INTRODUCTION

Blastocyst implantation into the uterus is a critical step in embryogenesis. This process requires preparation of the receptive uterus and activation of the blastocyst. Both of these processes proceed in a temporally and spatially coordinated manner. Implantation in the mouse is initiated by the attachment of the blastocyst trophectoderm to the uterine luminal epithelium at 22:00-23:00 hours on day 4 of pregnancy (Das et al., 1994). This is preceded by uterine luminal closure that results in the close apposition of the blastocyst with the luminal epithelium. Concurrently with the attachment reaction, the first signs of cellular changes are observed as increased endometrial vascular permeability at the site of implantation (Psychoyos, 1973). This event is followed by localized decidualization of the endometrial stroma, apoptosis of the luminal epithelium and subsequent invasion of the trophoblast cells through the basement membrane into the stroma (Enders, 1976; Parr et al., 1987).

SUMMARY

Blastocyst implantation requires molecular and cellular interactions between the uterine luminal epithelium and blastocyst trophectoderm. We have previously shown that heparin-binding EGF-like growth factor (HB-EGF) is induced in the mouse luminal epithelium solely at the site of blastocyst apposition at 16:00 hours on day 4 of pregnancy prior to the attachment reaction (22:00-23:00 hours), and that HB-EGF promotes blastocyst growth, zona-hatching and trophoblast outgrowth. To delineate which EGF receptors participate in blastocyst activation, the toxicity of chimeric toxins composed of HB-EGF or TGF-α coupled to Pseudomonas exotoxin (PE) were used as measures of receptor expression. TGF-α or HB-EGF binds to EGF-receptor (ErbB1), while HB-EGF, in addition, binds to ErbB4. The results indicate that ErbB1 is inefficient in mediating TGF-α-PE or HB-EGF-PE toxicity as follows: (i) TGF-α-PE was relatively inferior in killing blastocysts, 100-fold less than HB-EGF-PE, (ii) analysis of blastocysts isolated from cross-bred egfr+/− mice demonstrated that HB-EGF-PE, but not TGF-α-PE, killed egfr−/− blastocysts, and (iii) blastocysts that survived TGF-α-PE were nevertheless killed by HB-EGF-PE. HB-EGF-PE toxicity was partially mediated by cell surface heparan sulfate proteoglycans (HSPG), since a peptide corresponding to the heparin-binding domain of HB-EGF as well as heparitinase treatment protected the blastocysts from the toxic effects of HB-EGF-PE by about 40%. ErbB4 is a candidate for being an HB-EGF-responsive receptor since RT-PCR analysis demonstrated that day 4 mouse blastocysts express two different erbB4 isoforms and immunostaining with anti-ErbB4 antibodies confirmed that ErbB4 protein is expressed at the apical surface of the trophectoderm cells. It is concluded that (i) HB-EGF interacts with the blastocyst cell surface via high-affinity receptors other than ErbB1, (ii) the HB-EGF interaction with high-affinity blastocysts receptors is regulated by heparan sulfate, and (iii) ErbB4 is a candidate for being a high-affinity receptor for HB-EGF on the surface of implantation-competent blastocysts.

Key words: HB-EGF, TGF-α, ErbBs, Blastocyst, Mouse, Heparin, Growth factor, Proteoglycan

Heparin-binding EGF-like growth factor interacts with mouse blastocysts independently of ErbB1: a possible role for heparan sulfate proteoglycans and ErbB4 in blastocyst implantation

Bibhash C. Paria1, Klaus Elenius2*, Michael Klagsbrun2 and Sudhansu K. Dey1,‡

1Department of Molecular and Integrative Physiology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, KS 66160-7338, USA
2Department of Surgery, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
*Present address: Medicity Research Laboratories, University of Turku, 20520 Turku, Finland
‡Author for correspondence (e-mail: sdey@kumc.edu)

Accepted 2 February; published on WWW 6 April 1999
These dormant blastocysts can be activated to implant in the progesterone-primed uterus with a single injection of estrogen (Yoshinaga and Adams, 1966; Paria et al., 1993a,b). There are also negative signals that have inhibitory effects on implantation and downregulation of their expression may permit the implantation to proceed. For example, a glycoprotein mucin-1 (Muc-1) that is thought to form an anti-adhesive surface on mouse uterine epithelium disappears at the time of implantation (Survevour et al., 1995).

