Role of membrane-bound heparin-binding epidermal growth factor-like growth factor (HB-EGF) in renal epithelial cell branching

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Background. The developing metanephros is characterized by growth and differentiation of the ureteric bud and the surrounding mesenchymal tissue. These processes can be influenced by several growth factors, including epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). We examined whether another member of the EGF family of growth factors, heparin-binding epidermal growth factor (HB-EGF), might act as a morphogen in renal epithelial tubulogenesis.

Methods. Expression of HB-EGF mRNA and immunoreactive protein were examined in fetal, neonatal and adult rat kidneys. For in vitro studies of tubulogenesis, a rat renal epithelial cell line (NRK52E) stably transfected with proHB-EGF (NRKproHB-EGF) was treated with TPA for 30 minutes, washed with 2 mol/L NaCl to remove soluble HB-EGF trapped by cell surface heparan sulfate proteoglycan and replated onto plastic dishes in the absence of fetal calf serum. In further experiments, NRKproHB-EGF were suspended in a type I collagen gel in serum-free media.

Results. Northern blot analysis indicated that HB-EGF was strongly expressed in embryonic rat kidney (embryonic days 18-20) and was still increased in the neonatal kidney (day 10), compared to the low basal levels in adult kidney. Immunohistochemical analysis confirmed that immunoreactive HB-EGF expression in the fetal rat kidney was localized predominantly to the ureteric bud. When NRKproHB-EGF were plated onto plastic substrata, they became progressively flattened and enlarged and exhibited filopodia. By 10 hours after plating, NRKproHB-EGF began to migrate and subsequently developed cell-cell contact and fully established tubular-like structures. Immunoelectron microscopy revealed that the initial recovery of cellular proHB-EGF was localized predominantly to areas of cell-cell attachment. No tubule-like structures were observed in similarly treated NRK52E cells transfected with the vector alone.

Conclusions. These results indicate that membrane-bound HB-EGF can mediate both epithelial cell branching and cell motility. Localization of proHB-EGF to the site of cell-cell contact and development of tubule-like structures in collagen gels suggests that proHB-EGF may be an important morphogen for renal epithelial cells.

During metanephric kidney development, tubular structures arise as a result of the initiation of tubulogenesis from epithelial and mesenchymal precursors and subsequent branching morphogenesis [1]. These processes are regulated by mutually inductive epithelial-mesenchymal interactions [2, 3]. In addition, interactions between epithelial cells and extracellular matrix also have been implicated in renal tubular development [4].

Previous studies have suggested that locally produced growth factors, including glial cell-derived neutrophic factor (GDNF), osteopontin-1 (OP-1), hepatocyte growth factor (HGF), insulin-like growth factors (IGF)-I and -2, transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), may play a role in nephrogenesis and/or differentiation during renal development and in renal regeneration following acute tubular injury [5–18]. Following the initial identification of EGF and the EGF receptor, other growth factors have subsequently been identified that also signal through the EGF receptor, the so-called EGF family of growth factors [19–21]. One of these growth factors, heparin-binding EGF-like growth factor (HB-EGF) is a 22 kD glycoprotein that was origi-
nally purified from conditioned media of U-937 cells, a macrophage-like cell line [22]. In addition to macrophages, HB-EGF mRNA is expressed in other hematopoietic cells, endothelial cells, vascular smooth muscle cells and epithelial cells [23–26]. HB-EGF is a potent mitogen for smooth muscle cells, fibroblasts, keratinocytes, intestinal epithelial cells and renal epithelial cells and is a motility factor for smooth muscle cells and keratinocytes [27–30]. Our previous studies found that HB-EGF expression increased in rat kidney after acute ischemic insult [30, 31], that HB-EGF was expressed predominantly in the ureteric bud during rat metanephric development [31], and in normal adult rat kidney, immunoreactive HB-EGF expression was predominantly confined to tubules [30]. When NRK 52E cells, an immortal, non-transformed rat renal epithelial cell line that expresses EGF receptors, were transfected with proHB-EGF, these cells were resistant to apoptotic injury by H2O2 or etoposide, and cell attachment was significantly increased [32].

