American Journal of Pathology, Vol. 149, No. 6, December 1996

Early Expression of All the Components of the Renin-Angiotensin-System in Human Development

Susanne Schütz,* Jean-Michel Le Moullec,† Pierre Corvol,* and Jean-Marie Gasc*

Collège de France and INSERM U36, Paris, and Roussel-UCLAF,† Romainville, France*

Increasing evidence suggests that the renin-angiotensin system (RAS) is not only a potent regulator of blood pressure and fluid and electrolyte homeostasis, but that it also plays an important role in growth and differentiation in development as well as in pathological states. We, therefore, investigated the expression of all components of the RAS in the human embryo and fetus by* in situ *hybridization or immunohistochemistry. This study is the first to demonstrate the presence of all components of the RAS in very early human development (30–35 days of gestation). Angiotensinogen mRNA is expressed in very high amounts in the yolk sac, liver, and kidney, whereas renin mRNA and angiotensin-converting enzyme are expressed in the chorion, kidney, and heart, thus allowing fetal production of angiotensin II. This effector molecule of the RAS mediates its effects through binding to specific receptor types, AT_1 and AT_2. Both of these receptors are also expressed very early in development (24 days of gestation), suggesting a role for angiotensin II in organogenesis. Based on the expression pattern of these receptors, angiotensin II likely plays a role in the growth and differentiation of the kidney, adrenal gland, heart, and liver, all organs that are of major importance for the regulation of blood pressure later in life. (Am J Pathol 1996, 149:2067–2079)

The renin-angiotensin-system (RAS) is best known for its role in the control of blood pressure, salt, and water homeostasis. There is also a growing body of evidence showing the involvement of RAS in cell growth. In multiple studies, growth-promoting effects of angiotensin II (AngII), including hypertrophy and hyperplasia in different cell types, have been reported *in vivo* or in cultured cells.[1–3] In addition, AngII stimulates extracellular matrix production, potentiates actions of several growth factors, and increases transcription of proto-oncogenes (for a review see ref. 4). These proliferative effects of the octapeptide AngII are mediated by the AngII receptor type I, or AT_1.[2,5] Inhibitory effects on growth have been observed and seem to be signaled by the second AngII receptor type, or AT_2,[6,7] which suggests that the growth effect of AngII depends on the receptor subtype present on a given cell.

Embryonic and fetal development is an integrated program of growth and differentiation processes resulting in organogenesis. It has been proposed that AngII may play a regulatory role in these processes, based on AngII properties, as a growth factor in adult and fetal cells[4] and the expression of RAS components in the rodent fetus.[8] The recent inactivations of the genes for angiotensinogen (Ago), angiotensin-converting enzyme (ACE), and the AT_1 and AT_2 receptors have yielded interesting and unexpected results. Most animals with a homozygous disruption of the Ago locus die around the time of weaning.[9,10] Surviving animals exhibit severe kidney abnormalities. ACE −/− mice have similar histological changes of their kidney architecture.[11] Surprisingly, neither disruption of the AT_{1A} (leaving intact the AT_{1B}) nor the AT_2 receptor gene show serious effects on survival or morphology even though AngII receptors are widely distributed and expressed early in the developing rodent fetus.

Accepted for publication July 30, 1996.

Address reprint requests to Dr. J.M. Gasc, Collège de France, Laboratoire de Médecine expérimentale, 3 rue d'Ulm, 75005 Paris, France.

Presented at a poster session of the 28th Annual Meeting of the American Society of Nephrology, San Diego, CA, November 5–8, 1995, and published as an abstract in the Journal of the American Society of Nephrology (1995, 6:710).

The hypothesis for a role of AngII in organogenesis is supported by reports of severe malformations in the fetus consequent to the use of ACE inhibitors in hypertensive women during pregnancy. These malformations include kidney anomalies, patent ductus arteriosus, bony malformations, pulmonary insufficiency, and even neonatal death (for a review see ref. 12). Fetal renal failure, another consequence of ACE inhibition, resulted in oligohydramnios, which was reported to be a marker of adverse perinatal outcome related to intrauterine growth retardation.[13] These observations gave indirect evidence for a function of the RAS in the human fetus and made the use of ACE inhibitors and AT_1-receptor antagonists a contraindication in pregnant women. It is worth noting that the gene inactivations of several components of another hormonal system, the endothelin system, directly demonstrate the essential roles of these peptides and their receptors in normal gut and cardiovascular organogenesis.[14]