Members of the epidermal growth factor (EGF) family have been considered to be possible regulators of blastocyst implantation. In mice, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC) and epiiregulin (ER) are all expressed in the uterus at the time of implantation, although EGF itself is not expressed in the mouse uterus at this stage (reviewed in Das et al., 1997). TGF-α is expressed in the peri-implantation mouse uterus (Tamada et al., 1991), but its role in implantation is questionable because TGF-α null mice are apparently fertile (Bruce-Mann et al., 1993; Luetteke et al., 1993). The AR gene is induced in the uterine epithelium throughout day 4 and at the time of blastocyst attachment, but it seems to be a more potent activator of the EGF-receptor (EGFR/ErbB1) in the uterus than the ErbB1 in the blastocyst, implying a role in intrauterine signaling (Das et al., 1995). Both ER and BTC are induced in the luminal epithelium and stroma at the site of blastocyst apposition during implantation (Das et al., 1997). HB-EGF seems to be an EGF-like growth factor with an expression pattern highly relevant to the implantation process. Its expression is upregulated solely in the uterine luminal epithelium at the site of blastocyst apposition 6-7 hours before the initial attachment of the blastocyst to the uterus (Das et al., 1994). This implies that the blastocyst signals the uterine epithelial cells to express their HB-EGF gene at the site of subsequent implantation and suggests a central role for HB-EGF in this process. In vitro experiments have shown that soluble HB-EGF can stimulate proliferation, zona-hatching, outgrowth of trophoblasts and tyrosine phosphorylation of the ErbB1 in mouse blastocysts (Das et al., 1994). Furthermore, cells expressing the transmembrane form of HB-EGF can adhere to active mouse blastocysts (Raab et al., 1996). Consistent with the findings in mice, HB-EGF is expressed in the human endometrium during the ‘window’ of uterine receptivity for implantation (Yoo et al., 1997) and soluble HB-EGF improves the development of in vitro fertilized human embryos into blastocysts and their zona-hatching (Martin et al., 1998).

Four receptor tyrosine kinases (RTKs), ErbB1 (HER1), ErbB2 (HER2/c-Neu), ErbB3 (HER3) and ErbB4 (HER4) have been identified that are activated by members of the EGF family (Hynes and Stern, 1994; Earp et al., 1995). EGF-like growth factors bind these receptors in a specific manner. For example, ErbB1 is activated by EGF, TGF-α, AR, HB-EGF, BTC and ER (Shoyab et al., 1989; Carpenter and Wahl, 1991; Higashiyama et al., 1991; Shing et al., 1993; Toyoda et al., 1995). ErbB2 is an orphan receptor that is activated only in heterodimeric complexes with other ErbBs (Hynes and Stern, 1994). ErbB3 and ErbB4 are receptors for a novel family of EGF-like growth factors known as the neuregulins (NRG) (Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993, Carraway et al., 1997; Chang et al., 1997; Higashiyama et al., 1997, Zhang et al., 1997). ErbB4 can also be activated by other members of the ligand family, including BTC (Riese et al., 1996a), HB-EGF (Elenius et al., 1997c), ER (Komurasaki et al., 1997) and EGF (Wang et al., 1998). ErbB4 variants have been described that differ in their susceptibility to phorbol ester-mediated release of soluble receptor ectodomain (Elenius et al., 1997b) and in their ability to activate phosphatidyl inositol 3-kinase (PI3-K)-mediated intracellular signaling cascades (Elenius et al., 1999).

Besides binding to high-affinity RTKs, some EGF family members including HB-EGF and AR, but not BTC, ER, EGF or TGF-α, also bind to heparan sulfate proteoglycans (HSPG) associated with the cell surface. HB-EGF has a heparin-binding domain that mediates interactions with cellular HSPG (Higashiyama et al., 1991, 1993; Thompson et al., 1994). The ability of HB-EGF to bind to HSPG enhances its chemotactic activity for smooth muscle cells, as compared to TGF-α, and heparitinase and a 21 amino acid peptide (P21) corresponding to the heparin-binding domain of HB-EGF inhibit HB-EGF activity (Higashiyama et al., 1993). The presence of HSPG on the surface of the implantation-competent mouse blastocysts has been observed (Smith et al., 1997).

Given the probability that HB-EGF plays an important role in blastocyst growth and implantation (Das et al., 1994), we were interested in delineating the complement of receptors on the surface of blastocysts that interact with HB-EGF. While ErbB1 is known to be expressed by the trophectoderm cells of the implanting blastocyst (Paria et al., 1993b), a majority of the receptor population is polarized to the basolateral surface of trophectoderm cells, thus facing the inside of the blastocyst (Dardik et al., 1992). This suggests that other ErbB receptors could be involved in the interaction between the blastocyst and the luminal epithelium. As an assay to measure growth factor/receptor interactions, blastocysts were treated with chimeric toxins composed of HB-EGF or TGF-α coupled to Pseudomonas exotoxin (PE). To kill cells, the PE needs to be internalized and translocated into the cytosol where it arrests protein synthesis (Fitzgerald et al., 1980). In chimeric toxins, the cell-binding domain of the PE is mutated leaving the growth factor domains responsible for the internalization and subsequent cellular responses (Mesri et al., 1994). The killing of blastocysts by chimeric toxins is a convenient measure of receptor expression and is more quantitative than assays measuring blastocyst growth. In this report, we provide evidence that HB-EGF does not require ErbB1 to interact with blastocysts and that ErbB4 and HSPG are candidate HB-EGF receptors on the surface of implantation stage mouse blastocysts.

