[Frontiers in Bioscience 3, d288-299, March 1, 1998]

STRUCTURE AND FUNCTION OF HEPARIN-BINDING EGF-LIKE GROWTH FACTOR (HB-EGF)

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Received 2/20/98 Accepted 2/25/98

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1. ABSTRACT

Heparin-binding EGF-like growth factor (HB-EGF) is a 22 kDa, *O*-glycosylated protein that is mitogenic for fibroblasts, smooth muscle cells (SMC) and epithelial cells. This review describes the primary structure of HB-EGF, as well as its processing. The structure of the mouse and human HB-EGF genes is also discussed. Finally, this review summarizes HB-EGF expression patterns, receptor-mediated signaling, and role in several important biological systems.

2. INTRODUCTION

The first description of HB-EGF appeared in 1990 when it was shown that cultures of human macrophages secreted a novel heparin-binding growth factor (1). The conditioned media (CM) of cultured human macrophages derived from peripheral blood mononuclear cells was analyzed by heparin-sepharose affinity chromatography. Eluted proteins were tested for growth factor activity using DNA synthesis assays on BALB/c 3T3 fibroblast cells. A peak of activity that eluted from heparin-sepharose columns by 1.0M NaCl was detected. This macrophage-derived heparin binding growth factor was found to be heat-resistant and cationic, and had a molecular weight of 14,000-25,000 (1). Several lines of evidence suggested that this growth factor was novel, including the NaCl concentration required for its elution from heparin, persistent bioactivity in the presence of specific neutralizing antisera to other known heparin-binding growth factors (including acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF)), lack of reactivity on Western blots probed with aFGF, bFGF, and PDGF antisera, and mitogenic activity for fibroblasts and SMC, but not vascular endothelial cells. These properties did not correlate with any other known heparin-binding growth factors which elute from heparin-sepharose columns in the 1.0M NaCl range, most notably aFGF (1).

Since the initial observations, a tremendous amount of knowledge has been gained regarding the structure and function of HB-EGF. This review will summarize that information to provide a more complete picture of the mechanism of HB-EGF action and its potential functions in processes such as wound healing and tumorogenesis.

3. DISCUSSION

3.1. HB-EGF Primary Structure

HB-EGF was ultimately purified to homogeneity from the conditioned medium of U-937 cells, a human macrophage-like cell line (2). After obtaining an *N*-terminal amino acid sequence of the HPLC-purified

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  1  M K L L P S V V L K L F L A A V L S A L
 21  V T G E S L E R L R R G L A A G T S N P
 41  D P P T V S T D Q L L P L G G G R D R K
 61  V R D L Q E A D L D L L R V T L S S K P
 81  Q A L A T P N K E E H G K R K K K G K G
101  L G K K R D P C L R K Y K D F C I H G E
121  C K Y V K E L R A P S C I C H P G Y H G
141  E R C H G L S L P V E N R L Y T Y D H T
161  T I L A V V A V V L S S V C L L V I V G
181  L L M F R Y H R R G G Y D V E N E E K V
201  K L G M T N S H
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Figure 1. Predicted HB-EGF primary translation product. Two strongly hydrophobic regions representing a presumptive secretion signal peptide and a presumptive transmembrane domain are underlined. The *N*-terminal amino acid sequence of U-937-derived HB-EGF determined by Higashiyama, et. al. (2) is shown in italics. The underlined threonine residues (T) in the italicized sequence indicate sites of *O*-linked glycosylation.

protein, a degenerative oligonucleotide probe was designed and used to screen a U-937 cDNA library. A 2.36-kb clone was isolated and analyzed by DNA sequencing. The predicted amino acid sequence represented a 208-residue protein, of which amino acids 74-93 correlated with the *N*-terminus of the original purified protein (figure 1). This suggested that HB-EGF was processed from an initial primary translation product that is cleaved to produce a mature protein. The predicted span of hydrophobic residues following the translation-initiating methionine was consistent with a secretion signal sequence. Two threonine residues (Thr^{75} and Thr^{85} were potential sites for *O*-glycosylation.

Searches within known protein databases revealed that while the predicted amino acid sequence was novel, it possessed a domain that shared sequence identity with members of the EGF growth factor family. Because of its relationship to EGF and its ability to bind heparin, the new growth factor was named “heparin-binding EGF-like growth factor” or “HB-EGF”. Within the EGF-like domain of HB-EGF, the six cysteine residues that are characteristic of the EGF family and their spacing are highly conserved. At the amino acid level, the identity of HB-EGF to other EGF family members is 40-53% between the first and sixth cysteine in this domain (figure 2). Like many EGF family members, HB-EGF contains a strongly hydrophobic region corresponding to a transmembrane domain downstream of its EGF-like domain. HB-EGF most closely resembles amphiregulin (AR) in overall structure. AR was discovered in 1988 as a secretory product of phorbol 12-myristate 13-acetate (PMA)-treated MCF-7 (human breast carcinoma) cells (3) and later characterized as a member of the EGF family (4). The mature polypeptides for HB-EGF and AR have a similar number of amino acids and both molecules are heparin-binding, which correlates with each having a highly hydrophilic stretch of amino acid residues just upstream of their EGF-like domains (2).

