Kidney development starts when an epithelial tubule called the ureteric bud invades a mass of loosely associated mesenchymal cells called the metanephrogenic mesenchyme. The ureteric bud "induces" mesenchymal cells to proliferate and convert to epithelia. Although it has long been known that rescue from apoptosis (7, 23) and epithelialization (47) of the metanephrogenic mesenchyme requires signaling by the ureteric bud, the signaling molecules remain to be identified. Attempts to identify them by screening growth factors has yielded either inactive molecules (transforming growth factors [TGFs], insulin-like growth factors, platelet-derived growth factors, and retinoic acid; see Ref. 55) or factors that are mitogenic but have not been shown to be expressed by the ureteric bud during its invasion of the mesenchyme (epidermal growth factor, fibroblast growth factor [FGF], Wnt-1, hepatocyte growth factor; Refs. 1, 16, 55, 57).

To identify the molecular mechanisms by which the ureteric bud induces the metanephrogenic mesenchyme, we isolated ureteric bud cell lines (UB cells) from embryonic mice transgenic for SV40 T-antigen (1). We found that these cells act as appropriate surrogates for the ureteric bud, since they induce the mesenchyme to grow and differentiate into epithelia that form tubules. We also found that mesenchymal "induction" can be separated in two steps. In one step, soluble molecules secreted by UB cells rescue the mesenchyme from apoptosis and stimulate it to proliferate without inducing epithelialization. In the other step, UB cells induce mesenchymal-epithelial conversion and tubulogenesis by directly contacting the mesenchyme (1). These data imply that induction of the metanephrogenic mesenchyme by the ureteric bud in vivo is divisible into a step of obligatory rescue from apoptosis and growth, mediated by diffusible growth factors, and a step of mesenchymal-epithelial conversion, signaled by contact-dependent mechanisms.

To identify the diffusible molecules secreted by UB cells that rescue the metanephrogenic mesenchyme from apoptosis and cause it to proliferate, we have begun an analysis of media conditioned by UB cells. As an initial analytical step, we have used heparin-Sepharose chromatography, since many growth factors bind this ligand. We found that UB cells secrete at least four heparin binding factors that rescue the metanephrogenic mesenchyme from apoptosis. We have identified one of these factors as basic FGF (bFGF). We have also found that native ureteric bud, like UB cells, synthesizes bFGF. bFGF rescues nephron and vascular precursors from apoptosis, as well as the metanephrogenic mesenchymal cells that in turn signal the ureter to grow and branch.

Materials and Methods

UB Cells

UB cells were generated from mice transgenic for SV40 T-antigen (20). The cells express molecules typical of the ureteric bud in vivo. These include epithelial proteins (that are not expressed by the mesenchyme), as well as Dolichos biflorus binding sites and ret and c-met. The consistent expression of ret during serial passage of the UB cells was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) (upstream 5' CTCCGTGGCGACATTT; downstream 5'CTTCAGACCAGCTG; generating a 488-bp product). The authenticity of this product was confirmed by sequencing.

Media Conditioned by UB Cells

UB cells were grown in minimal essential media (MEM; Gibco, Grand Island, NY) with 10% fetal calf serum (FCS; Hyclone, Logan, UT) supplemented with γ-interferon (10 U/ml; Genzyme, Cambridge, MA). At 80% confluence, the cells were rinsed with MEM, and the medium was then
chymes, because controls rapidly die after an initial

This is a convenient time point to begin labeling the mesenchyme. Assay of Mesenchymal Growth Activity

Mesenchymal growth activity was assayed with a thymidine incorporation assay.

To assay the activity of the fractions, 100 µg of proteins of each fraction eluted from the heparin-Sepharose column were free of ureteric contamination, as assessed by immunodetection. Desalted conditioned media was introduced into two heparin-Sepharose columns placed in series, and the flowthrough was collected. We monitored contamination of the flowthrough with heparin binding proteins by assaying for bFGF with immunobLOTS (see below). We found that two heparin-Sepharose columns in series were required to deplete bFGF to less than 1–2 ng/100 µg protein (the limit of detection of the immunoblots). This concentration of bFGF is insufficient to rescue metanephrogenic mesenchyme from apoptosis.

Fractionation of Conditioned Media by Heparin Sepharose Chromatography

Conditioned media (30 mg) was equilibrated with 20 mM sodium phosphate (pH 7.0) by Sephadex G-25 columns (Pharmacia, Piscataway, NJ) and fractionated using heparin-Sepharose columns (5 ml, Pharmacia). Elution was done with a step gradient of NaCl in 20 mM sodium phosphate, pH 7.0.

In addition to the heparin binding fractions, we generated a non-heparin binding fraction that was essentially free of heparin binding proteins. Desalted conditioned media was introduced into two heparin-Sepharose columns placed in series, and the flowthrough was collected. We monitored contamination of the flowthrough with heparin binding proteins by assaying for bFGF with immunoblots (see below). We found that two heparin-Sepharose columns in series were required to deplete bFGF to less than 1–2 ng/100 µg protein (the limit of detection of the immunoblots). This concentration of bFGF is insufficient to rescue metanephrogenic mesenchyme from apoptosis.

