Expression of AT_2 Receptors in the Developing Rat Fetus

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Abstract

Angiotensin II is known primarily for its effects on blood pressure and electrolyte homeostasis, but recent studies suggest that angiotensin II may play a role in the regulation of cellular growth. This study was undertaken to identify the angiotensin II receptor subtypes expressed during fetal and neonatal development and to characterize their cellular localization. Using an in situ receptor binding assay on sagittal frozen sections of fetal and neonatal rats, bound ^{125}I-[Sar^1,Ile^8]-angiotensin II was visualized by film and emulsion autoradiography. Bound radioligand was detected by E11 (embryonic day 11) and maximal binding occurred by E19–21. Radioligand binding remained unaltered 30 min after birth, whereas a noticeable and stable decrease was observed 12 h postparturition. The highly abundant angiotensin II receptors were shown to be AT_2 by the marked reduction in radioligand binding achieved with PD123177 (10^{-7} M), a specific AT_2 receptor antagonist, whereas DuP 753 (10^{-5} M), an AT_1 receptor antagonist, had little effect. Emulsion autoradiography showed radioligand binding in the undifferentiated mesenchyme of the submucosal layers of the intestine and stomach, connective tissue and choroid surrounding the retina, subdermal mesenchyme adjacent to developing cartilage, diaphragm, and tongue. Residual AT_2 receptors were found on the dorsal subdermal region of the tongue 72 h after birth. AT_1 receptors were detected in the placenta at E13 and in the aorta, kidney, lung, liver, and adrenal gland at E19–21, consistent with an adult distribution. The transient expression of AT_2 receptors in the mesenchyme of the fetus suggests a role of angiotensin II in fetal development. (*J. Clin. Invest.* 1991. 88:921–933.) Key words: development • DUP 753 • PD123177 • mesenchyme • embryogenesis

Introduction

Angiotensin II receptors are present in a wide variety of tissues in the adult vertebrate (1–3). Acting through these receptors, angiotensin II maintains circulatory homeostasis (4, 5), stimulates the secretion of prolactin (6), aldosterone (7), adrenocorticotropic hormone (8), and corticotropin releasing hormone (9), acts as a neurotransmitter in the central nervous system (10), and has a less well defined role in the function of reproductive organs (11, 12). All the components of the renin-angiotensin system (RAS),[1] including angiotensin II receptors, have been colocalized within many adult organs (13, 14) and in the developing fetal-placental unit (15, 16). Because both the renin and angiotensinogen genes (14, 17) and angiotensin II receptors (18, 19) are regulated in a tissue-specific fashion, it has been suggested that the RAS has autocrine/paracrine functions that may be independent from its function in cardiovascular homeostasis. Angiotensin II is also one of a growing number of peptide hormones that have been implicated in the regulation of cellular growth (20). Angiotensin II potentiates the mitogenic effect of epidermal growth factor (21), increases expression of platelet-derived growth factor (22) and the proto-oncogenes c-*fos* (23, 24), c-*myc* (22), and c-*jun* (25). The RAS is active during fetal development and high concentrations of angiotensin II receptors are expressed transiently in mouse, rat, and primate fetuses (26–29).

Two distinct angiotensin II receptor subtypes (AT_1 and AT_2) have been identified by both sensitivity to dithiothreitol (30, 31) and by their ability to bind the nonpeptide antagonists DuP 753 (AT_1) and PD123177 (AT_2) (32, 33). In the adult rat, the majority of the hemodynamic actions of angiotensin II are mediated through the AT_1 receptor (33–35). The type and cellular localization of angiotensin II receptors expressed in the developing rat fetus and neonate are not known. This study demonstrates that this highly abundant and transiently expressed angiotensin receptor is AT_2 and identifies the cellular sites of expression in the developing fetus and neonate.

Methods

Animals. Rats were maintained on rat chow and tap water ad lib., with 12-h light/dark cycles. Timed pregnant and neonatal Sprague Dawley rats were killed by carbon dioxide asphyxiation and the fetuses (embryonic day [E]11, 12, 14, 16, 19, 20, and 21) or neonatal pups (6, 12, 24, 48, and 72 h of age) were placed in optimal cutting temperature mounting medium, snap frozen in liquid nitrogen, and stored at −80°C before sectioning. The day after a vaginal plug was observed was considered to be embryonic day 0.

In situ angiotensin II receptor assay. Angiotensin II receptors were localized using the method of Mendelsohn et al. (36, 37). Briefly, midsagittal frozen sections (4 μm) of whole fetuses or pups were cut on a cryostat at −20°C, thaw-mounted onto poly-L-lysine coated slides, dried in vacuo overnight at −5°C over silica gel, and stored with silica gel in sealed boxes at −80°C. Immediately before use, the sections were dried for an additional 2–4 h in vacuo at room temperature. The endog-

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1. *Abbreviations used in this paper:* E, embryonic day; RAS, renin-angiotensin system; TGF, transforming growth factor.

