Hereregulin Induces Glial Cell Line-derived Neurotrophic Growth Factor-independent, Non-branching Growth and Differentiation of Ureteric Bud Epithelia*  

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We have purified a protein present in a conditioned medium derived from the metanephric mesenchyme that supports non-branching growth and epithelial differentiation of the isolated ureteric bud (UB) independent of glial cell line-derived neurotrophic growth factor (GDNF). By sequential liquid chromatography, together with protein microsequencing, the protein was identified as heregulin (HRG) α. The addition of recombinant HRG to the isolated UB grown in three-dimensional culture confirmed the proliferative activity of HRG. In branching UBs induced by whole metanephric mesenchyme cell-conditioned medium, proliferating cells were localized at ampullae, where a binding receptor for GDNF, GFRα1, was found. In HRG-induced UBs, however, the expression of GFRα1 was down-regulated, and proliferating cells were distributed throughout the structure. Electron microscopic examination of the HRG-induced UB revealed the presence of structurally mature and polarized epithelial cells reminiscent of the epithelial cells found in the stalk portion of the branching UB. cDNA array analysis further revealed that genes ontologically classified as developmental were down-regulated by HRG, whereas those involved in transport were up-regulated. For example, the mRNA for the GDNF receptors, GFRα1 and ret9, was down-regulated, whereas the mRNA for collecting duct transporters, such as urea transporter2, aquaporin3, and sodium-hydrogen exchanger2 was up-regulated in HRG-treated UBs compared with UBs grown in the presence of branch-promoting factors. Moreover, HRG promoted growth of UBs cultured in the absence of GDNF. Taken together, the data suggest that HRG supports UB epithelial cell differentiation and non-GDNF-dependent growth, raising the possibility that this kind of activity plays a role in the growth and differentiation of the stalk portion of the branching epithelial UB. Branching morphogenesis is essential for the development of many epithelial organs including the lung, salivary gland, mammary gland, and kidney. In the kidney, growth factors such as glial cell line-derived neurotrophic growth factor (GDNF), pleiotrophin (PTN), fibroblast growth factor (FGF)s, and transforming growth factor-β superfamily have been implicated in this process. However, beyond cell proliferation and survival, how the actions of these growth factors translate into forming a branched tubular or ductal structure is not well understood.  

To form a branching structure, it appears necessary to have clear tip and stalk regions. In fact, it has recently been shown that there are at least two distinct cell types in the developing ureteric bud, ret-expressing tip cells and ret-negative stalk cells (1). These cells clearly have different fates, tip cells are fated to remain within the tip and make new branches or to move toward the stalk region to become stalk cells, whereas stalk cells are fated to remain within the stalk portion. For ret-expressing tip cells, GDNF plays an important role in their maintenance. Bone morphogenetic protein 4 and FGF1 or FGF10 have been suggested to facilitate the elongation of the stalk (2, 3); however, factors that largely or exclusively cause proliferation and epithelial differentiation without branching, such as occurs in elongating stalks, have not been reported.  

For an isolated UB to grow and branch robustly in vitro, GDNF alone is not sufficient. Conditioned medium from metanephric mesenchyme derived cells (BSN-CM) is required. Previously, we isolated PTN from this conditioned medium and were able to demonstrate that a combination of PTN and GDNF is sufficient to induce in vitro branching morphogenesis of the isolated UB (4). During the fractionation of this medium and isolation of PTN, the presence of a potent non-branch-promoting yet growth-inducing factor was noted.  

Here, we have purified this factor from BSN-CM and have identified it as heregulin (HRG). Close examination of the HRG-induced UB revealed that the cells of these non-branching UBs displayed a predominant stalk cell-like morphology and expressed markers of maturation, raising the possibility that HRG may act as a stalk-promoting factor.  

MATERIALS AND METHODS  

Growth Factors  

Recombinant growth factors, GDNF, and HRGα were from R&D systems. FGF1 was purchased from Calbiochem.  

Cell Culture  

The metanephric mesenchyme-derived cell line (BSN cells) was cultured in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% fetal calf serum at 37 °C in an atmosphere of 5% CO2. Conditioned medium was collected after incubation with serum-free Dulbecco’s modified Eagle’s medium/F12 for 3–4 days.  

