An Endothelial Growth Factor Involved in Rat Renal Development

Juan A. Oliver and Qais Al-Awqati
Department of Medicine, Columbia University, College of Physicians & Surgeons, New York, New York 10032

Abstract

In the kidney, there is a close and intricate association between epithelial and endothelial cells, suggesting that a complex reciprocal interaction may exist between these two cell types during renal ontogeny. Thus, we examined whether metanephrogenic mesenchymal cells secrete endothelial mitogens. With an endothelial mitogenic assay and sequential chromatography of the proteins in the media conditioned by a cell line of rat metanephrogenic mesenchymal cells (7.1.1), we isolated a protein whose amino acid analysis identified it as hepatoma-derived growth factor (HDGF). Media conditioned with Cos-7 cell transfected with HDGF cDNA stimulated endothelial DNA synthesis. With immunoaffinity purified antipeptide antibodies, we found that HDGF was widely distributed in the renal anlage at early stages of development but soon concentrated at sites of active morphogenesis and, except for some renal tubules, disappeared from the adult kidney. From a 7.1.1 cells cDNA library, a clone of most of the translatable region of HDGF was obtained and used to synthesize digoxigenin-labeled riboprobes. In situ hybridization showed that during kidney development mRNA for HDGF was most abundant at sites of nephron morphogenesis and in ureteric bud cells while in the adult kidney transcripts disappeared except for a small population of distal tubules. Thus, HDGF is an endothelial mitogen that is present in embryonic kidney, and its expression is synchronous with nephrogenesis. (J. Clin. Invest. 1998. 102:1208–1219.) Key words: glomerulogenesis • angiogenesis • glomerular capillary • glomerular growth • hepatomas derived growth factor

Introduction

Development of an organ requires that morphogenesis of its parenchymal cells be synchronized with proliferation, migration, and morphogenesis of the endothelial cells of its vasculature. Anatomical examination of mature organs illustrates the complexity of this process. For example, in the kidney—the organ with the highest blood flow per unit mass in the body—there are three morphologically and functionally distinct capillary beds (glomerular, peritubular, and vasa recta) each in close contact with, and functionally linked to, a different nephron segment. An additional level of complexity arises from the fact that each nephron possesses its own vascular supply requiring that morphogenesis of its parenchymal and vascular components occur simultaneously. Each nephron is derived from a single terminal branch of a ureteric bud (an “ampulla”), which induces a group of metanephric mesenchymal cells to form the epithelial cells of the proximal part of the nephron. We (2) and others (3, 4) have found that as the ureteric bud generates new branches, each of these ampullae become tightly surrounded by endothelial cells, thereby providing a potential targeting mechanism for the vascularization of individual nephrons. This suggests that cells of the renal anlage might synthesize molecules regulating endothelial cell migration and location. Indeed, with monoclonal antibodies, we found an antigen synthesized by embryonic kidney parenchymal cells that colocalizes with migrating endothelial cells (2). This antigen likely provides embryonic renal endothelial cells with directional cues since addition of the antibody to developing kidney rudiments in vitro inhibited localization of these cells around ureteric bud ampulla (2).

Renal embryonic parenchymal cells also synthesize endothelial growth and chemotactic factors as well as molecules capable of regulating their phenotype. Some epithelial cells of the renal anlage synthesize vascular endothelial growth factor/vascular permeability factor (VEGF; 5), a very restrictive endothelial mitogen. Furthermore, while synthesis of VEGF markedly decreases as the kidney matures, synthesis continues in the glomerular epithelial cells of the adult kidney, likely contributing to the high permeability of the glomerular capillary (5–7).

In summary, the intricate arrangement between endothelial and epithelial cells in the adult kidney makes it likely that during its development there exists a complex reciprocal interaction between these two cell types. Accordingly, we postulated that embryonic renal parenchymal cells might secrete endothelial growth and chemotactic factors. We examined this hypothesis by using a cell line isolated from metanephrogenic mesenchymal cells (7.1.1 cells) at day 13 of embryonic age (E13). These cells have mesenchymal and epithelial markers (8) suggesting that they are mesenchymal cells transforming into epithelia, a process that occurs in the renal anlage at a time of dramatic endothelial growth and migration (1, 2, 9). We found that media conditioned with 7.1.1 cells contained endothelial chemotactic and growth activity. With an endothelial mitogenic assay and sequential chromatography of serum-free 7.1.1 cells–conditioned media, we isolated a protein whose amino acid sequence was identical to a recently cloned molecule derived from human hepatoma cells and named hepatoma-derived growth factor (HDGF; 10).

