Induction of Collecting Duct Morphogenesis In Vitro by Heparin-Binding Epidermal Growth Factor-Like Growth Factor

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Abstract. Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the epidermal growth factor family of growth factors, is synthesized as a membrane-anchored precursor (proHB-EGF) that is capable of stimulating adjacent cells in a juxtacrine manner. ProHB-EGF is cleaved in a protein kinase C-dependent process, to yield the soluble form. It was observed that HB-EGF acts as a morphogen for the collecting duct system in developing kidneys. HB-EGF protein was expressed in the ureteric bud of embryonic kidneys. Cultured mouse ureteric bud cells (UBC) produced HB-EGF via protein kinase C activation. After stimulation with phorbol ester (12-O-tetradecanoylphorbol-13-acetate) or recombinant soluble HB-EGF, UBC cultured in three-dimensional collagen gels formed short tubules with varied abundant branches. When proHB-EGF-transfected UBC were stimulated with 12-O-tetradecanoylphorbol-13-acetate and cultured in collagen gels, they exhibited linear growth, forming long tubular structures with few branches at the time of appearance of proHB-EGF on the cell surface. The structures exhibited a strong resemblance to the early branching ureteric bud of embryonic kidneys. When UBC were cultured in the presence of transforming growth factor-β and soluble HB-EGF, they formed long tubules and few branches, similar to the structures observed in proHB-EGF-transfected UBC. These cells exhibited apical-basolateral polarization and expression of the water channel aquaporin-2. These findings indicate that soluble HB-EGF and proHB-EGF induce branching tubulogenesis in UBC in different ways. Juxtacrine activation by proHB-EGF or the synergic action of soluble HB-EGF with transforming growth factor-β is important for well balanced morphogenesis of the collecting duct system.

The initial events in the process of mammalian kidney development are characterized by proliferation of ureteric bud cells (UBC) and tunneling of these cells into the surrounding undifferentiated mesenchyme (1–3). The ureteric bud forms branching tubules, with subsequent differentiation into the collecting ducts. Simultaneously, cells of the metanephric mesenchyme differentiate into epithelial cells that eventually develop into nephrons. These processes can be affected by several locally produced growth factors, including growth factor (EGF), transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF), glial cell line-derived neutrophic growth factor (GDNF), insulin-like growth factor-1 (IGF-1), and IGF-2. These growth factors are produced by the ureteric bud or metanephric mesenchyme (2,4–6).

Since the initial discovery of EGF and its receptor, other growth factors that signal through the EGF receptor, i.e., the so-called EGF family of growth factors, have been identified. Heparin-binding EGF-like growth factor (HB-EGF), a relatively new member of this family, was originally purified from conditioned medium from U-937 cells, which were derived from a human histiocytic lymphoma (7). HB-EGF demonstrates potent mitogenic functions in several types of epithelial cell lines, including renal and intestinal epithelial cells, through activation of the intrinsic tyrosine kinase of the EGF receptor (8–10). Mesenchymal cell-derived cells, such as mesangial cells, vascular smooth muscle cells, fibroblasts, and keratinocytes, can also be stimulated to proliferate (11–14). The mitogenic activity of HB-EGF is potentiated through binding to cell surface heparan sulfate proteoglycans (15). Similar to other EGF family members, HB-EGF is initially synthesized as a membrane-anchored precursor (proHB-EGF), which exists as a
206-amino acid transmembrane protein (29 kD) and undergoes processing to an 86-amino acid secreted protein (mature HB-EGF, 22 kD) in a protein kinase C (PKC)-dependent process (7,16,17). ProHB-EGF can contact and activate cognate receptors on adjacent cells through binding to EGF receptors, establishing a mode of stimulation known as juxtacrine stimulation (10,18). During the process of organ development, juxtacrine stimulation can be an efficient method to selectively deliver mitogenic signals to adjacent cells. We recently reported that, when rat renal epithelial cells (NRK 52E, a cell line with distal nephron characteristics) were stably transfected with proHB-EGF, they proliferated and underwent tubulogenesis (19).

Using UBC, we demonstrate here that HB-EGF can induce branching tubulogenesis in three-dimension collagen gel cultures. Differences in morphogenesis induced by HB-EGF with and without cooperation with TGF-β or proHB-EGF were also examined.

