Insulin-like Growth Factors I and II Are Produced in the Metanephros and Are Required for Growth and Development In Vitro

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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 3.5×10^{-9}M IGF I and 8.3×10^{-9}M IGF II 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.

Most fetal tissues contain extractable polypeptide growth factors or growth factor activities and express receptors and binding proteins for these agents. For this reason, members of one or more polypeptide growth factor families are thought to regulate processes of morphogenesis, differentiation and growth during mammalian development (20). Certain of these agents have been implicated as causative of organogenesis of the metanephric kidney (2,7). A role for insulin-like growth factors (IGFs)[1] in this process is supported by several lines of investigation. First, mRNAs for IGFs I and II are present in fetal kidneys, and both IGF I and IGF II can be extracted from the organ (15–17). Second, receptors for both IGF I and IGF II are present in fetal renal tissue, establishing the potential for IGF-mediated signal transduction (10, 21). In addition, IGF I is produced in vitro by cells removed from fetal kidneys, suggesting that synthesis of at least one of the IGFs can occur in the metanephros in vivo (11). Finally, IGF II, stimulates growth and promotes segmental differentiation of tubular segments when added as a growth supplement to explants of metanephroi (3).

Despite what is known about the presence of growth factors and mRNAs and receptors for these agents in developing tissues, there is a paucity of information relating directly the growth and development of any one specific organ system to endogenously produced growth factors. To address this issue, in view of what is known about IGFs and fetal kidney, we carried out studies to define the dependence of renal organogenesis on IGFs I and II. We removed metanephroi from 13-d-old rat embryos and cultured them in serum-free defined media for up to 6 d. We probed for IGF I and IGF II mRNAs in the tissues, measured synthesis of IGFs in organ culture, and characterized growth and development in the absence and presence of anti-IGF I or anti-IGF II antibodies or anti-IGF II receptor antibodies. We demonstrate that both IGF I and IGF II are synthesized by metanephroi from fetal rat and that each is required for growth and development to take place in vitro. It is likely that both members of this family of polypeptide growth factors are produced by the 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. Meta-

1. *Abbreviation used in this paper*: IGF, insulin-like growth factor.

nephric kidneys were surgically dissected from embryos. The age of the embryos 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 branches (1).

Cultures of whole metanephroi were carried out on sterile membranes (No. 110409; Nuclepore Corp., Pleasanton, CA) of 0.8 μm thickness and 13 mm diameter in a medium consisting of equal volumes of Dulbecco's modified Eagles medium and Hams F12 medium supplemented with 25 mM Hepes buffer, sodium bicarbonate (1.1 mg/ml), 10 nM $Na_2 \cdot SeO_3 \cdot 5H_2O$, prostaglandin E1 ($10^{-11}$ M), iron-saturated transferrin (5 μg/ml), gentamicin (50 μg/ml), Nystatin (50,000 U/ml), 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 Institute of Diabetes Digestive and Kidney Diseases (NIDDK) 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 antiserum (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 ^{125}I-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 carried out for 1–6 d. Media were removed from cultures every 24 h for measurements 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 radioimmunoassays. The primary antisera used in radioimmunoassays 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 C_{18} HPLC gel filtration column exactly as before (25). Fractions corresponding to those that contained ^{125}I-IGF I (~7,000–8,000 D) were collected, combined, concentrated, reconstituted in a radioimmunoassay buffer and assayed for IGF I, or IGF II radioimmunoactivities (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 ^{125}I-IGF I and ^{125}I-IGF II were used to detect IGF binding activity. To 75-μl samples suspended in radioimmunoassay buffer (25) were added ^{125}I-IGF I or II (~30,000 counts per minute, final concentration 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 present in the supernatant after centrifugation (nonspecific binding). We have used this technique to detect IGF binding activity originating in renal collecting duct from adult rats (25). In the present studies, six separate experiments were performed (three each using ^{125}I-IGF I and ^{125}I-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 metanephroi was isolated using the method of Chomczynski and Sacchi (8). The solution used in the isolation is available commercially as RNAzol B (CINNA/BIOTECX Laboratories, Friendwood, TX). 20 μg of yeast tRNA were added to metanephroi as carrier. First strand cDNA was synthesized from the RNA transcripts using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; GIBCO BRL, Gaithersburg, MD) and oligo dT as the primer. The cDNAs for IGF I and IGF II were amplified using the polymerase chain reaction (PCR) (28) using sequence specific oligomers. The 5′ and 3′ oligomers used in the amplification of IGF I cDNA correspond to nucleotides 280–298 of exon 2, and 584–568 of exon 5 of the rat gene, respectively (31). The IGF II cDNA was amplified with oligonucleotides corresponding to bases 25–45 of exon 1, and 148–130 of exon 2 (13). The oligomers chosen for PCR were located across introns in the IGF I and IGF II sequences to ensure that amplified products originated from mRNA sequences. The size of the amplified regions was visualized after polyacrylamide gel electrophoresis. Tris-borate (89 mM), 2 mM EDTA, pH 8.3, was used as the running buffer. The identity of the PCR products was confirmed by restriction digest analysis.