Assay of Mesenchymal Growth Activity

Each fraction eluted from the heparin-Sepharose column was concentrated by centrifugation in a Microcon-10 (Amicon) and washed once with phosphate-buffered saline (PBS). To assay the activity of the fractions, 100 µg of proteins of each was resuspended in 2 ml MEM with 10% “defined” FCS (HyClone) and sterilized by 0.2-µm filters. We used this FCS to assay column fractions, because it has no heparin binding proteins and did not support the viability of the mesenchyme.

Metanephric mesenchymes were dissected from embryonic day 13 (E13) rat kidney (vaginal plug = day 0). At this stage, the ureteric bud has entered the mesenchymal mass and initiated its first dichotomous branching. Mesenchymes were isolated from the ureteric bud using trypsin (1 mg/ml) and deoxyribonuclease I (ribonuclease free, 10 U/ml; Boehringer Mannheim, Indianapolis, IN) digestion (20 min, 37°C) in L-15 media ( GibCO), followed by mechanical separation with steel needles. Trypsin was then inactivated with soybean trypsin inhibitor (50 µg/ml; Sigma) for 1 h at room temperature in L-15 media. Ninety-eight percent of the isolated mesenchymes were free of ureteric contamination, as assessed by routine surveillance of the isolated mesenchymes by staining with D. biflorus (Vector Labs, Burlingame, CA), a lectin specific for the ureteric bud.

Four to five mesenchymes were plated on a collagen-coated Transwell (3425 filters; Costar, Acton, MA), and the filter was suspended in the MEM containing 10% defined FCS plus the fractionated proteins. Care was taken to maintain each mesenchyme at an air-fluid interphase. Mesenchymes plated in MEM with defined FCS, without UB proteins served as controls.

Mesenchymal growth activity was assayed microscopically at 12-h intervals. In addition, mesenchymal proliferation was assayed with a thymidine incorporation assay. [3H]Thymidine (10 µCi; 6.7 Ci/mmol; New England Nuclear, Boston, MA) was added 12 h after the initiation of the culture. This is a convenient time point to begin labeling the mesenchymes, because controls rapidly die after an initial 6 h in culture (48). At 48 h of culture, mesenchymes were photographed and then removed from the Transwell filters. Mesenchymes isolated from each culture were washed in MEM with 10% FCS (4°C) for 20 min three times, solubilized in 10 µl of 10% sodium dodecyl sulfate and counted in Ready Protein (Bio-Rad, Hercules, CA). This [3H]thymidine incorporation procedure is sensitive to less than 1 ng of bFGF. Similar results were obtained after precipitating the DNA by the addition of trichloroacetic acid (10%) and washing the pellet with a mixture of ethanol and ether (3:1).

FGF Expression by UB Cells and by Ureteric Bud

Fractions from the heparin-Sepharose column were assayed for bFGF by immunoblot using an anti-bFGF neutralizing antibody (R&D Systems, Minneapolis, MN). Recombinant human bFGF (R&D Systems) served as a positive control for the detection of bFGF. In addition, we assayed media conditioned by UB cells that had been grown in defined FCS (which is depleted of heparin binding proteins).

Proteins (50–100 µg) were separated on 10–25% gradient gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose filters. Membranes were first blocked in 10% nonfat milk in 0.1 M tris(hydroxymethyl)aminomethane (Tris), 0.9% NaCl, pH 7, washed with 0.1% Tween and anti-bFGF (2 µg/ml) applied in 1% milk. After extensive washing, the primary antibody was detected with anti-rabbit antibodies (1:10,000; Jackson Immunoresearch Labs, West Grove, PA) and enhanced chemiluminescence (ECL, Amersham). Intensity of the immunodetection was measured by laser densitometry.

Poly-A RNA was prepared from UB cell monolayers, from 500 ureteric buds, and from 200 isolated mesenchymes using RNAzol B (Tel-Test), followed by selection of poly-A RNA with oligo(dT) beads (Oligo-Tex; Qiagen, Santa Clarita, CA). For each RT-PCR assay, 0.3 µg of poly-A RNA was first reverse transcribed and then amplified with the GeneAmp kit (Perkin-Elmer, Foster City, CA) in HotStart tubes (MJ) for 35 cycles. Two primer sets, designed with the Prime (GCC) program, were used to assay each FGF family member. We chose primer pairs that were least homologous to other FGF species by comparing their sequences to every other FGF cDNA using the BestFit (GCG) program. To amplify FGF sequences from rat mesenchyme or ureteric bud, we used the PCR primers shown in Table 1. To amplify FGF sequences from mouse UB cells, we used the PCR primers shown in Table 2.