**Materials and Methods**

**Cell Culture**

UBC were isolated and established from the embryonic ureteric buds of mice transgenic for SV40 T antigen (20). The cells were identified as authentic UBC because they possessed epithelial- and ureteric bud-specific markers. UBC were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma Chemical Co., St. Louis, MO) containing 10% fetal calf serum (FCS) (Hyclone, Logan, UT), 100 U/ml interferon-γ (Genzyme, Cambridge, MA), 100 U/ml ampicillin, and 100 μg/ml streptomycin. ProHB-EGF-transfected UBC (UBCproHB-EGF) or empty vector-transfected UBC (UBCvector) were continuously grown in the aforementioned medium supplemented with geneticin (G418 sulfate, 400 μg/ml; Life Technologies, Gaithersburg, MD).

Immortalized metanephric mesenchymal cells (MS-7 cells) were obtained from an 11.5-d embryonic kidney from a homozygous mouse transgenic for H-2Kb-tsA (21). The collected embryonic kidney exhibited two ureteric bud branches. After trypsinization, mesenchymal caps were carefully isolated with a thin needle. After the cells were triturated, suspended, and placed on collagen-coated dishes, they were grown in minimal essential medium with 10% FCS and interferon-γ. The mesenchymal phenotype was confirmed by positive immunofluorescence for vimentin and absent immunofluorescence for Dolichos biflorus lectin.

**Production of Collagen Gels**

Five volumes of type I collagen solution (Cellmatrix IA; Nitta Zelatin Inc., Osaka, Japan) were mixed with 4 vol of 2.5-fold concentrated DMEM and 1 vol of 0.05 M NaOH with 2.2% NaHCO3 and 200 mM Hepes, followed by incubation at 37°C. Cells (105 cells/well plate) were resuspended in ice-cold gel mixture. In particular experiments, the gel mixture was supplemented with human recombinant soluble HB-EGF (rHB-EGF) (R & D Systems, Minneapolis, MN) and/or human recombinant TGF-β1 (R & D Systems). The solution was dispensed into plastic culture dishes (Falcon six-well plates; Becton-Dickinson, Franklin Lakes, NJ) in 3-ml aliquots and was allowed to solidify for 5 to 10 min at 37°C. The collagen gels were overlaid with DMEM containing 1% FCS and were incubated at 37°C.

**cDNA and Vectors**

Mouse membrane-anchored HB-EGF cDNA was prepared according to the previously described method (10). In brief, mRNA was extracted from kidneys from BALB/C mice (weighing 30 to 40 g) that had been treated with gentamicin. A 688-bp fragment representing the mouse membrane-anchored proHB-EGF coding region was amplified by PCR from the mRNA, using the primer pair of 5’-GGACCATT- GAAGCTGTGCGCGTCAAACTGCTCAGAAGT-3’ and 5’-TAACGAAACCACGTCGCTCTCAAGT-3’ (23). The PCR amplification was performed with 30 cycles of 94°C for 30 s, 72°C for 60 s, and 58°C for 45 s. The PCR product was then sequenced to confirm identity with the previously published sequence data for the mouse membrane-anchored proHB-EGF coding region (23). The PCR fragment was initially ligated into pPCRII vector (Invitrogen, San Diego, CA). A HindIII/NorI-digested cDNA fragment was then ligated into an eukaryotic expression vector (pRc/CMV plasmid; Invitrogen). Rat TGF-β cDNA was a gift from Dr. Enyu Imai (The First Department of Internal Medicine, Osaka University Faculty of Medicine, Osaka, Japan).

**Transfection**

Cultured UBC were stably transfected with proHB-EGF/pRc/CMV by the use of lipofectin (Life Technologies) and were subsequently selected by continuous growth in geneticin (G418, 400 μg/ml; Life Technologies). After five passages, 50 individual clones were isolated and screened for proHB-EGF expression by dot Northern blot analysis, using a 32P-labeled, EcoRI-digested, membrane-anchored HB-EGF cDNA fragment. Finally, five clones with high expression levels were identified. Two of these, UpHB 7 and UpHB 14, were used in this study. As a control, UBC were transfected with pRc/CMV plasmid alone and maintained in the presence of geneticin.