The binding of growth factors to cell surfaces and extracellular matrices is often mediated by proteoglycans. Many growth factors are known to interact with heparin and heparin sulfate proteoglycans (HSPG) including HB-EGF, AR, betacellulin (BTC), PDGF, aFGF, bFGF, vascular endothelial growth factor (VEGF) and granulocyte-macrophage-colony stimulating factor (GM-CSF). Therefore, the property of heparin-binding has been exploited previously and is currently being used in the identification and purification of a variety of novel growth factors such as connective tissue growth factor (CTGF) (5). The ability of HSPG to bind heparin-binding growth factors is an area of intense study, largely due to the fact that the binding of some of these growth factors, such as bFGF, to their signal-transducing receptors appears to be critically dependent on interactions with HSPG in some systems (6, 7, 8).

Several studies have demonstrated that the binding of HB-EGF to the EGF receptor is modulated by HSPG (9, 10, 11, 12, 13). Heparin appears to differentially regulate the interaction of HB-EGF with EGF receptor (EGFR) molecules and its effects appear to be cell type specific (14). For example, the mitogenic activity of HB-EGF on murine AKR-2B (fibroblast-like) cells was enhanced by co-incubation with heparin, whereas its activity on human dermal fibroblasts was not affected (14). Using a combination of synthetic peptide and site-directed mutagenesis studies, the heparin-binding domain of HB-EGF has been localized to residues 21-41 of the mature protein (residues 93-113 in precursor; refer to figure 1) (9, 15). Three stretches of basic amino acids within this region (^{21}KRKKGK,^{31}KKR, ^{38}RKYK) have been implicated in heparin-binding, the latter of which actually falls within the EGF-like domain of HB-EGF. In addition to that region, a *C*-terminal leucine residue (Leu^{76} in mature *E. coli*-derived HB-EGF; Leu^{148} in the precursor, refer to figure 1) may also play an important role in regulating the response of HB-EGF to heparin (13, 14). Analysis of recombinant forms of HB-EGF showed that a *C*-terminal truncation deleting this residue can convert HB-EGF to a heparin-inhibited molecule. Therefore, Leu^{148} appears to be required for heparin-dependent enhancement of HB-EGF mitogenic activity. These studies suggest that more than one region within the HB-EGF protein contains vital structural information for regulating responsiveness to heparin.

In 1992, a monkey cDNA encoding a diphtheria toxin (DT) sensitivity determinant was isolated by expression cloning in mouse L-M cells (16). Mouse L-M cells are resistant to DT, presumably because they lack functional receptors. Cells that were transfected with the DT sensitivity determinant cDNA were rendered DT sensitive. The cDNA was ultimately determined to encode a protein that is identical to the HB-EGF precursor. With the identification of the monkey HB-EGF homologue, a new function for the HB-EGF precursor as a receptor for a bacterial toxin was defined. A processed 75-amino acid form of HB-EGF was shown to be sufficient for DT binding, suggesting that at least part of the DT-binding site lies within the mature growth factor (17). Later, two independent studies involving chimeric proteins verified that the DT binding region of HB-EGF was localized between Asp^{106} and Leu^{148} (refer to figure 1) (18, 19). The

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HB-EGF      1      VTLSSKP--QALATPNKEEHGKRKKKGKGLG------KKRDPCLR
AR          1   SVRVEQVVKPPQNKTESENTSDKPKRKKKGGKNGKNRRNRKKKNPCNA
TGF-alpha   1                                          VVSHFNDCPD
EGF         1                                            NSDSECPL

HB-EGF     38  KYKDFCIH-GECKYVKELRAPSCICHPGYHGERCHGLSL
AR         49  EFQNFCIH-GECKYIEHLEAVTCKCQQEYFGERCGEK
TGF-alpha   9  SHTQFCFH-GTCRFLVQEDKPACVCHSGYVGARCEHADLLA
EGF        11  SHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR
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Figure 2. Comparison of predicted HB-EGF sequence with mature forms of other EGF family members. Amino acid number one for mature HB-EGF corresponds to amino acid 74 in the 208-residue precursor and the *C*-terminus is assumed to be Leu[148]. To maximize alignments dashes were inserted into the sequences. Underlined amino acids are shared between HB-EGF and AR. Amino acids in bold type are identical in at least three of the proteins.

monkey HB-EGF precursor was compared to the mouse precursor (which does not bind DT) and three clusters of differing residues were identified; residues 43-62, 65-76 and 122-135 termed "clusters I, II and III" respectively (18). The third cluster was found to be the most important region for toxin binding.