1. Abbreviations used in this paper: DEPC, diethyl pyrocarbonate; HDGF, hepatoma-derived growth factor; HMG-1, high mobility group 1 protein; PBS C/M, PBS with calcium and magnesium; VEGF, vascular endothelial growth factor.
Methods

Cell culture. The metaneprogenic mesenchymal cell line (7.1.1) has been reported (8). Bovine aortic endothelial cells were isolated and maintained as described (11). Rat aortic endothelial cells were isolated as described (12).

Cell growth assay. Growth stimulation activity was assayed by [3H]thymidine incorporation into bovine or rat (where noted) aortic endothelial cells. In brief, 105–106 cells were seeded in each well of a 24-well plate (GIBCO BRL, Gaithersburg, MD) and the next day were placed in MEM containing 0.5% FCS. 24–48 h later, the fractions to be tested were added to the cells in triplicate. After 18 h, the wells were rinsed with MEM, and 0.5–1.0 μCi/well of [3H]thymidine in MEM was added for 2–6 h. Incubation was ended by rinsing with ice-cold PBS and incubating the cells with 10% TCA at 4°C. After washing the wells with 1:3 ethanol/chloroform (vol:vol), they were incubated with 200 μl of 0.4 N NaOH at 60°C, and after neutralizing with 200 μl of glacial acetic acid, the samples were mixed with liquid scintillation fluid and counted.

Endothelial cell growth activity of the 7.1.1 cells–conditioned media was also assayed by determining the effect of the media on cell number. In brief, 3 × 104 bovine aortic endothelial cells in MEM with 10% FCS were seeded in 24-well plates, and 1 d later, the medium was changed to MEM containing 0.5% FCS. The next day, 10–100 μl of 10% FCS were seeded in 24-well plates, and 1 d later, the medium was changed to MEM containing 0.5% FCS. After washing the wells with 1:3 ethanol/chloroform (vol:vol), they were incubated with 200 μl of 0.4 N NaOH at 60°C, and after neutralizing with 200 μl of glacial acetic acid, the samples were mixed with liquid scintillation fluid and counted.

Isolation of HDGF. Confluent 7.1.1 cells growing in 150-cm2 flasks were rinsed twice with MEM (GIBCO) and incubated at 31°C with 5% CO2. MEM containing 5% FCS was added to MEM containing 0.5% FCS and 1 d later, the medium was changed to MEM containing 0.5% FCS. The next day, 10–100 μl of 7.1.1-conditioned media concentrated ~15-fold (or the same volumes of MEM) were added to wells in triplicate. 5 d later, the cells were trypsinized and counted in Trypan blue.

Transfection of Cos-7 cells with HDGF cDNA. HDGF cDNA was constructed by one of two methods. In one method, PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA) was performed with primers 5’-CCC.GCA.ATG.TCG.CGA.TCC.AAC.CGG.CAG.AAG.AGG.TAC-3’ (sense nucleotides 309–345) and 5’-GGT.TCC.CAG.TTT.GCA.GGC.CAT.GG-3’ (antisense nucleotides 1107–1129) containing, respectively, EcoRI and XhoI restriction sites. Analysis of the nucleotide sequence of the isolated product confirmed its identity as the cDNA for HDGF (not shown). After digestion with these enzymes, the PCR product was ligated into vector pCDNA3.1 (Invitrogen). Rat aortic endothelial cells (Invitrogen) was screened with a digoxigenin-labeled probe generated by PCR (Boehringer Mannheim, Indianapolis, IN) with the primers described above. Three positive clones were isolated, but none included the 5’ end of the HDGF cDNA. Accordingly, the clone containing the stop codon (with nucleotides 712–1080 of the HDGF cDNA) was digested with XhoI and MaeII (not shown) and was ligated to vector pCDNA3.1 (Invitrogen, Carlsbad, CA) and used to transfect Epicurian Coli XL1-Blue supercompetent cells (Stratagene). In the second method, a 7.1.1 cDNA library constructed from poly(A) RNA from 7.1.1 cells (Invitrogen) was screened with a digoxigenin-labeled probe generated by PCR (Boehringer Mannheim, Indianapolis, IN) with the primers described above. Three positive clones were isolated, but none included the 5’ end of the HDGF cDNA. Accordingly, the clone containing the stop codon (with nucleotides 712–1080 of the HDGF cDNA) was digested with XhoI and MaeII and ligated with a fragment of the PCR product described above after its digestion with EcoRI and MaeII (nucleotides 309–816 of HDGF cDNA). The resulting product was inserted into the pCDNA3.1 vector.