**MATERIALS AND METHODS**

**Isolation of blastocysts**

CD-1 female mice (48 days old, Charles River Laboratories, Raleigh, NC) were mated with males of the same strain to induce pregnancy (day 1=vaginal plug). To induce delayed implantation, pregnant mice were ovariectomized at 09:00 hours on day 4 of pregnancy and maintained by daily injections of P4 (2 mg/mouse) for days 5-7 (Paria et al., 1993a,b). To obtain day 4 blastocysts of egfr<sup>−/−</sup>, egfr<sup>+/−</sup> and egfr<sup>+/+</sup> genotypes, egfr<sup>−/−</sup> mice on a CD-1 background (Threadgill et al., 1995) were cross-bred. Normal and dormant blastocysts were recovered in Whitten’s medium by flushing the uterus on day 4 of pregnancy.
pregnancy and day 8 of delayed implantation, respectively. Day 4 normal blastocysts were freed of zona pellucidae by a brief exposure to 0.5% pronase solution (Paria et al., 1993a). Zona-free embryos were washed and cultured for 2 hours in Whitten’s medium before subjected to any treatment. Day 8 dormant blastocysts were already zona-free.

**Toxin effects on blastocysts**

To study the effects of growth factor-*Pseudomonas* toxin (PE) fusion proteins on normal day 4 or day 8 dormant blastocysts, they were cultured in groups of 6-10 in 25 μl of Whitten’s medium under silicon oil in an atmosphere of 5% CO₂ in air at 37°C for 18 hours with or without various concentrations of HB-EGF-PE, TGF-α-PE or HB-PE. These fusion proteins were produced as previously described (Mesri et al., 1994). In order to digest cell surface heparan sulfate, a group of day 4 blastocysts were incubated for 1 hour with 0.01 U/ml of heparitinase (Seikagaku America, Inc., Rockville, MD). A second aliquot of heparitinase was added to bring the concentrations to 0.02 U/ml and the incubation was allowed to proceed for another 2 hours. Blastocysts were then cultured with HB-EGF-PE for 18 hours. For competitive inhibition analysis, day 4 blastocysts were preincubated with P21 (25 μg/ml), an HB-EGF heparin-binding domain peptide, for 1 hour prior to culture with HB-EGF-PE for 18 hours (Raab et al., 1996). To examine whether HB-EGF-PE toxicity on blastocysts is specific for HB-EGF, blastocysts were cultured with increased concentrations of HB-EGF together with HB-EGF-PE. After termination of culture, the morphology of blastocysts were examined under an inverted microscope with a Hoffman module for any sign of lysis and [14C]leucine uptake into protein was measured.

**Uptake of radiolabeled leucine by blastocysts or A431 cells**

To determine the [14C]leucine uptake into protein by the blastocyst after different treatments, blastocysts in groups of 6-10 were incubated in drops of 25 μl of Whitten’s medium containing [14C]leucine (200,000 cts/minute/drop) under silicon oil in an humidified atmosphere of 5% CO₂ in air at 37°C for 3 hours. At the end of incubation, blastocysts were washed four times in Whitten’s medium and placed on glass fiber filters (GF-C). The filters were rapidly washed with 1 ml of 10% trichloroacetic acid (TCA) followed by 20 ml of 5% TCA under vacuum. The filters were then washed with absolute ethanol, dried at 50°C for 5 minutes and the radioactivity was determined in a β-scintillation counter.

The [3H]leucine uptake into protein by A431 cells was measured as described previously (Mesri et al., 1994). Briefly, cells were grown to confluency in 96-well plates, toxins were added for 16-18 hours and during the last 2 hours, 5 μCi/ml of [3H]leucine was added to cells in a leucine-free medium. Incorporated radioactivity was assessed.