Authenticity of the products amplified from isolated ureteric buds was confirmed by sequencing the PCR products after isolation (Qiagen II, Qiagen). The authenticity of bFGF amplified from UB cells with the third and fourth primer sets was confirmed by digestion with either Dde I or Rsa I. Digestion of products amplified by the third primer set yielded the predicted fragments of 217 and 151 bp after Dde I digestion and 180 and 188 bp after Rsa I digestion. Digestion of products amplified by the fourth primer set yielded the predicted fragments of 193 and 107 bp after Dde I digestion and 156 and 144 bp after Rsa I digestion. Control PCR reactions were performed without initial reverse transcription of RNA and were reported as –RT.

Rescue of Renal Progenitors with bFGF

Mesenchymes were treated with bFGF (R&D Systems) in increasing doses, and the incorporation of [3H]thymidine was measured, as described above. In some experiments, anti-bFGF neutralizing antibodies (1–30 µg; R&D Systems) were used to block FGF signaling. The morphology of these mesenchymes was assessed by fixation in 4% glutaraldehyde, 0.1 M phosphate buffer (pH 7.2), postfixation with 1% OsO4, and embedding in Epon-812.
Table 1. PCR primers for amplification of rat mesenchyme or ureteric buds

<table>
<thead>
<tr>
<th>FGF Species</th>
<th>Primer Set</th>
<th>Sequences</th>
<th>Bases</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF1</td>
<td>1</td>
<td>upstream 5' GGGAGATCACACACCTTTGC</td>
<td>277–715</td>
<td>439</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>downstream 5' AGGGGAGAACAAGATGG</td>
<td>282–667</td>
<td>386</td>
</tr>
<tr>
<td></td>
<td></td>
<td>upstream 5' ATACACACACCTTTGCAGCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bFGF2</td>
<td>1</td>
<td>downstream 5' CAGGAGTTGAGTATGCTTTC</td>
<td>699–986</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>upstream 5' ACCACACACCAAAACATACT</td>
<td>732–982</td>
<td>251</td>
</tr>
<tr>
<td></td>
<td></td>
<td>downstream 5' CAGGATGAGAAAGAAACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF5</td>
<td>1</td>
<td>upstream 5' TTCTGTTTCTGCCTTTC</td>
<td>159–587</td>
<td>429</td>
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<tr>
<td></td>
<td>2</td>
<td>downstream 5' CTTGTTTCTGCTGCAAAC</td>
<td>11–593</td>
<td>583</td>
</tr>
<tr>
<td></td>
<td></td>
<td>upstream 5' AGGTGAAGTTGGGAGC</td>
<td>235–655</td>
<td>421</td>
</tr>
<tr>
<td></td>
<td></td>
<td>downstream 5' TTCTGRTTCTGTTTACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>upstream 5' TTCTGTTTCTGCTGAAAC</td>
<td>112–442</td>
<td>331</td>
</tr>
<tr>
<td></td>
<td></td>
<td>downstream 5' CTTGTTTCTGCTGCAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF7</td>
<td>1</td>
<td>upstream 5' AGGTGAAGTTGGGAGC</td>
<td>333–632</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>downstream 5' TTCTGTTTCTGCTGAAAC</td>
<td>187–631</td>
<td>445</td>
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<tr>
<td></td>
<td></td>
<td>upstream 5' TTCTGTTTCTGCTGAAAC</td>
<td></td>
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</tbody>
</table>

PCR, polymerase chain reaction; bFGF and aFGF, basic and acidic fibroblast growth factor.

Mesenchymes rescued from apoptosis with bFGF (100 ng) were assayed for capillary precursors by fixation in 4% paraformaldehyde in PBS followed by extensive washing in PBS with 50 mM NH₄Cl. Mesenchymes were then permeabilized in 0.1% Triton X-100 in PBS for 10 min, rinsed with 0.05% Tween 20 for 10 min, and stained with either antibodies to von Willebrand factor (vWF, 1:50; Dakopatts, Carpinteria, CA) or to fit (1:100; Santa Cruz Biochemical, Santa Cruz, CA) in PBS with 0.05% Tween 20 and 1% bovine serum albumin for 1 h. Subsequently, the mesenchymes were washed in PBS containing NaCl (2.7%) and with 0.05% Tween 20 for 10 min, and stained with either antibodies to von Willebrand factor (vWF, 1:50; Dakopatts, Carpinteria, CA) or to fit (1:100; Santa Cruz Biochemical, Santa Cruz, CA) in PBS with 0.05% Tween 20 and 1% bovine serum albumin for 1 h. Subsequently, the mesenchymes were washed in PBS with additional NaCl (2.7%) and with 0.05% Tween 20 and then stained with anti-rabbit antibodies coupled to fluorescein (1:200, Jackson Immunoresearch Labs) in PBS containing 5% rat serum.

Mesenchymes maintained in culture with bFGF for 4 days were also assayed for the survival of epithelial precursors. To visualize these cells, fragments of spinal cord (a heterologous inducer) obtained from E13 rat embryos were cultured next to the rescued mesenchymes. After an additional 4 days, the tissues were fixed and embedded in Epon-812 as above. This bioassay is necessary because reliable lineage markers of preepithelial cells are not available, and histological evidence of mature epithelia is required. Mesenchymes were also assayed for the expression of uromorulin by immunocytochemistry (Sigma) after fixation in 100% methanol (−20°C) for 5 min.