Immunohistochemistry. Embryonic kidneys were isolated and processed as described (2). Immunostaining with HDGF antibodies was done by incubating 4–6-μm sections with 50 μM NH4Cl followed by 10-min incubation with PBS C/M containing 0.1% Triton × 100. Antibody (10 μg/ml) was next added in PBS C/M containing 1% bovine serum albumin for 2 h. After extensive washing with PBS C/M containing 2.7% NaCl and 0.05% Tween 20, FITC or rhodamine-coupled goat anti–rabbit antibody (Jackson ImmunoResearch) in PBS C/M.
with 4% rat serum and 4% goat serum was incubated for 1 h. After washing, sections were mounted and examined with a fluorescence microscope. Control slides by incubating the primary antibody in the presence of a 100-fold molar excess of the peptide used to generate the antibody gave no signal.

**Reverse transcription PCR for HDGF.** RNA from 7.1.1 cells was isolated with RNazol (Tel-Test Inc., Friendswood, TX). 15 μg of total RNA were reverse transcribed with random hexamers with the GeneAmp PCR kit (Perkin Elmer, Norwalk, CT). PCR was then performed using primers derived from the human cDNA (13): 5'-AAC.CGG.CAG.AAG.GAG.GAG.TAC.CAA.A.A.TGC.C-3' (sense nucleotides 328–351) and 5'-GTT.CCC.AGT.TTG.CAG.GCC.ATG.G-3' (antisense nucleotides 1107–1128).

**PCR for HDGF in cDNA libraries of 7.1.1 cells and of E14 rat kidneys.** The rat E14 embryonic kidney cDNA library was a gift of Jonathan Barasch (Columbia University, New York, NY). Plasmids were purified as instructed in the QIAprep Spin Kit (QIAGEN, Valencia, CA). 500 ng of plasmid DNA were PCR with the GeneAmp PCR Reagent kit (Perkin Elmer) with the two primers described above. Nested PCR was performed with the product obtained with the two initial primers and the following internal primers: 5'-TTC.TTT.TTC.GAC.CCA.GAG.AC-3' (sense nucleotides 460–479); 5'-TTC.TTT.CTC.TTG.CCT.GGC.CTC-3' (antisense nucleotides 742–761); 5'-TTC.TGC.CTC.CTT.GGG.ACG.TT-3' (antisense nucleotides 814–834).

**Preparation of RNA probes and Northern blotting.** The 801-bp PCR product obtained from the 7.1.1 cells cDNA library was ligated into vector pCR II (Invitrogen) and cloned. The plasmid was linearized with EcoRV and BamHI, and sense and antisense RNA labeled with digoxigenin were synthesized with the DIG RNA labeling kit (Boehringer Mannheim). After measuring their concentration by serial dilutions on a nylon membrane, the probes were purified by precipitation with 4 M LiCl and ethanol washed (16).

The antisense probe was first tested in Northern blot hybridization with polyadenylated RNA from 7.1.1 cells. In brief, total RNA from 7.1.1 cells obtained as described above was incubated with Oligotex beads (QIAGEN), and polyadenylated RNA was isolated. 10 μg of poly(A)+ RNA were subjected to electrophoresis in a 1% agarose gel containing formaldehyde as described (16) and transferred onto Nytran nylon membrane (Schleicher & Schuell, Keene, NH). Hybridization was performed as described (16) with the antisense probe synthesized above, and chemiluminescent detection was performed with the Genius 7 Luminescent Detection Kit (Boehringer Mannheim).

**In situ hybridization.** 7.1.1 cells were washed with 0.1% DEPC-treated PBS and suspended by incubating them with PBS containing 0.2% EDTA. Except when incompatible, such as the presence of Tris, all solutions were treated with 0.1% diethyl pyrocarbonate (DEPC).