Members of the EGF family of growth factors are synthesized as membrane-anchored precursors [33]. The precursor for HB-EGF (proHB-EGF) exists as a 206 amino acid transmembrane protein and undergoes processing to an 86 amino acid secreted protein (mature HB-EGF) [22, 29]. Both the membrane bound TGF-α precursor and preproEGF have been shown to activate EGF receptors in adjacent cells and to stimulate the cells to proliferate in a juxtacrine manner [19]. Recent studies by others and us also have indicated that proHB-EGF may be capable of juxtacrine stimulation [32–34]. Juxtacrine stimulation has been suggested to be a mechanism by which adjacent cells can directly exchange biological signals necessary for cell survival and which can contribute to maintenance of cell-cell contact [32]. Therefore, the present studies were designed to determine whether membrane-anchored HB-EGF play a role in renal tubular morphogenesis.

**METHODS**

**Animal studies**

Timed pregnancies were performed in Sprague-Dawley rats. Samples from adult male rats (weighing 150 to 200 g) were also obtained.

**cDNA and vectors**

Male Sprague-Dawley rats were given gentamicin 200 mg/kg every 12 hours for two days in order to increase renal HB-EGF mRNA expression [31]. Following sacrifice, kidneys were removed for total RNA extraction by ISOGEN regent (Nippon Gene, Toyama, Japan). A rat membrane-anchored HB-EGF cDNA was developed by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification from an extracted RNA of a 692 bp fragment of the rat membrane-anchored HB-EGF coding region [35]. The sequences of the forward primer: GGACCAT GAAGCTGCTGCGTGCG and reverse primer: TAA GGAAACGCCATCCTCGAGAT were based on the published rat HB-EGF cDNA sequence [33]. The amplified PCR fragment was initially ligated into pPCR II vector (Invitrogen, San Diego, CA) and sequenced, and the Hind III-Not I digested cDNA fragment of membrane-anchored HB-EGF from this plasmid was subsequently ligated into a eukaryotic expression vector (pRc/CMV plasmid; Invitrogen).

**Transfection**

NRK 52E cells were purchased from ATCC (ATCC No. CRL-1571; Rockville, MD, USA) and were stably transfected by lipofectin (Life Technologies, Gaithersburg, MD, USA) with proHB-EGF/pRc/CMV and selected by continuous growth in G418 [400 μg/mL Dulbecco’s modified Eagle’s medium (DMEM); Geneticin; Life Technologies, Grand Island, NY, USA]. After seven passages, sixty individual clones were isolated and screened for proHB-EGF expression by dot northern blot analysis using 32P-labeled EcoR I digested membrane-anchored HB-EGF cDNA fragment. Three clones (E15, E24 and E56) expressing high levels of message were identified and were utilized in these studies. All responses to growth conditions, agonists and antagonists were confirmed in all three clones and were qualitatively indistinguishable. Unless otherwise indicated, the representative figures are results obtained from clone E15. As a control, NRK 52E cells also were transfected with pRc/CMV plasmid alone and grown and maintained in G418. Details of these clones have been previously reported [32].

**Cell culture**

Non-transfected NRK 52E (wt), vector transfected (NRKvector) and membrane-anchored HB-EGF transfected cells (NRKproHB-EGF) were routinely grown in DMEM (Sigma Chemical Co., St. Louis, MO, USA), containing 10% fetal calf serum (FCS; HyClone, Logan, UT, USA) supplemented with 100 U/mL ampicillin and 100 μg/mL streptomycin. Transfected cells were routinely grown in Geneticin (G418 sulfate, 400 μg/mL; Life Technologies). To activate protein kinase C, 12-O-tetradecanoylphorbol 13-acetate (TPA; 10⁻⁷ mol/L) was added to quiescent cells for 30 minutes prior to trypsinization and replating on the indicated substratum, using a protocol previously reported to promote angiogenesis [36].

**Production of collagen gels**

Five volumes of type I collagen solution (Cellmatrix IA; Nitta Zelatin Inc., Osaka, Japan) were mixed with four volumes of 2.5 × concentrated DMEM and 1 volume of 0.05 NaOH with 2.2% NaHCO₃ and 200 mmol/L HEPES and heated to 37°C. The wells were first coated with 5 mL collagen. Cells (10⁵ cells/well plate) were
then mixed in a volume of 5 mL of collagen and overlaid. Following gelation, 3 mL of DMEM was overlaid.