Establishing the expression and the tissue distribution of the different components of the RAS during development is a prerequisite to assessing their functional roles. Several studies have demonstrated expression of some of the components of RAS during development in rodents, though the concomittant presence of all components early in development has not been described. In the mouse and rat fetus the expression of Ago in the yolk sac, liver, and brain[15] and of renin in the developing kidney (for a review see ref. 16) have been shown. Moreover, AT_1 and AT_2 receptors are also present in the rodent fetus with a distribution suggesting a role in growth or differentiation in addition to the regulation of fluid and electrolyte homeostasis (for a review ref. 8, 17). The AT_1 receptor is expressed in the rat in classical target organs of AngII, such as liver, kidney, adrenal gland, heart, large blood vessels, pituitary, and in several specific undifferentiated mesenchymes.[18] AT_2 is much more abundant and displays a more widespread distribution than AT_{1A}; it is expressed in the mesenchyme of the kidney, adrenal medulla and zona glomerulosa, large arterial ducts, bronchi, tongue, diaphragm, and connective tissues surrounding cartilage and bone.[19–21] A feature common to AT_2 expression in most developing organs is its early appearance in the mesenchymal component during organogenesis followed by its disappearance as functional differentiation occurs. In contrast, the AT_1 receptors expressed in mesenchymal or epithelial components at the undifferentiated stage continue to be expressed throughout differentiation and their presence persists through to adulthood in AngII target tissues.

In humans, very few data are available concerning expression of the RAS components in early fetal life. Only the expression of renin[22,23] and ACE[24] have been studied by immunohistochemistry in the developing kidney. The presence of AngII binding sites in the late primate and one human fetus has been reported.[25]

The aim of the present study was to investigate the expression of all the components of the RAS in early human fetal development. To that end a systematic study of the expression of Ago, renin, ACE, and AT_1 and AT_2 receptors was carried out in human embryo and fetal samples, starting at 3 weeks of gestation, a period during which most organs undergo organogenesis. The expression of the RAS components was detected either by *in situ* hybridization or by immunohistochemistry. The observations demonstrate the presence of the RAS early in development supporting a role in organogenesis.

Materials and Methods

All techniques used in this study were previously described.[26] Only the outline of each of them and specific details are given below.

Human Tissue

Human embryos were obtained from two different sources. Twenty-two morphologically normal intact embryos (3–6 weeks of gestational age) were obtained after legal abortions by Mifepristone (RU486) performed at the Hôpital Broussais (Paris, France). Written maternal consent was obtained after information about the research project was given and the abortion had been performed. INSERM and the ethics committee were informed about the entire procedure. Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline solution (PBS), microdissected from the whole trophoblast, staged following the Carnegie classification,[27] dehydrated, and embedded in paraplast before sectioning. A summary of the correspondence between gestational ages and the stages of development is shown in Table 1. For comparison, the equivalent ages in the rat are also given. In addition, kidneys, adrenal glands, hearts, and genital organs from 37 fetus from spontaneous or induced abortions (8 to 39 weeks of gestational age) were obtained from Hôpital Necker (Paris, France). They were fixed in 10% formalin and embedded in paraffin following a routine automated process at the fetal pathology laboratory of Hôpital Necker. Sections were cut at 5–7 μm and mounted on silanated slides.

Table 1. *Comparative Table of Human and Rat Development*

Rat age, days	Human age, days	Stages
10	20	9
10.5	21–22	10
10.5	23–24	11
10.5–11.5	25–27	12
11.5–12.5	28–30	13
12.5–13.0	30–35	14
13.0–14.0	35–37	15
14.0–14.5	37–42	16
14.5–15.0	42–44	17
15.0–16.0	44–48	18

Comparison of gestational ages of rat and human embryos. The gestational age is defined as days after conception.