**RNA Isolation and Northern Blot Analysis**

Collagen gels containing UBC were homogenized in 2 vol of Isogen solution (Nippon Gene, Tokyo, Japan), followed by RNA extraction as described elsewhere (24). RNA from monolayer cultured cells on plastic dishes was also collected and subjected to Northern blot analysis, according to previously described methods (10,25). Aliquots of total RNA (each containing 15 to 20 μg) were subjected to electrophoresis, transferred to S & S Nytran nylon membranes (Schleicher & Schuell, Keene, NH), and hybridized to the membranes by exposure to ultraviolet light (CL-1000 ultraviolet crosslinker; Funakoshi Co., Tokyo, Japan). The membranes were prehybridized at 42°C in a solution containing 30% formamide, 5× SSC, 5× Denhardt’s solution (Wako Pharmaceutical Co., Osaka, Japan), and 100 μg/ml sonicated salmon sperm DNA (Invitrogen). The blots were hybridized overnight at 42°C with 0.5 to 1.0 cpm/ml 32P-labeled cDNA. After two washes each with 2× SSC for 15 min at room temperature, one wash with 1× SSC/0.1% sodium dodecyl sulfate (SDS) for 15 min at 65°C, and finally one wash with 0.2× SSC/0.1% SDS for 15 min at 65°C, 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 cDNA (Clontech, Palo Alto, CA), using the conditions described above. mRNA levels were quantitated by densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

**Antibodies**

Goat anti-mouse HB-EGF antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). This antibody, which was raised against a peptide corresponding to an amino acid sequence located at the carboxyl terminus of proHB-EGF of mouse origin, can recognize both proHB-EGF and the soluble form of HB-EGF and also crossreacts with rat HB-EGF (according to the information provided by the manufacturer). The following antibodies were also used: mouse...
monoclonal antibody to the EGF receptor (Transduction Laboratories, Lexington, KY), mouse monoclonal antibody to phosphorytrosine (clone PY20; Transduction Laboratories), mouse monoclonal antibody to phosphoinositide-3-kinase (PI-3-kinase) (Transduction Laboratories), rabbit anti-EGF-β antibody (R & D Systems), rabbit antibody to matrix metalloproteinase-1 (MMP-1) (Chemicon, Temecula, CA), goat antibody to MMP-2 (Santa Cruz Biotechnology), rabbit antibody to rat aquaporin-2 (26), alkaline phosphatase-conjugated goat anti-mouse IgG (EY Laboratories, San Mateo, CA), rabbit anti-goat IgG antibody (Cappel Research Products, Durham, NC) labeled with FITC, and goat anti-rabbit IgG antibody (Cappel).

**Immunoblotting**

UBC and MS-7 cells cultured in the medium described above were solubilized in RIPA buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 50 mM NaF, 100 μM NaVO₄, 5 mM ethylenediaminetetraacetate, 0.1% SDS, 0.5% sodium deoxycholate, 1% aprotonin). The protein concentration in the lysates was measured by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The lysates, containing equal amounts of protein (300 μg), were incubated with the primary antibody, followed by incubation with Pansorbin (Calbiochem, La Jolla, CA). After electrophoresis on 6% SDS-polyacrylamide gels, the proteins were transblotted onto Immobilon-P membranes (Millipore Japan, Yonezawa, Japan) in transfer buffer (25 mM Tris-HCl, pH 8.5, 192 mM glycine, 20% methanol) at 30 V for 16 h. After incubation at room temperature in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 3% bovine serum albumin, the membranes were incubated again with the primary antibody (the antibody used for immunoprecipitation) for 2 h at room temperature, followed by incubation with the appropriate secondary antibody conjugated with alkaline phosphatase. The reaction products were detected using a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Phosphorylation of the EGF receptor in UBC was demonstrated by immunoprecipitation with anti-EGF receptor antibody at the first step described above and detection with anti-phosphotyrosine antibody on the blotted membranes at the second step.