106 cells in 100 μl were cytopsin onto baked slides (ETHO cleaned, DEPC–H2O rinsed), allowed to dry and fixed in 3% paraformaldehyde in DEPC-treated PBS for 1 h at room temperature. After rinsing with PBS, slides with the cells were placed in 70% ethanol and progressively dehydrated with ethanol (85%, 100%) and xylene. Thereafter, they were processed as the tissue sections (see below).

Kidneys were microdissected and placed in 4% paraformaldehyde in PBS for 18 h at 4°C. After rinsing three times with PBS, they were placed into 70% ethanol. They were embedded in paraffin according to standard procedures. Sections of 4 μm were cut, and after drying the slides overnight at 50°C, they were dewaxed with xylene and rehydrated with sequential incubations with ethanol (100%, 95%, 70%, and 50%) and finally with H2O. Before hybridization, the slides were subjected to the following treatments: incubation with 0.2 N HCl for 5 min; rinsed with H2O; incubation with 10 μg/ml proteinase K (Boehringer Mannheim) in 50 mM EDTA, 100 mM Tris (pH 8.0) for 15 min at 22°C; rinsed with PBS; fixation with 4% paraformaldehyde in PBS for 10 min; rinsed with PBS; acetylation with 0.5% acetic anhydride in 0.1 M triethanolamine (pH 8.0) at 22°C for 10 min; rinsed with 0.1 M triethanolamine (pH 8.0) at 22°C for 40 min; and finally 0.1 M triethanolamine (pH 8.0) at 22°C for 15 min, and twice rinsed with SSC (0.15 M NaCl; 15 mM sodium citrate, pH 7.0).

**Table I. 3H-Thymidine Incorporation into Endothelial Cells Induced by 7.1.1 Cells–Conditioned Media**

<table>
<thead>
<tr>
<th>Cell</th>
<th>% above control</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAEC</td>
<td>57±6</td>
</tr>
<tr>
<td>RAEC</td>
<td>46±11</td>
</tr>
</tbody>
</table>

BAEC and RAEC, bovine and rat aortic endothelial cells. Mean±SE. n = 5 for each cell type with each experiment being the average of three wells.

**Results**

**Endothelial growth activity of 7.1.1 cells–conditioned media.** In initial studies, we examined whether 7.1.1 cells–conditioned media had mitogenic activity for endothelial cells. As shown in Table I, 50 μl of a 15-fold concentrate of 7.1.1 cells–conditioned media increased [3H]thymidine incorporation into bovine and rat aortic endothelial cells. Moreover, as shown in Table II, increasing volumes of conditioned media progressively increased the cell number of growth arrested bovine aortic endothelial cells.

**Purification of HDGF from 7.1.1 cells–conditioned media.** To isolate endothelial mitogens secreted by the 7.1.1 cells, the proteins in the media conditioned by the cells were subjected to sequential chromatography while the endothelial mitogenic activity was assayed by [3H]thymidine incorporation into bovine and rat aortic endothelial cells. Mean±SE; n = 4 with each experiment being the average of three wells. *P < 0.01.
vine aortic endothelial cells. First, 7.1.1 cells–conditioned media were applied to a heparin-Sepharose column (Fig. 1 A) and eluted with increasing concentration of NaCl. Two peaks of \(^{3}H\)thymidine incorporation activity eluted from the column and the peak eluting at 1 M NaCl (second peak of activity in Fig. 1 A) was injected into a cation exchange chromatography column. Two peaks of activity eluted (at 0.2 M and 0.4 M NaCl) from this column (Fig. 1 B) and the first peak (fractions 38 and 39) was processed further. The endothelial growth activity in these fractions showed a high degree of hydrophobicity and, unlike most other proteins in the sample, bound to the hydrophobic interaction column at low salt concentration (1 M NaCl). As shown in Fig. 1 C, a single peak of endothelial growth activity eluted from the hydrophobic interaction column. Radio-iodination of the proteins present in this peak revealed a prominent band with an apparent molecular mass of \~14 \text{ kD} in SDS/PAGE (Fig. 2 A). Because only limited amounts of active protein could be prepared from the hydrophobic interaction column, the active fractions eluting at 0.2 M NaCl from the cation exchange column (37 and 38 in Fig. 1 B) were subjected to SDS/PAGE, and after staining with Coomassie brilliant blue, the 14-kD band was excised from the gel and processed for protein sequencing as described in Methods. The sequences of two proteolytic fragments (Fig. 2 B) were identical to amino acids 45–61 and 81–95 of the recently identified human HDGF (10).