To assay whether bFGF rescues mesenchymal cells that permit survival and branching of the ureteric bud, nine rat mesenchymes were cultured in three adjacent clusters on Transwell filters in the presence or absence of bFGF (100 ng) for 4 days. Ureteric buds were then obtained either from E11.5 mice, transgenic for β-galactosidase (Rosa J R2073 animals; Jack- son Labs, Bar Harbor, ME), or from E13 rat kidney. Eight ureteric buds from Rosa mice or else single rat ureteric buds were placed on the mesenchymal clusters, then cultured for an additional 4–7 days. In some experiments, bFGF was withdrawn at the time the exogenous ureteric buds were applied. Cultures were fixed in 2% paraformaldehyde and processed for β-galactosidase histochemistry (15) or for D. biflorus lectin staining. In the latter case, the tissues were washed in 0.1% saponin in PBS at 37°C and then stained with the lectin (50 µg/ml; Vector Labs) in 0.1% saponin in PBS for 30 min.

Poly-A RNA was isolated from freshly dissected mesenchymes and from mesenchymes rescued from apoptosis with bFGF (100 ng/ml) as above. Glial cell line-derived neurotrophic factor (GDNF) was amplified using two different primer sets obtained with the Prime (GCG) program: primer 1, upstream 5' AGCCACCATCAGAAAAGAC and downstream 5' TCAGATACTCCACGCG, yielding a 431-bp product; and primer 2, upstream 5' CAGGACAGAGTAAGGTTT and downstream 5' TGTTGTCAGATACCATC, yielding the predicted 654-bp product (6).

Table 2. PCR primers for amplification of mouse UB cells

<table>
<thead>
<tr>
<th>FGF Species</th>
<th>Primer Set</th>
<th>Sequences</th>
<th>Bases</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF2</td>
<td>1</td>
<td>upstream 5' ACCACACAGCTCAAAACTC</td>
<td>167–454</td>
<td>288 bp</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>downstream 5' CAGGCTTGGAAGAACAG</td>
<td>166–465</td>
<td>300 bp</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>upstream 5' CACCAGCCACCTCTCAC</td>
<td>61–429</td>
<td>368 bp</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>downstream 5' TTCTGAGTTCACCC</td>
<td>86–385</td>
<td>300 bp</td>
</tr>
<tr>
<td>FGF9</td>
<td>1</td>
<td>upstream 5' TGTTGTCAGATAAGCACC</td>
<td>230–552</td>
<td>323 bp</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>downstream 5' TTCTGAGTTCACCC</td>
<td>143–594</td>
<td>452 bp</td>
</tr>
</tbody>
</table>

FGF, fibroblast growth factor; UB, ureteric bud.
RESULTS

UB Cells Secrete Many Metanephrogenic Mesenchymal Growth Factors

The metanephrogenic mesenchyme can be separated from the ureteric bud at day E13, the time when the bud invades the mesenchyme and branches. In contrast to the E13 kidney anlage which grows well in vitro, isolated E13 mesenchymes die by apoptosis within 48 h. Thus in vitro cultures of isolated mesenchyme were used to detect molecules that rescue it from apoptosis.

We have previously shown that medium conditioned by UB cells induces the growth of isolated metanephrogenic mesenchymes (1). As an initial step to identify the responsible molecules, serum-free medium conditioned by UB cells was fractionated by heparin-Sepharose chromatography. The fraction of proteins that did not bind to heparin-Sepharose had inconsistent mesenchymal growth activity, indicating that most of the activity bound to heparin.

The heparin binding proteins comprised 35 ± 7% of the proteins present in conditioned media. By using a shallow NaCl gradient for elution, we found four distinct fractions capable of rescuing the isolated metanephric mesenchyme from apoptosis (eluting at 0.3, 0.5, 0.7, and 1.5 M). All mesenchymes cultured with these fractions enlarged over a 48-h period of observation, whereas all mesenchymes cultured in basal media and all mesenchymes incubated with inactive column fractions involuted over the same period. The dying mesenchymes were nearly entirely replaced by apoptotic bodies (see Fig. 4D), whereas mesenchymes treated with active fractions were densely cellular (see Fig. 4C). In agreement with these visual observations, mesenchymes treated with the active fractions incorporated [3H]thymidine up to 400% in excess of mesenchymes incubated in basal media (Fig. 1, A and B). Fractionation of growth activity into distinct peaks was reproducible using many separate collections of conditioned media, obtained over many months (n = 12). Moreover, the peaks of activity are likely due to different proteins secreted by UB cells, since they also could be easily separated by both anion exchange and by hydrophobic interaction chromatography (not shown).