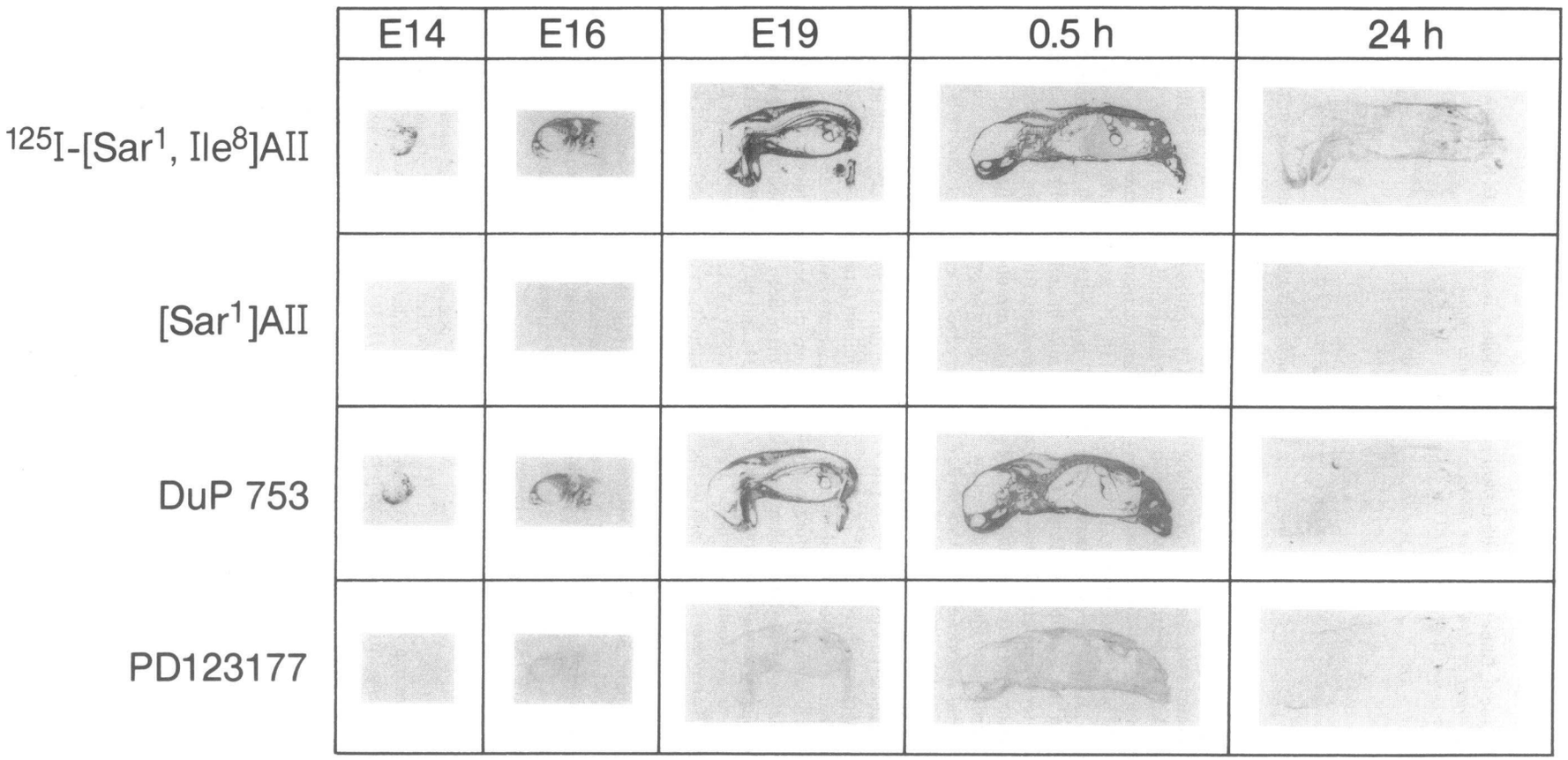


Figure 1. Developmental regulation of angiotensin II receptors. Binding of ^{125}I-[Sar^1,Ile^8]-angiotensin II to fetal and neonatal midsagittal rat sections was visualized with film autoradiography (*top panel*). Autoradiographs were obtained after 72 h of exposure, except at E14, which required 7 d of exposure for optimal visualization. [Sar^1]-angiotensin II (10^{-5} M) (*second panel*) and PD123177 (10^{-6} M) (*fourth panel*) eliminated radioligand binding, while little effect was detected with competition by DuP 753 (10^{-5} M) (*third panel*).

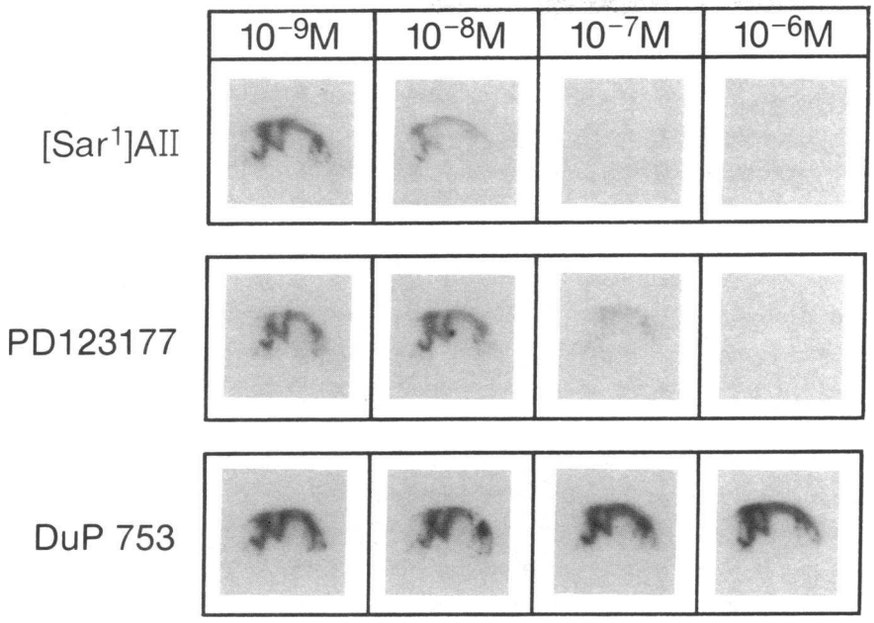


Figure 2. Receptor antagonist competition curves. Using E16 fetal sections, radioligand binding in the presence of increasing concentrations (10^{-10}–10^{-3} M) of [Sar^1]-angiotensin II, (*top panel*), PD123177, (*middle panel*), and DuP 753, (*bottom panel*), was visualized by film autoradiography in three separate experiments. Depicted above is a representative autoradiograph showing the concentrations of PD123177 and [Sar^1]-angiotensin II necessary to achieve maximal radioligand inhibition. DuP 753 at 10^{-4} M was necessary to achieve any visible binding inhibition.

enously bound ligand was removed by preincubation with 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 5 mM Na_2EDTA, 0.3 mM bacitracin, and 0.2% BSA for 20 min at room temperature. This was replaced with the same buffer containing 250 pM ^{125}I-[Sar^1,Ile^8]-angiotensin II (2,200 Ci/mmol, DuPont NEN Research Products, Boston, MA) and the sections were incubated for 60 min at 16°C in a humidified chamber. Sections were washed four times for 60 s each in 50 mM Tris, pH 7.4, at 0°C, dried in a stream of cool air, and exposed to Ultrofilm (Reichert-Jung, Heidelberg, Germany) for 4–14 d. The receptor-angiotensin II complexes were fixed by exposure to paraformaldehyde vapors at 80°C for 2 h in a closed chamber under vacuum. The vapors were evacuated from the chamber and the sections left under a drafted hood overnight to ensure removal of residual vapors. The sections were subsequently coated with NTB2 photographic emulsion (Eastman Kodak Co., Rochester, NY) and air dried lying flat (37, 38). The sections were exposed for 4–8 d at 4°C, stained with hematoxylin-eosin, and examined by bright/dark-field microscopy using a Zeiss Universal microscope equipped with a dark-field condenser. Routine controls to evaluate the emulsion for altered chemography included coating a blank slide (to detect background levels of silver grains in the emulsion) and coating sections which had no radioligand in the receptor binding buffer. In addition, specificity of binding was evaluated with [Sar^1]-angiotensin II (10^{-5} M) (Peninsula Laboratories, Inc., Belmont, CA) while receptor subtypes were identified by inhibition of radioligand binding with the receptor antagonists DuP 753 (10^{-5} M, AT_1) and PD123177 (10^{-6} M, AT_2), generously provided by E. I. du Pont de Nemours & Co., Wilmington, DE. Each experiment was repeated a minimum of three times with highly reproducible results. The autoradiographs presented are representative.

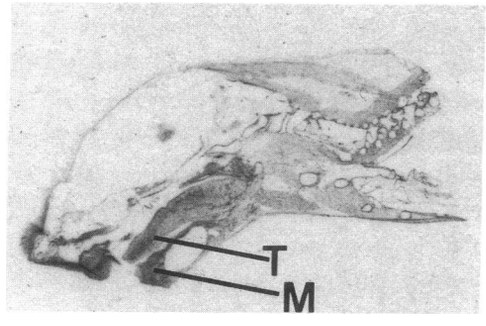


Figure 3. Angiotensin II radioligand binding in the head of a rat 72 h after birth. An autoradiograph of radioligand binding to midsagittal frozen sections obtained 72 h postparturition reveals residual binding to AT_2 receptors in the tongue (*T*) and lower mandible (*M*).