Measurement of Phospholipase C Activity

Phospholipase C activity was evaluated by measuring levels of inositol trisphosphate (Ins-P_3) in suspensions of canine renal proximal tubular basolateral membranes exactly as described in detail before (23). Activities were measured in membranes incubated with or without 10^{-9} M human recombinant IGF II (hIGF II) (Bachem Inc., Torrance, CA) or 10^{-9} M IGF II originating from metanephroi (mIGF II). To obtain sufficient quantities of mIGF II, HPLC fractions that contained immunoreactive IGF II originating from 25 metanephroi were pooled and concentrated as described previously (25). Phospholipase C activities were measured in the presence and absence of 5 mM mannose 6-phosphate. Fractions of incubation media alone subjected to HPLC corresponding to fractions that contained mIGF II in media removed from metanephroi were used in control experiments where indicated (Media; see Table I).

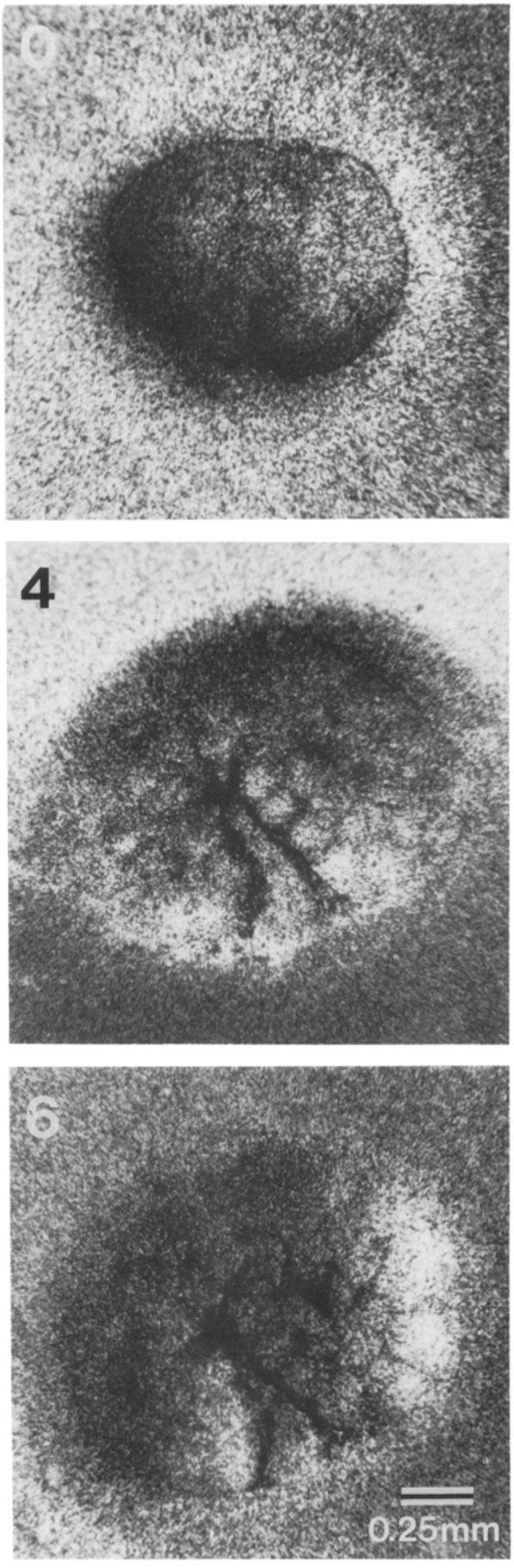
Processing of Cultured Metanephroi for Histological Studies