Fig. 1. Fractionation of media conditioned by ureteric bud cells (UB cells, 30 mg) by heparin-Sepharose chromatography. A: chromatogram of proteins eluted with a gradient of NaCl (broken line). Protein concentration in the eluant was measured by absorbance at 280 nm (solid, spiky trace). Activity of each pooled fraction (100 µg) of eluant was measured by [3H]thymidine incorporation in 4–5 metanephrogenic mesenchymes cultured for 48 h (solid line, ○). Mesenchymal growth activity was measured in 12 separate fractionations of conditioned media, and a representative curve is reported as a percentage of the [3H]thymidine incorporated by mesenchymes incubated in basal media. Fractions 1–11 contain non-heparin binding proteins. Four distinct peaks of activity are found: fractions 21–22 eluting at 0.3 M NaCl, fractions 33–34 eluting at 0.5 M NaCl, fractions 43–44 eluting at 0.7 M NaCl, and fractions 49–55 eluting at 1.5 M NaCl. B: photomicrographs of representative mesenchymes incubated with fractions from the heparin-Sepharose column for 48 h. Four distinct peaks of mesenchymal growth activity are found corresponding to the peaks of [3H]thymidine incorporation. Ascending numbers correspond to the fractionation shown in Fig. 2A; mesenchyme in control (C) is incubated in basal media. Clear zones around each mesenchyme are media above the collagen-coated filter; magnification, ×55. C: representative immunoblot for basic fibroblast growth factor (bFGF) of proteins eluting from the heparin-Sepharose column. Fraction numbers correspond to the fractionation shown in A. The 18 × 10^3 M, form of bFGF elutes from the heparin-Sepharose column at 1.5 M NaCl. bFGF standards (2.5–25 ng) are also shown.
Histological analysis of metanephrogenic mesenchymes incubated with the active fractions for up to 5 days showed that none of these growth factors induce formation of epithelia. Sections of the mesenchymes showed a homogeneous organization with no evidence of aggregation or alignment of cells typical of the earliest nephron (renal vesicles). In addition, none of the active fractions activated the expression of uvomorulin (not shown), an adhesion protein expressed by mesenchymal cell undergoing mesenchymal-epithelial transition.

These data demonstrate that UB cells secrete multiple factors that can rescue the metanephric mesenchyme from apoptosis without inducing the transition of mesenchyme to epithelia. Because screening of large panels of known growth factors has failed to identify mesenchymal mitogens that are also expressed by the ureteric bud (55), our results suggest that UB cells secrete unidentified metanephrogenic growth factors.

**UB Cells Secrete bFGF**

In addition to the three active fractions eluting from the heparin-Sepharose column at low salt concentration, there was an active fraction eluting at 1.5 M NaCl. Since this condition is typical of bFGF, we analyzed the fraction by immunoblot with anti-bFGF antibodies. As shown in Fig. 1C, bFGF was present in the fraction eluting at 1.5 M NaCl. bFGF was not present in the earlier fractions (limit of detection 1 ng/ lane), confirming that they contain distinct growth factors.

Unfractionated media conditioned by UB cells contained $3.4 \pm 0.8$ ng bFGF/100 μg protein (or 1–2 ng/ml; n = 12). RT-PCR confirmed that UB cells express bFGF; identity was demonstrated by four sets of primers and by restriction digestion of two of the amplified products with two different enzymes (Fig. 2).

**Native UB Expresses bFGF**

The secretion of bFGF from UB cells suggests that native ureteric buds should also synthesize this molecule. However, UB cells are grown in vitro and are immortalized with T-antigen, conditions which may alter the expression of growth factors including bFGF (58).

To determine whether native UB synthesizes bFGF when the metanephrogenic mesenchyme starts proliferating at E13, we isolated poly-A RNA from 500 E13 rat ureteric buds. RT-PCR with two different primer sets showed that the ureteric bud expresses bFGF (Fig. 3). Authenticity of the PCR products was confirmed by sequencing (not shown).

To rule out the possibility that expression of bFGF in the ureteric bud preparation was due to contamination with mesenchymal cells, we determined whether E13 metanephrogenic mesenchyme expresses bFGF. As shown in Fig. 3, we found only trace amplification of bFGF by RT-PCR with 200 dissected mesenchymes (perhaps due to contamination by a few UB cells). To obtain additional evidence that the bFGF originated from ureteric cells in the ureteric bud preparation, we used RT-PCR to determine whether the expression pattern of several members of the FGF family differed in ureteric buds and mesenchymes (Fig. 3). Whereas ureteric buds expressed bFGF and FGF9, the mesenchymes expressed acidic FGF, trace bFGF and FGF5, and no FGF9. The different patterns of expression of FGFs indicate that there was little if any mesenchymal contamination in the UB preparation. This, together with the weak mesenchymal expression of bFGF, indicates that ureteric bud in vivo synthesizes bFGF.