**Genotyping of blastocysts derived from cross-breeding of egfr+/− mice after exposure to TGF-α-PE and/or HB-EGF-PE**

Zona-free day 4 blastocysts from each egfr+/− female mated with an egfr−/− male were cultured with TGF-α-PE (100 ng/ml), HB-EGF-PE (10 ng/ml) or TGF-α-PE followed by HB-EGF-PE in 25 μl Whitten’s medium at 37°C for various periods. After termination of cultures, blastocysts were examined under an inverted microscope for signs of lysis. At the end of culture, each blastocyst was subjected to genotyping by polymerase chain reaction (PCR). Tubes containing the individual blastocysts in 3 μl purified water were heated for 3 minutes at 95-100°C and PCR amplifications of the genomic DNA were performed in a 25 μl volume. PCR reaction mixture contained 14.3 μl H₂O, 2 μl of dNTP mix (10 mM), mixtures of three oligonucleotide primers: 5′-TGCAACGACATCCCCCTTTC-3′ (0.1 μl, 20 units/μl), 5′-CTCTCTGCTGTTTCTGCAC-3′ (0.1 μl, 20 units/μl), 5′-GCCCTGCTTCTTTCTCCACAT-3′ (0.3 μl, 7 units/μl) and 5 μl of 5x buffer (300 mM Tris-HCL, pH 8.5, 75 mM (NH₄)2SO₄ and 7.5 mM MgCl₂). Reaction mixture was added into each tube containing the blastocyst extracts and the tubes were overlaid with two drops of light mineral oil. Tubes were heated to 95°C for 5 minutes. Temperature was then set at 85°C and 0.2 μl of AmpliTaq polymerase (5 U/μl) (Perkin-Elmar) was added to each tube. PCR extension was performed for 40 cycles: of 96°C, 20 seconds and 65°C, 30 seconds. After the last cycle, the PCR products were allowed to extend at 72°C for 7 minutes. PCR product (10 μl) was run on 2% agarose gel, visualized by staining the gel with ethidium bromide and photographed under UV light.

**RT-PCR analysis of blastocyst ErbB4 mRNA**

To prepare total RNA, 68 day 4 blastocysts were lysed with 500 μl RNAzol B reagent (Tel-Test, Inc., Friendswood, TX) and combined with 20 μg of carrier RNA (ribosomal RNA from *E. coli*; Boehringer Mannheim, Mannheim, Germany). The RNA extraction was completed according to the manufacturer’s instructions. The reverse transcription reaction was performed using random oligonucleotide primers (GIBCO-BRL). PCR analysis with primers specific for the juxtamembrane domain of mouse ErbB4 (sense: 5′-GAAATGTCCT-AGATGGCCCTACAGGG-3′ and antisense: 5′-CTTTTTTGCTC-TTTCTCTGAC-3′) and β-actin (sense: 5′-CTACAATGAGCTGC-GTGTTG-3′ and antisense: 5′-TAGCTCTTCTCCAGGGAGA-3′) was subsequently performed as previously described (Elenius et al., 1997b).

**Immunostaining of ErbB4 in the blastocyst**

Zona-free day 4 blastocysts and day 8 dormant blastocysts were fixed in 2% paraformaldehyde in phosphate-buffered saline for 15 minutes and cytospun onto poly-L-lysine-coated glass slides. Blastocysts were incubated in blocking solution (10% goat serum) for 10 minutes before incubation with affinity-purified rabbit anti-epitope antibodies to ErbB4 (5 μg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 18 hours at 4°C. A peptide (amino acid 1291-1308) corresponding to an amino acid sequence mapping at the carboxy terminus of the human ErbB4 precursor was used as the antigenic peptide. This antibody cross-reacts with mouse ErbB4. Immunostaining was performed using a Histostain-SP Kit for rabbit primary antibody (Zymed Lab, San Francisco, CA) (Paria et al., 1993a). Blastocysts were counterstained lightly with hematoxylin. The red stain indicated the sites of immunoreactive ErbB4. Day 4 blastocysts incubated without the primary antibody served as negative controls.

**RESULTS**

**Toxicity of HB-EGF-PE and TGF-α-PE for mouse blastocysts**

The toxicity of growth factor chimeric toxins for cells is a measure of the expression of cell surface growth factor receptors (Elenius et al., 1997a). Blastocysts were incubated with HB-EGF-PE or TGF-α-PE chimeric toxins and toxic effects as determined by shrinkage, blebbing and disintegration of blastomeres were analyzed (Fig. 1). Day 4 normal and day 8 dormant blastocysts cultured for 18 hours (Fig. 1A,B) and 36 hours (not shown) in the absence of chimeric toxins were maintained with perfectly preserved morphology. When isolated day 4 mouse blastocysts were treated with 10 ng/ml of HB-EGF-PE for 18 hours, dramatic morphological changes were observed indicative of cell death (Fig. 1C). The adverse effects of HB-EGF-PE on day 4 blastocysts were even more severe at 36 hours (data not shown). By comparison, there did not appear to be any HB-EGF-PE toxic effects on dormant mouse blastocysts isolated from ovarioctomized and progesterone-treated delayed implanting mice (Fig. 1D). In dormant blastocysts, ErbB1 is downregulated and not available.
to bind EGF family ligands (Paria et al., 1993a) suggesting that the toxic effects of HB-EGF-PE are ErbB1-mediated. In contrast, TGF-\( \alpha \)-PE showed toxicity only at a concentration of 100 ng/ml for normal blastocysts (Fig. 1E), but again did not appear to be toxic for dormant blastocysts (Fig. 1F).