**Immunodetection of HDGF in 7.1.1 cells and embryonic kidney.** Two polyclonal rabbit antibodies were generated from a fragment (EGLWETENNPT) of peptide 2 (underlined in Fig. 2 B) and used for immunodetection of HDGF. 7.1.1 cell homogenates immunoblotted with immunopurified antibody (Fig. 3) showed an \~40 \text{ kD} band (7.1.1 cells lane). As shown in Fig. 3, an identical band was also detected in developing kidney. In serum-free 7.1.1 cells–conditioned media harvested after 3 d of incubation (Fig. 3, 3 days lane), the same \~40-kDa band was also detected. However, as shown in Fig. 3, an additional band of \~25 kDa and a less prominent band of \~35 kDa were also detected by the antibody. Furthermore, if conditioned media were harvested after more than 3 d of incubation, additional bands were detected by the antibodies.
culture (6 days lane), the ∼ 40-kD band disappeared, and only the prominent ∼ 25-kD and faint ∼ 35-kD bands could be detected. When the peptide used to generate the antibodies was added to the antibody during its incubation with the immunoblot (+ peptide lanes), neither the ∼ 40- nor the ∼ 25-kD bands were detected (Fig. 3).

Expression of HDGF cDNA in Cos-7 cells. The media conditioned by Cos-7 cells transfected with either the pCDNA3.1 vector (mock transfection) or the vector containing the cDNA for HDGF were analyzed by immunoblots. As shown in Fig. 4 A, while no protein was detected by the immunopurified antibody in the conditioned media of the mock-transfected cells, two proteins were apparent in the media from the cells transfected with HDGF cDNA. The most abundant protein had an apparent molecular mass of ∼ 40 kD (identical to that found in media from 7.1.1 cells) while the other was ∼ 35 kD. This smaller protein was also present, albeit in small amounts, in the 7.1.1 cells–conditioned media (see Fig. 3).

To test the mitogenic effect of the expressed HDGF, the media conditioned with the mock Cos-7–transfected cells and the media from the cells transfected with HDGF cDNA were injected into heparin-Sepharose columns to isolate the 1.0 M NaCl fractions. Interestingly, as shown in Fig. 4 B, immunoblots of the sample containing HDGF revealed a new immunoreactive band of ∼ 14 kD, the molecular mass of our initially isolated protein (see Fig. 2 A).

Fig. 5 shows the effect on [3H]thymidine incorporation into BAEC of the fractions eluting at 1.0 M NaCl from the heparin-Sepharose column. Whereas the fractions from the conditioned media of the mock transfection had no effect, those from the media of the cells transfected with HDGF cDNA increased [3H]thymidine incorporation. Similar results were obtained with Swiss albino 3T3 cells (not shown; 10).

Immunohistochemistry. Fluorescence immunocytochemistry with affinity-purified rabbit antibodies to HDGF detected this protein in the cytoplasm of all 7.1.1 cells, whereas nuclei showed no signal (Fig. 6). In these cells grown in vitro, staining was most intense in the perinuclear space, with a morphological appearance suggestive of Golgi distribution.

Immunohistochemistry of embryonic kidneys showed that location of HDGF changed markedly during kidney development. Upon arrival of the ureteric bud to the metanephrogenic...
Hepatoma-derived Growth Factor in Renal Development

Figure 5. Effect of conditioned media by Cos-7–transfected cells on [3H]thymidine incorporation into bovine aortic endothelial cells. The fraction eluting at 1.0 M NaCl from heparin-Sepharose chromatography of supernatants of Cos-7 cells transfected with HDGF cDNA increased [3H]thymidine incorporation, but the same fraction from mock-transfected Cos-7 (vector) did not show activity (n = 3). Assuming that the immunoblot signal of HDGF in Fig. 4 represents 5–20 ng, the concentration of recombinant protein used in these experiments was ~15–60 ng/ml.