Protein Purification  

2–3 liters of BSN-CM was concentrated ~50-fold by Ultratase tangential flow devices (5K molecular weight cutoff; Gelman Sciences). Morphogenetic activity of BSN-CM was retained in the ~5,000 Dalton fraction, but not in the <5,000 Dalton flow-through. The buffer of the
concentrated BSN-CM was changed to 0.4 M NaCl, 50 mM Hepes, pH 7.2, buffer and applied to a heparin-Sepharose column (HiTrap Heparin 5 ml, Amersham Bioscience). Heparin-bound proteins were eluted via increasing concentrations of NaCl (0.4-2 M), and individual 5-ml fractions were collected. The 0.9–1.2 M NaCl heparin eluate fractions were adjusted to 1.7 M ammonium sulfate in 50 mM Hepes, pH 7.2, buffer and applied to a Resource Phenyl hydrophobic interaction column (Amersham Bioscience). Bound proteins were eluted via decreasing concentrations of ammonium sulfate (1.7-0 M) in 50 mM Hepes, pH 7.2, buffer. The 1.5–1.6 M ammonium sulfate fractions were dialyzed against 50 mM Hepes, pH 7.2, buffer and applied to a Resource S cation exchange column (Amersham Bioscience), followed by elution with increasing concentrations of NaCl (0–1 M). The morphogenetic activity of each fraction collected from this column was assayed using the isolated rat UB culture system (see below). An apparently homogenous protein band (see Fig. 1, lane 2) was excised from Sypro-Ruby (Bio-Rad) stained Tris-glycine SDS-polyacrylamide gels (Cambrex) and analyzed by in gel digestion followed by microcapillary HPLC and tandem mass spectrometry (Harvard Microchem).

**In Vitro Morphogenesis Assay**

**Isolated Rat UB Culture**—As previously described (4, 5), UBs were microdissected from E13 rat embryonic kidney and suspended within extracellular matrix gels (1:1 mixture of type 1 collagen and growth factor reduced Matrigel, BD Biosciences), applied to the inner wells of a 24-well Transwell insert (Costar, 0.4-µm pore size). UBs were cultured in either whole BSN-CM (positive control), Dulbecco’s modified Eagle’s medium/F12 (negative control), dialyzed fractions (to Dulbecco’s modified Eagle’s medium/F12) or recombinant HRG (250–500 ng/ml); 125 ng/ml of GDNF, 250 ng/ml of FGF1, and 10% fetal calf serum, were also added to the outer wells in most cases. These cultures were placed in a 37°C, 5% CO2 incubator. Phase-contrast pictures were taken with Spot RT digital camera attached to Nikon microscope. In some cases, cultures were stained with rhodamine-conjugated phalloidin (Molecular Probes) and observed by scanning laser confocal microscopy (Zeiss LSM-510). Image analysis was done by Image Pro software.

**Electron Microscopy**—Isolated UBs cultured for 5 days in the presence of HRG as described above were fixed in Karnovsky’s fixative for 1 h at room temperature and processed for ultrastructural analysis as described previously (3). Thin sections were mounted onto mesh grids, stained with lead citrate and uranyl acetate, and examined with a Zeiss EM109 microscope.

**BrdUrd Incorporation Assay**—As previously described (3), cultured ureteric buds grown for 4–5 days were labeled with 10 µM BrdUrd for 30 min. After extensive washing with phosphate-buffered saline, the UBs were fixed in 4% paraformaldehyde in phosphate-buffered saline. The incorporated BrdUrd was detected following a protocol of the Cell Proliferation kit (Amersham Biosciences) except for the use of fluorescein-conjugated anti-mouse IgG (Jackson ImmunoResearch) as a secondary antibody. After counterstaining with rhodamine-conjugated Dolichos biflorus lectin (Vector Lab), the samples were analyzed by scanning laser confocal microscopy (Zeiss LSM-510).

