**Abstract.** The role(s) of one family of polypeptide growth factors in a developing organ system was examined. Renal anlagen (metanephroi) were surgically removed from 13-d-old rat embryos and grown in organ culture for up to 6 d. Over this period of time when placed in serum-free defined media, the metanephroi increased in size and morphologic complexity. Messenger RNAs for both insulin-like growth factors (IGFs), IGF I and IGF II, were present in the metanephroi. Immunoreactive IGF I and IGF II were produced by the renal anlagen and released into culture media. Levels were relatively constant during the 6 d in culture and averaged 3.5 \times 10^{-8} \text{M} \text{IGF I} \text{ and } 8.3 \times 10^{-8} \text{M} \text{IGF II} \text{ in media removed from metanephroi after contact for 24 h. IGF binding protein activity was not detected in culture media. Growth and development of metanephroi in vitro was prevented by the addition of anti-IGF I or anti-IGF II antibodies to organ cultures. IGF II produced by metanephroi was active in an IGF II biological assay system and addition of anti-IGF II receptor antibodies to organ cultures prevented growth and development, consistent with the action of IGF II in metanephroi being mediated via the IGF II receptor. The data demonstrate production of both IGF I and IGF II by developing rat metanephroi in organ culture. Each of these peptides is necessary for growth and development of the renal anlage to take place in vitro. Our findings suggest that both IGF I and IGF II are produced within the developing metanephros in vivo and promote renal organogenesis.

**Materials and Methods**

**Removal of Embryos from Rats, Removal of Kidneys from Embryos, Dissection of Kidneys, and Organ Culture of Whole Metanephroi**

Embryos were removed from anaesthetized pregnant female Sprague Dawley rats (Harlan, Indianapolis, IN) on day 13 of the pregnancy. Metadata...
nephric kidneys were surgically dissected from embryos. The age of the em-
bryos was confirmed by examination of the shape of the ureteric bud. At 13 d of development the ureteric bud has differentiated into a metanephric duct that ends in a pelvis with two branchlets (1).

Cultures of whole metanephroi were carried out on sterile membranes (No. 110409; Nuclocorp Corp., Pleasanton, CA) of 0.8 μm thickness and 13 mm diameter in a medium consisting of equal volumes of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 25 mM Hepes buffer, sodium bicarbonate (1.1 mg/ml), 10 mM Na₃SeO₃·5H₂O, 10 mM N₂H₄, 10 mM NaCl, 50 μM gentamicin, and 50 μg/ml Nystatin, and penicillin/streptomycin (50 U/ml). The following additions were made to cultures when indicated in the text: monoclonal anti-human IGF I antibodies, kindly provided through the National Hormone and Pituitary Program by Dr. Robert Baxter, University of Sydney, Sydney, Australia, or an equal volume and protein content of mouse IgG3 kappa in clarified ascites fluid (Sigma Chemical Co., St. Louis, MO); rabbit anti-human IGF II antisemur (Calbiochem-Behring Corp., San Diego, CA) or an equal volume and protein content of nonimmune rabbit serum; or affinity-purified anti-bovine cation-independent mannose 6-phosphate receptor/IGF II receptor antibodies, kindly provided by Dr. William Sly, St. Louis University School of Medicine, St. Louis, MO. The monoclonal anti-IGF I antibody is an IgG3 (4). Its specificity is such that there is little or no competition for binding of 125I-IGF I to antibody by rat IGF II (4). The cross-reactivity of the anti-IGF II antiserum for IGF I is 0.5% (Calbiochem-Behring Corp.). Protein was measured by the method of Lowry et al. (19).