RNA isolation and Northern blot analysis

Total RNA from cultured cells was extracted and subjected to Northern blot analysis according to previously described methods [30]. Aliquots of total RNA, each containing 10 to 15 μg, were subjected to electrophoresis, transferred to S & S Nytran nylon membranes (Schleicher & Schuell, Keene, NH, USA), and fixed to the membrane by exposing to UV (CL-1000, Ultraviolet Crosslinker; Funakoshi Co., Tokyo, Japan). The membranes were prehybridized in a solution of 30% formamide, 5 × standard sodium citrate (SSC), 5 × Denhardt’s (Wako Pharmaceutical Co., Osaka, Japan), and 100 μg/mL sonicated salmon sperm DNA (Invitrogen) at 42°C. Following prehybridization, blots were hybridized with 0.5 to 1.0 cpm/mL of 32P-labeled cDNA overnight at 42°C. The cDNA probes utilized included a 686 bp EcoR I fragment of rat membrane-anchored HB-EGF, 1.3 kb PstI-BamH I fragment of rat type I collagen, 2.23 kb EcoR I-BamH I fragment of rat type III collagen and a 760 bp EcoR I fragment of rat EGF receptor, which were labeled to >106 cpm/μg by the random priming procedure using a commercially available kit (Amersham, Arlington Heights, IL, USA). After washing with 2 × SSC for 15 minutes at room temperature twice, and at 65°C with 1 × SSC/0.1% sodium dodecyl sulfate (SDS) for 15 minutes, and finally with 0.2% SSC/0.1% SDS for an additional 15 minutes, the membranes were exposed to Kodak X-Omat AR film at −70°C with an intensifying screen. The blots were stripped and reprobed with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA utilizing the conditions of labeling, hybridization, and washing as described above. The levels of mRNA were quantitated by densitometry and normalized to GAPDH expression.

Antibodies and chemicals

Goat anti-rat HB-EGF antibody was kindly provided by Judith Abraham of Scios, Inc. (Mountain View, CA, USA), rabbit anti-EGF receptor antibody was from Calbiochem (La Jolla, CA, USA), HB-EGF and rabbit anti-human HB-EGF antibody was from Santa Cruz Biochemicals (Santa Cruz, CA, USA). Anti-F-actin was purchased from Molecular Probe Inc (Oregon, Eugene, USA), and anti-α-tubulin was from Calbiochem. Rhodamine-labeled sheep anti-rabbit and FITC-labeled donkey anti-goat antibodies were from Cappel Laboratories (Durham, NC, USA). 12-O-tetradecanoylphorbol 13-acetate was purchased from Sigma.

Immunohistochemistry

Direct or indirect immunofluorescent staining was performed as described previously [37, 38]. For indirect immunofluorescent staining, cells were incubated with the primary antibody after fixation with 1% glutaraldehyde/PBS, washed in phosphate buffered saline (PBS), and then reacted with the appropriate secondary antibody labeled with fluorescein-isothiocyanate (FITC). Immunoperoxidase staining was carried out as reported previously [36, 37]. Briefly, tissues fixed with paraformaldehyde-lysine periodate (PLP) were cut into thin sections, and treated with periodate and sodium borohydroxide to inhibit endogenous peroxidase. Each section was reacted with anti-HB-EGF antibody, then with secondary antibody labeled with horseradish peroxidase (HRPO; Cappel). As controls, kidney sections or cultured NRK 52E cells were incubated with nonimmune mouse sera or with unrelated mouse IgG monoclonal antibody, followed by incubation with FITC or HRPO-labeled goat anti-mouse IgG, or with secondary antibody alone. These controls were entirely negative.

For immunoelectron microscopy, cells were rinsed with ice-cold PBS and fixed with PLP fixative solution and treated progressively with 10 to 20% sucrose in PBS. They were then incubated with periodate and sodium borohydrate to inhibit endogenous peroxidase and reacted with HB-EGF antibody, followed by the peroxidase-labeled F(AB)2 fragment of rabbit anti-goat IgG. The cells were fixed with 1% glutaraldehyde in PBS, incubated with diaminobenzidine/hydrogen peroxide and post-fixed with osmic acid. They were then treated with graded ethanol solutions (50, 70, 80, 90, 100%) for five minutes each and embedded in Epon 812. Ultrathin sections were observed under an electron microscope (H-7100; Hitachi, Tokyo, Japan) without lead citrate or aqueous uranyl acetate staining.