Cultured metanephroi and filters were fixed in 10% phosphate-buffered formalin, pH 7.4 for 30 min at room temperature, and then stored in 60% ethanol at 4°C until processing could be performed. Metanephroi were embedded in paraffin, sliced into 5-μm sections, and placed on gelatin-coated slides in preparation for staining with hematoxylin-eosin as before (6).

Results

Metanephroi placed in organ culture underwent growth and development in vitro such that there were increases in size and morphologic complexity. This is illustrated in Fig. 1 that shows a metanephros removed from a 13-d-old embryo immediately 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 amplified IGF I region there are two Alu I sites (30). The predicted 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).



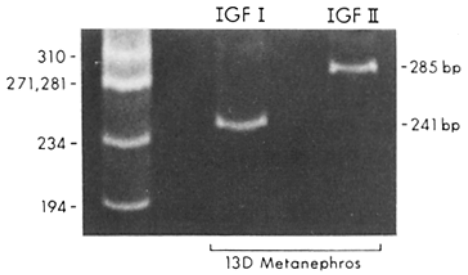


Figure 2. IGF I and IGF II mRNAs are present in the metanephros. Shown is a 5% polyacrylamide gel stained with ethidium bromide. On the left are molecular size markers expressed in base pairs. On the right are the sizes of the IGF I (241 bp) and IGF II (285 bp) cDNAs amplified using the polymerase chain reaction. The gel is representative of three experiments.

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 3.5×10^{-9} and 8.3×10^{-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/play 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

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.

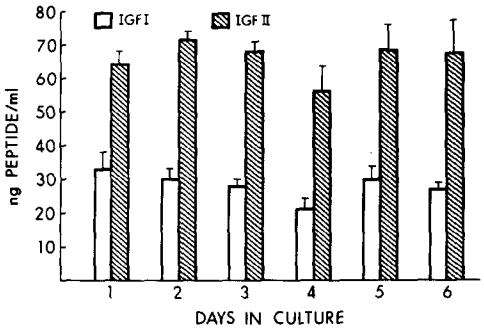


Figure 3. Levels of IGF I and IGF II in media removed from developing rat metanephroi. The volumes of the media were 1 ml per explant. Media were removed after 24 h of contact with metanephroi. Data are expressed as mean ± SE of seven experiments (seven metanephroi).

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.