The HB-EGF precursor (proHB-EGF) has been shown to associate with DRAP27/CD9, a membrane-spanning protein that appears to up-regulate functional DT receptors and DT sensitivity (17). CD9 was also demonstrated to up-regulate proHB-EGF juxtacrine activity to a great extent (20). The mechanism by which this occurs is still unknown. DRAP27/CD9 does not seem to increase the amount of HB-EGF mRNA or proHB-EGF protein. It does increase the number of DT binding sites, but does not appear to increase their affinity (17). It has been determined that DRAP27/CD9 interacts with HB-EGF through a heparin-binding domain (21).

3.2. HB-EGF Gene Structure

The human HB-EGF gene, which spans 14-kb of DNA and contains 6 exons and 5 introns, was mapped to chromosome 5 (22). Exon I contains the 5′ untranslated region and encodes the first 16 amino acids of the 208-amino acid primary translation product including the hydrophobic signal peptide; exon II encodes amino acids 17-74 of precursor HB-EGF; exon III encodes amino acids 75-133 which includes the heparin-binding domain and the first two disulfide loops of mature HB-EGF; exon IV encodes amino acids 134-185 which contains the third disulfide loop and the hydrophobic transmembrane domain; exon V encodes amino acids 186-208 which encompasses the cytoplasmic domain of HB-EGF; and exon VI contains the 3′ untranslated region.

Like the human HB-EGF gene, the mouse gene spans 14-kb of DNA, contains 6 exons, 5 introns and is localized to chromosome 18 (23, 24). While the human and mouse genes are similar in organization, DNA sequence analysis of introns failed to show a high degree of identity (24).

cDNA clones have been obtained that encode the mouse and rat forms of HB-EGF (25). The region of these cDNAs corresponding to the mature growth factor was determined to be the least conserved, having only 73-76% identity when compared to human or monkey HB-EGF. However, the putative heparin-binding region within mature HB-EGF was highly conserved, demonstrating evolutionary conservation of this domain. The proposed transmembrane and juxtamembrane domains were also regions of high sequence conservation. When the predicted amino acid sequences of the HB-EGF precursors are compared, there is 97% homology between those of monkey and human, 82% between human and rodent, 80% between monkey and rodent and 92% between rat and mouse. In addition, a partial HB-EGF precursor cDNA clone of 166 amino acids isolated from pig endometrial tissues was shown to encode a polypeptide that is, respectively, 85%, 85% and 78% homologous to the corresponding region of human, monkey and rodent HB-EGF (26).

3.3 HB-EGF Processing

The discovery that the CM of U-937 cells contained multiple forms of HB-EGF resulted in a more detailed structural analysis of human HB-EGF. These studies revealed additional information about its processing to produce a mature, secreted growth factor, as well as its post-translational modifications (27). Amino acid sequencing analysis demonstrated that the secreted protein contains at least 86 amino acids, but that forms missing 10, 11, 14 and 19 amino acids at the *N*-terminus were also present in CM. The form truncated by 11 amino acids, initiating at Val[74], was the protein originally sequenced and used to design a cDNA probe with which the entire HB-EGF cDNA was isolated (2). Sequencing analysis using tryptic fragments revealed that the most *C*-terminal tryptic fragment extended through at least Leu[148] of the HB-EGF precursor. Amino acid sequencing of HB-EGF isolated from burn wound fluid (BWF) showed that its *N*-terminus was truncated by 24 amino acids (28).

A comparison of the bioactivities of U-937-cell derived HB-EGF demonstrated that the truncated forms

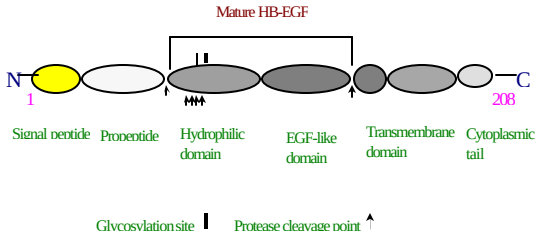

Figure 3. HB-EGF precursor structure. The predicted domains of the 208-residue HB-EGF precursor are illustrated.

appear to be equally mitogenic to the longest form identified (27). It is unclear if the *N*-terminal truncations observed for HB-EGF isolated from a variety of sources (27, 28) are the result of true proteolytic processing events mediated by the producer cells/tissues, or if they are artifacts of the purification protocols that were utilized. Therefore, no definitive conclusions can yet be drawn regarding the physiological relevance of the various truncated forms of HB-EGF.

