adhesion results are consistent with our previous demonstration that the chemotactic and mitogenic activities of HB-EGF for SMC are HSPG-dependent (Higashiyama et al., 1993). In these studies, it was found that HB-EGF was a much more potent mitogen and chemotactic factor for SMC than TGF-α or EGF. The enhanced activity was attributed to the ability of HB-EGF, but not TGF-α or EGF, to bind to cell surface HSPG since after incubation with heparinase or P21, HB-EGF SMC chemotactic activity and HB-EGF binding to high affinity SMC EGFR was diminished by 60-70%. Thus, in the absence of available cell surface HSPG, HB-EGF SMC activity was comparable to that of EGF and TGF-α. Based on our previous and present studies, we speculate that cells expressing HB-EGF adhere to blastocysts by interaction with EGFR and possibly other EGFR-related receptors (e.g. HER-2,-3,-4), and that this interaction is greatly enhanced by blastocyst HSPG. Thus, by virtue of its HSPG-binding properties, HB-EGF, among the various EGF family ligands, may be particularly suited for mediating binding to blastocysts. Previous studies have shown that heparin-binding growth factors such as HB-EGF (Higashiyama et al., 1993), VEGF (Cohen et al., 1995) and bFGF (Yayon et al., 1991) interact with target cells to stimulate proliferation using a dual receptor system of high affinity tyrosine kinase receptors and HSPG (Baird and Klagsbrun, 1991). Our results with HB-EGF-blastocyst interactions extends this dual receptor concept to mediating adhesion as well.

We have found that HB-EGF mRNA and HB-EGF are present in uterine luminal epithelium only during the implantation period and that HB-EGF is capable of mediating adhesion of cells to mouse blastocysts. Mature HB-EGF can stimulate blastocyst EGFR phosphorylation, zona hatching, and trophoblast outgrowth in vitro (Das et al., 1994). Transmembrane HB-EGF has growth promoting properties indicating that this form is an active growth factor (Ono et al., 1994; Higashiyama et al., 1995). Thus, we speculate that HB-EGF might be a physiologically relevant juxtacrine factor that acts as an adhesion factor and as an inducer of signal transduction in the mouse blastocyst. It is possible, however, that the transmembrane form of other EGF-like ligands, such as TGF-α and EGF could also mediate adhesion of blastocyst to the uterine wall via EGFR. However, a physiological role for TGF-α and EGF in temporal regulation of adhesion and EGFR signaling...
is less likely than HB-EGF. TGF-α gene expression in the uterus, unlike that of HB-EGF, is not limited to the site of implantation, and occurs even in pseudopregnant mice (Paria et al., 1994). Furthermore, homozgyous TGF-α ‘knock out’ mice are fertile (Luetteke et al., 1993; Mann et al., 1993). EGF is not detected in uterine epithelium on day 4 of pregnancy (Huet-Hudson et al., 1990).

Precisely regulated blastocyst-uterine interactions are critical for implantation and subsequent embryonic development. Although integrins, proteoglycans and extracellular matrix components have been suggested, the factors that regulate initial attachment of the blastocyst to the receptive uterine epithelium are still not well understood (Cross et al., 1994). Based on our studies, we suggest that the transmembrane form of HB-EGF might be an important regulator of blastocyst-uterus adhesion.

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