**cDNA Array**—For each treatment (HRG, BSN-CM, PTN), three isolated UBs were cultured in the presence of FGF1, GDNF, and indicated

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**FIGURE 1. Identification of non-branching growth inducing activity.** A, active fractions from a Resource Phenyl hydrophobic interaction column (Fraction 3) were applied to a Resource S cation exchange column. Numbers (1–4) in the chromatogram indicate fraction number. B, fractions 1–4 were subjected to SDS-PAGE, followed by Sypro-Ruby staining (upper panel). Fraction 2 has a band between 40 and 50 kDa, which was subsequently identified as HRGs by mass spectrometry-based method. Western blotting of these fractions probed with anti-HRG antibodies confirmed the identification. C, morphogenetic activity of these fractions and indicated purified proteins was assayed by isolated rat UB culture. Photographs were taken at 5– 6 days of culture. Bars = 100 µm.
factor for 4 days. For each condition, 3 separate wells were prepared. RNA was isolated using PicoPure RNA isolation kit (Arcturus). Double IVT reaction and hybridization to Affymetrix rat gene chip 230A was performed at UCSD Cancer center GeneChip core facility. GCOS (Affymetrix) and Genesifter (VizX lab) software was used for data analysis. The gene expression data are available in the Gene Expression Omnibus website (ncbi.nlm.nih.gov/projects/geo/, accession number GSE3394).

**Immunohistochemistry**—Whole-mount staining of isolated UBs in three-dimensional culture was performed as described elsewhere (6). Antibodies/staining reagents were as follows: Topro-3 nuclear staining dye (1:1000, Molecular Probes), goat anti-rat GFRα1 antibody (1 μg/ml, R&D systems), rabbit anti-occludin antibody (1:100, Zymed Laboratories Inc.), and mouse anti-β-catenin monoclonal antibody (1:200, BD Transduction Lab). The samples were observed with Zeiss LSM-510 confocal microscope.

**RESULTS**

BSN-CM Contains Non-branching Growth-inducing Activity for the Isolated UB—Fractionation of BSN-CM by multiple sequential liquid column chromatography has consistently revealed the presence of several growth-inducing activities in BSN-CM including a non-branching yet growth-inducing activity as well as a branch-promoting activity and an inhibitory activity. Thus far, a branch-promoting factor (PTN) (4) and growth/branch-inhibiting factors (transforming growth factor-β superfamily) (7) have been identified. In this study, we have purified a non-branching growth-inducing factor, which on a heparin-Sepharose column was found to elute prior to the branch-promoting activity (data not shown). This fraction was further separated on a Resource Phenyl-Sepharose column, and the non-branching growth-inducing activity was found to elute around 1.5–1.6 mM ammonium sulfate (Fig. 1A).

Heregulin α in BSN-CM Induces Non-branching Growth of the Isolated UB—This fraction from the Resource Phenyl column was further separated by Resource S cation exchange chromatography, and an apparently homogenous protein containing fraction with non-branching growth-inducing activity was obtained (Fig. 1). The homogenous protein band eluted from the Resource S column at 0.6 mM NaCl (in Fig. 1B, a band between 40 and 50 kilodalton present in fraction 2) was subsequently identified as HRGα by mass spectrometry. As expected, recombinant human HRGα induced non-branching growth of the isolated UB comparable with that seen with the native purified protein (Fig. 1C).

Heregulin-α Induces Non-branching Polarized Epithelial Proliferation of the Ureteric Bud in Three-dimensional Culture—To further evaluate the morphogenetic effect of HRGα, isolated UBs were cultured in the presence and absence of HRGα for several days. HRG stimulated overall growth of the isolated UB in the presence of GDNF and FGF1, compared with growth in the presence of only GDNF and FGF1, leading to the formation of non-branching structures morphologically distinct from BSN-CM-treated UBs (Fig. 1C). Confocal microscopic examination of phalloidin-stained UBs revealed the presence of a clear lumen with a much larger caliber in HRG-treated UBs compared with controls (Fig. 2A). As shown in Fig. 2B, electron microscopy demonstrated that the cells comprising these structures were epithelial in nature with characteristics usually associated with polarized epithelial cells. For example, the nuclei were uniformly localized to the basal portion of the cells, and intercellular junctions were readily apparent, presumably leading to the separation of basolateral and apical membrane domains.
### A