The relative toxic effects of HB-EGF-PE or TGF-\( \alpha \)-PE were quantitated by analyzing the signs of degeneration microscopically for each individual blastocyst (Table 1). Day 4 normal or day 8 dormant mouse blastocysts were incubated in the absence (A,B) or presence of 10 ng/ml HB-EGF-PE (C,D), 100 ng/ml TGF-\( \alpha \)-PE (E,F) and 100 ng/ml HB-PE (G,H). Blastocysts were photographed with an inverted microscope using a Hoffman module at 400x magnification. ICM, inner cell mass; TE, trophoectoderm.

![Fig. 1. Photomicrographs of mouse blastocysts after an 18 hour exposure to HB-EGF-PE, TGF-\( \alpha \)-PE or HB-PE. Day 4 normal (left panels) and day 8 dormant (right panels) blastocysts were incubated in the absence (A,B) or presence of 10 ng/ml HB-EGF-PE (C,D), 100 ng/ml TGF-\( \alpha \)-PE (E,F) and 100 ng/ml HB-PE (G,H). Blastocysts were photographed with an inverted microscope using a Hoffman module at 400x magnification. ICM, inner cell mass; TE, trophoectoderm.](image)

Table 1. Effect of chimeric toxins on the morphology of day 4 normal and day 8 dormant blastocyst

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 4 Blastocysts</th>
<th>Day 8 Dormant blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Degenerated no. (%)</td>
<td>Degenerated no. (%)</td>
</tr>
<tr>
<td>No addition</td>
<td>70 (0)</td>
<td>26 (0)</td>
</tr>
<tr>
<td>HB-EGF-PE (ng/ml)</td>
<td>0.01</td>
<td>51 (24)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>53 (46)</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>68 (68)</td>
</tr>
<tr>
<td>TGF-( \alpha )-PE (ng/ml)</td>
<td>1.0</td>
<td>35 (0)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>42 (12)</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>45 (40)</td>
</tr>
<tr>
<td>HB-PE (ng/ml)</td>
<td>1.0</td>
<td>26 (0)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>26 (4)</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>26 (10)</td>
</tr>
</tbody>
</table>

Zona-free day 4 normal or day 8 dormant blastocysts were cultured in groups (6-10 blastocysts/group) in 25 \( \mu \)l of Whitten’s medium in an atmosphere of 5% CO\(_2\) in air at 37°C for 18 hours with indicated concentrations of HB-EGF-PE, TGF-\( \alpha \)-PE or HB-PE. After termination of cultures, morphology of blastocysts was examined under an inverted microscope and signs of lysis were recorded. Each experiment was repeated three to seven times.

Fig. 2. Toxic effects of chimeric toxins on blastocyst protein synthesis. (A) Day 4 blastocysts; (B) day 8 dormant blastocysts. Zona-free day 4 normal or day 8 dormant blastocysts were cultured in 25 \( \mu \)l of Whitten’s medium in groups (6-10 blastocysts/group) for 18 hours with indicated concentrations of HB-EGF-PE, TGF-\( \alpha \)-PE or HB-PE as described in the legend to Fig. 1. At the end of culture, blastocysts were incubated with \(^{14}\)C]leucine for 3 hours and incorporation into protein was measured.

A more quantitative approach was to measure the inhibition of protein synthesis (Fig. 2). The concentration of HB-EGF-PE required to inhibit protein synthesis in blastocysts as measured by incorporation of \(^{14}\)C]leucine into protein was at least two orders of magnitude less than that of TGF-\( \alpha \)-PE (Fig. 2A). The inhibition caused by 1 ng/ml HB-EGF-PE was equivalent to about 100 ng/ml TGF-\( \alpha \)-PE. On the contrary, the chimeric toxins failed to inhibit dormant blastocyst protein synthesis (Fig. 2B). These results suggest that putative cell surface receptors on dormant blastocysts for interaction with HB-EGF-PE are also downregulated.
As a control to demonstrate that TGF-α-PE was indeed capable of toxicity, A431 cells, which produce abundant amounts of ErbB1 (Ullrich et al., 1984), were treated with TGF-α-PE or HB-EGF-PE (Fig. 3). TGF-α-PE was significantly more potent than HB-EGF-PE in inhibiting A431 cell protein synthesis, with an ED50 of 0.02 ng/ml compared to 0.3 ng/ml, respectively, confirming that TGF-α-PE is a potent toxin. Taken together, these results suggested that blastocysts have a complement of receptors that interact with HB-EGF differently than they do with TGF-α.

The role of HSPG in mediating HB-EGF-PE toxicity for blastocysts

The relative differences in HB-EGF-PE- and TGF-α-PE-mediated blastocyst toxicity suggested differential interactions with blastocyst receptors. HB-EGF has two domains capable of interacting with cell surface molecules, an EGF-like domain that binds to EGF receptors and a heparin-binding domain that binds to cell surface HSPG molecules (Higashiyama et al., 1993; Thompson et al., 1994). In contrast, TGF-α has only the EGF-receptor-binding domain and does not interact with HSPG. A chimeric toxin containing only the heparin-binding domain of HB-EGF fused to PE (HB-PE) was tested for toxicity towards blastocysts. HB-PE had little adverse effects on blastocysts as ascertained by examining degenerative morphology (Fig. 1G,H; Table 1) and blastocyst protein synthesis (Fig 2). These results suggested that binding to HSPG was insufficient to result in the internalization of the toxin.