While it is obvious that at least part of the difference between the various HB-EGF forms identified was due to *N*-terminal truncations, it was also possible that they differed at their *C*-termini and/or state of glycosylation. It was consistently observed that no clear residues could be identified in positions 75 and 85 of the HB-EGF protein by amino acid sequencing. This suggested that these residues may be modified by post-translational modifications such as *O*-glycosylation. Further experimentation involving enzymatic digestion revealed that native and recombinant forms of HB-EGF were extensively modified by *O*-glycosylation (27, 29). Based on data compiled from the structural analysis of HB-EGF purified from U-937 cell CM, predictions regarding the overall domain structure of the HB-EGF precursor and its processing could be made (figure 3).

Recombinant HB-EGF produced in a baculovirus system has also been purified and characterized (30). Insect cells were demonstrated to secrete two forms of HB-EGF: a 15 kDa protein termed "SEC-I" and a 18 kDa protein termed "SEC-II". In addition, 21.5 kDa and 24 kDa transmembrane forms termed "TM-I" and "TM-II" respectively were also identified. TM-II had an *N*-terminal sequence beginning with Asp^{63} and most likely corresponded to a 148-amino acid protein ending at His^{208}. The *N*-termini of TM-II and SEC-II were the same as that of the longest form of U-937-cell secreted HB-EGF. This implies that secreted and transmembrane forms of HB-EGF are both generated by processing at Arg^{62}/Asp^{63}. The preceding amino acids to those residues within HB-EGF (^{57}RDRKVR) correlate with the consensus sequence for the protease furin, indicating that this membrane-bound protease may be responsible for processing HB-EGF at its *N*-terminus (31). TM-II was shown to be biologically active in both 3T3 and SMC mitogenic assays. An *N*-terminal sequence was not obtained for TM-I. The SEC-I and SEC-II proteins secreted by insect cells had a lower molecular weight (15-18 kDa) than that observed for U-937-cell secreted HB-EGF (20-22 kDa). U-937-cell secreted HB-EGF treated with *O*-glycanase (an enzyme which digests *O*-glycan moieties from proteins) decreased the molecular weight to 14-16 kDa. Insect-cell derived HB-EGF is unaffected by *O*-glycanase. This suggests that recombinant HB-EGF produced in baculovirus is not properly modified by *O*-glycosylation.

Although native HB-EGF is *O*-glycosylated, recombinant HB-EGF produced in *E. coli* is able to bind to and stimulate autophosphorylation of EGFR and to stimulate DNA synthesis in target cells (15, 32), suggesting that *O*-glycosylation is not essential for biological activity. However, since there are other possible effects of glycosylation on HB-EGF function and since no previously described systems had enabled a functional analysis of the *O*-linked glycosylation of HB-EGF, a recombinant vaccinia virus designed to express human HB-EGF in HeLa cells was generated (29). Site-directed mutagenesis of the HB-EGF cDNA was performed to prevent *O*-glycosylation at Thr^{75} and Thr^{85}. Purification and characterization of the mutant proteins demonstrated that both potential *O*-glycosylation sites of HB-EGF are utilized, *O*-glycosylation does not appear to be required for HB-EGF secretion or processing, and HB-EGF produced in HeLa cells is heavily sialylated (97). Although localization of HB-EGF does not appear grossly affected by *O*-glycosylation state (97), additional studies are required to establish whether the lack of *O*-glycosylation affects the rate of HB-EGF trafficking or secretion.

HB-EGF is present in human MDA MB 231 (breast adenocarcinoma) cells primarily as a cell-associated precursor, the processing of which was rapidly induced following treatment of the cells with PMA (33). Similar results were obtained after treatment of Vero cells that were transfected with HB-EGF cDNA (34). Phorbol esters such as PMA have been previously demonstrated to induce the processing of other membrane-anchored precursors including that of transforming growth factor (TGF-alpha) (35). TGF-alpha cleavage involves a protein kinase C (PKC)-dependent mechanism of activation of elastase-like enzymes. Although the HB-EGF cleavage site(s) are not consistent with the elastase-like consensus sequence, a similar PKC-dependent activation may be necessary for HB-EGF proteolysis. The effect of PMA on HB-EGF-transfected Vero cells was abrogated by the addition of TAPI, a specific inhibitor of matrix metalloproteases (36) which suggested that the enzyme responsible for HB-EGF processing may be a metalloprotease. A more recent study has implicated matrix-metalloprotease-3 (MMP-3/stromelysin-1) as the enzyme that converts HB-EGF from a membrane-bound growth factor to a secreted protein (37).