**bFGF Rescues the Mesenchyme From Apoptosis**

Since bFGF is secreted by UB cells, it is synthesized in vivo by the ureteric bud, and is present in a fraction eluting from the heparin-Sepharose column that rescues the mesenchyme from apoptosis (Fig. 1C), we
examined whether pure bFGF is also active. Indeed, we found that mesenchymes treated with as little as 1 ng bFGF/2 ml culture demonstrated enhanced [3H]thymidine incorporation (Fig. 4A) and that at higher doses (2.5–5 ng/2 ml culture) the mesenchymes enlarged (Fig. 4B). These tissues were highly cellular, whereas mesenchymes cultured in basal media showed an abundance of fragmented nuclei and apoptotic bodies (Fig. 4, C and D). The growth-promoting effect of bFGF was inhibited by an anti-bFGF neutralizing antibody, where greater than 5 µg of antibody abolished the activity of 5 ng bFGF per 2 ml of culture media. (Fig. 4, A and B).

To determine the class of receptor that bFGF activates in the metanephric mesenchyme, we examined the activity of several FGF family members. Mesenchymes were rescued from apoptosis by FGF4 but had little response to acidic FGF and no response to FGF5, FGF6, and FGF7 (100 ng; n = 4–6). These data indicate that bFGF rescues the mesenchyme by activating the β-isofrom of the flg receptor (FGFR1, IIIC; see Refs. 27, 45, 56). This receptor is highly expressed in embryonic mesenchyme (22).

We conclude that bFGF is a potent mesenchymal survival factor and mitogen expressed by UB cell lines and by ureteric bud in vivo. We suggest that this factor is secreted by the invading ureteric bud in vivo, rescuing the mesenchyme from apoptosis by the activation of the flg IIIc receptor. Our results are in agreement with...
recent studies showing that bFGF is able to rescue mesenchyme from apoptosis (21, 37) and contrast with prior failures to detect its mesenchymal growth activity (5, 55, 38).

Characterization of the Cell Types Rescued From Apoptosis by bFGF

Several cell types are present in E13 metanephrogenic mesenchyme. These include the precursors of tubular epithelia and precursors of endothelial cells (34) and stromal cells (14, 46). In addition, the mesenchyme contains cells that provide an essential signal for kidney development; these as yet unidentified cells trigger the invasion and branching of the ureteric bud. We examined whether these cell types survive in metanephrogenic mesenchymes treated with bFGF.

Rescue of nephron precursors. Mesenchymes treated with bFGF appear homogeneous, without histological evidence of mesenchymal-epithelial transition (formation of aggregates, renal vesicles or S-shaped bodies; Fig. 4C). Thus, to assay whether nephron precursors have been rescued from apoptosis by bFGF, we determined whether bFGF-treated mesenchymes were competent to produce tubules. We did this by first treating mesenchymes for four days with bFGF (mesenchymes cultured without bFGF involuted by 2 days in culture) and then introducing fragments of embryonic spinal cord to serve as a heterologous “inducer.” We found that the mesenchymes rescued by bFGF were capable of generating tubules upon contact with the spinal cord (in 80%, n = 10; Fig. 5, A and B). These data unequivocally demonstrate that bFGF rescues epithelial progenitors. In addition, the data reveal that bFGF does not prevent the expression of the mature epithelial phenotype, as it does in some cells (2, 19, 35).

Rescue of capillary precursors. At an early stage of metanephrogenic development (E13), endothelial cells are already present. The cells are identified by the expression of vWF (34) or vascular endothelial growth factor receptors, flk (17, 18, 24, 33, 42, 43) and flt (10, 39). As expected, in isolated E13 metanephrogenic mesenchymes we found cells expressing vWF and flt.
a fundamental function of metanephrogenic mesenchyme is, in turn, to induce growth and branching of the ureteric bud (47).

To determine whether bFGF rescued metanephrogenic mesenchymal cells that could support the growth and branching of the ureteric bud, we cocultured ureteric buds with mesenchymes that had been maintained for 4 days with bFGF. As shown in Figs. 7, B and C, mesenchymes rescued with bFGF stimulated the branching of ureteric buds (n = 4 experiments), whereas control mesenchymes could not even support their structural integrity (Fig. 7D). In addition, we found that mesenchymes treated with bFGF had cells expressing GDNF, a growth factor produced by the E13 mesenchyme (Fig. 8) that is required for ureteral branching and growth (29, 41, 44).

To establish that the ureteric bud branched in response to the rescued mesenchyme and not in response to bFGF, we withdrew bFGF at the time the ureteric buds were cocultured with the rescued mesenchymes. Despite removal of bFGF, the ureteric buds grew over a 4-day period, suggesting that the rescued mesenchyme and not bFGF stimulated branching of the ureteric bud.

As an additional control, we tested whether bFGF (100 ng) could enhance the survival of ureteric buds in culture. Both in the presence and absence of bFGF, some ureteric cells grew from the explanted buds. However, all of the cells died after 4 days in either bFGF-treated or control cultures. This demonstrates that bFGF does not directly enhance survival of ureteric epithelia.