Rat embryonic kidneys (embryonic days 14 and 18) and kidney tissues after birth (1 d, 10 d, and 12 wk after birth) were collected and homogenized with a Dounce homogenizer in RIPA buffer. After centrifugation, the supernatants were dialyzed against phosphate-buffered saline (PBS) and freeze-dried. The materials were then dissolved in RIPA buffer. The fractions (300 μg of protein) were subjected to immunoprecipitation with anti-HB-EGF antibody and then to Western blotting, as described above.

**Determination of [³H]Thymidine Incorporation**

Cells were cultured in 24-well plates until confluent and were incubated in FCS-free DMEM for 72 h before the addition of rsHB-EGF (10⁻⁷ M). Subsequently, 1 μCi/ml [³H]thymidine was added and the cells were incubated for an additional 4 h. After two washes with ice-cold PBS, the cells were incubated for 30 min on ice with 10% TCA and were rinsed with ethanol. The acid-precipitated pellets were dissolved in 0.25 M NaOH/0.1% SDS and quantitated by scintillation counting.

**Immunocytochemical Staining**

Rat kidney tissues were fixed in paraformaldehyde-lysine periodate, treated sequentially with 10, 15, and 20% sucrose in PBS, and finally embedded in paraffin solution. UBC were cultured in six-well plastic dishes, coated with CELL-TAK cell and tissue adhesive solution (Collaborative Biomedical Products, Bedford, MA) until confluent, and then fixed in 1% paraformaldehyde/PBS for 15 min. The rat kidney tissue sections or cultured cells were examined by indirect immunofluorescence or immunoperoxidase staining methods, using the antibodies described above, as previously reported (27,28). As controls, tissue sections or cells were incubated with nonimmune goat or rabbit serum, followed by incubation with the appropriate secondary antibody, or were incubated with the secondary antibody alone. These controls yielded completely negative results.

**Electron Microscopic Observations**

Collagen gels containing UBC were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.4) for 12 h. After extensive washing with cacodylate buffer, collagen gels were scraped from the culture dishes, cut into 3-mm x 3-mm sections, postfixed with 1% osmic acid/PBS (pH 7.2) for 60 min, and stained with uranyl acetate in 50% ethanol. The stained sections were then dehydrated in graded ethanol and embedded in Epon 812 (Taab, Alderdmaston, UK). Ultrathin sections (600 to 900 Å) were prepared and examined by electron microscopy.

**Results**

**HB-EGF mRNA and Protein Expression in UBC**

Immunocytochemical staining of rat kidney tissues revealed HB-EGF protein expression in the ureteric bud of the rat metanephros (embryonic day 16). However, staining was negative in the remaining structures (Figure 1A). In rat kidneys after birth, immunoreactivity for HB-EGF was almost absent. Western blot analysis of kidney tissues with anti-HB-EGF antibodies revealed the presence of a single band of 22 kD in fetal kidneys, with weak or absent staining in kidney tissues after birth (Figure 1B). In UBC monolayers cultured in plastic dishes, HB-EGF mRNA expression was minimal at baseline levels and increased after 12-O-tetradecanoylphorbol-13-acetate (TPA) incubation (0.1 nM TPA for 30 min) (Figure 1C). HB-EGF mRNA expression was completely blocked by coinoculation with staurosporine (an inhibitor of the PKC pathway). Immunofluorescence analysis demonstrated that, after stimulation with TPA, HB-EGF protein appeared on the surface of UBC, condensed in areas of cell-cell attachment, suggesting the synthesis of membrane-anchored HB-EGF (Figure 1D).

**EGF Receptor Phosphorylation, PI-3-Kinase Expression, and Cell Growth Induced by rsHB-EGF**

UBC were cultured in the presence of rsHB-EGF (10⁻⁷ M). In Western blot analyses, the UBC lysate immunoprecipitates exhibited EGF receptor phosphorylation (Figure 2A) and PI-3-kinase upregulation (Figure 2B). After exposure to rsHB-EGF, UBC exhibited an approximately threefold increase in [³H]thymidine incorporation (Figure 2C).