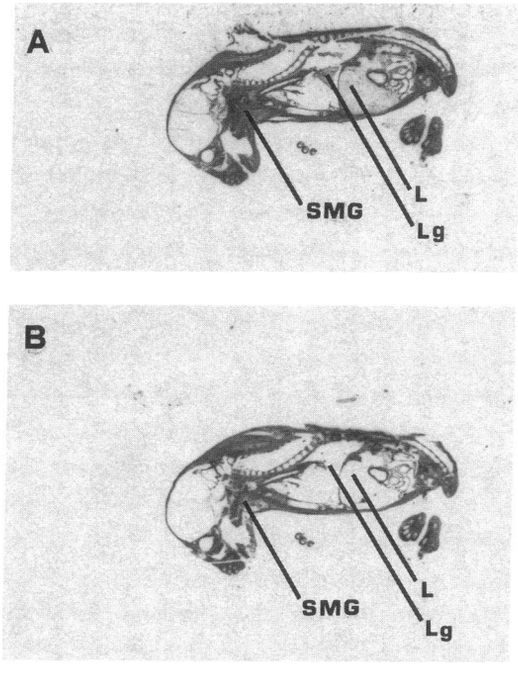


Figure 4. AT_1 angiotensin II receptors in the rat fetus at E19. Autoradiography performed on frozen sections of an E19 rat fetus shows the distribution of radioligand binding to angiotensin II receptors (panel *A*). Diminution in AT_1 receptor binding is found in the liver (*L*), submaxillary gland (*SMG*), and lung (*Lg*) after competition with DuP 753 (10^{-5} M) (panel *B*) (×1.5).

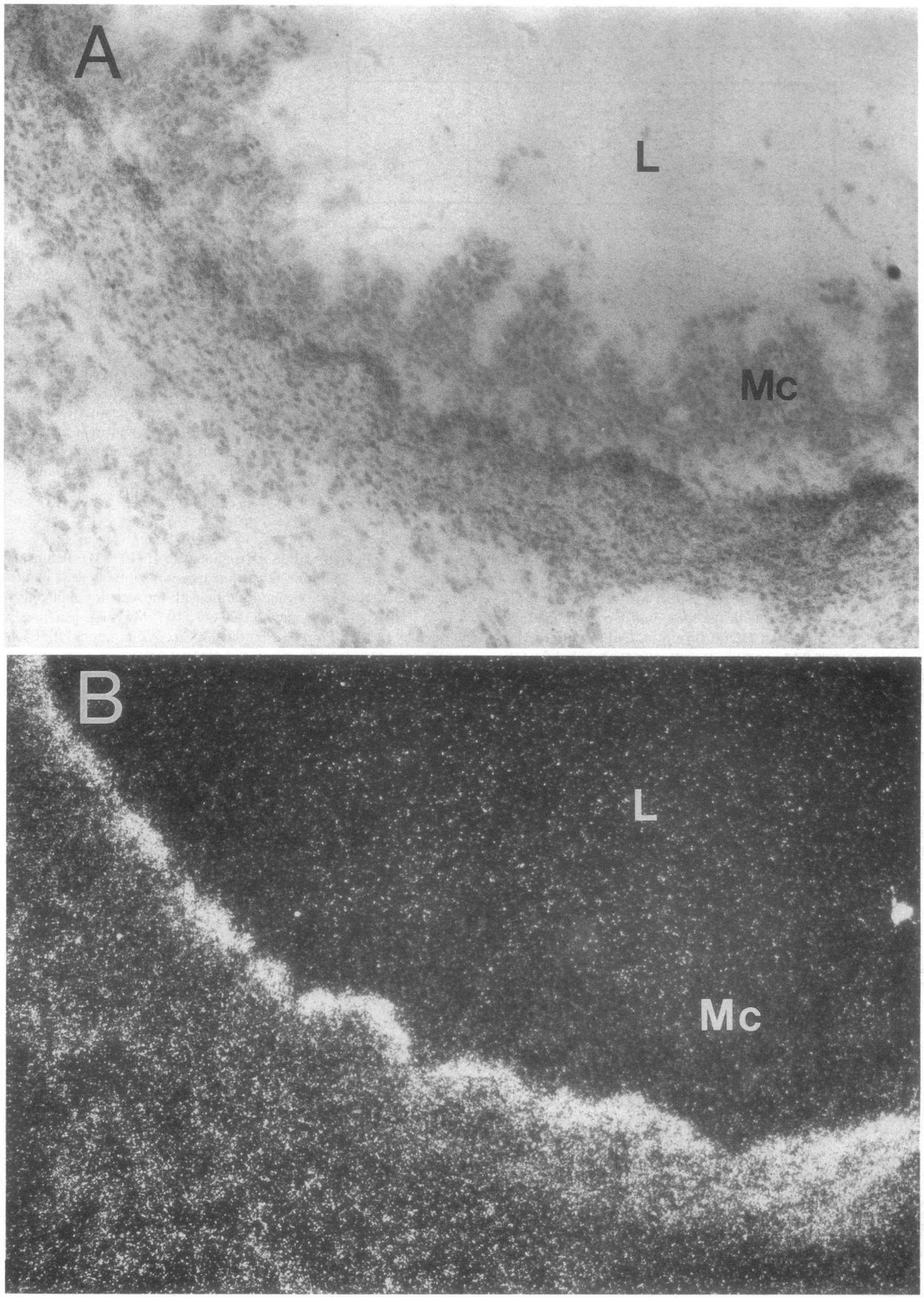
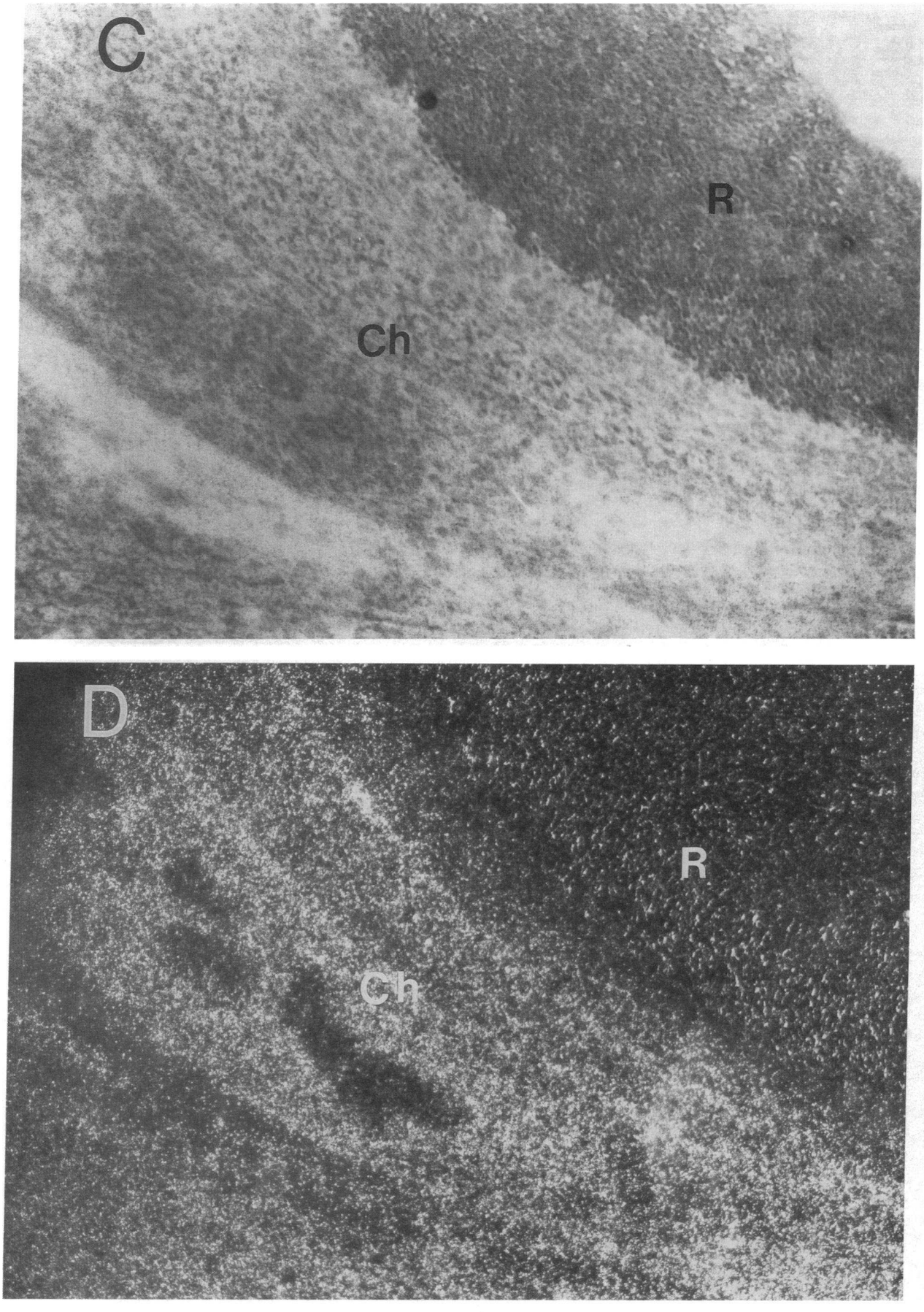