| Ontology                                                                 | List | Up  | Down | Array  | z-up  | z-down |
|--------------------------------------------------------------------------|------|-----|------|--------|-------|--------|
| physiological process                                                   | 363  | 156 | 207  | 3257   | 0.14  | 2.26   |
| metabolism                                                               | 257  | 121 | 136  | 2007   | 3.81  | 2.17   |
| cellular process                                                         | 238  | 114 | 124  | 2444   | -0.39 | -3.1   |
| protein metabolism                                                       | 95   | 56  | 39   | 821    | -1.11 | -1.67  |
| cell communication                                                       | 92   | 43  | 49   | 1201   | -2.32 | -3.34  |
| development                                                              | 83   | 51  | 32   | 770    | 2.7   | -2.38  |
| nucleoside, nucleotide and nucleic acid metabolism                      | 78   | 47  | 31   | 623    | 3.55  | -1.15  |
| transport                                                                | 73   | 19  | 54   | 907    | -4.32 | -0.04  |
| signal transduction                                                      | 67   | 28  | 39   | 888    | -2.57 | -2.27  |
| cell proliferation                                                       | 61   | 47  | 14   | 244    | 8.11  | -1.56  |
| morphogenesis                                                            | 60   | 37  | 23   | 543    | 2.41  | -1.85  |
| organogenesis                                                            | 57   | 35  | 22   | 512    | 2.36  | -1.72  |
| protein modification                                                     | 56   | 37  | 19   | 406    | 4.34  | -1.17  |
| cell cycle                                                               | 44   | 37  | 7    | 237    | 8.08  | -2.03  |
| phosphate metabolism                                                     | 40   | 23  | 17   | 308    | 2.32  | -0.35  |
| phosphorous metabolism                                                   | 40   | 23  | 17   | 308    | 2.32  | -0.35  |
| carboxylic acid metabolism                                               | 34   | 9   | 25   | 183    | 0.1   | 4.49   |
| organic acid metabolism                                                  | 34   | 9   | 25   | 183    | 0.1   | 4.49   |
| cell surface receptor linked signal transduction                         | 33   | 17  | 16   | 516    | -1.69 | -2.96  |
| phosphorylation                                                          | 33   | 20  | 13   | 260    | 2.29  | -0.69  |

### B

- **Ret9**
- **GFRa1**
- **GFRa2**
- **AQP3**
- **Sle14a2 (Urea transporter)**
- **Sle9a2 (NHE2)**
aspect of the cells contained multiple secretory granules, which can be seen in contact with the luminal surface (Fig. 2B, c, arrows). As expected, the adherens junction-associated protein, β-catenin, was localized at the site of cell-cell contact, and the tight junction protein, occludin, was localized at the luminal side (Fig. 2C).

Heregulin Treatment Does Not Maintain GFRα1 Expression and Leads to GDNF-independent Growth and Maturation of the Ureteric Bud—We next sought to investigate how HRG induced non-branching growth. To address this question, the gene expression profile of either whole BSN-CM or PTN-treated branching UBs was compared with the HRG treated UBs on cDNA arrays. First, the overall gene expression profile among HRG-treated, non-branching UBs, and either BSN-CM- or PTN-treated branching UBs was compared using the MAPPFinder algorithm built into the Genesifter application (8). This analysis provides information on the probability (i.e., Z score) that a change in expression of a group of genes (i.e., ontological class) is because of random chance or is likely to be a real effect. A gene was included in the analysis when the gene was determined to be either present in all three

FIGURE 4. A, whole mount immunostaining of isolated UB cultured in the presence of HRG or BSN-CM plus FGF1 and GDNF. a, GFRα1 (green) and β-catenin (red) staining of isolated UB grown in the presence of HRG or BSN-CM and FGF-1 and GDNF. B, BrdUrd (Brd2) incorporation in isolated UBs in three-dimensional culture. Rat isolated UBs were cultured in the presence of either HRG or BSN-CM plus FGF1 and GDNF for 5 days at which time the UBs were labeled with BrdUrd, which was detected with mouse anti-BrdUrd antibodies and fluorescein isothiocyanate-conjugated anti-mouse IgG (green). UBs were counterstained with rhodamine-conjugated lectin D. biflorus (DB) (red). Pictures presented here are three-dimensional reconstruction images from confocal microscopy. Bar = 100 µm. B, left, phase contrast pictures of rat isolated UBs cultured in the presence of HRG (500 ng/ml) and FGF1 (250 ng/ml) or FGF1 alone (control) for 1 week. Note that UBs in these experiments were cultured in the absence of GDNF. Bar = 100 µm. The graph shows quantification of UB area between HRG-treated UBs and control UBs. Error bars indicate S.E. n = 5 for each condition. Right, confocal microscopic analysis of HRG-treated UBs in the absence of GDNF stained with β-catenin. Arrows indicate lumens. Bar = 20 µm.