**Branching Tubulogenesis of UBC Treated with TPA and Cultured in Type I Collagen Gels**

After stimulation with TPA for 30 min, UBC were immersed in type I collagen gels in medium containing 1% FCS. The cells developed short branches by day 1 and extended into the surrounding collagen matrix, exhibiting tubular structures with...
varied diffuse branches, by day 2 or 3 (Figure 3, a to d). When TPA-treated UBC were cultured in the presence of neutralizing anti-HB-EGF antibody, branching differentiation was partially inhibited (Figure 3e). Northern blot analysis of mRNA from UBC grown in collagen gels demonstrated that HB-EGF mRNA expression was upregulated after treatment with TPA and peaked in the subsequent 24 to 48 h (data not shown), concomitant with the morphologic changes of branching cord elongation.

Branching Tubulogenesis of UBC Grown in Collagen Gels Supplemented with rsHB-EGF

When UBC were cultured in collagen gels supplemented with rsHB-EGF (30 ng/ml) in medium containing 1% FCS, they began to develop short branching cords by day 1 and gradually formed abundant arborization by days 2 to 4 (Figure 4A, a and b). The structures resembled those found in UBC stimulated with TPA. Expression of MMP-1 was upregulated in these cells (Figure 4B). Addition of neutralizing anti-HB-
EGF antibody to collagen gels inhibited tubulogenesis and branching (Figure 4A, c).

**Tubulogenesis of UBC Transfected with Membrane-Anchored HB-EGF (ProHB-EGF)**

To investigate the role of proHB-EGF (the membrane-anchored form of HB-EGF) in the process of branching tubulogenesis, UBC constitutively expressing proHB-EGF (UBCproHB-EGF) were established and cultured in collagen gels. HB-EGF expression at the mRNA and protein levels by UBCproHB-EGF was confirmed by Northern blotting and immunodetection (Figure 5A). Because cleavage of proHB-EGF to its soluble form occurs within 60 min after activation of the PKC pathway and membrane-anchored proHB-EGF is recovered within the subsequent 6 to 12 h (29), we incubated UBCproHB-EGF with 0.1 mM TPA for 30 min. The cells were then extensively rinsed with medium containing 2 M NaCl, to remove soluble HB-EGF trapped by cell surface heparan sulfate proteoglycans. UBCproHB-EGF cultured in monolayers were stained with anti-HB-EGF antibody. After plating for 12 h, proHB-EGF protein appeared on the surface membrane, especially at cell-cell contact sites (Figure 5B).

After incubation with TPA and washing with medium containing 2 M NaCl, UBCproHB-EGF were immersed in collagen gels and cultured in medium containing 1% FCS. The controls, i.e., wild-type UBC and UBCvector, were treated in a similar manner. UBCproHB-EGF formed short epithelial branching cords during the first 12 h and then elongated, exhibiting linear tubular structures within 24 to 36 h (Figure 6). After 48 to 96 h, they formed branches. Compared with UBC cultured in the presence of TPA or rsHB-EGF, UBCproHB-EGF exhibited longer tubules with minimal branches, accompanied by well organized luminal structures. Electron microscopic analyses revealed luminal and basolateral polarization, similar to the findings presented in Figure 7D. The cells exhibited microvillus-rich apical surfaces. Aquaporin-2, which is normally present in collecting ducts (26), was immunocytochemically detected in the UBC (Figure 5C). Wild-type UBC and UBCvector in culture exhibited only clusters of round cells. There were no differences in the levels of TGF-β mRNA expression among the three cell types (data not shown).

**UBC Culture with rsHB-EGF and TGF-β**

When UBC were cultured in collagen gels containing rsHB-EGF (30 ng/ml) and recombinant TGF-β1 (2 ng/ml), they developed single branching cords by 12 h, which subsequently differentiated to long tubules with little branching (Figure 7, A to D). Finally, the UBC formed straight tube-like structures with less branching and well organized lumina, resembling structures that developed in UBCproHB-EGF. UBC cultured with rsHB-EGF alone, without recombinant TGF-β1, formed abundant short branches (Figure 7E). Addition of TGF-β1 alone to UBC did not induce tubulogenesis. To determine the source of TGF-β, Northern blot analysis of mRNA from UBC and MS-7 cells was performed. TGF-β mRNA and protein expression...
was detected in mesenchymal cells (MS-7 cells) but not in UBC (Figure 8). TGF-β1 suppressed the expression of MMP-1 in rsHB-EGF-stimulated UBC (Figure 4B).