Figure 5. Bright-field (panels *A*, *C*, and *E*) and dark-field (panels *B*, *D*, and *F*) photomicrographs of angiotensin II radioligand binding visualized using emulsion autoradiography. Radioligand binding to angiotensin receptors in the fetal and neonatal sagittal sections was visualized by silver grains overlying cells expressing angiotensin II receptors. Abundant AT_2 receptors are identified in the intestine (panels *A* and *B*; *L*, lumen; *Mc*, mucosa), choroid surrounding the retina (panels *C* and *D*; *R*, retina; *Ch*, choroid) and tongue (panels *E* and *F; T*, tongue; *G*, gustatory papillae, *U*, upper palate) (×687).



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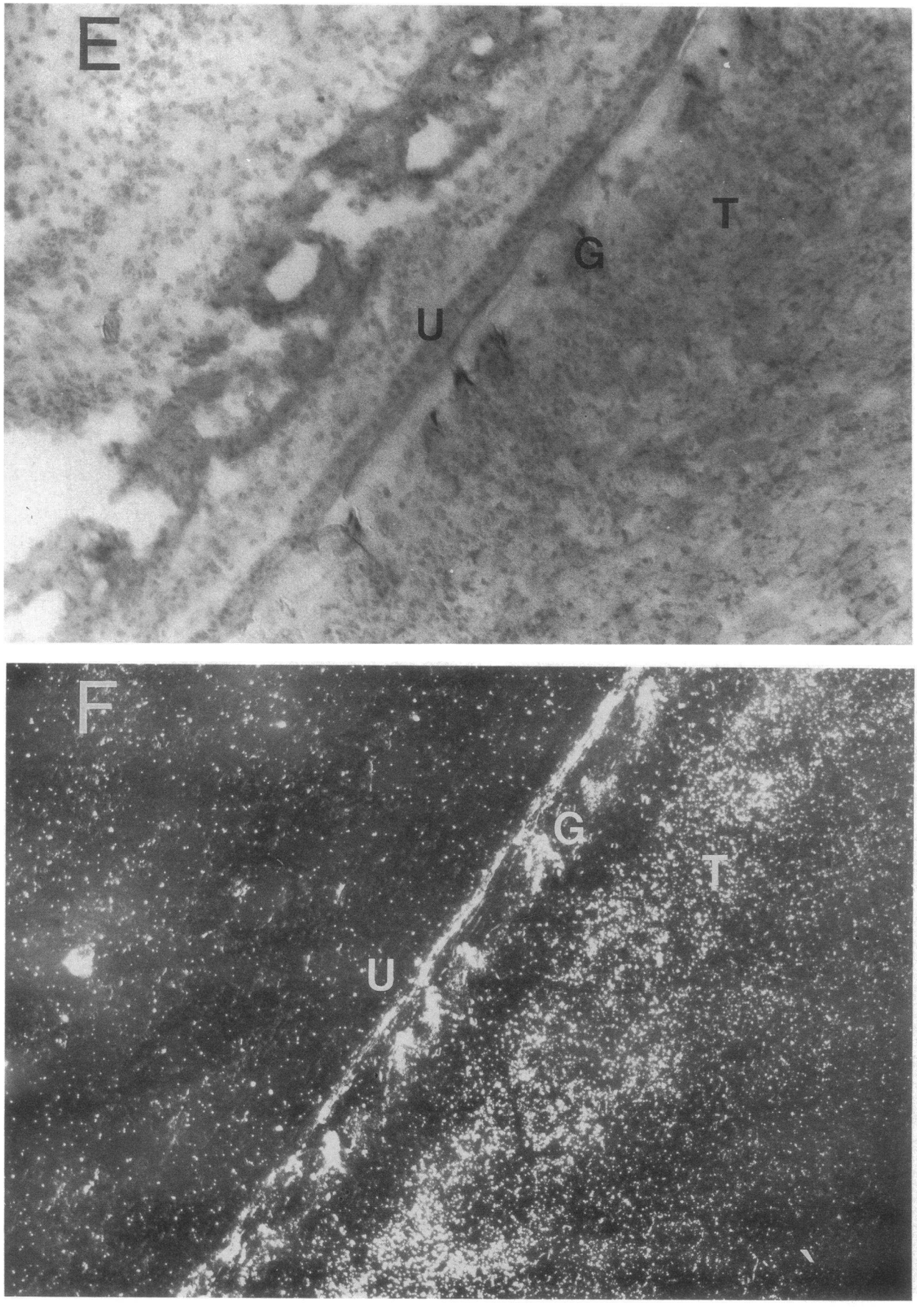

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Results

Developmental regulation of angiotensin II receptors. Autoradiographic visualization of radioligand binding to angiotensin II receptors was evident along the spine of the E14 fetus as depicted in Fig. 1 (*top panel*), but with longer exposures, angiotensin II receptors could also be detected at E11 (data not shown). Binding increased markedly by E16, was maximal by E19–21, and remained unaltered 30 min postparturition. Thereafter, radioligand binding decreased markedly by 12 h after birth and reached a stable nadir from 24 to 72 h (Fig. 1, *top panel*).