The possibility still remained, however, that HB-EGF-PE interactions with HSPG contribute to HB-EGF-PE toxicity. To assess this, blastocysts were pretreated with P21, a peptide corresponding to the heparin-binding domain of HB-EGF (Thompson et al., 1994) or heparitinase, an enzyme that digests the HS-like sequences in HSPG, prior to exposure to HB-EGF-PE. P21 (25 μg/ml) and heparitinase (0.02 U/ml) inhibited the toxic effects of 10 ng/ml HB-EGF-PE on blastocyst protein synthesis by 36% and 41%, respectively (Fig. 4). These reagents had no effect on TGF-α-PE-mediated toxicity (data not shown). These results indicate that HB-EGF-PE binding to HSPG contributes to HB-EGF-PE toxicity for blastocysts and might be in part responsible for the enhanced toxicity of HB-EGF-PE compared to TGF-α-PE. The complete inhibition of HB-EGF-PE toxicity by 50 ng/ml HB-EGF is shown (Fig. 4, lane 3) as a control for non-specific PE effects.

The role of ErbB1 in mediating HB-EGF-PE toxicity for blastocysts

Another possible explanation for the differential toxicity of HB-EGF-PE compared to TGF-α-PE is that they bind to different complements of blastocyst ErbB receptors. To date TGF-α is known to bind only to ErbB1 (Carpenter and Wahl, 1991; Riese et al., 1996b), while HB-EGF has been found to bind to ErbB1 and ErbB4 (Elenius et al., 1997c). To determine whether ErbB1 is required for the binding of HB-EGF-PE or TGF-α-PE, toxicity was analyzed in mouse blastocysts that did not express ErbB1. egfr<sup>+/−</sup> blastocysts were produced by cross-breding egfr<sup>+/−</sup> heterozygous mice (Threadgill et al., 1995). In theory, 25% of these blastocysts should be homozygous for egfr (<sup>egfr</sup>+/+), 50% heterozygous missing one egfr allele (<sup>egfr</sup>+</sup> and 25% homozygous egfr null mutants (<sup>egfr</sup>−/−). Blastocysts were treated with TGF-α-PE at 100 ng/ml, a high concentration at which it is toxic (Table 2). Most of the blastocysts (34/46, 74%) showed signs of lysis, consistent with the ability of TGF-α-PE to be toxic to <sup>egfr</sup>+/+ and <sup>egfr</sup>−/− blastocysts only. These TGF-α-PE-treated blastocysts, both intact and those undergoing lysis, were then genotyped by PCR. Of the blastocysts that exhibited signs of degeneration in response to TGF-α-PE, none (0/23) were egfr<sup>−/−</sup> but instead had the egfr gene, either one (16/23) or two (7/23) alleles. On the contrary, of the blastocysts that survived TGF-α-PE...
treatment, 75% (9/12) were egfr<sup>−/−</sup>, 25% (3/12) were egfr<sup>+/−</sup> and none (0/12) were egfr<sup>+/+</sup>, suggesting that the lack of ErbB1 expression increased the chances of survival in response to TGF-α-PE. Taken together, these results suggest that expression of ErbB1 is necessary to mediate TGF-α-PE toxicity.

HB-EGF-PE, at a concentration of 10 ng/ml, was toxic for 92% (49/53) of mouse blastocysts obtained after cross-breeding, a percentage higher than expected for toxicity mediated by EGFR alone, suggesting that HB-EGF-PE was able to adversely affect egfr<sup>−/−</sup> blastocysts (data not shown). To demonstrate more directly that HB-EGF-PE could damage egfr<sup>−/−</sup> blastocysts, a pool of 105 blastocysts derived from cross-breeding of egfr<sup>−/−</sup>-mice were first exposed to 100 ng/ml TGF-α-PE and the surviving blastocysts (34/105) were then exposed to 10 ng/ml HB-EGF-PE (Table 3). All of the blastocysts (34/34) surviving TGF-α-PE were killed by HB-EGF-PE. Genotyping of these 34 blastocysts indicated that 50% (17/34) were egfr<sup>−/−</sup>, 41 % (14/34) were egfr<sup>+/−</sup> and 9% (3/34) were egfr<sup>+/+</sup>. Thus HB-EGF-PE was readily toxic (17/34) for egfr<sup>−/−</sup> blastocysts, unlike TGF-α-PE which was not toxic (0/23) for any egfr<sup>+/−</sup> blastocysts. Taken together, these results suggest that unlike TGF-α-PE, HB-EGF-PE binds to blastocysts via receptors other than ErbB1 on the surface of day 4 mouse blastocysts.