Stages of human development are according to the Carnegie classification.[27]

Cloning of Human AT_2 Receptor

The AT_2 receptor was recloned by RT-PCR from an adult human adrenal gland using antisense and sense primers corresponding to the previously published sequence of the human AT_2-receptor cDNA: ATG.AAG.GGC.AAC.TCC.ACC.CTT.GCC (nucleotides 1–24) and TTA.AGA.CAC.AAA.GGT.CTC-.CAT.TTC (nucleotides 1069–1092).[28] After reverse transcription, the 1092-bp fragment containing the entire coding sequence of the AT_2 receptor, was amplified with 38 cycles of denaturation at 95°C, 1 minute; annealing at 62°C, 1 minute; and extension at 72°C, 1 minute, followed by a 10-minute incubation at 72°C. This PCR product was subcloned into the *Xho*I and *Hin*dIII sites of the pcDNA3 plasmid (In Vitrogen, San Diego, CA). The sequence of the insert was checked using a Sequenase kit (US Biochemical, Cleveland, OH).

The other clones used were gifts from Dr. E. Clauser (angiotensinogen cDNA; ref. 29), Dr. F. Soubrier (renin cDNA, clone pGRh14[30]), and Dr. E. Davies (AT_1-receptor cDNA, clone BSh2A).

Riboprobe Transcription

Recombinant plasmids were linearized by the following restriction enzymes to generate the antisense and sense *in situ* hybridization probes, respectively, that contain the AT_2 receptor with *Hin*dIII and *Xho*I, AT_1 receptor with *Sal*I and *Hin*dIII, renin with *Hin*DIII and *Sac*I, and angiotensinogen with *Hin*dIII and *Xba*I. *In vitro* transcription was carried out in the presence of ^{35}S-UTP (Amersham, Les Ulis, France) with the following RNA polymerases (Boehringer, Mannheim, Germany) to generate the antisense and sense probes, respectively: SP6 and T7 for angiotensinogen, T7 and SP6 for renin, T7 and T3 for AT_1 receptor, and T7 and SP6 for AT_2 receptor. Probes were purified by ammonium acetate-ethanol precipitation and dissolved in 100 μl TE-DTT (10 mM Tris, 1 mM EDTA, 20 mM dithiothreitol). Starting from 100 μCi^{35}S-UTP and approximately 1 μg of cDNA, 80–90% of all radiolabeled UTP was recovered in the precipitate. The average transcription reaction yielded 1–2 × 10^6 cpm/μl probe. Their length was verified by electropheresis on a 1% agarose gel followed by autoradiography on X-omat AR films (Kodak). Before use the labeled probes were diluted to 10–13 × 10^3 cpm/μl in a hybridization mixture containing 50% formamide, 2X SSC (0.3 M NaCl, 0.03 M Na citrate), 1 mg/ml salmon sperm DNA, 10% dextran sulfate, and 70 mM DTT.

In Situ *Hybridization*

The detailed protocol for *in situ* hybriduzation has been recently published.[26] Deparaffinized sections were pretreated by boiling for 12 minutes in a citric acid buffer in a microwave oven followed by digestion for 20 minutes with proteinase K to enhance the hybridization signal. *In situ* hybridization was performed by covering the sections with 30–50 μl diluted riboprobe in hybridization mixture (3–4 × 10^5 cpm/section) and incubating overnight in a humid chamber at 50°C. Slides were washed in solutions of varying degrees of stringency (from 5X SSC with 50% formamide at 55°C to 0.01X SSC at room temperature) and digested with RNase A (20 μg/ml; Sigma, Saint-Quentin, France) to remove the unhybridized single-stranded RNA. Macroscopic autoradiographic pictures were obtained by apposition of slides to a Biomax film (Kodak) for 1–3 days and microscopic autoradiographic pictures were obtained by dipping the same slides in NTB2 liquid emulsion (Kodak). Exposure times varied from 1 to 8 weeks. Exposed slides were developed and fixed, then stained with toluidin-blue. Examination was under bright and dark field with a Leitz microscope.

Immunostaining

Slides were deparaffinized in toluol and rehydrated in decreasing concentrations of ethanol. Sections were prepared by incubation in 3% normal horse serum for 15 minutes followed by the primary antibody specific for somatic ACE (Ciba I, ref. 31) diluted 1:300 (90–120 minutes). Antigen-antibody complexes formed on the section were detected with the ABC-peroxidase complex (Vector, Burlingame, CA) and revealed with DAB-H_2O_2. Control sections were incubated with a preimmune rabbit IgG and