Recently, a cDNA encoding a novel, short form of HB-EGF (SF HB-EGF) was cloned and characterized (38). SF HB-EGF mRNA is believed to be the result of an alternative splicing event. Multiple forms of EGF-like growth factors are frequently generated by alternative splicing mechanisms (39). SF HB-EGF was found to

contain the signal peptide, propeptide, heparin-binding domain, the first two disulfide loops of the EGF-like domain, and a nine amino acid tail. It has yet to be determined if SF HB-EGF has different biological properties than HB-EGF.

3.4 HB-EGF Expression Patterns

To date, at least two HB-EGF transcripts have been identified; a 2.5-kb transcript (HB-EGF mRNA) originally identified in U-937 cells (2) and a 1.2-kb transcript (SF HB-EGF mRNA) generated by alternative splicing (38).

Northern blotting experiments using mouse, rat and human cDNA clones as probes revealed HB-EGF transcript expression in multiple tissues including lung, skeletal muscle, brain and heart (25). Relatively low levels of the 2.5-kb HB-EGF transcript were detected in liver in all three species. HB-EGF mRNA distribution was also analyzed by RT-PCR in a variety of porcine tissues (40). The principal sites of HB-EGF synthesis were skin, kidney, lung, heart, brain and male reproductive tissues. Expression was also detected in various regions of the adult central nervous system. Lower but detectable signals were found in spleen, lymph node and thymus. Later, it was shown that HB-EGF is present in pig uterine luminal flushings and is produced by pig endometrial tissues (26). Taken together, these results indicated that HB-EGF mRNA is present in a wide range of body tissues.

The expression of SF HB-EGF was investigated in various tissues and cell types using RT-PCR and Northern blotting (38). COS (SV40 transformed African green monkey kidney), HeLa (human cervical carcinoma), Vero (African green monkey kidney), BeWo (human choriocarcinoma), U-2 0S (human osteogenic sarcoma), Raji (human Burkitt lymphoma) and Daudi (human Burkitt lymphoma) cells all produced SF HB-EGF. It was also expressed in human pancreas, placenta, heart and skeletal muscle. Interestingly, the presence of additional bands on Northern blots suggested that more than one alternatively spliced transcript may exist (38).

3.5 HB-EGF Biology

The ability of HB-EGF to evoke a mitogenic response from a variety of cell types and its expression in a large number of tissues suggests that a vast array of potential roles may exist for HB-EGF *in vivo*. This growth factor may not only be an important part of many normal developmental processes, but it may also be relevant to the progression of many disease states. As a result, numerous studies have been designed to attempt to define the role(s) of HB-EGF *in vivo*.

3.5.1 Myogenesis

HB-EGF gene expression and protein localization were analyzed during myogenic differentiation in mice (41). It was demonstrated that the HB-EGF gene is induced during myogenesis and that MyoD (a DNA-binding transcription factor known to be involved in myogenesis) plays a key role in modulating HB-EGF transcription during this process. Skeletal muscle-derived HB-EGF could be interacting with EGFR or HSPG on adjacent cells to facilitate contact, signaling or adhesion between cells to promote myotube and myofibril formation.

3.5.2 Reproductive Tract

Production of HB-EGF in the uteri of several species including rat, mouse and pig is well documented (26, 42, 43, 44). HB-EGF was shown to be induced in the mouse uterus by the blastocyst at the site of apposition (43) and there is evidence that HB-EGF is an adhesion factor for mouse blastocysts (44). *In vitro* studies showed that HB-EGF could induce blastocyst EGFR tyrosine autophosphorylation and promote blastocyst growth. Later studies investigated the temporal and spatial expression of human endometrial HB-EGF using immunohistochemistry and RNase protection assays in nonpregnant and pregnant endometrial tissues and in chorionic villi during placentation (45). It was shown that human endometrial HB-EGF mRNA expression increased immediately prior to implantation, HB-EGF is localized to the luminal epithelium during the implantation window, and HB-EGF mRNA expression levels were high in placental tissues at the early stages of placentation (45). Taken together, these results suggest that HB-EGF is important in the process of embryo implantation and placentation, possibly by facilitating cell-cell interactions between the endometrium and conceptus during pregnancy. HB-EGF is also differentially regulated by estradiol and progesterone in rat uterine stromal and epithelial cells, suggesting that it may be involved in mediating the activity of steroid hormones in the uterus of the rat (42).