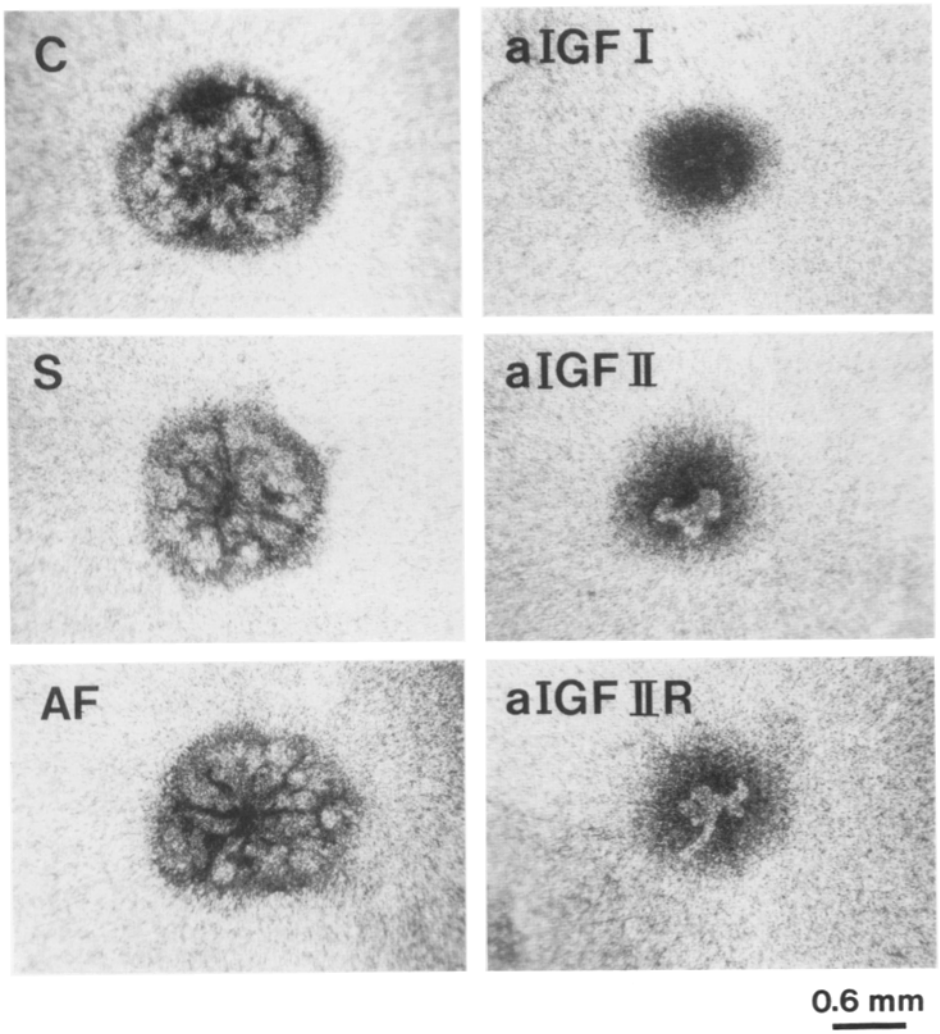


Figure 4. Photomicrographs of metanephroi. Shown are metanephroi cultured for 4 d in the serum-free chemically defined media (control, *C*) or in media to which the following additions were made: mouse ascites fluid (*AF*); normal rabbit serum (*S*); anti-IGF I antibodies (*aIGF I*); anti-IGF II antibodies (*aIGF II*); or anti-IGF II receptor antibodies (*aIGF IIR*). The pictures are representative of 10 separate experiments.