Using fetal sections obtained at E16, the specificity of radioligand binding was demonstrated by the ability of unlabeled [Sar^1]-angiotensin II to compete for the majority of angiotensin II receptors at concentrations of 10^{-8} M or greater (Fig. 2, *top panel*). This result correlates well with the known binding affinity of angiotensin II for its receptor. As also shown, radioligand binding was markedly reduced with PD123177 (10^{-7} M) (Fig. 2, *middle panel*), while substantially higher concentrations of DuP 753 (10^{-4} M) were necessary to achieve any binding inhibition (Fig. 2, *bottom panel*). These results indicate that the vast majority of the fetal angiotensin II receptors present at E16 are AT_2. Radioligand binding at other fetal (E14, 16, and 19) and neonatal (0.5 and 24 h) stages was eliminated with [Sar^1]-angiotensin II (10^{-5} M) and PD123177 (10^{-6} M), while DuP 753 (10^{-5} M) had little effect (Fig. 1). These results demonstrate that the angiotensin II binding is specific and that throughout fetal development, the highly abundant receptors are AT_2. Residual radioligand was found at all ages after competition with PD123177 and at 24 h after competition with either DuP 753 or PD123177 (Fig. 1). Combined competition with PD123177 and DuP 753 did not decrease the autoradiographic signal further (data not shown). In the neonatal animal, abundant radioligand binding to AT_2 receptors remained on the dorsal surface of the tongue and mesenchyme surrounding the mandible as late as 72 h after birth (Fig. 3).

With longer exposure of sections obtained at E19, radioligand binding could be seen within the liver, lung, and submaxillary gland (Fig. 4 *A*) that was eliminated with DuP 753 (Fig. 4 *B*). Additional sites of AT_1 receptor binding included the adrenal cortex, aorta, and kidney (data not shown). This indicates that AT_1 receptors are present in the fetus with a tissue distribution identical to that found in the adult animal. Radioligand binding to AT_2 receptors was also found in the placenta of E13 fetuses.

Cellular localization of AT_2 angiotensin II receptors. Using emulsion autoradiography, abundant silver grains, indicative of bound radioligand, were evident by both bright (Fig. 5, *A*, *C*, and *E*) and dark-field (Fig. 5, *B*, *D*, and *F*) microscopy. The greatest number of grains were found overlying undifferentiated mesenchyme in the submucosal layers of the intestine (panels *A* and *B*; *L*, lumen; *Mc*, mucosa) and stomach, connective tissue and choroid surrounding the retina (panels *C* and *D*; *R*, retina; *Ch*, choroid), dorsal subdermal region of the tongue (panels *E* and *F*; *T*, tongue; *G*, gustatory papillae; *U*, upper palate), and subdermal mesenchyme adjacent to developing cartilage, adrenal medulla, and diaphragm (data not shown). Fewer silver grains were found overlying well differentiated skeletal muscle while none were detected overlying bone. Radioligand binding was competed with the following antagonists: [Sar^1]-angiotensin II (10^{-5} M), PD123177 (10^{-6} M), and DuP 753 (10^{-5} M) (Fig. 6). Highly abundant silver grains were found overlying the intestinal mucosa (Fig. 6, *A* and *B*) in sections incubated with radioligand alone. These silver grains were diminished drastically by both [Sar^1]-angiotensin II (Fig. 6, *C* and *D*) and PD123177 (Fig. 6, *E* and *F*). Competition with DuP 753 had no effect on the levels of silver grains (Fig. 6, *G* and *H*). These data confirm the specificity of the emulsion chemography.

Discussion

Highly abundant angiotensin II receptors were detected along the spine of the E11 rat fetus. This developmental expression agrees with membrane binding studies in which angiotensin II receptors were identified at about E10 (28). At this stage of development, the fetus is coiled in a C-shape, major organs are completing organogenesis, and the neural groove is open (39). By E14–16, when angiotensin II radioligand binding was easily detected, organogenesis is complete except for the urogenital tract, and the first skeletal cartilage appears in the ribs. The maximal radioligand binding that occurred at E19–20 correlates with ongoing mesenchyme differentiation into bone, vascular structures, muscle, and fat (39). 30 min after birth, receptors were still abundant in the mesenchyme, declining sharply thereafter and by 72 h, persisted only in the subdermal layer of the tongue.

That the highly abundant receptors in the rat fetus are AT_2 was demonstrated by the ability of PD123177, an AT_2 receptor antagonist, to effectively eliminate radioligand binding. These AT_2 angiotensin receptors were most abundant in the cells localized in the mesenchyme (Fig. 5). These cells have the potential to differentiate into smooth and striated muscle, connective tissue sheaths surrounding the muscle, blood vessels, and ligaments, and other cells such as chondroblasts, osteoblasts, and fibroblasts. The mesenchyme also induces regional epithelial differentiation as exemplified by the role palatal mesenchyme plays in inducing mandibular epithelium to differentiate in a palatal pattern (40). In addition, mesenchyme can itself be modified by the juxtaposed epithelium. For example, 1-d old murine uterine mesenchyme develops increased amounts of well organized myometrium only when it is grown with uterine epithelium (41). It is likely that the complex developmental decisions made by the multipotent mesenchymal cells are influenced by many environmental factors acting in sequence. Therefore, it is relevant that receptors for insulin-like growth factor I (42) and progesterone (43), and insulin-like growth factor I mRNA (42) are present in the mesenchyme of the fetus in a pattern similar to that of the angiotensin II receptor. In contrast, transforming growth factor (TGF)-β mRNA and protein has been localized to the mesenchyme during murine embryogenesis (44). This growth factor is thought to be coupled to mesenchymal remodeling involved in limb and digit formation and may be important in stimulating angiogenic activity. The AT_2 receptors are localized to subdermal mesenchyme whereas TGF-β is localized more to the mesenchymal cells in the dermis and epidermis (44). Serial section analysis will be necessary to determine accurately their comparative cellular localization. Even if AT_2 receptors do not colocalize with TGF-β, the known reciprocal inductive influence epithelium and mesenchyme have on each other during devel-