3.5.3 Gastrointestinal Tract

The gastrointestinal tract mucosa is renewed constantly (98). Epithelial cell renewal, which is important to the integrity of the mucosa, involves many processes including cell proliferation, migration, differentiation, senescence and loss of gastrointestinal epithelial cells (46, 47). The expression and production of HB-EGF were examined by Northern blotting and immunohistochemistry in human gastric mucosa (48). These studies suggested that HB-EGF is synthesized in parietal and gastrin cells and may act in an autocrine or paracrine fashion to regulate the proliferation and differentiation of gastric mucosal cells (48). Later studies involving a rat gastric mucosal cell line (RGM1) demonstrated that HB-EGF is a potent mitogen for these cells, confirming that it may act as an autocrine growth factor (49). This further substantiated the hypothesis that HB-EGF may be playing a crucial role in mucosal repair of the stomach.

Studies using a rat intestinal epithelial cell line (IEC 18) revealed that HB-EGF is able to protect these cells from the deleterious effects of hypoxia (50). The cytoprotective effect of HB-EGF on these cells was mediated, at least in part, by the ability of the growth factor to preserve cytoskeletal integrity via preservation of cellular ATP stores. Further *in vivo* studies using a rat model of isolated segmental intestinal ischemia showed that enteral administration of HB-EGF was able to significantly reduce the mortality associated with the

ischemia/reperfusion event (51). Thus, it appears that HB-EGF may play an important role in protecting the small bowel from ischemic injury.

The effects of oxidative stress on HB-EGF expression was also investigated in RGM1 cells (52). In response to stimulation with hydrogen peroxide, HB-EGF gene expression was immediately increased in a dose-dependent manner. The hydrogen peroxide induced expression of HB-EGF could be blocked by pretreatment with an antioxidant and significantly inhibited by pretreatment with an EGF receptor-specific tyrosine kinase inhibitor, suggesting that the effect is mediated by the EGF receptor. The immediate up-regulation of HB-EGF mRNA in response to oxidative stress in RGM1 cells indicates that HB-EGF may be involved in the series of reparative events following gastric injury.

The role of HB-EGF during pancreas development has been examined by immunostaining of HB-EGF in neonatal, fetal, and adult rat pancreas (53). Endocrine cells associated with primitive ducts or ductular cells in developing organs produced HB-EGF, whereas, the exocrine pancreas lacked HB-EGF protein. The HB-EGF gene was shown to contain a functioning PDX-1 binding site. The PDX-1 homeodomain-containing transcription factor is known to be essential for pancreas development. These observations suggest that HB-EGF, regulated in part by PDX-1, is likely to be involved in pancreatic development.

3.5.4 Vasculature

The SMC hyperplasia in atherosclerosis has been linked to growth factors of macrophage, endothelial, SMC and platelet origin (54, 55). To date, HB-EGF is recognized to be produced by all of the aforementioned cell types with the exception of platelets. HB-EGF has been shown to be induced in endothelial cells treated with tumor necrosis factor-alpha (TNF-alpha) and was initially identified from human peripheral monocytes that had differentiated into macrophages (1, 56). In addition, it is a potent mitogen for SMC and is synthesized and secreted by cultured fetal human vascular SMC (57). Several endogenous growth factors synthesized by SMC are believed to be important in initiating SMC migration and proliferation following arterial injury (58). Expression of HB-EGF mRNA was induced in rat balloon-injured carotid arteries, suggesting that HB-EGF may foster vascular remodeling in response to arterial injury by an autocrine or paracrine mechanism (59). Taken together these data suggest that HB-EGF may be playing an important role in regulating SMC growth *in vivo.*

To directly examine the role of HB-EGF in the pathogenesis of atherosclerosis, immunohistochemical localization was performed on human aortic walls and atherosclerotic plaques (60). HB-EGF was expressed in the SMC and macrophages of normal human aortic walls and individuals with varying stages of atherosclerosis showed significantly higher levels of protein. A study examining the production of HB-EGF in human coronary arteries showed that medial SMC expression of HB-EGF gradually decreased with age when comparing neonates, infants and children to adults (61). In adult nonatherosclerotic arteries with diffuse intimal thickening, the SMC of the intima were highly positive for HB-EGF protein. Atherosclerotic plaques of coronary arteries with eccentric intimal thickening showed high expression of HB-EGF in macrophages and SMC (61). Additional studies showed that macrophages in human atherosclerotic plaques were positive for HB-EGF mRNA and protein expression (62).

Lysophosphatidylcholine (lyso-PC), a component of oxidatively modified low-density lipoprotein, is increased in the plasma of hypercholesterolemic patients and has been correlated with the development of atherosclerosis (61). Lyso-PC has been shown to up-regulate the level of HB-EGF mRNA in human monocytes, T lymphocytes, and endothelial cells (63, 64, 65). Therefore, lyso-PC may be inducing HB-EGF expression and contributing to the migration and proliferation of various cell types *in vivo*. All of these data suggest that HB-EGF may be involved in the proliferation and migration of SMC and other cell types which occurs during normal development of arterial walls and other tissues, and in the formation of atherosclerotic plaques.