RESULTS

Localization of HB-EGF protein in the embryonic rat metanephros. Heparin binding-EGF mRNA expression in the embryonic developing rat kidneys and the adult rat kidneys was determined by Northern blot analysis. As shown in Figure 1A, significant HB-EGF mRNA expression was observed in rat metanephric kidney at embryonic (e) days e18 and e20. By postnatal day 10, HB-EGF mRNA expression was decreased compared to fetal expression but was still greater than that seen in kidneys for adult rats. Immunological localization using an anti-HB-EGF polyclonal antibody confirmed our previous observation [31] that the HB-EGF protein in the metanephric kidney (e18) was initially expressed in the ureteric buds (Fig 1B). In the present studies, we determined that HB-EGF immunoreactivity persisted in the kidney during late embryogenesis in structures derived from the ureteric buds. In later fetal and postnatal kidneys (through postnatal day 28), HB-EGF immunoreactivity was also detected in the smooth muscles of the interlobu-
These cells were then subcultured on plastic dishes at a density $1 \times 10^5$ cells/mL of medium in the absence of fetal calf serum (FCS). Wild type (Wt) and vector-transfected cells (NRK<sup>vector</sup>) underwent identical treatment. Initially after plating, TPA-pretreated NRK<sup>proHB-EGF</sup> remained rounded (Fig 2A). At this time, both F-actin filaments (Fig. 2B) and α-tubulin (Fig. 2C) were weakly distributed in the perinuclear region and sequestered around the canalicular domain. After six hours of culture, there was increased expression of F-actin and α-tubulin, with more widespread distribution throughout the cytoplasm. During the period from 6 to 12 hours, there was progressive cell attachment and elongation, with development of filopodial extensions. By 10 hours, there was evidence of initial contact by TPA-pretreated NRK<sup>proHB-EGF</sup> (Fig. 3A). By 12 to 16 hours, single epithelial cell strands were observed (Fig. 3B, C). By 16 to 20 hours, the initial epithelial branches had extended and begun to join to adjacent branching cell aggregates (Fig. 3D, E), which persisted over the subsequent 16 hours (Fig. 3F-H). When NRK<sup>proHB-EGF</sup> were plated in FCS deficient media without pretreatment with TPA, they did not form tubule-like structures (Fig. 3I). In NRK<sup>proHB-EGF</sup>, but not in NRK<sup>vector</sup>, EGF receptor mRNA expression increased progressively within the first 12 hours after plating (Fig. 4A, B). By 12 hours after plating, immunofluorescent EGF receptors were concentrated at areas of cell-cell attachment (Fig 4C). A similar pattern of immunofluorescent HB-EGF localization was noted (Fig 4D). Immunoelectron microscopy with HRP-labeled anti-rat HB-EGF further indicated that the initial recovery of HB-EGF in NRK<sup>proHB-EGF</sup> was localized mainly to areas of cell-cell attachment (Fig. 4E).

**Tubule formation in NRK 52E cells grown in collagen gels**

To examine growth in collagen gels, cells were again pretreated with TPA for 30 minutes prior to replating in the gel. When NRK<sup>proHB-EGF</sup> were suspended in a type I collagen gel in serum-free media, they developed epithelial branching cords by day 1, and by day 2, exhibited cytoplasmic processes forming small luminal spaces (Fig. 5). Beginning at day 3, the cells extended into the surrounding collagen matrix, became progressively elongated and began to branch, with arborized structures present by day 6 (Fig. 5, A–F). Electron microscopy indicated polarized tubules, with a smooth basal surface in contact with the collagen matrix and a microvillus-rich apical surface facing the lumen (Fig 5G).