The dilution of anti-IGF I antibodies added to cultures was 1:4,000. The choice of this dilution was based upon the observation that a 1/4,000 dilution of a different monoclonal antibody to IGF I maximally inhibited DNA synthesis in platelet-derived growth factor–primed BALB/c3T3 cells (27). The dilution of anti-IGF II antiserum was also 1:4,000. The quantity of anti-IGF II receptor antibodies added to cultures was 1 μg/ml. The choice of the latter two dilutions was based upon availability of antibodies. Cultures were car-
bled for 1–6 d. Media were removed from cultures every 24 h for measure-
ments of peptides or IGF binding proteins and replaced with fresh media.

**Measurement of IGF I, IGF II, and IGF Binding Protein Contents of Media**

IGF I and IGF II contents of media were determined using radioimmunoas-
says. The primary antiserum used in radioimmunoaassays were a polyclonal rabbit anti-human IGF I antiserum provided through the NIDDK by Drs. Louis E. Underwood and Judson J. Van Wyk (University of North Carolina, Chapel Hill, NC), and anti-human IGF II antiserum (Calbiochem-Behring Corp.). Before measurement of peptides, media (1 ml vol) were extracted with 9 vol of 0.2 M acetic acid containing 0.1 M trimethylamine pH 2.8, to separate IGF binding protein activity from IGF I (25). The mixture was allowed to incubate at 4°C for 2 h, concentrated, resuspended in acetic acid/trimethylamine, and injected into a C18 HPLC gel filtration column exactly as before (25). Fractions corresponding to those that contained 125I-IGF I (~7000–8000 D) were collected, combined, concentrated, re-
constituted in a radioimmunoassay buffer and assayed for IGF I, or IGF II radioimmunactivity (25).

IGF binding protein activity was measured in samples reconstituted after HPLC using a competitive charcoal-binding assay exactly as in previous studies (25) except both 125I-IGF I and 125I-IGF II were used to detect IGF binding activity. To 75-μl samples suspended in radioimmunoassay buffer (25) were added 125I-IGF I or II (~30,000 counts per minute, final concen-
tration 10 pM). The resulting suspension was mixed, and incubated for 2 h at 22°C after which charcoal was added to suspensions. The suspensions were mixed, incubated for 8 min at 2°C, and subjected to centrifugation. Radioactivity in the supernatant was measured using a gamma counter. In suspensions that contained only buffer, ~2% of the radioactivity was pres-
ent in the supernatant after centrifugation (nonspecific binding). We have used this technique to detect IGF binding activity originating in renal col-
lecting duct from adult rats (25). In the present studies, six separate experi-
ments were performed (three each using 125I-IGF I and 125I-IGF II) that used supernatants obtained from three metanephroi on the fourth day of culture.

**Detection of IGF I and IGF II mRNAs in Metanephroi**

RNA from single freshly dissected metanephrolph was isolated using the

**Results**

Metanephroplc placed in organ culture underwent growth and development in vitro such that there were increases in size and morphological complexity. This is illustrated in Fig. 1 that shows a metanephros removed from a 13-d-old embryo im-
mediately after placement in organ culture (top) and the same renal anlage 4 days later (center). Typically, the long axis of the metanephros increased by ~50% over this period of time and the surface of the metanephros changed from a smooth to a more convoluted appearance. There was little additional change in gross morphology between 4 and 6 d in culture (bottom).