**Expression of ErbB4 by mouse blastocysts**

Since HB-EGF, but not TGF-α, has been shown to interact with ErbB4, the expression of ErbB4 by mouse day 4 blastocysts was analyzed. RT-PCR analysis of total RNA isolated from these blastocysts demonstrated the presence of two ErbB4 isoforms (Fig. 5, lane 2). These isoforms correspond to the two

| Table 3. Genotypes of mouse blastocysts treated first with TGF-α-PE and the survivors treated subsequently with HB-EGF-PE |
|---|---|---|---|---|
| egfr | (+/+) | (+/−) | (+/−) |
| 3 (9%) | 14 (41%) | 17 (50%) |

Day 4 blastocysts from egfr<sup>(+/−)</sup> crossings were made zona-free and were cultured in pools in 25 µl Whitten’s medium under silicon oil in an atmosphere of 5% CO<sub>2</sub> in air with 100 ng/ml TGF-α-PE for 8 hours. The blastocysts were examined individually under an inverted microscope and examined for morphological changes. Of the 105 original blastocysts, 34 appeared intact and these were cultured individually with 10 ng/ml HB-EGF-PE for another 8 hours and then genotyped. All of the 34 blastocysts showed signs of lysis.

**DISCUSSION**

Several previous reports describing HB-EGF expression in the uterus and its biological effects on blastocysts suggest that this growth factor may play an important role in mediating blastocyst growth and implantation (Das et al., 1994; Raab et al., 1996; Yoo et al., 1997; Martin et al., 1998). The identity of the receptors on the surface of blastocysts that interact with HB-EGF is not clear. Studies with vascular cells and cells engineered to express individual members of the ErbB receptor family have demonstrated that HB-EGF can bind to ErbB1, ErbB4 and HSPG (Higashiyama et al., 1993; Elenius et al., 1997c). To analyze the interactions of HB-EGF with blastocysts, the effects of chimeric HB-EGF-PE toxins were determined. These fusion proteins are toxic only if the growth factor moieties bind to their cognate receptors and are internalized (FitzGerald et al., 1980; Mesri et al., 1994). The chimeric toxin assay has been shown to be a direct measure of functional receptor expression (Elenius et al., 1997a). This assay has advantages over the mitogenic assays which are difficult to carry out with blastocysts and which sometimes do not correlate with receptor levels. For example, EGF is not mitogenic for A431 cells even though these cells express about 10<sup>6</sup> copies of ErbB1 per cell (Ullrich et al., 1984).
However, HB-EGF-PE and TGF-α-PE readily kill these cells (Mesri et al., 1994).

Using the toxin approach, it was determined that HB-EGF interacts with blastocysts preferentially via receptors other than ErbB1. One piece of evidence is that HB-EGF-PE chimeric toxin is 100-fold more potent than TGF-α-PE in causing lysis and in inhibiting protein synthesis in day 4 mouse blastocysts. However, TGF-α-PE readily killed A431 cells showing that it is an active toxin acting via ErbB1. In addition, HB-EGF-PE, but not TGF-α-PE, was toxic for egfr-/- null blastocysts. Blastocysts that survived TGF-α-PE treatment were all killed subsequently by HB-EGF-PE and at least half of these blastocysts were egfr-/-/- null blastocysts. Together, these observations suggest that HB-EGF either interacts with ErbB1 on the surface of day 4 mouse blastocysts with higher affinity than TGF-α or that HB-EGF binds to receptors that are different from ErbB1 and cannot bind to TGF-α. However, the former alternative seems unlikely since previous studies with cells expressing ErbB1 demonstrate that HB-EGF and TGF-α have similar affinities, about $10^{-10}$ M, for this receptor (Mesri et al., 1994). A possible explanation for inefficient killing of blastocysts by TGF-α-PE via ErbB1 is that a large population of ErbB1 on trophectoderm cells is polarized to the basolateral surface, and thus not facing the uterine epithelium (Dardik et al., 1992). It is interesting to note that both TGF-α-PE and HB-EGF-PE failed to affect dormant blastocysts. Like ErbB1, ErbB4 was downregulated in dormant blastocysts. Thus, the failure of HB-EGF-PE to exert toxic effects on dormant blastocysts is consistent with the speculation that ErbB4 is downregulated during delayed implantation and/or that HSPG which is known to be downregulated in dormant blastocysts (Smith et al., 1997) is required for high-affinity binding of HB-EGF to ErbB4.