Discussion

In epithelial tissues, including lung, pancreas, salivary gland, and kidney ureteric bud, proper tubulogenesis (ductogenesis) and branching morphogenesis are essential for organ development (2,30). In the metanephric kidney, branching of the ureteric bud and invasion into the surrounding undifferentiated mesenchymal tissues, followed by interactions between invading UBC and mesenchymal cells, are crucial events (1–3). Several soluble factors, such as HGF, EGF, TGF-α, IGF-1, GDNF, and bone morphogenic protein-7, have been demonstrated to induce epithelial cell tubulogenesis. On the basis of accumulating evidence (1–3), it is likely that the balance between growth-promoting factors (e.g., HGF), branching-promoting factors (e.g., GDNF), and growth-inhibiting factors (e.g., TGF-β) regulates branching tubulogenesis. Available data also suggest redundancy in these regulatory pathways, because genetically engineered mice that lacked HGF, c-Met (HGF receptor), TGF-α, or IGF-1 exhibited mostly normal metanephric development (31–33). EGF receptor-knockout mice developed dilated collecting ducts and impaired renal function (34). Tyrphostin AG1478, an inhibitor of EGF receptor tyrosine kinase, blocked tubulogenesis of mIMCD3 cells grown in collagen gels (5). Culture studies using embryonic kidney rudiments demonstrated that sulfated glycosaminoglycans were upregulated during nephron development (35). Furthermore, removal of these molecules, although it did not affect c-Met expression, inhibited ureteric bud growth and branching (35), suggesting that ureteric bud growth and morphogenesis are regulated by distinct signaling pathways mediated via sulfated glycosaminoglycans. These data collectively indicated that growth factors that belong to the EGF family, other than EGF or TGF-α, and have an affinity for sulfated molecules might be important for collecting duct morphogenesis.

Figure 5. (A) Expression of HB-EGF mRNA in UBC transfected with mouse proHB-EGF cDNA. Hybridization with a mouse proHB-EGF cDNA probe revealed a 1.0-kb band in proHB-EGF-transfected UBC (UBCproHB-EGF) (lane c) but not in wild-type (lane a) or empty vector-transfected (lane b) cells. In Western blot analyses, proHB-EGF protein (29 kD) expression was detected in UBCproHB-EGF (lane f) but not in wild-type (lane d) or empty vector-transfected (lane e) cells. By immunocytochemical staining, HB-EGF was demonstrated to be present on the surface of UBCproHB-EGF (g). (B) Recovery of membrane-anchored proHB-EGF in UBCproHB-EGF. After reaching confluency, UBCproHB-EGF were treated with TPA, followed by washing with 2 M NaCl-containing Dulbecco’s modified Eagle’s medium to remove cleaved soluble HB-EGF trapped on the cell surface heparan sulfate proteoglycans. The cells were then plated on plastic dishes coated with type I collagen (see Materials and Methods). Twelve hours after plating, cells formed attachments with neighboring cells, with subsequent appearance of single epithelial cell strands (a). HB-EGF protein was detected on the cell surface, particularly intensely at the areas of cell-cell attachment (b). (C) Expression of aquaporin-2 in UBCproHB-EGF. UBCproHB-EGF cultured for 72 h and stained for aquaporin-2 exhibited positive immunofluorescence (b). The control sample, which was stained with nonimmune rabbit serum and the secondary antibody, was negative (a). Magnifications: ×200 in A; ×100 in B and C.

Figure 6. Branching tubulogenesis of UBCproHB-EGF in three-dimensional collagen gels. Tubule-like structures appeared 24 h after plating. The cells progressively elongated and formed minimal arborization with 48 to 96 h of incubation. Magnification, ×100.
Therefore, we examined the role of HB-EGF in ureteric bud growth and branching. In the initial study, we noted that HB-EGF protein was immunocytochemically evident in the ureteric bud and that HB-EGF mRNA expression was upregulated in cultured UBC after TPA stimulation. The exogenous addition of HB-EGF to quiescent UBC stimulated cell proliferation, with tyrosine phosphorylation of the EGF receptor. Next, we observed that, after TPA incubation, UBC formed tubule-like structures in collagen culture. Similar morphologic features were observed when UBC were grown in the presence of rsHB-EGF alone, without recombinant TGF-β1, formed abundant short branches (E). Magnifications, ×100 in A, B, C, and E; ×3000 in D.