![Fig. 7. Metanephrogenic mesenchymes rescued from apoptosis with bFGF induce the branching of exogenous ureteric buds. Ureteric buds were isolated from embryonic day E13 (E13) rat kidneys (A and B) or from E11 mouse kidneys (C and D) and placed on top of mesenchymes that had been maintained in culture for 4 days with bFGF (100 ng). Cultures were then fixed and stained after an additional 4–7 days of incubation. A: a freshly isolated ureteric bud is branched once. Bar = 55 µm. B: a ureteric bud has branched twice after coculture for 4 days on a cluster of mesenchymes rescued by bFGF. Ureteric bud is visualized with D. biflorus lectin; the mesenchyme is beneath and surrounding the ureteric bud and not stained by the lectin. Bar = 80 µm. C and D: 8 ureteric buds from E11 Rosa mice were placed upon a cluster of 9 mesenchymes that had been maintained by bFGF (C) or had been cultured in basal media (D). Ureteric buds placed on mesenchymes rescued from apoptosis with bFGF are viable and undergo branching morphogenesis over 7 days (C), whereas ureteric buds do not survive when cultured upon involuting mesenchymes (D). Ureteric buds were visualized by histochemistry for β-galactosidase. For C and D, bar = 270 µm.]

![Fig. 8. Metanephrogenic mesenchymes rescued from apoptosis with bFGF express glial cell line-derived neurotrophic factor (GDNF). Freshly dissected mesenchymes (mesenchyme) and mesenchymes maintained in culture for 4 days with bFGF (mesenchyme + bFGF) were analyzed for the expression of GDNF by RT-PCR using two different primer sets. –RT, PCR reactions without prior reverse transcription of RNA.]

MESENCHYME MESENCHYME + bFGF

-RT #1 #1 #2 #1 #1 #2 #2
Taken together, these data demonstrate that bFGF rescues mesenchymal cells, which in turn are competent to promote survival and branching morphogenesis of the ureteric bud.

DISCUSSION

Authentic Mesenchymal Growth Factors

At early stages of nephrogenesis, the ureteric bud rescues the metanephrogenic mesenchyme from apoptosis. This is demonstrated by the rapid death of the mesenchyme when invasion of the ureteric bud is defective in vivo (29, 41, 44, 49) or when the mesenchyme is mechanically separated from the ureteric bud in vitro (23).

To identify signals produced by the ureteric bud that rescue the mesenchyme from apoptosis, we previously isolated UB cells (1). We showed that UB cells are adequate surrogates for the native ureteric bud; they express proteins typical of the ureteric bud, and they induce the mesenchyme to grow and differentiate into epithelia that form tubules. These results suggest that our UB cells express the same factors as does the ureteric bud in vivo. Supporting this conclusion, we now find that bFGF, which is secreted by UB cells, is also expressed by the ureteric bud at the time of its invasion of the mesenchyme (E13 in the rat). Thus bFGF is an authentic mesenchymal growth factor expressed by the ureteric bud at a time when it rescues the mesenchyme from apoptosis.

Induction: Proliferation vs. Morphogenesis

In classic experiments with isolated metanephrogenic mesenchymes and spinal cord as an inducer, mesenchymal growth and epithelialization occurred simultaneously, suggesting that these two processes may result from the same inducing signal (48).

In contrast, we have found that four soluble factors secreted by UB cells induce growth but not epithelialization of the mesenchyme, whereas as previously shown, contact with UB cells induces mesenchymal-epithelial conversion (1). This suggests that growth and mesenchymal-epithelial conversion are mediated by different molecules. It is of course possible that withdrawal of the growth factors secreted by the ureteric bud could be the mechanism that triggers the expression of the mature phenotype, as occurs elsewhere (3, 19, 31, 35, 51). However, we found that bFGF does not inhibit epithelialization, since nephrons could be induced in the presence of bFGF by the addition of spinal cord. Furthermore, we found in preliminary experiments that the withdrawal of bFGF from rescued mesenchymes does not cause maturation but results in the fulminating death of the mesenchyme. These results show that bFGF neither stimulates nor inhibits mesenchymal-epithelial conversion but rather maintains epithelial precursors available for a secondary signal that triggers mesenchymal-epithelial conversion. Thus the data demonstrate that mesenchymal growth and conversion to epithelia are regulated by independent signaling mechanisms.

The sequence of nephrogenesis in vivo is consistent with the proposal that distinct molecules trigger proliferation and epithelialization. A localized proliferation of mesenchymal cells occurs around each branch of the ureteric bud (a process that has been called “condensation”) prior to the appearance of epithelia (52). This suggests that the ureteric bud first stimulates proliferation of mesenchymal cells and then induces epithelialization. This of course could occur first by the exposure of mesenchymal cells to diffusible growth factors followed by the contact of mesenchymal cells with the ureteric bud.