Figure 6. Immunocytochemistry of 7.1.1 cells. 7.1.1 cells were fixed with 4% paraformaldehyde and permeabilized with 1% triton × 100. After incubation with immunopurified antibody, bound antibody was detected with FITC-conjugated anti-rabbit IgG. Control slides with a 100 molar excess of the peptide used to obtain the antibody gave no signal.

mesenchymal mesenchymal cells called “vesicles” (v; Fig. 7 B: E14). As kidney development advanced (Fig. 7 C; E15) HDGF preferentially located at sites of epithelial morphogenesis such as “comma” (c) and “s” bodies (s) as well as the tip of developing nephrons (Fig. 7 D; E17), including the glomerular tuft (g). Fig. 7 E (E19) shows that as glomeruli (g) developed, ureteric bud cell ampullae (ub) remained strongly positive. As glomeruli matured (Fig. 7 F; E19), HDGF became less prominent in the glomerular tuft (g) but was easily detectable in the glomerular parietal epithelial cells (pe). As shown in Fig. 7, A–F, encompassing renal anlages from E13 to E19, during the early and middle periods of renal development, HDGF is characteristically found in the periphery of the cells, at sites of cell-to-cell contact, perhaps suggesting that HDGF acts in a paracrine manner.

As development advanced (E20–21), HDGF became hardly detectable in the glomerular tuft (g; Fig. 7 G; E21) but was prominent in some renal tubules. In the adult (Fig. 7 H), glomeruli (g) had no detectable HDGF, but some renal tubules contained the factor. Most tubules had HDGF in well-defined vesicles and by their morphology could be identified as proximal tubules (pt; Fig. 7 H). Much weaker staining was present in fewer tubules that had a diameter ~28 μm and were thus identified as distal tubules (dt, Fig. 7 H). In contrast with the findings during the early phases of kidney development where HDGF located in areas of cell-to-cell contact, in the later stages of development and in the adult kidney, HDGF location had a vesicular characteristic suggesting that it is either released by a different mechanism or might even be endocytosed into the tubule from the blood stream.

HDGF expression in 7.1.1 cells and E14 kidneys. As assessed by RT–PCR, the predicted 801-bp transcript was detected in 7.1.1 cells (Fig. 8 A, lane 1), and its identity was confirmed by the presence of an Xmn I restriction site (lane 2). Furthermore, the appropriate 801-bp product comprising almost all translatable region of HDGF cDNA was also obtained by PCR of a 7.1.1 cDNA library (Fig. 8 B, lane 1) and a cDNA library from E14 rat kidneys (Fig. 8 C, lane 1). Identity of these products was established by nested PCR with internal primers (Fig. 8 B, lanes 2 and 3 and Fig. 8 C, lane 2). Furthermore, partial sequence of the product obtained with the PCR of the 7.1.1
library cDNA confirmed its identity as the cDNA for HDGF (not shown).

In situ hybridization. The riboprobe was first tested in Northern blot hybridization with polyadenylated mRNA from 7.1.1 cells. As shown in Fig. 9 A the riboprobe hybridized with a mRNA that is the same size as the mRNA of human HDGF (10). Fig. 9 B shows that in situ hybridization of 7.1.1 cells gave a strong signal with the antisense probe (top) and no signal with the sense probe (bottom).

Similarly, the sense probe gave no significant hybridization signal over renal tissue from different embryonic ages (Fig. 10, C, I, N, and Q show examples). In early stages of kidney development (E13-14), HDGF transcripts were detected both in the metanephrogenic mesenchyme (m) and ureteric bud (ub; Fig. 10 A; E14; Fig. 10 B; E15) albeit the hybridization signal was weak except in the outer part of the mesenchyme at E15 (Fig. 10 B).

There was a marked increase in the hybridization signal in the middle period of kidney development (E17-19). At E17 (Fig. 10, D, E, and F), hybridization was particularly strong in cells of the branching ureteric bud (ub; Fig. 10, D and E) and the mesenchymal cells of the mesenchymal-epithelial cells (pe). (G) E19, kidney (date of birth) with a glomerulus (g) and several proximal tubules (pt). (H) Adult kidney with several glomeruli (g), proximal (pt), and distal (dt) tubules.