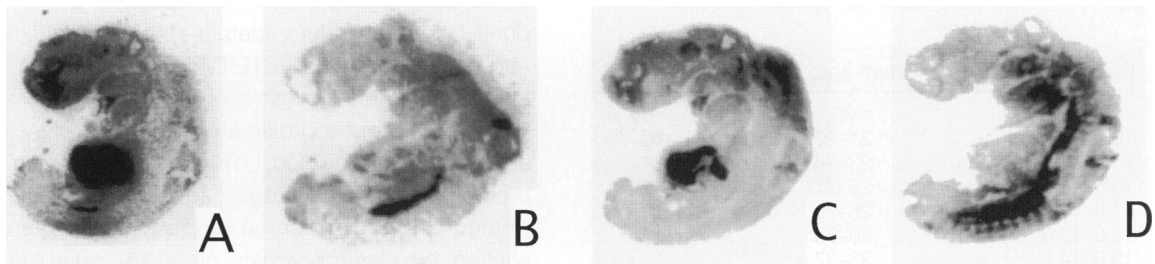


Figure 1. *Localization by* in situ *hybridization of RAS components on whole body sections of human embryos at stage 16 of gestation. Ago mRNA* (**A**) *is expressed at high levels in the liver and mesonephros. Renin-mRNA expression* (**B**) *is confined to the mesonephros. AT_1-receptor mRNA* (**C**) *can be detected in liver and mesonephros, whereas AT_2-receptor mRNA* (**D**) *is more widely distributed and can be detected in the mesonephros, adrenal gland, heart, sympathoganglionar chain, tongue, and several mesenchymal tissues. The labeling of the head* (**A** *and* **C**) *is artifactual. Magnification:* ×*4.*

showed no specific staining. Slides were examined under bright field with a Leitz microscope.

Results

As early as stage 16, RAS components could be observed at a macroscopic level (Figure 1). Ago mRNA was present in the liver in high amounts (Figure 1A). Renin was expressed in the mesonephros (the vestigial embryonic kidney) (Figure 1B). ACE mRNA could not be shown in the mesonephros at this level of observation, but could be detected by immunohistochemistry (see below). AT_1-receptor mRNA was expressed in the liver (Figure 1C) and AT_2-receptor mRNA in the mesonephros, heart, adrenal gland, sympathetic ganglion chain, tongue, and intervertebral mesenchyme (Figure 1D).

Mesonephros

The mesonephros is present in the fetus from stage 11 on. It is organized segmentally and formed of the Wolffian duct, primitive glomeruli, and tubules. By microscopical examination RAS components are observed in the mesonephros and have a specific distribution. Ago was detected by stage 12–13 in the proximal portion of the primitive tubules (Figure 2A). Renin was expressed very strongly from stage 13 on in capillaries within glomeruli (Figure 2B) as well as in the wall of arteries in the interstitium and in arterioles up to the aorta (not shown). ACE was detected by immunohistochemistry at stage 14 in the apical membrane of the mesonephric tubule cells (Figure 2C). The AT_1-receptor mRNA was detected in glomeruli, probably in mesangial cells, beginning at