**Messenger RNAs for both IGF I and IGF II were present in whole metanephroi removed from 13-d-old embryos. The sizes of the amplified regions for IGF I (241 bp) and IGF II (285 bp) are consistent with amplification of IGF I and IGF II mRNA sequences (Fig. 2). The 241-bp amplified region for IGF I reflects mRNA that does not contain the 52-base insert or any sequence from exon 4 as described by Roberts et al. (22). The identities of the amplified regions were confirmed by restriction enzyme digestions. Within the am-
plified IGF I region there are two Alu I sites (30). The pre-
dicted three fragments of 117, 68, and 56 bp were produced upon digestion. The IGF II sequence contains a Pvu II site (13). Digestion yielded the predicted fragments of 201 and 84 bp (data not shown).
Both immunoreactive IGF I and IGF II were synthesized by the renal anlage and released into culture media. This is shown in Fig. 3 that illustrates levels of IGFs in media removed from metanephroi on days 1–6 after initiation of culture. Levels of IGF II in media were approximately twice those of IGF I. Levels of each peptide were relatively constant during 6 d of culture. The concentrations of IGFs I and II in culture media averaged $3.5 \times 10^{-9}$ and $8.3 \times 10^{-9}$ M respectively over this period of time. In additional experiments we determined that the radioimmunoassay we employed for IGF I was completely insensitive to $10^{-8}$ M recombinant hIGF II (Bachem Inc.) and that the radioimmunoassay that we used for IGF II was completely insensitive to $10^{-8}$ M hIGF I (Amgen Biologicals, Thousand Oaks, CA) added to media (data not shown). This demonstrates that cross-reactivity of one or both assays for the other member of the IGF family cannot explain the findings of radioimmunoactivity for both peptides in the media. Our observations establish that metanephroi synthesize both IGF I and IGF II in vitro. In contrast to the presence of IGF I and IGF II, IGF binding protein activity was not detectable in supernatants of metanephroi (data not shown).

To determine whether IGF I, IGF II, or both peptides produced by the renal anlage plays a role in growth and developmental processes in vitro, we cultured metanephroi removed from 13-d-old rat embryos in the serum-free defined media (control) (C) or in the presence of monoclonal anti-IGF I antibodies (aIGF I) or anti-IGF II antiserum (aIGF II). Dilutions containing the same amount of protein of mouse ascitic fluid (AF) and nonimmune rabbit serum (S) were substituted for the IGF I and IGF II antibodies, respectively, in additional control experiments. After 4 d in culture, metanephroi were photographed, then fixed, embedded in paraffin and sliced into 5-μm sections. Fig. 4 illustrates the appearance of whole metanephroi cultured under control (C, S, and AF) and experimental conditions (aIGF I, and aIGF II). Fig. 5 shows microscopic sections sliced perpendicular to the plane of dissection and stained with hematoxylin and eosin.

**Figure 1.** Photomicrographs of metanephroi. (Top) Metanephros dissected from a 13-d-old rat embryo (day 0). (Center) The same metanephros after 4 d in organ culture using serum-free defined media. (Bottom) The same metanephros after 6 d in organ culture. Pictures are representative.
to plane of the filter and perpendicular to the axis of the original ureteric bud midway between the top and bottom of the renal anlage. Cells within control metanephroi grow and differentiate such that the renal anlage enlarge and the original two branches of the collecting system undergo extensive arborization. Neither the ascites fluid, nor the nonimmune serum affected growth and differentiation of metanephroi. In contrast, if anti-IGF I or anti-IGF II antibodies were added to cultures, growth of the metanephros and development of the ductal system were markedly inhibited (Fig. 4 and 5). These findings demonstrate a dependence of metanephroi in organ culture upon IGF I and IGF II.

Controversy exists regarding whether physiological actions of IGF II are mediated via interaction of this peptide with the IGF I receptor or with the IGF II receptor (26). Bondy et al. demonstrated early and widespread expression of the IGF I receptor gene in rat embryos, in contrast to a relatively limited and localized pattern of IGF I gene expression. They suggested that their findings were consistent with actions of both IGF I and IGF II being mediated via the IGF I receptor (5).

We addressed the question of whether the action of IGF II to permit development of metanephroi in vitro is exerted through the IGF I receptor or through the IGF II receptor by first determining whether IGF II of metanephric origin (mIGF II) is biologically active. To this end we measured phospholipase C activity in canine renal proximal tubular baso-lateral membranes incubated with and without hIGF II or mIGF II. As shown in Table I, both hIGF II and mIGF II stimulated phospholipase C activity in the membranes.
IGF I does not activate this enzyme in basolateral membranes (23). Therefore the stimulation by mlGF II shown in Table I could not have resulted from IGF I of metanephric origin that was also present in the mlGF II preparations. Additional evidence that mlGF II is the agent in the preparations that stimulates phospholipase C, was provided by determining whether mannose 6-phosphate potentiates this effect.