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FIGURE 3. Differential mRNA expression profile of non-branching UBs (HRG-treated) and branching UBs (BSN-CM or PTN-treated) obtained from cDNA array analysis. A, differentially expressed genes (expression level > 1.5-fold at p < 0.05) were categorized according to their ontology as obtained by Genesifter software. HRG-treated condition was compared with either PTN-treated (upper) or BSN-CM treated (lower) conditions. Numbers in the List column indicate total number of differentially regulated genes. Ontological classes containing more than 32 differentially expressed genes were listed. Up/Down columns indicate the numbers of genes up-regulated or down-regulated in branching (PTN or BSN-CM treated) conditions in comparison with non-branching (HRG-treated) condition. Numbers in the Array column indicate total number of genes in each ontology class on cDNA array. Extreme numbers (>2 or < -2) in the Z score columns indicate that a certain process is either over-represented or under-represented among the differentially expressed genes. B, expression levels of GDNF receptor genes (Ret and Gfra1) and those encoding transporters expected to be present in the mature collecting duct (aquaporin3 (AQP3), urea transporter, and sodium-hydrogen exchanger2 (NHE2)) were compared among branching and non-branching UBs. Note that another Gfra family gene, Gfra2 was not differentially expressed among these conditions. Expression level was normalized to median of all experiments. Error bars indicate S.E.
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experiments or present in two experiments and one marginal in at least one treatment condition. Of these genes, the differentially regulated (i.e. >1.5-fold at p < 0.05) genes were categorized according to their ontology (Fig. 3A). Genes up-regulated by PTN treatment were over-represented (Z score > 2) by genes thought to be involved in cell proliferation and development, whereas transport-related genes were under-represented (Z score < −2). Genes thought to be involved in acid metabolism were over-represented in the group of genes down-regulated by PTN. Genes involved in cell communication, signal transduction, and developmental processes were under-represented in groups down-regulated by PTN. Similar to the comparison of HRG versus PTN, genes involved in cell proliferation and development were over-represented among those genes up-regulated by BSN-CM treatment. Genes involved in metabolism were over-represented among those genes down-regulated by BSN-CM treatment, whereas genes involved in cell communication, signal transduction, and development were under-represented in this group of genes. From this data analysis, we hypothesized that HRG treatment decreases the overall proliferation of isolated UBs compared with pro-branching factors such as BSN-CM and PTN and directs them toward maturation. When we examined specific gene expression, it was noted that the expression level of both the binding receptor for GDNF, GFRα1, and the signal transducing receptor, ret, were down-regulated in the HRG-treated condition (Fig. 3B). On the other hand, consistent with the ultrastructural finding that mature appearing epithelial cells were lining HRG-treated UBs, the expression level of transporter genes expressed in the mature collecting duct, such as aquaporin3, the urea transporter, and sodium hydrogen exchanger 2, was up-regulated in HRG-treated condition (Fig. 3B).

The down-regulation of GFRα1 was confirmed at the protein level by immunostaining (Fig. 4A, a). In the BSN-CM-treated branching UB, GFRα1 was localized predominantly at the UB tip, whereas its expression was dramatically reduced in the HRG-treated UB. This expression pattern of GFRα1 raised the possibility that the “tip cell phenotype” is diminished in the HRG-treated UB. Because most of the proliferating cells found in the UB have been shown to be localized at the tip (1, 3, 6, 9), we examined the pattern of proliferation by BrdUrd incorporation. In contrast to the BSN-CM-treated branching UB, in which most proliferating cells are localized at the tip, proliferating cells in HRG-treated UB displayed no specific localization (Fig. 4A, b). Together with down-regulation of tip-localized GDNF receptors, GFRα1 and Ret, HRG is likely to stimulate non-GDNF-dependent UB cell proliferation that may be operational in stalk growth. The fact that HRG-treated UBs did not grow as large as BSN-CM-treated UBs (Fig. 4A), together with overall down-regulation of cell proliferation genes in the HRG-treated condition versus BSN-CM-treated condition, suggests that this proproliferative effect is weaker than that of tip proliferation-inducing factors.