To determine possible mechanisms by which proHB-EGF expression and protein kinase C (PKC) activation interact to promote tubulogenesis in NRK-52E cells, we examined expression of the metalloproteinase, matrix metalloproteinase-1 (MMP-1). Under baseline conditions, NRK<sup>proHB-EGF</sup> expressed minimal MMP-1 mRNA.
or immunoreactive protein (Fig. 6). After pretreatment of these cells with TPA, there was increased expression of MMP-1 mRNA by 24 hours, and a corresponding increase in immunoreactive MMP-1, with an apparently maximal expression by 48 hours. Addition of the metalloproteinase inhibitor 1,10-phenanthroline greatly reduced tubulogenesis of the TPA-treated NRK<sup>protHB-EGF</sup> cells grown in collagen gels.

To further investigate the interactions of proHB-EGF and protein kinase C in promoting tubulogenesis in collagen gels, we compared tubule formation in clone E15 of NRK<sup>protHB-EGF</sup> cells grown in collagen gels in the absence or presence of TPA treatment (Fig. 7). As indicated in Figure 7A, without PKC activation, the transfected cells form small tubule-like structures compared to the TPA-treated tubules (Fig. 7B). However, addition of the relatively selective EGF receptor tyrosine kinase inhibitor AG 1478 (0.3 μmol/L) blunted the tubule formation in TPA-treated NRK<sup>protHB-EGF</sup> cells (Fig. 7C). Furthermore, addition of an anti-rat HGF antibody (T. Nakamura, Osaka University, Osaka, Japan) or an anti-GDNF antibody (R & D Systems, Minneapolis, MN, USA) did not affect tubule formation in TPA-treated NRK<sup>protHB-EGF</sup> cells. Quantitation of the percentage of colonies with branching processes is indicated in Figure 8. As also indicated in Figure 8, neither TPA-treated wt nor NRK vector formed branching processes when plated under the identical conditions. In addition, no tubulogenesis was ob-
Fig. 3. Branching morphogenesis in NRK<sup>proHB-EGF</sup>. After plating on plastic substratum in the absence of FCS, TPA-treated NRK<sup>proHB-EGF</sup> underwent branching morphogenesis, (A, 10 h; B, 12 h; C, 14 h; D, 16 h; E, 20 h). Over the ensuing 16 hours, further tubule-like development was noted (F, 24 h; G, 30 h; H, 36 h). In contrast, without TPA pretreatment, NRK<sup>proHB-EGF</sup> plated on plastic in the absence of FCS presented as characteristic epithelial cell colonies (I, 24 h).

served if Wt or NRK<sup>vector</sup> were plated in collagen gels in the presence of soluble HB-EGF (10<sup>−7</sup> mol/L).

DISCUSSION

Cell proliferation, motility, differentiation and extra-cellular matrix production are all critical events during embryonic tissue development [41]. Metanephric kidney development is characterized by epithelial cell growth and differentiation from the ureteric buds and induction of the surrounding mesenchymal cells [3]. Although molecular mechanisms underlying these processes have not been completely clarified, inductive signals regulating renal tubulogenesis may be mediated by both cell-cell and cell-extracellular matrix (ECM) interactions [42]. Studies in genetically engineered mice have demonstrated an essential role for GDNF/c-ret interactions in induction of nephrogenesis [5–8], and other studies have also indicated an essential role for bone morphogenic protein-7 (BMP-7)/OP-1 [9, 10]. Other growth factors such as HGF and IGF-1 have been implicated in metanephric development in studies utilizing cultured metanephroi or in vitro tubulogenesis assays [11–13], although genetically engineered mice deficient in either HGF or insulin-like growth factors have not exhibited phenotypic abnormalities in renal development [43–45], suggesting redundancy in these regulatory pathways.

Previous studies also have indicated a role for EGF or EGF family members in renal development [14–18, 46–53]. Both the number and extent of tyrosine phosphorylation of EGF receptors increase in the developing kidney during late fetal development, co-incident with the timing of tubulogenesis and glomerulogenesis [14, 15]. Addition of EGF to uninduced metanephrogenic mesenchyme in culture induces differentiation into stroma but not epithelial cells, arguing against an essential role for EGF family members in induction of the metanephric blastema [46]. However, EGF or TGF-α has been shown to be important for branching tubulogenesis of renal epithelial cell in matrigel, and in complete metanephric organ culture, in which induction of the mesenchyme by ureteric bud occurs, administration of EGF increases growth and differentiation of collecting ducts but decreases differentiation of glomeruli and proximal tubules.
Furthermore, administration of tyrosine kinase inhibitors, EGF receptor blocking antibodies or anti-TGF-α antibodies to metanephric cultures all block differentiation of the structures arising from ureteric bud [48, 49]. Homozygous mice engineered with a targeted disruption of the EGF receptor also have evidence of abnormalities in differentiation of structures derived from ureteric bud [50]. Of interest, two groups have reported that in fetal kidney, EGF administration decreases apoptotic cell death in the nephrogenic zone of the developing kidney cortex and in the developing medullary papilla [54, 55].