Hyperoxia was used to create a model of pulmonary hypertension in rats that facilitated the analysis of the temporal expression of growth factors by resident and infiltrating lung cells (66). Northern blot analysis and *in situ* hybridization demonstrated that HB-EGF mRNA levels were increased by 100-fold by day 7 of hyperoxia compared with normal rat lungs. In addition, HB-EGF was expressed by eosinophils that are localized around microvessels in hypertensive lungs. These findings are significant because eosinophils are believed to be a source of potent mitogens for vascular cells in pulmonary hypertension and in other diseases characterized by a chronic inflammatory cell process (66, 67, 68, 69). The localization of HB-EGF mRNA in rat eosinophils during hyperoxia suggests that HB-EGF may be playing an important role in vascular remodeling in the hypertensive lung.

3.5.5 Renal System

To investigate the role of HB-EGF in the renal system, the distribution of HB-EGF mRNA and protein in normal rat kidneys was determined (70). Immunohistochemical and *in situ* hybridization studies showed that the tubular epithelial cells of the S3 segment of the outer stripe in the outer medulla were the major renal source of HB-EGF. It was also ubiquitously present in the epithelial cells of the proximal tubules and the arterial SMC. HB-EGF expression was not detected in the glomeruli or other parts of the kidney. These results suggested that HB-EGF may play a role in the function of proximal tubules in normal kidneys.

The importance of HB-EGF in the diseased kidney has also been investigated. Increased expression of HB-EGF mRNA in the kidneys of diabetic rats suggests that it may be involved in the development of diabetic nephropathy (71). Additional studies have implicated EGF

and other members of the EGF ligand family in the proliferation of renal tubular epithelial cells which occurs during recovery from injury to the kidney (72). Northern blotting and *in situ* hybridization demonstrated that HB-EGF mRNA is induced in the kidney *in vivo* after acute tubular injury (73). This suggests that HB-EGF may act as an autocrine or paracrine growth factor involved in the proliferation of tubular epithelial cells during repair of the kidney. HB-EGF protein is produced in response to renal injury induced by either ischemia/reperfusion or aminoglycosides (74). Additionally, the membrane-bound form of HB-EGF appears to promote the survival of cultured renal epithelial cells (75). These data support the idea that HB-EGF is involved in renal cell repair and proliferation and may contribute to regeneration and nephrogenesis.

3.5.6 Urinary Tract

The mammalian urinary tract, including the urethra, bladder, ureters, and renal pelvis, is lined by urothelium, a multi-layered transitional epithelium. HB-EGF has been shown to act as an autocrine growth factor for normal human urothelial cells (76). Several *in vivo* sites of HB-EGF synthesis within the human bladder were also identified by immunohistochemical localization (76). These data supported the first example of a functional role by an EGF family member within a normal human bladder. The proposed physiologic function of HB-EGF within the urothelium is as a regulator of cell cycling or regeneration in response to mucosal injury (76). The ability of cell surface heparin-like molecules to coordinate the functional activity of soluble and membrane-bound HB-EGF suggests that HB-EGF may act in concert with proteoglycans and glycosaminoglycan components of bladder mucins to mediate cellular responses *in vivo* (76).

3.5.7 Hepatic System

HB-EGF is a mitogen for rat hepatocytes in primary culture (77). It was also observed that the level of HB-EGF mRNA was increased in regenerating rat liver after partial hepatectomy (78). An additional study examined changes in the amount of HB-EGF mRNA in rat liver after injury induced by hepatotoxins (78). The level of HB-EGF mRNA was very low in normal rat liver, but increased significantly in the liver of rats injured by various hepatotoxins. Western blots revealed that levels of HB-EGF protein also appeared to be increased. These results suggested that HB-EGF may play a role in liver regeneration following hepatotoxic injury or hepatectomy.

3.5.8 Wound Healing

HB-EGF is a large growth factor component in wound fluid analyzed from porcine partial-thickness excisional wounds (11). Human burn wound fluid also contains 10-15 kDa forms of HB-EGF, one of which commenced at Gly^{98} (28). Immunohistochemical analysis demonstrated that whereas HB-EGF is restricted to the basal epithelium of normal skin, it was uniformly distributed throughout surface epithelium of partial thickness burn wounds (28). HB-EGF was present in the advancing epithelial margin, islands of regenerating epithelium within the burn wound, and in the ducts and proximal tubules of eccrine sweat glands. Human monocytes, which are known to play an important role in wound healing, were recently shown to release HB-EGF upon activation (79). Taken together these data support a role for HB-EGF in wound healing. Furthermore, using a murine model of a partial-thickness scald burn, topical application of HB-EGF was found to accelerate burn wound healing, by both a direct action of the growth factor, and via HB-EGF-induction of TGF-alpha mRNA (80).