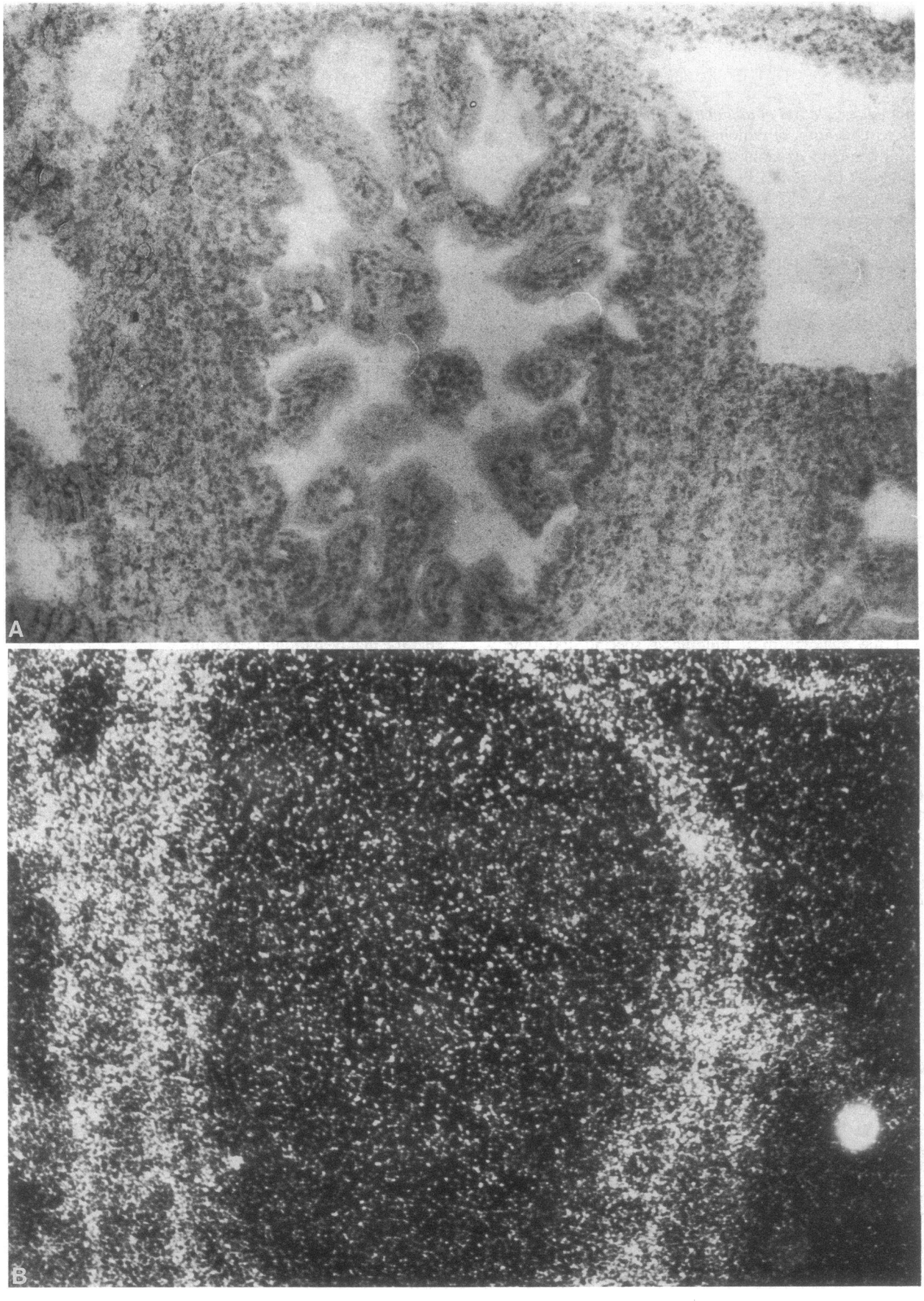
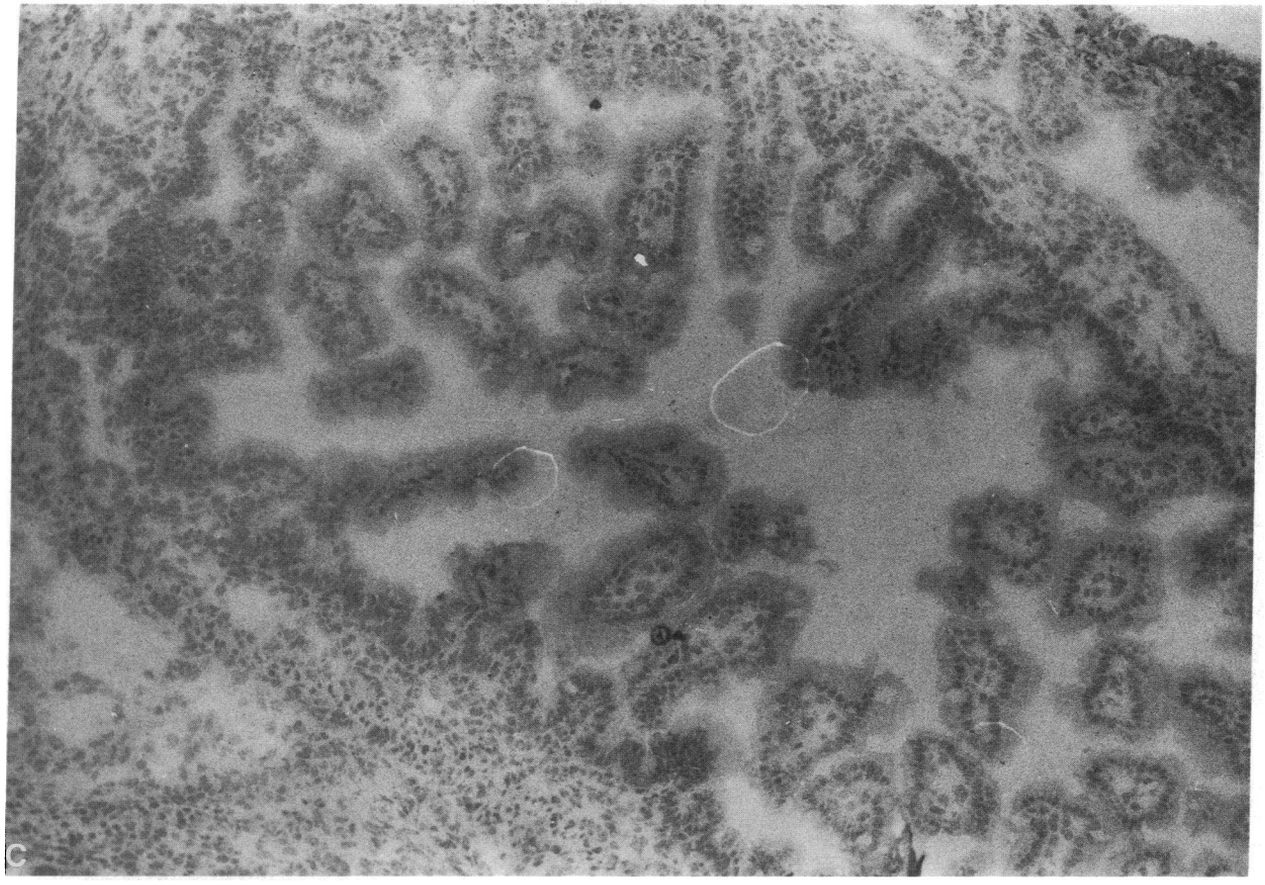


Figure 6. Bright-field (panels *A*, *C*, *E*, and *G*) and dark-field (panels *B*, *D*, *F*, and *H*) photomicrographs demonstrating the specificity of angiotensin II radioligand binding visualized using emulsion autoradiography. Radioligand binding to angiotensin receptors in 1/2-h postnatal intestine was visualized by silver grains overlying cells expressing angiotensin II receptors (panels *A* and *B*). Competition of radioligand binding with either [Sar^1]-angiotensin II (panels *C* and *D*) or PD123177 (panels *E* and *F*) dramatically diminished the number of silver grains to background levels. In contrast, DuP 753 (panels *G* and *H*) had no significant effect on the number or distribution of silver grains (×687).