Dichotomous control of mesenchymal proliferation and epithelialization by the ureteric bud also implies that dysfunction of each could occur. For example, defects in growth factor signaling should result in hypocellular kidneys with few nephrons, whereas defects in triggering mesenchymal-epithelial conversion would allow mesenchymal cell proliferation but not nephrogenesis. This could explain some of the phenotypes of recent gene deletion experiments. For example, the deletion of BMP-7 (expressed initially by the ureteric bud; see Ref. 54) results in hypocellular kidneys with a few nephrons (11, 26), suggesting that this factor predominantly acts as mesenchymal proliferative factor. We would predict that deletion of a ureteric molecule that converts mesenchyme to epithelia would result in a large number of mesenchymal cells clustered at each branch of the ureteric bud but no nephrons. This phenotype is found after deletion of Wnt-4, a mesenchymal protein that is necessary for mesenchymal-epithelial transition (50).

The Function of Multiple Ureteric Growth Factors

Since UB cells secrete many activities that rescue the mesenchyme from apoptosis, the question arises as to whether each has a unique function. There are two possibilities: each ureteric factor rescues a specific cell lineage, or alternatively, each factor stimulates the progenitors of multiple cell lineages. Distinguishing these possibilities for each ureteric growth factor will require their identification.

bFGF is the first growth factor secreted by UB cells that we have identified and confirmed to be produced by the ureteric bud in vivo. By examining the composition of mesenchymes rescued by bFGF from apoptosis, we found that this factor maintains progenitors of multiple cell lineages including nephron and capillary precursors as well as cells that stimulate branching of the ureteric bud, including GDNF-expressing mesenchymal cells (29, 41, 44). bFGF may rescue each of these progenitors directly or may only rescue a single type of mesenchymal cell that then mediates the effects of bFGF on other cells. The former possibility is more likely, since FGF receptors are widely expressed in the mesenchyme (22, 36, 39), and bFGF is a mitogen for at least some endothelia (12). Regardless, our data indicate that multiple progenitors present in the metanephrogenic mesenchyme at the stage of the invasion of the ureteric bud survive because of bFGF. Similarly, in other developing tissues, such as in the developing limb.
and during body axis elongation (8, 30), as well as during cardiac (28) and skeletal muscle proliferation (19), FGFs maintain multiple precursors without selecting subsets of cells.

Although the other, yet unidentified, growth factors secreted by UB cells may regulate the survival of specific precursor cells, they may be similar to bFGF and rescue multiple types of progenitors from apoptosis. In this case, the factors could have additive effects on their targets. At the stage in which the ureteric bud extends from the Wolffian duct to enter the mesenchyme, this organ contains only 10^3 cells. These cells must generate 10^4 nephrons (each initially composed of 100 cells; see Ref. 9) as well as a complex vasculature and stroma. Thus many soluble ureteric factors may be required to generate enough precursor cells in a short time. Estimates of total number of cells in the developing kidney in fact suggest a brief period for rapid cell multiplication; expansion of cell number occurs only in the first few days after the ureteric bud contacts the mesenchyme (55) and for only 48 h in mesenchymes grown in vitro with an inducer (48). In other systems, multiple growth factors are required to rescue precursors from apoptosis and to induce their proliferation (2, 4, 13, 53).

Recently, Karavanova et al. (21) found that conditioned media from a UB cell line triggers tubulogenesis when enriched with exogenous TGF-α and bFGF (21). These results suggest that soluble proteins in their media can trigger growth and tubulogenesis in metanephrogenic mesenchymes, that TGF-α is a ureteric factor, and that both TGF-α and bFGF are either not present or are present at limiting concentrations in their UB-conditioned media. It is likely that our UB cells differ from those used by Karavanova et al. (21) in their ability to secrete inducing or growth factors, since we found abundant bFGF but no TGF-α in our conditioned media (not shown). Furthermore, although the results of Karavanova et al. (21) indicate that TGF is a mesenchymal mitogen, it is of note that deletion of TGF-α in vivo does not inhibit renal development (25). Furthermore, although both Karavanova et al. (21) and we found that bFGF is a mesenchymal growth factor, neutralization of bFGF with anti-bFGF antibodies (100 μg/ml) does not block development of E13 kidneys (unpublished experiments). These data suggest that neither TGF-α nor bFGF is limiting to mesenchymal growth and development, but perhaps TGF-like or bFGF-like molecules are required in vivo.

In sum, our data suggest that the classic view of the ureteric bud as an inducer of the metanephrogenic mesenchyme should be expanded. We propose that the ureteric bud in vivo not only induces mesenchymal-epithelial conversion but independently determines the number of mesenchymal cells available to form nephrons and, ultimately, nephron number. In addition, by rescuing endothelial cells from apoptosis, the ureteric bud could locally regulate the vascularity of each nephron. Finally, by supporting the survival of mesenchymal cells that trigger its own growth, the ureteric bud indirectly regulates its own growth and thus the nephron and capillary precursors that it rescues from apoptosis. We therefore suggest that variable expression of one or more of these ureteric factors could account for variations in the number of nephrons found in human kidneys (32) and could be responsible for cases of congenital renal hypoplasia, where nephron number, but not nephron architecture, is disturbed.

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