It has not yet been determined which EGF-like growth factors may be mediating the EGF receptor-mediated responses in renal development. Salido et al reported that EGF was expressed substantially only at the end of differentiation [48, 49]. Homozygous mice engineered with a targeted disruption of the EGF receptor also have evidence of abnormalities in differentiation of structures derived from ureteric bud [50]. Of interest, two groups have reported that in fetal kidney, EGF administration decreases apoptotic cell death in the nephrogenic zone of the developing kidney cortex and in the developing medullary papilla [54, 55].

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Fig. 5. Growth of TPA-treated NRK(proHB-EGF) in collagen gels at (A) day 0, (B) day 1, (C) day 2, (D) day 3, (E) day 4, (F, G) day 6. In panels D–F, arrows indicate developing lumen. In panel G, Lu is the lumen and JC the junctional complex; small arrows show the microvilli.

Fig. 6. Expression of MMP-1 in TPA-treated NRK(proHB-EGF) in collagen gels. Either mRNA or protein was extracted at the indicated time and probed for expression of MMP-1 mRNA or immunoreactive protein.

In addition to EGF and TGF-α, other members of the EGF family of growth factors include amphiregulin, vaccinia growth factor, betacellulin, and HB-EGF [20–22, 32]. A common structural feature of these peptides is single or multiple EGF-like repeats in the extracellular domain, consisting of six cysteine residues that form three intramolecular disulfide bonds [32]. As an initial investigation of whether other EGF family members might be involved in renal development, we evaluated the role of HB-EGF in epithelial tubular morphogenesis. Immunohistochemical staining and northern blot analysis revealed that both HB-EGF protein and mRNA were strongly expressed in the embryonic kidney at day 14 to 18, with persistence of high expression in the neonatal period. Kidney by PCR but was expressed at low levels (60 cycles were required for detection) [49].

TGF-α mRNA was detected by in situ hybridization in day 8 to 10 mouse embryonic mesonephric kidneys but was absent at later times in the developing metanephric kidney, nor was there any detection of TGF-α mRNA by Northern blot analysis in rat embryos after embryonic day 10 [52, 53]. TGF-α mRNA was detected in cultured metanephric kidney by PCR but was expressed at low levels (60 cycles were required for detection) [49].
Fig. 7. Effect of an EGF receptor tyrosine kinase inhibitor on growth TPA-treated NRKproHB-EGF in collagen gels. NRKproHB-EGF were plated in collagen gels without TPA treatment (A), which gave shorter, less well developed tubule-like structure than in cells plated in the presence of TPA (B). When TPA treated cells were simultaneously exposed to AG1478 (0.3 μmol/L), tubulogenesis was blunted (C).

NRKvector when grown in 1% FCS, and when NRKproHB-EGF were plated in the absence of FCS, NRKproHB-EGF had greater attachment, spreading and cell-cell contact than NRKvector [32]. Our previous studies also demonstrated that expression of proHB-EGF in NRK-52E cells protected against acute cell injury-induced apoptosis in the absence of serum or other exogenous growth promoting agents [32].

Goishi et al previously demonstrated that the phorbol ester, TPA, can cleave membrane-associated HB-EGF to produce soluble HB-EGF, with subsequent recovery of membrane anchored HB-EGF after 12 to 24 hours [39]. In the present studies, when NRKproHB-EGF were treated with TPA, washed with 2 mol/L NaCl to remove adherent soluble HB-EGF and then replated in FCS-deficient medium, they formed branching tubule-like structures. PKC activation initiates a variety of cell signaling processes in addition to proHB-EGF cleavage. It should be noted, however, that the addition of TPA did not induce tubule formation in vector transfected cells, indicating that PKC activation alone was not a sufficient stimulus for tubulogenesis. Our previous studies with NRK-52E cells, in which neither expression of proHB-EGF or addition of soluble HB-EGF alone induced tubule formation [32], indicates the importance of simultaneous activation...
of PKC-mediated pathways and EGF receptor-mediated pathways