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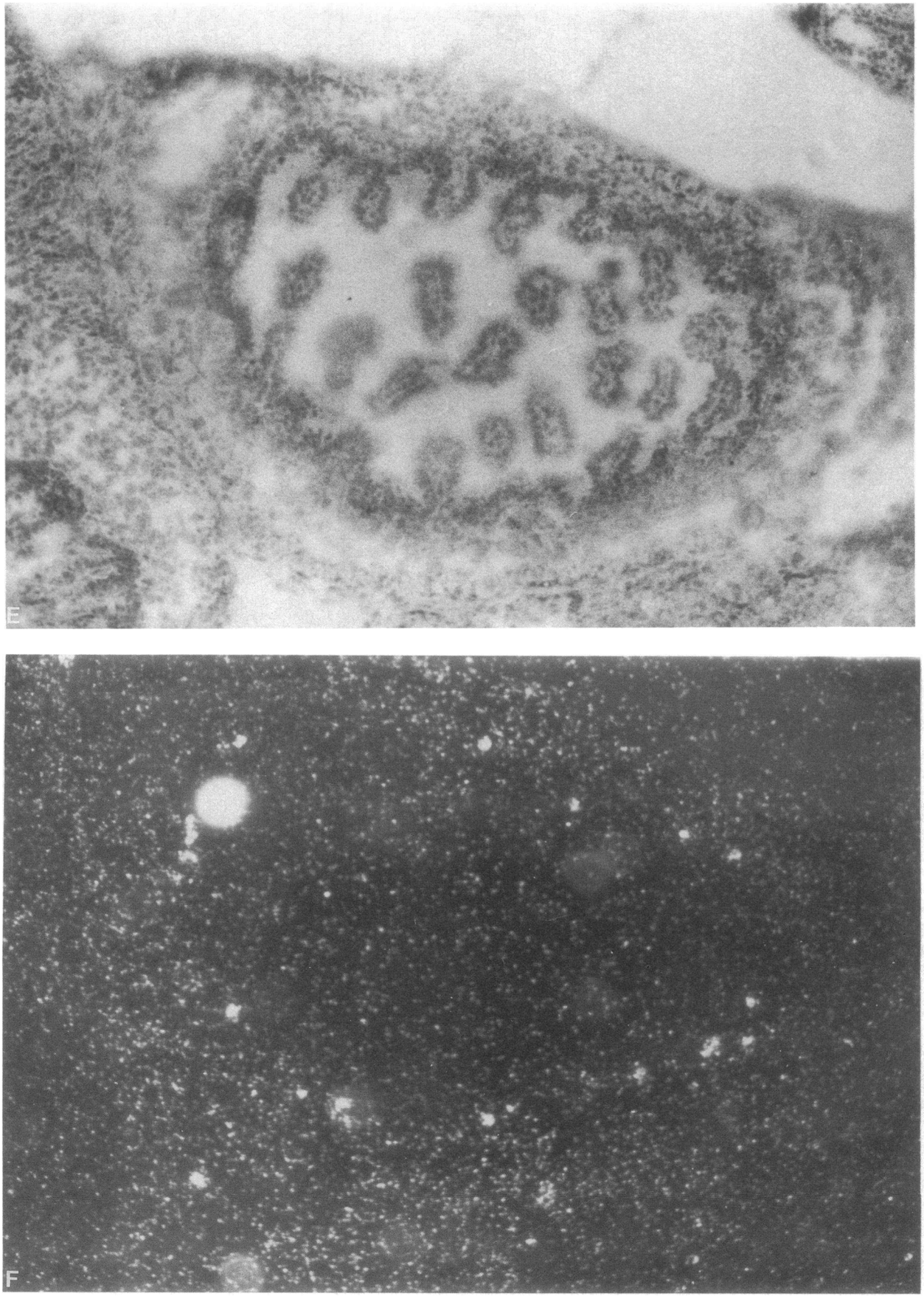

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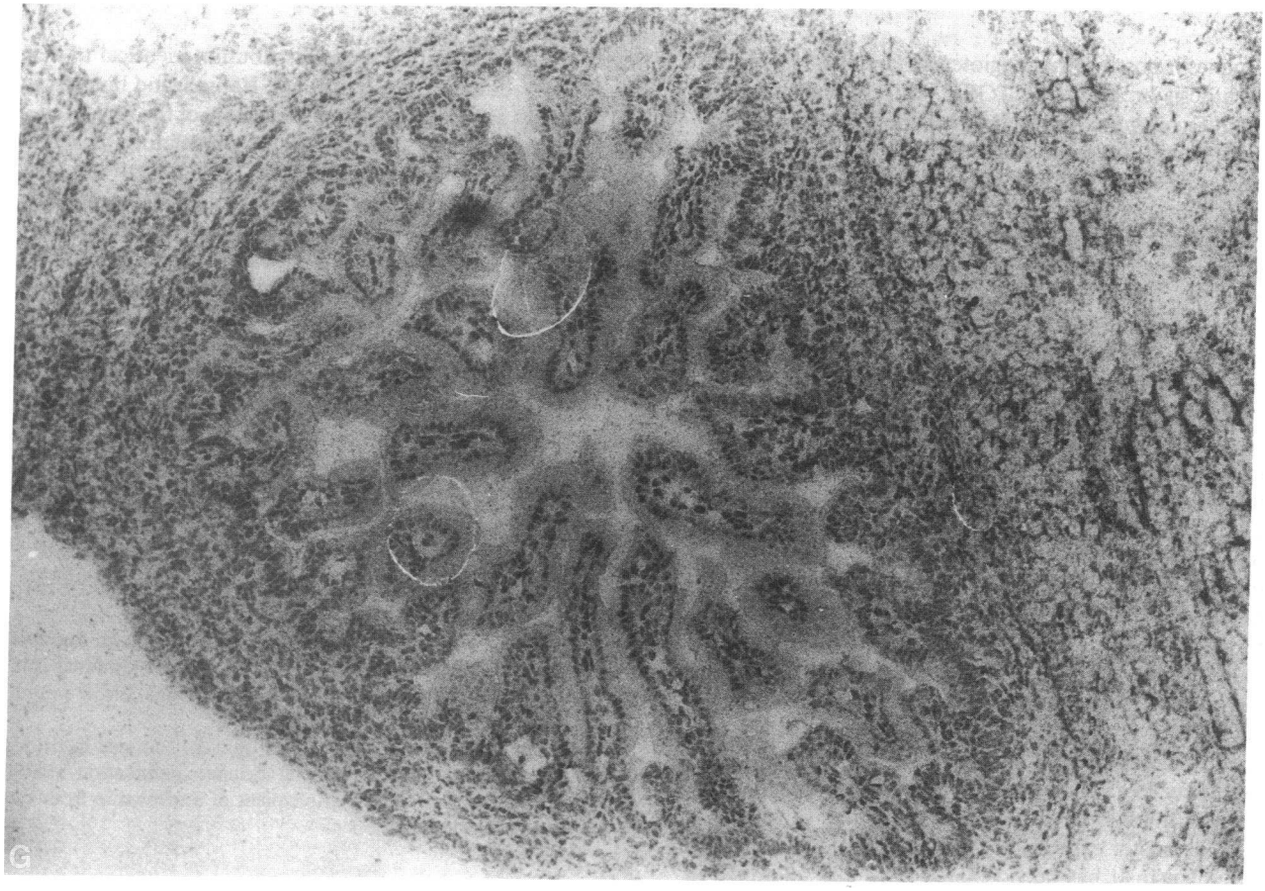