3.5.9 Carcinogenesis

HB-EGF mRNA is increased in human pancreatic cancer tissues in comparison to normal pancreas, suggesting that HB-EGF may be an important contributor to pancreatic cancer cell growth (81). It is also highly expressed in hepatoma tissues but not in normal liver, indicating that HB-EGF is associated with the progression of hepatocarcinogenesis (82). By Northern blot analysis, HB-EGF mRNA levels were increased by 4.7-fold in human gastric cancers compared with normal gastric tissues (83). Immunohistochemical studies involving human skin carcinomas showed that both basal and squamous cell carcinomas stain positively for HB-EGF expression, while melanomas were negative for HB-EGF staining (84). The expression pattern of HB-EGF was also examined using immunohistochemistry in a murine skin carcinogenesis model (85). HB-EGF production appeared to be induced in all epidermal layers in contrast to normal skin. Studies involving human non-small cell lung cancers (NSCLC) monitored HB-EGF expression using RT-PCR and Northern blotting (86). HB-EGF was not expressed in either tumor tissue or normal lung tissue. These data suggest that HB-EGF appears to play no role in the growth of NSCLC.

The adhesion of cancer cells to vascular endothelium is an important step in the metastasis of cancer and the expression of integrin molecules are known to be important in that process (87, 88, 89). HB-EGF has been shown to induce integrin expression in both human breast and esophageal cancer cells (90, 91). Taken together these data suggest that HB-EGF may be an important factor in the progression of some forms of cancer.

3.6 HB-EGF Signaling

The biological activity of each member of the EGF family is mediated by binding and activating tyrosine kinase receptors. Four distinct receptors for ligands of the EGF family have been identified and designated as ErbB-1/HER1, ErbB-2/HER2, ErbB-3/HER3 and ErbB-4/HER4. Ligand binding to each of these receptors causes receptor dimerization, receptor autophosphorylation, phosphorylation of numerous intracellular substrates and recruitment of SH2 domain-containing signaling molecules to the phosphorylated tyrosine residues of receptor cytoplasmic tails (92). In T47D breast tumor cells, EGF ligands appeared to differ in their ability to induce tyrosine phosphorylation of the various ErbB receptors and activation of different receptor subsets resulted in distinct biological activities on cultured mammary epithelial cell lines (93). Additionally, the interaction of some EGF ligands with cell-surface heparin molecules may also serve to determine the specificity of biological responses.

HB-EGF binds and activates ErbB-1/HER1 (2, 12, 20, 27, 94). NIH 3T3 cells expressing each receptor subtype independently (95) were used to examine the ability of HB-EGF to bind additional EGF receptor subtypes (96). HB-EGF binds ErbB-4/HER4, induces tyrosine phosphorylation, and stimulates phosphatidyl-inositol 3-kinase (PI3-K) associated activity. HB-EGF is chemotactic but not mitogenic for cells expressing ErbB-4/HER4. However, HB-EGF appears to be both a chemotactic factor and a mitogen in cells expressing ErbB-1/HER1. This suggests that different EGF receptor subtypes mediate different biological activities in response to HB-EGF.

Similar to other growth factors, HB-EGF elicits biological responses at extremely low concentrations. HB-EGF is a potent mitogen and chemotactic factor for bovine aortic smooth muscle cells (BASMC) with half-maximal activity of 100 pg/ml (2) and 150 pg/ml (10) respectively. Importantly, HB-EGF is a more potent mitogen for SMC than EGF (2). HB-EGF is also mitogenic for BALB/c 3T3 (mouse fibroblast) cells with half maximal activity of 250 pg/ml (2). It was not mitogenic for capillary endothelial cells, even at concentrations in 20-fold excess of active doses used on BASMC cells (2).

4. SUMMARY

HB-EGF is believed to play a crucial role in many key biological processes *in vivo,* such as SMC growth, skeletal muscle myogenesis, gastrointestinal tract mucosa maintenance, embryo implantation, wound healing and injury repair. It has also been implicated as a factor in the progression of some diseases such as cancer, atherosclerosis and diabetes. A function for the HB-EGF precursor as a receptor for a bacterial toxin has also been defined. Future molecular and cellular studies will allow a more complete understanding of the regulation and physiological manifestations of HB-EGF bioactivity.

5. ACKNOWLEDGMENT

We thank David Brigstock and Srikimar Pillai for reviewing the manuscript.

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Key words: Diphtheria toxin, Diphtheria toxin receptor, EGF family, EGF receptor, ErbB-1/HER1, ErbB-4/HER4, Heparin-binding

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