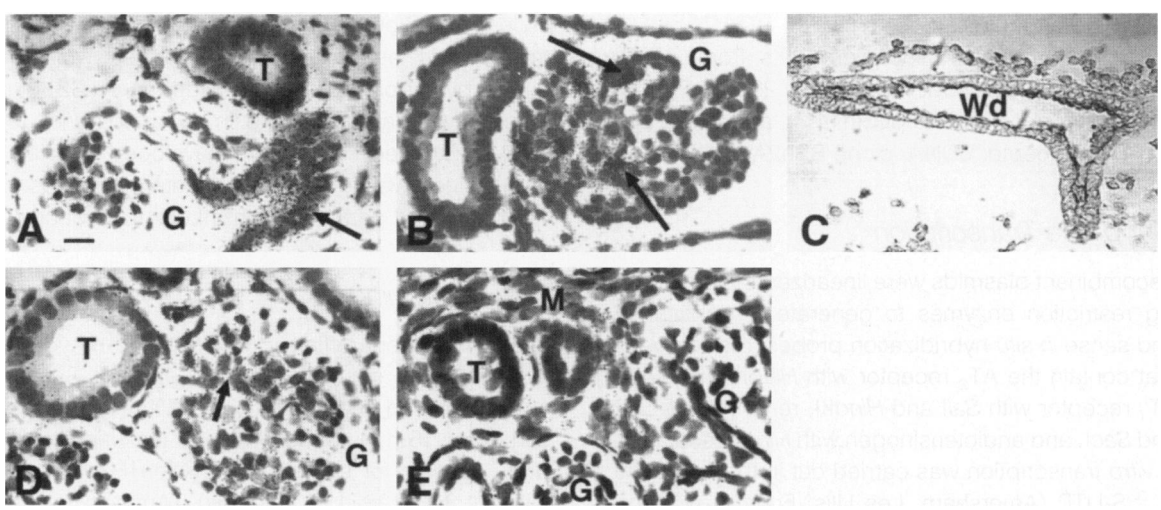


Figure 2. In situ *hybridization and immunohistochemistry of RAS components on sections of human mesonephros at stage 15–16 of gestation.* **A:** *Ago mRNA is confined to the epithelium of proximal tubules* (**arrow**). **B:** *Renin-expressing cells are located throughout the glomerular tuft in the mesonephros* (**arrows**). **C:** *ACE protein is localized by immunohistochemistry to the apical membrane of the epithelium of the Wolffian duct.* **D:** *AT_1-receptor mRNA is expressed in distinct cells* (**arrow**) *of mesonephric glomeruli and also in cells of mature glomeruli in the metanephros throughout gestation.* **E:** *AT_2-receptor mRNA is confined to the undifferentiated mesenchyme* (*M*) *of the mesonephros. G: glomerulus; M: mesenchyme; T: Tubule; Wd: Wolffian duct; Scale bars: 20 μm. Stained with toluidine blue.*

stage 12 (Figure 2D). The AT_2-receptor mRNA was the component to be expressed first (stage 11) and was confined to the undifferentiated mesenchyme of the mesonephros surrounding the preliminary tubules and glomeruli (Figure 2E). The simultaneous expression of the five RAS components could be demonstrated at stage 14 in the mesonephros.

Metanephros

The metanephros arises as the ureter branches out from the lowest segment of the Wolffian duct. The ureter induces the mesenchyme to condense and transform into the epithelial structures of the definitive kidney, the comma and S-shaped bodies, the precursors of glomeruli, and the proximal and distal tubules. The collecting ducts are formed from the final ureter branchings and their elongation.

The RAS components were distributed in the metanephros in a fashion similar to that in the mesonephros. Ago mRNA showed only a faint labeling macroscopically and was expressed in proximal tubules from 8 weeks of and throughout gestation (Figure 3, A and a). The distribution of renin mRNA appeared slightly different from that in the mesonephros as it had become confined to the juxtaglomerular apparatus at the vascular pole of the glomerulus and to dispersed cells of arteries located next to glomeruli (Figure 3, B and b). Immunoreactive ACE was detected in proximal convoluted tubules and collecting ducts in increasing amounts until birth (Figure 3e). A hybridization signal for AT_1-receptor mRNA was observed in glomeruli, probably in mesangial cells, and in proximal tubular epithelium (Figure 3, C and c), which might explain the diffuse macroscopic image noted at the microscopic level. Juxtaglomerular cells expressed AT_1 mRNA as soon as they differentiated, whereas vasa recta bundles did not express this receptor at any stage of prenatal development. AT_2-receptor mRNA expression was highest in the metanephros at 8 to 9 weeks of gestational age. It could be detected in undifferentiated mesenchyme, surrounding tubules and immature glomeruli (Figure 3, D and d), and in a few cells at the vascular pole of the glomerulus that resembled mesangial cells (not shown). The AT_2-receptor mRNA signal in the metanephros declined after 20 weeks of gestation but remained detectable until birth.

Adrenal Gland

The adrenal gland plays an important role in the RAS because AngII regulates the secretion of aldosterone by the zona glomerulosa. The AT_1 receptor could be detected in the neocortex, which differentiates into the zonae glomerulosa, fasciculata, and reticularis around the time of birth, at 8 weeks (Figure 4A) and throughout gestation. The expression of the AT_2 receptor starts earlier than the AT_1 receptor and is detected in a mass of condensed cells above the developing kidney in embryos at stage 16 (5–6 weeks). This condensation represents the fetal zone (Figure 4B). At 7 weeks of gestation, sympathoblasts of neural crest origin migrate into the fetal cortex as condensed clusters that assemble and form the adrenal medulla. They are characterized as sympathoblasts by their immunoreactivity to an antityrosine hydroxylase antibody (data not shown). These clusters of medullary cells show no hybridization signal for AT_2-receptor mRNA (Figure 4C).