Previous in vitro experiments with cell types overexpressing different combinations of ErbB receptors have shown that HB-EGF can bind to both ErbB1 and ErbB4, whereas TGF-α binds only to ErbB1 (Elenius et al., 1997c; Graus-Porta et al., 1997). Thus ErbB4 is a good candidate for being a high-affinity blastocyst receptor for HB-EGF-PE. In support of this possibility, we have demonstrated that ErbB4 is expressed by day 4 blastocysts both at the protein and mRNA levels and that the ErbB4 immunoreactivity is associated with blastocyst cell surface. RT-PCR analysis indicated that the blastocysts expressed two different ErbB4 isoforms that differ in their extracellular juxtamembrane domains. These isoforms, designated ErbB4 JM-a and ErbB4 JM-b, have been shown to differ functionally in that ErbB4 JM-a is susceptible to phorbol ester-stimulated processing and release of the receptor ectodomain, whereas ErbB4 JM-b is not (Elenius et al., 1997b). In addition, previous cross-linking analysis showed that radioiodinated HB-EGF interacted with two 170-180 kDa proteins of day 4 mouse blastocysts consistent with the size of ErbB4 (Elenius et al., 1997a). However, the lack of available murine ErbB4 antibodies and lack of adequate quantities of blastocyst samples prevented unambiguous identification of blastocyst ErbB4 in the cross-linked complex. Another possible target for HB-EGF on blastocysts would be integral cell surface HSPG. HSPGs, such as syndecans are expressed on the surface of all adherent cells (Bernfield et al., 1992) and do get constitutively internalized (Yanagishita and Hascall, 1992). HB-EGF, but not TGF-α, binds to HSPG (Higashiyama et al., 1991). Treatment of cultured blastocysts with heparitinase, or with a synthetic peptide corresponding to the heparin-binding domain of HB-EGF (P21) reduces the adhesion of cells expressing transmembrane HB-EGF to cultured blastocysts (Raab et al., 1996). Heparitinase or P21 peptide reduced HB-EGF-PE toxicity by about 40%, suggesting that the interaction of HB-EGF with the high-affinity receptors at the surface of blastocyst is enhanced by its interaction with HSPG. However, it does not seem that interaction with HSPG is sufficient to internalize HB-EGF since a chimeric toxin composed of the heparin-binding domain and PE (HB-PE) is not toxic for blastocysts.

While expression of ErbB1 in mouse blastocysts has been described (Paria et al., 1993a), the expression of ErbB4 in day 4 blastocysts is novel. However, blastocysts with null mutation for egfr or erbB4 have no obvious defects during the initial stage of implantation (Gassmann et al., 1995; Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Sibilia et al., 1998), suggesting that the functions of these two ErbB receptors are redundant. The status of blastocysts with double mutations for egfr and erbB4 has not yet been described. Besides HB-EGF, the luminal epithelium also expresses BTC and ER as ligands at the site of implantation that can interact with ErbB1 and ErbB4 (Das et al., 1997; Riese et al., 1996a; Komurasaki et al., 1997). Thus, the contribution of these various ligands to implantation may be redundant. In general, the expression of multiple ligands and multiple ErbB family receptors might be a protective mechanism to ensure high probability of blastocyst development and implantation.
Among several growth factors examined, HB-EGF is the most potent growth factor for enhancing the development of human IVF-derived embryos to blastocysts (71%) and subsequent zona-hatching (82% of the blastocysts developed) (Martin et al., 1998). This is a significant finding and likely to improve the pregnancy success rate, since there is increasing evidence that transfer of blastocysts rather than 2- to 8-cell embryos improve pregnancy rates in humans (Menezo et al., 1992). The peak expression of HB-EGF in the human endometrium during the ‘window’ of implantation (days 19-21) is consistent with an important role of this growth factor in blastocyst functions and implantation (Birdsall et al., 1996; Yoo et al., 1997). In summary, we suggest that HB-EGF expressed by the luminal uterine epithelium interacts with the trophectoderm cells of the implanting blastocyst by binding to ErbB4 and HSPG.

We are grateful to Terry Magnuson (Case Western Reserve University, Cleveland, OH) for providing us with the egfr mutant mice. Our sincere thanks to David Threadgill (Vanderbilt University, Nashville, TN) for the protocol and advice in genotyping of single blastocysts. Growth factor-toxin conjugates were kindly provided by Ira Pastan (NIH, Bethesda, MD) and Enrico Mesri (Cornell University Medical College, New York, NY). We also thank H. Lim for immunostaining of ErbB4 and Michelle Lewis for breeding and genotyping of egfr mutant mice. This work was supported by the Academy of Finland (to K. E.), NIH grants HD 12304 and HD 29968 (the National Cooperative Program on Markers of Uterine Receptivity for Blastocyst Implantation grant) (to S. K. D.), HD 35114 (to B. P.), GM 47397 (to M. K.) Center grants on Reproductive Biology (HD 02528) provided access to various core facilities (to S. K. D.).

REFERENCES


Graus-Porta, D., Beerli, R. R., Daly, J. M. and Hynes, N. E. (1997). ErbB-2, the preferred heterodimerization partner of all ErB receptors, is a mediator of lateral signaling. EMBO J. 16,1647-1655.