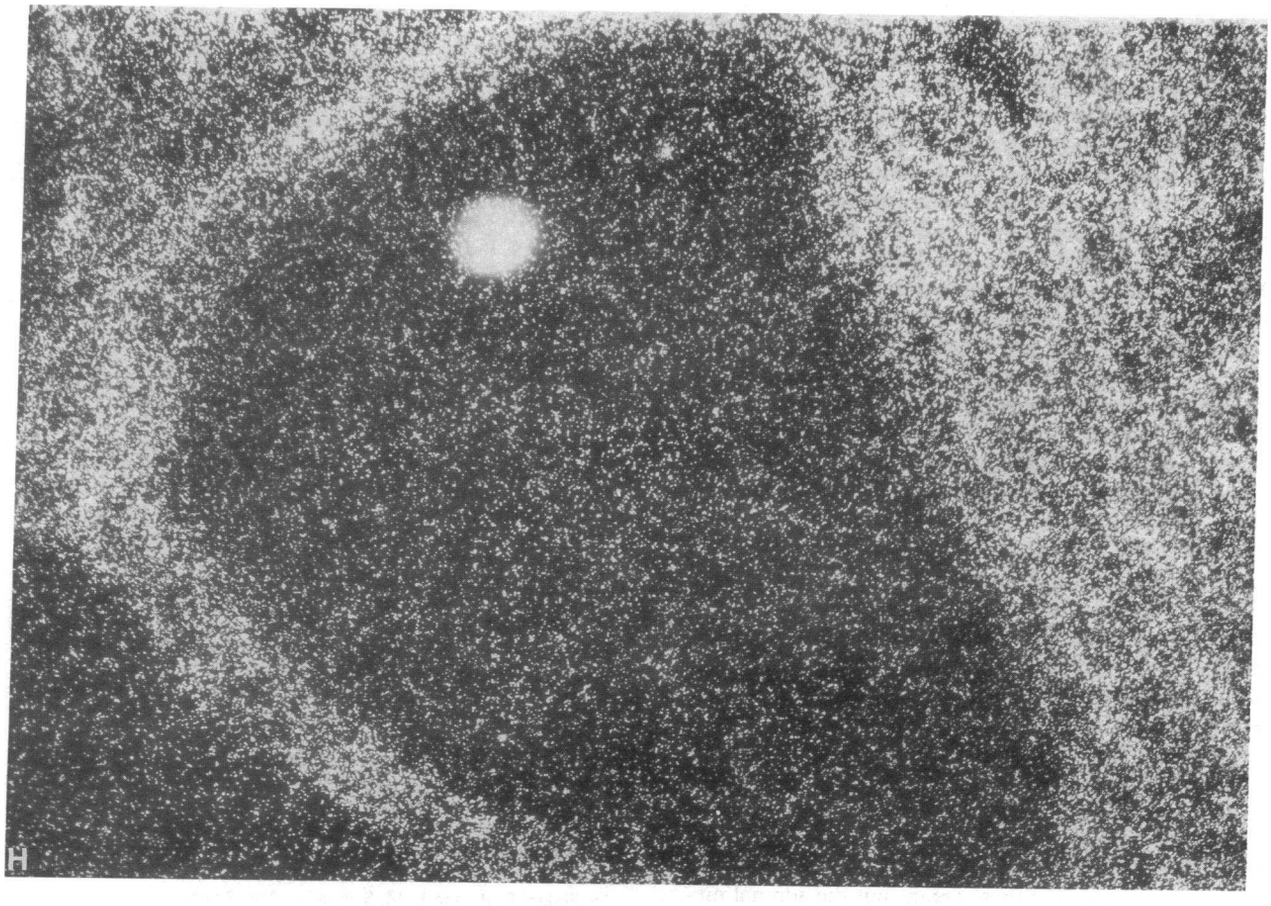

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opment makes it possible that angiotensin II may be modulating the synthesis or actions of TGF-β. Our finding of highly abundant AT_2 receptors on the less differentiated mesenchymal cells of the rat fetus also suggests that angiotensin II may have a role in mesenchymal regeneration, differentiation, and/or as a potentiator of other growth factors.

Our findings indicate that the majority of the angiotensin II receptors expressed in the mesenchyme of the developing rat embryo are AT_2. It is also evident with both film and emulsion autoradiography that residual, albeit low levels, of radioligand binding remain after PD123177 competition. Combined DuP 753 (10^{-5} M) and PD123177 (10^{-6} M) was equally ineffective in eliminating this residual binding. These data suggest, although do not prove, that a third angiotensin II receptor subtype may be present. Further studies will be necessary to investigate this possibility.

The presence of a receptor is functionally unimportant if its ligand is not present. Therefore it is important to note that all the components of the RAS have been found in the developing fetal-placental unit (45, 46). Renin and angiotensin converting enzyme have been detected in the second third of gestation (47–50). Lee et al. (51) detected angiotensinogen mRNA in the body of the rat embryo as early as day 9 with mRNA levels reaching a maximum by day 15. Day 15 is when the AT_2 receptors become easily detected, but they do not reach their maximum until day 19–21. Whether the increased synthesis of either angiotensinogen or angiotensin II is the stimulus for greater AT_2 receptor expression remains to be determined. Finally, angiotensin II has been isolated from and identified in the eviscerated rat fetus by using a combination of chromatographic elution and radioimmunoassay and displacement of radioligand binding from adrenal membranes (28).

Numerous studies indicate that angiotensin II plays a role in the regulation of cell growth. Angiotensin II increases protein synthesis, content, and cell size in murine proximal tubule cells (52) and augments epidermal growth factor-induced proliferation (21). In vitro, angiotensin II induces similar increases in protein synthesis and hypertrophy of vascular smooth muscle cells (53). In addition, angiotensin converting enzyme inhibitors, which decrease circulating angiotensin II levels, suppress the proliferative response of rat vascular smooth muscle cells to injury, again suggesting that angiotensin potentiates a growth response (54). Finally, the treatment of vascular smooth muscle cells with angiotensin II increases expression of c-*fos* (23, 24) and c-*myc* (22), platelet-derived growth factor A chain (22), thrombosporin (55), and TGF-β mRNAs (55). These growth factors have all been shown to be upregulated in early stages of cell growth.

In contrast to the AT_2 receptor which predominates in the mesenchyme of the fetus and is only found in the adrenal medulla (32), uterus (30), a subset of brain nuclei, (56, 57) and ovary (58) of the adult animal, the AT_1 receptor is more abundant and widespread in the mature animal. In fact, the majority of the cardiovascular and hormone modulating actions of angiotensin II in the adult animal are mediated through the AT_1 receptor (33–35). Longer autoradiographic exposure of the E19 fetal sections showed that there was specific, although lower, levels of radioligand binding to the following organs: liver, lung, submaxillary gland, adrenal cortex and medulla, and kidney. DuP 753, an AT_1 receptor antagonist, eliminated radioligand binding in all of these organs but the adrenal medulla. This demonstrates that these receptors are AT_1 and are present in the fetus with a tissue distribution identical to that found in the adult animal (32, 59–61). Radioligand binding to AT_1 receptors was also found in the placenta of the E13 fetuses where they are thought to function in modulating placental circulation (45).

The cellular and biochemical mechanisms involved in embryogenesis are complex and a variety of polypeptides appear to be necessary to regulate cell growth, differentiation, and functional maturation. In addition, it has been proposed that some of the mechanisms involved in embryogenesis may be reiterated in the adult, functioning in tissue repair, angiogenesis, or as a factor involved in carcinogenesis. The presence of angiotensin II during fetal development in conjunction with the transient expression of the AT_2 receptor in the developing rat fetus, suggests to us that angiotensin II, acting through the AT_2 receptor, may be one of the peptide hormones involved in fetal development.

Acknowledgments

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