Heart

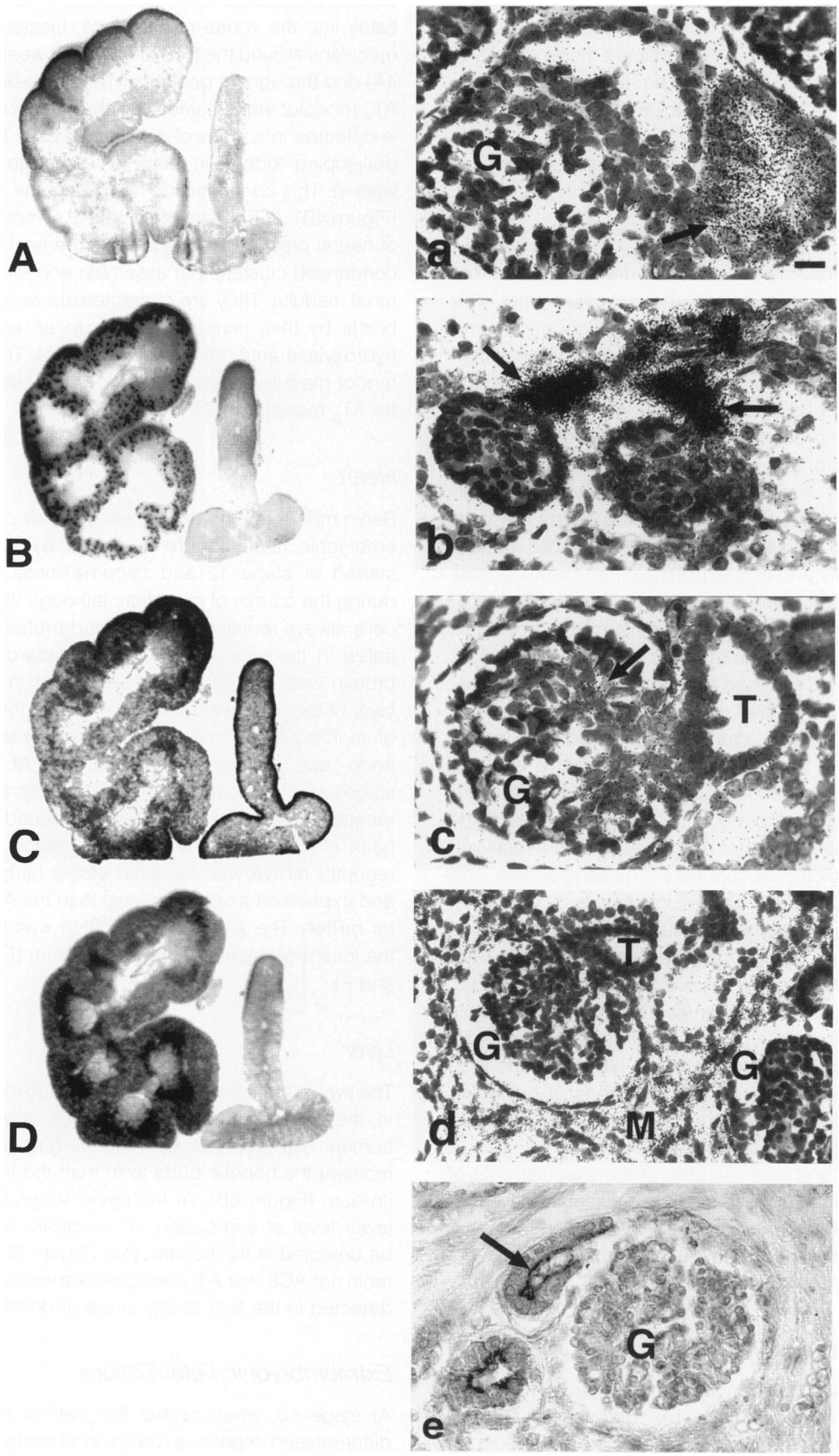
Renin mRNA could be detected in a few cells of the embryonic heart (Figure 5, A and B). Expression started at stage 12 and became more abundant during the course of gestation, although the positive cells always remained isolated and preferentially located in the inner parts of the myocardium. ACE protein was first detected at stage 14 in the inner layer of the heart wall, corresponding to the myocardium in adults, but not in cells destined to form the endo- and pericardium (not shown). At the early stages studied a weak hybridization signal for AT_1-receptor mRNA was diffusely distributed over the heart muscle (Figure 5, C and D, stage 15). AT_2-receptor mRNA was detected earlier (stage 11–12) and expressed at a higher level than the AT_1-receptor mRNA. The AT_2-receptor mRNA was located in the innermost layers of the myocardium (Figure 5, E and F).