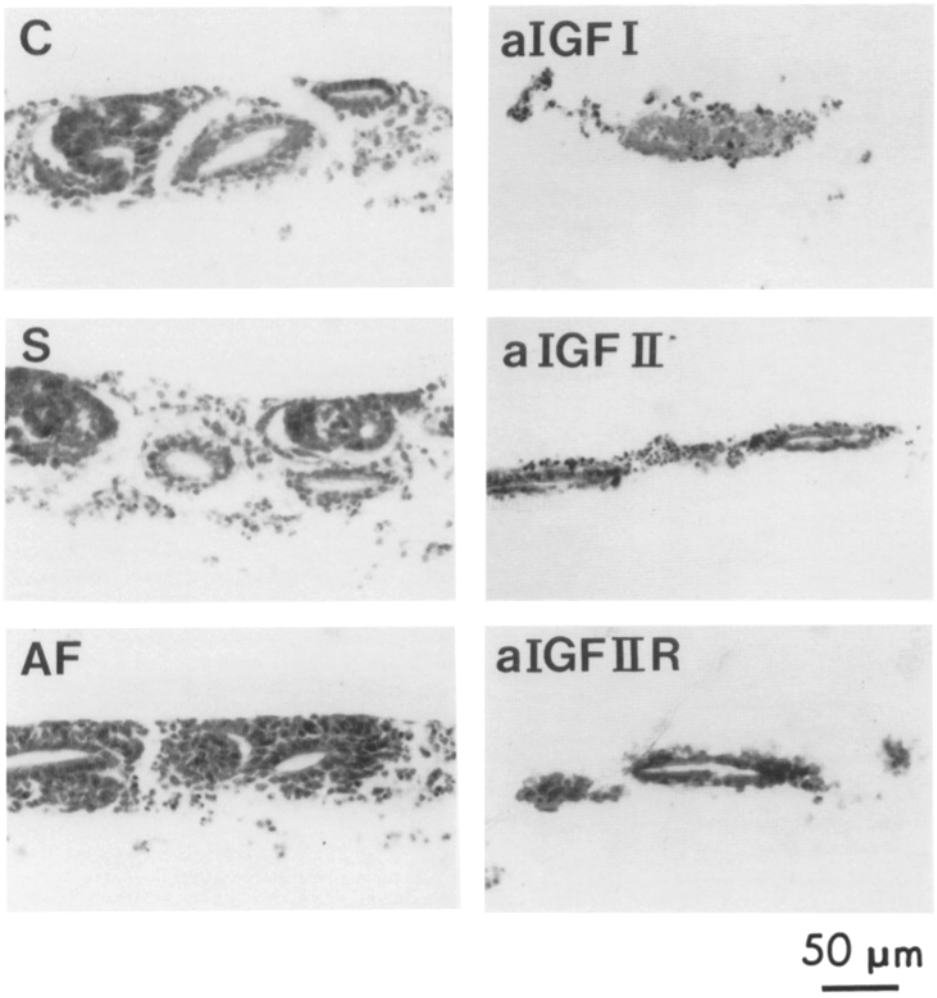


Figure 5. Photomicrographs of metanephroi. Shown are hematoxylin and eosin-stained sections originating from metanephroi cultured for 4 d in media described in the legend to Fig. 5. The pictures are representative of 10 separate experiments.

IGF I does not activate this enzyme in baso-lateral membranes (23). Therefore the stimulation by mIGF II shown in Table I could not have resulted from IGF I of metanephric origin that was also present in the mIGF II preparations. Additional evidence that mIGF 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 mIGF II in basolateral membranes. This observation indicates that mIGF II is biologically active and capable of signal transduction via the IGF II receptor.

Table I. Production of Ins-P_3 in Basolateral Membrane Suspensions

Addition to suspension	Ins-P_3
	nmol/mg protein/15 s
None	0.780 ± 0.05
Media	0.835 ± 0.09
M6P	0.895 ± 0.05
hIGF II	2.10 ± 0.25
hIGF II and M6P	4.64 ± 0.44
mIGF II	2.29 ± 0.21
mIGF II and M6P	5.12 ± 0.84

Shown are results of four experiments. Additions to basolateral membrane suspensions were 5 mM mannose 6-phosphate (M6P), 10^{-9} M human recombinant IGF II (hIGF) or 10^{-9} M IGF II originating from metanephroi (mIGF II). hIGF II > none, $P < 0.01$; hIGF II and M6P > hIGF II, $P < 0.01$; mIGF II > media, $P < 0.01$; mIGF II and M6P > mIGF II, $P < 0.05$ (Dunnett's multiple comparison procedure) (12). Data are expressed as mean ± SE.

In other experiments, metanephric anlage were cultured in the presence of anti-human IGF II receptor antibodies (aIGF IIR). 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 disialoganglioside G_{D3}, and establishment of epithelial cell polarity during tubule development is inhibited by antibodies to the basement membrane glycoprotein, laminin (18). These observations indicate that G_{D3} 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.

We acknowledge the administrative assistance of Ms. Lynn Wesselmann. We are grateful to Dr. Alan Perantoni (University of Colorado, School of Medicine, Denver CO), and Dr. George F. Schreiner Jr. (Washington University School of Medicine) for help with embryo dissection and organ culture.

S. A. Rogers, G. Ryan, and M. R. Hammerman were supported by grants DK-27600, DK09976, and DK-42958 from the National Institutes of Health.

Received for publication 14 December 1990 and in revised form 25 February 1991.

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