Figure 7. Immunohistochemical analysis of developing kidney with immunopurified antibody to HDGF peptide. (A) E13 renal anlage with ureteric bud (ub) surrounded by metanephrogenic mesenchyme (m). (B) E14 renal anlage showing condensates of mesenchymal cells (vesicles, v) indicating mesenchymal-epithelial conversion. (C) E15 renal anlage with a comma (c) and s (s) bodies. (D) Early nephron with glomerular tuft (g) and proximal tubule (pt) at E17. (E) More mature glomerulus (g) and ureteric bud ampulla (ub) at E16. (F) Glomerulus (g) in an E19 kidney showing parietal epithelial cells (pe). (G) E21 kidney and several proximal tubules (pt). (H) Adult kidney with several glomeruli (g).
glomerular tuft developed, their hybridization signal became weaker (g, Fig. 10 K), but HDGF mRNA expression remained strong in the semicircular cluster of parietal glomerular epithelial cells (pe; Fig. 10 K) and in what appeared to be distal tubules.

1 wk after birth (Fig. 10, L and M), development is completed in the inner part of the kidney, and the hybridization signal there was negative except in tubules that entered the renal medulla, likely collecting ducts. In contrast, there was a weak hybridization signal in the outer cortex (oc) of the kidney where renal development continues in the rat; note embryonic epithelial structures such as “s” bodies (s, Fig. 7 M). In the adult kidney (Fig. 10, O and P) all glomeruli (g) were negative, and only a small group of renal tubules had a positive hybridization signal. The diameter of these tubules averaged 30±2 μm (n = 26) and were thus identified as distal tubules (dt, Fig. 7 P), which by their morphological appearance, appeared most likely to be collecting ducts.

Discussion

When the cells of the renal anlage organize to form mature nephrons they are surrounded by invading and proliferating endothelial cells (1, 2, 9). Fig. 11 shows a schematic diagram of the initial events in the development of the renal vasculature; endothelial cells invade the metanephrogenic mesenchyme from the ilium and periphery (A) and quickly proliferate throughout the developing kidney, simultaneously with the
condensation and epithelialization of the mesenchymal cells under the influence of the branching ureteric bud (B). As nephron morphogenesis advances, endothelial cells invade the crevice of the “comma” and “s” bodies (C). Morphogenesis and proliferation at these sites leads to development of the glomerular capillary bed in early (D and E) and mature glomeruli (F). The close association and parallel morphogenesis of renal parenchymal and endothelial cells makes it likely that a reciprocal interaction exists between these two cell types. With this in mind, we used media conditioned by a line of metanephrogenic mesenchymal cells to isolate an endothelial mitogen. Two proteolytic peptides of the isolated factor had complete identity with two segments of the amino acid sequence predicted from the cDNA of human HDGF (10). Immunoblots using antipeptide antibodies showed that HDGF from 7.1.1 cells and from E15 kidneys had an apparent molecular mass of \( \approx 40 \) kD. As the cDNA sequence of human HDGF predicts a protein of 240 amino acids (10), the \( \approx 40 \)-kD molecular mass is likely due to co-/post-translational modification of the protein. Indeed, in preliminary studies, we found HDGF to be both \( N \) - and \( O \)-glycosilated (C. Hikita, J. Oliver, and Q. Al-Awqati, unpublished observations).

Whereas the antipeptide antibody recognized a \( \approx 40 \)-kD protein in immunoblots of homogenates of 7.1.1 cells and rat E15 kidneys, in the media conditioned with 7.1.1 cells, the HDGF antibody recognized the \( \approx 40 \)-kD protein as well as a protein of an apparent molecular mass of \( \approx 25 \) kD and a less prominent protein of \( \approx 35 \) kD. In the initial isolation of HDGF by Nakamura et al. (10), the protein obtained from media conditioned with hepatoma cells also had an apparent molecular mass of \( \approx 25 \) kD. The smaller forms of HDGF could either be secreted forms of the intracellular protein (i.e., processed before secretion) or result from proteolysis after secretion. The second possibility is more likely because while both the \( \approx 40 \)-kD form of HDGF as well as the smaller forms of the protein could be detected in media conditioned for 3 d, only the \( \approx 25 \)-kD protein (as well as the less prominent \( \approx 35 \) kD) could be detected in media conditioned for 6 d. Furthermore, 7.1.1 cells only contained the \( \approx 40 \) kD form of HDGF regardless of the time of incubation in serum-free media (not shown). We thus conclude that the \( \approx 25 \)-kD form of HDGF is a fragment of the mature protein generated by extracellular proteolysis.