We addressed the role of proHB-EGF (the membrane-anchored form of HB-EGF) in collecting duct morphogenesis. It is known that cleavage of membrane-anchored proHB-EGF to its soluble peptide is regulated by PKC-dependent proteolysis. Maximal loss of cell surface proHB-EGF occurs within 30 to 60 min after PKC activation, and membrane-anchored proHB-EGF is restored within the subsequent 6 to 12 h (29). We treated UBC(proHB-EGF) with TPA, removed soluble HB-EGF, and then placed the cells in collagen gels. ProHB-EGF reappeared at the cell surface membrane, particularly at cell-cell contact sites. Without addition of any other growth factors, the cells formed linear tubules with minimal branches. The superficial structures of the cultured cells exhibited a strong resemblance to the early branching ureteric bud of embryonic kidneys. In contrast, the soluble form of HB-EGF induced varied diffuse growth, with branching. The difference between the branching caused by soluble HB-EGF and that produced by the membrane-bound form may be explained by juxtacrine activation, which is a unique mode of action exhibited by proHB-EGF. In general, juxtacrine activation has an advantage with respect to constant signaling exclusively to adjacent cells. In our previous studies, proHB-EGF-transfected NRK 52E cells (derived from rat distal nephron) exhibited TPA-dependent differentiation in vitro, resulting in the formation of single tubule-like structures (19). The cells exhibited increased migration on the plates and remarkable changes in shape associ-
ated with the rearrangement of F-actin and α-tubulin. UB-C
proHB-EGF in this study or proHB-EGF-transfected NRK 52E
cells in our earlier study (10) exhibited no differences in the
levels of TGF-β mRNA expression, compared with wild-type
or vector-transfected cells. Tubulogenesis mediated by proHB-
EGF is different from that mediated by HGF (6,36,37) or
soluble HB-EGF and is also independent of TGF-β mRNA
expression. Therefore, it is speculated that juxtacrine activa-
tion, which is a unique action of proHB-EGF, is related to the
formation of long tubules. In addition, our preliminary studies
suggested that proHB-EGF, compared with soluble HB-EGF,
tends to promote cell survival by inhibiting apoptosis (41),
which might facilitate differentiation to form tubular structures
in UBC. However, the precise mechanisms involved in juxta-
crine activation by membrane-anchored growth factor, eliciting
cellular morphogenesis responses, as distinct from activation
by the soluble form of the growth factor, are still unclear.

We observed that UBC cultured in the presence of both
rsHB-EGF and TGF-β exhibited long tubules with few
branches, similar to those formed in UBCproHB-EGF. TGF-β is
a multifunctional peptide that exerts its effects in the suppres-
sion of cell proliferation, the stimulation of extracellular matrix
formation, which is a unique action of proHB-EGF, is related to the
expression of MMP-1 in HB-EGF-stimulated UBC. Previous
studies from other laboratories revealed that, in
in situ hybridization analyses, TGF-β mRNA signals were most prominent
in the mesenchymal/stromal cells adjacent to branching tubules and
TGF-β protein expression seemed to be greatest near the
ureteric bud (43.44). In mMCD3 cells grown in collagen gels
in the presence of HGF, TGF-β inhibited the expression of
MMP-1 and urokinase and stimulated the synthesis of their
inhibitors (24). Therefore, mesenchymal cells can be a source
of TGF-β. It seems that TGF-β contributes to the formation
of long linear tubules by inhibiting branching via modulation of
the balance between matrix-degrading enzymes and their
inhibitors.

In summary, these results indicate that HB-EGF may be
one of the regulators involved in the localization of branches
determination of the stalk length between branches. The
soluble form of HB-EGF can facilitate branching. In con-
trast, membrane-anchored HB-EGF can induce linear tubu-
lar structures with few branches, which resemble those that
form in the presence of rsHB-EGF and TGF-β. These data
provide a basis for future research to produce highly differ-
etiated tubules. The refinement of in vitro tubule culture

techniques would make it possible to obtain artificial tu-
bules for the treatment of developmental disorders of the
kidney or acute tubular injury.

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