Nevertheless, we further went on to confirm the ability of HRG to induce UB cell proliferation independent of GDNF. It was possible to maintain isolated UB survival in the presence of FGF1 without significant growth (Fig. 4B, control). Addition of HRG (without GDNF present) to this condition facilitated the growth as indicated by the increase in UB area (Fig. 4B). These HRG-treated UBs appeared to retain tubular epithelial structure with lumen as shown by confocal microscopy (Fig. 4B).

**DISCUSSION**

Taken together, our data suggest that HRG does not maintain the tip cell phenotype but promotes differentiation of the isolated UB, making it all stalk phenotype. Moreover, non-branching growth of the UBs induced by HRG treatment is independent of GDNF, raising the possibility that tip and stalk cells in the branching epithelial tissue are under the control of different sets of growth factors.

BSN-CM, derived from metanephric mesenchyme cells, contains at least three apparently independent morphogenetic activities that stimulate the isolated UB in three-dimensional culture: 1) a branch-promoting activity, 2) a non-branching growth-inducing activity, and 3) an inhibitory activity (4, 7). The presence of these multiple activities is consistent with a model of UB branching where multiple soluble factors secreted from the metanephric mesenchyme, perhaps under different spatial and temporal conditions, regulate the growth and shape of the UB-derived tubular tree. This is very different from cell culture models of branching in which a single growth factor (e.g. hepatocyte growth factor in MDCK cells or epidermal growth factor receptor ligands in mIMCD3 cells) is sufficient to induce growth and branching. Previous studies have identified proteins likely to be responsible for the branch-promoting (3–5) and inhibitory activities (7) present in BSN-CM. In this study, we have isolated a non-branching yet growth-inducing factor from BSN-CM and have identified it as HRGα. Both natural and recombinant HRG reproducibly induced the non-branching growth of the isolated UB in three-dimensional culture.

HRGα was originally isolated from a breast cancer cell line as an activator of the oncoogene/transmembrane tyrosine kinase receptor erbB2 (10, 11). HRGα is 1 of at least 15 isoforms differentially spliced from the neuregulin1 transcript (12). These neuregulin1 isoforms signal through their receptors erbB2, -3, and -4 (13) and have been shown to be critical in development of the central and peripheral nervous system as well as the heart (14–16) and mammary gland (17). Neuregulin1 transcripts have been detected in mesenchyme of the lung, intestine, stomach, kidney, and the genital ridge in early mouse development (18). In organ culture of the mammary gland, HRG has been shown to stimulate alveolar differentiation, whereas different growth factors stimulate branching morphogenesis (19, 20).

Our results using the isolated UB culture system are potentially compatible with this observation. HRG did not stimulate branching morphogenesis, but it promoted growth and maturation of the UB. Cells comprising these structures were epithelial in nature with characteristics associated with tight polarized epithelial cells (Fig. 2). We demonstrated that HRG-induced growth could be explained by loss of total and differential expression of GFRα1 (Figs. 3 and 4). We speculate that the presence of GFRα1 in cells at the branching tip might be a prerequisite for the UB to undergo branching morphogenesis. Thus, if GFRα1 were ubiquitously expressed along the UB at a relatively high level, the UB would be expected to display an “all-tip” phenotype, resulting in globular growth without any apparent stalk formation. On the other hand, if GFRα1 expression were reduced or non-existent, tip cells would not be able to respond to GDNF and would be expected to display an “all-stalk” phenotype. We suggest this is the case in UBs treated with HRG. Moreover, losing tip phenotype from the UB seems to confer maturation toward the collecting duct epithelium. Other conditions that lead to down-regulation of ret and GFRα1 (e.g. FGF1 alone or FGF1 and GDNF) also induced increased transporter/water channel expression as seen with HRG treatment.

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