The cell growth assay we used during isolation of HDGF prevented the use of proteolytic inhibitors and proteolysis of the protein.
factor is the likely reason for our isolation of a 14-kD fragment of it. Indeed, the findings with the Cos-7 cells expressing HDGF are consistent with this view. Media conditioned with Cos-7 cells transfected with HDGF cDNA contained the ~40-kD form of HDGF and a smaller ~35-kD form (present only in small amounts in the media conditioned with 7.1.1 cells; Fig. 3). However, after heparin-Sepharose chromatography, a new form with an apparent molecular mass of ~14 kD was detected (Fig. 4).

The data from immunoblots of E15 kidney homogenates, PCR of the rat E14 kidney cDNA library as well as immunohistochemistry and in situ hybridization of embryonic kidney sections all indicate that HDGF is present and synthesized in the developing kidney and that its location as well as its synthesis is highly regulated during renal ontogeny. Several lines of evidence suggest that HDGF has an important role as a growth factor in nephrogenesis and perhaps in the morphogenesis of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the renal vasculature. First, HDGF is a growth factor. Though we isolated it after its endothelial growth activity, it is also effective in fibroblasts and some hepatoma cells (10) suggesting that like several other growth factors, it has some promiscuity in its effect, at least in cells in vitro. Needless to say, elucidation of HDGF-specific targets during renal ontogeny in vivo must await identification of its receptor and development of probes for its study, but the complex pattern of expression of HDGF mRNA suggests that HDGF may have a multifunctional role. Second, HDGF is synthesized by many cells of the developing kidney, and it is widely distributed through this organ, but except for some distal tubules, mRNA for HDGF disappeared from the kidney with completion of nephrogenesis. Third, mRNA for HDGF was highest during the most active period of nephron differentiation (E16–21), and both HDGF and its mRNA were found in segments such as “s” bodies that develop into the proximal part of the nephron. This part of the
nephron is heavily vascularized with an intricate arrangement between capillaries and epithelial structures likely to require many mechanisms of control during its development. mRNA for the specific endothelial mitogen, VEGF is also found in “s” bodies (5). Platelet-derived growth factor, another potential endothelial mitogen has also been localized in comma and s-shaped bodies (17), but its sites of synthesis in developing kidney remain to be determined. Of note is that HDGF and its mRNA were present in the parietal glomerular epithelial cells of developing glomeruli and disappeared in mature glomeruli. This temporal location suggests that HDGF might act as a growth factor for the final development of the glomerular capillary loop.

In embryonic kidney mRNA for HDGF was frequently found in the same cells that contain immunoreactive protein. However, during the early stages of development (E15-15) while the immunoreactive signal was quite prominent, the hybridization signal was weak suggesting that HDGF immunoreactive cells may accumulate protein from the circulation or that the half-life of the protein is longer. Similarly, proximal tubular cells in the adult kidney (with no detectable mRNA for HDGF) had vesicles with abundant HDGF. As many filtered proteins are endocytosed by these cells into vesicles, it is possible that the HDGF detected by the antibody in the proximal tubules may have originated in plasma.

HDGF has a 36% amino acid homology with the high mobility group 1 protein (HMG-1) and like this protein it lacks a signal peptide (10). HDGF has an internal hydrophobic region (located in peptide 1 of our isolate; Fig. 2) that could function as an internal signal sequence, but its mechanism of secretion is unknown. Interestingly, HMG-1 has been identified as amphoterin (18) a protein that increases neurite outgrowth (19) and appears to be involved in neural development (19, 20). HMG-1/amphoterin is located on cell surfaces and leading edges of neurons and other cell types suggesting that it plays a role in cell invasion (19, 21, 22). As shown in Fig. 5, during early stages of kidney development, HDGF is also found in the periphery of metanephrogenic mesenchymal and ureteric bud cells suggesting a role for this factor in cell-to-cell interaction and cell movement.
In summary, HDGF is present and synthesized during kidney development in a synchronous manner with nephrogenesis. Whether the role of HDGF during kidney development is restricted to its action on endothelial cells or it has other targets requires identification of its receptor and location of it in embryonic kidney. As the receptor for HMG-1/amporphin is a member of the immunoglobulin superfamily (20, 23), one of its members is a potential candidate for the receptor of HDGF. Its identification and location during kidney development will provide invaluable information on the role of HDGF in this process.

**Acknowledgments**

This work was supported by National Institute of Diabetes and Digestive Diseases Grant DK-46934.

**References**