We have shown that mannose 6-phosphate enhances activation of phospholipase C in basolateral membranes by IGF II via the IGF II/mannose 6-phosphate receptor (24). As illustrated in Table I, mannose 6-phosphate potentiated the activity of both hIGF II and mlGF II in basolateral membranes. This observation indicates that mlGF II is biologically active and capable of signal transduction via the IGF II receptor.

In other experiments, metanephric anlage were cultured in the presence of anti-human IGF II receptor antibodies (aIGF IIIR). As shown in Fig. 4 and 5 (lower right), growth and differentiation of metanephroi were inhibited by the antibodies. These findings provide strong additional evidence that the actions of IGF II to promote these activities are mediated via its own receptor.

**Discussion**

The formation of metanephric kidney is initiated by the interaction of the ureteric bud with metanephric blastema (29). This event occurs at 12.5 d of embryological development in the rat (1). The metanephric blastema differentiates into all of the tubular structures of the adult nephron with the exception of collecting duct that arises from the ureteric bud. Differentiation of metanephric blastema and the ureteric bud
is dependent upon an inductive event (or events) that occur when the ureteric bud encounters the metanephric blastema. The nature of this event (events) is unknown.

After induction of the metanephros, the kidney develops through an orderly process of tissue differentiation. The agents that control and regulate this postinductive process are unknown. It is proposed that a number of cell adhesion molecules, components of the extracellular matrix, and polypeptide growth factors coordinate the differentiative events (7). The roles of several putative coordinators of differentiation have been investigated using antibodies directed against these agents. Antibodies to the epithelial cell adhesion molecule, uvomorulin, do not perturb the development of polarized kidney tubules in embryologic organ culture (31). In contrast, postinductive differentiation of metanephric blastema is prevented by antibodies to the cell surface disialo-ganglioside GD3, and establishment of epithelial cell polarity during tubule development is inhibited by antibodies to the basement membrane glycoprotein, laminin (18). These observations indicate that GD3 and laminin participate in differentiation and development. Our present findings provide compelling evidence for roles of both members of the IGF family as well.

Growth and development of metanephroi in culture were markedly inhibited by anti-IGF I or by anti-IGF II antibodies. Because of the very low or absent reactivity for IGF II of the anti-IGF I mAbs used in our experiments, it is unlikely that they act to inhibit metanephric growth and development by rendering IGF II inactive. Therefore, our data strongly suggest a necessary role for IGF I in these processes. Because the anti-IGF II antibodies are relatively specific for IGF II, and because anti-IGF II receptor antibodies also inhibit growth and development, it is probable that IGF II is required for growth and development to occur in addition to IGF I. The only sources for either peptide in metanephric cultures are the metanephroi themselves. Therefore, our observations establish roles for both peptides of metanephric origin in renal growth and organogenic processes in vitro. It is likely that these findings reflect roles of endogenously produced IGFs for metanephrogenesis in vivo.

Levels of IGF II mRNA and protein in a number of tissues are higher during gestation than are levels of IGF I mRNA and peptide and, in rodents, the former decline within a few weeks postpartum. In contrast, IGF I exhibits the opposite pattern of expression. For this reason, it has been proposed that IGF II is the predominant fetal mitogen (20). Our data show that IGF I as well as IGF II is produced in developing metanephros. Assuming that the peptides present in media are in equilibrium with peptides within metanephroi, the levels of both IGF I and IGF II in developing kidneys would be sufficiently high so as to result in half-maximal binding to IGF receptors in most cellular systems including kidney (14). Therefore, it is likely that each peptide is present within the metanephros in concentrations that could regulate metabolic, growth, and developmental events.

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