It is noteworthy that the timing of reappearance of membrane associated HB-EGF coincided with the initiation of branching, and that membrane associated HB-EGF was found predominantly at the sites of cell-cell attachment. This finding suggests that juxtacrine or tightly coupled autocrine interactions of HB-EGF with adjacent EGF receptors may be crucial for the development of the observed branching. Therefore, a combination of PKC activation and expression of membrane associated HB-EGF may be necessary to initiate tubule formation in NRK52E cells.

Although these initial studies did not elucidate the cellular mechanisms underlying this interaction, it is noteworthy that TPA treatment induced expression of the metalloproteinase, MMP-1, in the proHB-EGF transfected cells. Previous studies have suggested that phorbol ester-induced metalloproteinase activity is also important for in vitro angiogenesis [36]. Since proHB-EGF is a survival factor for NRK-52E cells [32], and EGF appears to inhibit apoptosis during nephrogenesis [54, 55], it is also possible that the predominant effect of proHB-EGF was to prevent apoptosis and thereby allow other PKC-dependent tubulogenic processes to proceed. However, in preliminary studies, we determined that in cells cultured in 1% FCS, in which no detectable apoptosis was observed, tubulogenesis again occurred in response to TPA treatment only in NRK52E and not in NRKvector.

It has been suggested that membrane-anchored growth factor precursors are biologically active, and may contact and activate cognate receptors on adjacent cells, establishing a mode of stimulation known as juxtacrine stimulation [32]. In recent studies, we detected increased tyrosine phosphorylation in A431 cells after addition of formalin-fixed NRK 52E cells transfected with proHB-EGF but not transfected with vector alone [32], demonstrating that membrane-attached proHB-EGF expressed in NRK 52E cells can potentially activate EGF receptors on adjacent cells. Whether juxtacrine receptor activation of the EGF receptor by proHB-EGF or other EGF-like growth factors elicits different cellular responses than activation by soluble growth factors has not yet been determined.

Previous studies have indicated an important role for growth factors in control of cell motility. In this regard, EGF has been shown to stimulate the nondirected movement of keratinocytes [56, 57]; although Higashiyama et al have shown that HB-EGF can act as a motility factor for smooth muscle cells [28], no previous reports have addressed the effect of HB-EGF on motility for renal epithelial cells. In the present study, we demonstrated that in addition to increased migration on the plate, cells transfected with proHB-EGF exhibited remarkable changes in shape, associated with the rearrangement of F-actin and α-tubulin. These findings suggest that HB-EGF may be a mediator of cell motility in NRK 52E cells.

Finally, it is important to remember the inherent limitations of in vitro systems in investigating tubulogenesis. As an example, HGF induces tubulogenesis in MDCK cells, while EGF ligands are ineffective [17]. In contrast, EGF ligands effectively promote tubulogenesis in IMCD-3 cells [18], as well as in NRK52E cells. Sakurai et al have evaluated the tubulogenic activity of cultured medium derived from cultured metanephric mesenchymal cells and have determined that only approximately 50% of tubulogenic activity was inhibited by a combination of AG1478 and anti-HGF antibody treatment [58] suggesting that EGF receptor ligands may be only one of a number of participant factors that induce and/or modulate tubulogenesis in the developing kidney.

In summary, these results indicate that membrane-anchored HB-EGF initiates and facilitates renal epithelial cell branching, possibly by promoting cell-cell and cell-matrix interactions and regulating cell movement. We speculate that HB-EGF may play a role in distal tubule growth and differentiation during metanephric development. Furthermore, in the adult kidney, in response to tubular injury, soluble HB-EGF may be cleaved from its membrane-anchored precursor by proteases and participate in recovery of the injured cells and cell proliferation. As cells recover and differentiate, proHB-EGF will be restored to the cell surface and may act as a juxtacrine or tightly coupled autocrine factor to mediate reorganization of cell-cell and cell-ECM contact. Further studies will be needed to define the intracellular signaling pathways activated in response to such interactions.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant DK51265 and by funds from the Department of Veterans Affairs.

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