Liver

The liver is the site of production for Ago in the adult. In the human embryo, hepatocytes show an extremely high level of Ago mRNA (Figure 6A) the moment the hepatic buds form from the foregut epithelium (Figure 6B). At the same stage, but with a lower level of expression, AT_1-receptor mRNA can be detected in the hepatocytes (Figure 6C). Neither renin nor ACE, nor AT_2-receptor expression could be detected in the liver at any stage of development.

Extraembryonic Fetal Tissues

At stage 10, when neither the liver or kidney are differentiated organs, expression of components of


A
B
C
D
G
a
b
T
G
c
T
G
G
d
M
G
e

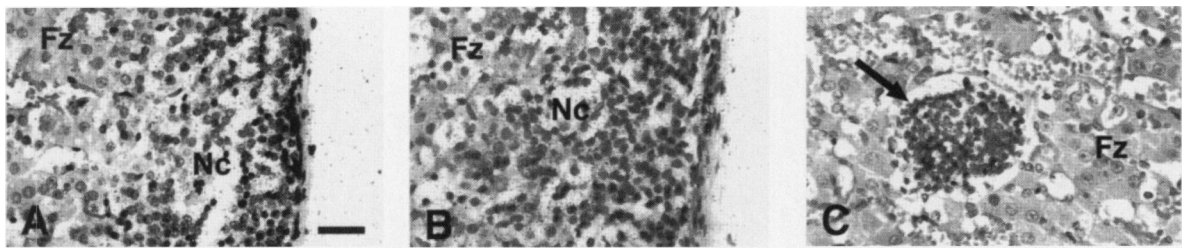


Figure 4. *Distribution of AT_1 and AT_2 receptor mRNA in human fetal adrenal gland. AT_1-receptor mRNA* (A) *is expressed in the neocortex* (*Nc*) *of the fetal adrenal gland, whereas AT_2-receptor mRNA* (B) *is expressed in both the fetal zone* (*Fz*) *and neocortex. AT_2 mRNA could not be detected in the medullary cells* (C) *which migrate in clusters* (arrow) *into the fetal zone of the adrenal gland. Scale bar: 40 μm. Stained with toluidine blue.*

the RAS can be detected in other locations in the human fetus. The yolk sac, which is composed of two layers, one of endodermal origin and the other mesodermal, expressed Ago mRNA in the inner endodermal layer (Figure 7A) and the AT_1 receptor in the outer mesodermal layer (Figure 7B). Both renin and ACE were detected in the chorion. Although renin mRNA was confined to isolated cells of the chorionic mesenchyme (Figure 7C), ACE protein was detected by immunohistochemistry in the epithelium of the chorion (not shown). AT_2-receptor mRNA could not be detected in any of these structures.

Other Organs and Tissues

Ago and renin were located exclusively in the usual sites of the RAS. Surprisingly, ACE, AT_1, and AT_2 receptors showed additional expression in a variety of tissues not directly involved in blood pressure control and water and salt metabolism. Immunostaining for ACE protein was observed in the developing nervous system and dermomyotome (stages 14–16, not shown). AT_1 receptor could be visualized in gut epithelium and parts of the developing brain. The hybridization signal observed for AT_2 receptor was the most widespread. Distinct cells, probably neural crest cells based on their location, around the neural groove and somites were labeled at stage 15–16 of development. Later during development, the sympathoganglionar chain showed a strong hybridization signal for the AT_2 receptor. The thyroid, the subdermal layer of the buccal cavity, the anlage for the palatine bones, and the tongue—all organs that contain components of neural crest origin—were labeled at stages 14–16. AT_2-receptor expression was observed in genital organs in mesenchymal cells surrounding ductuli efferentes in testis and in mesenchyme at the hilus of the ovary at the earliest stages studies (14 and 19 weeks, respectively). The AT_1 receptor was expressed in a few cells surrounding the developing lungs at stage 16, whereas AT_2 receptor could be clearly detected in mesenchymal cells surrounding bronchi by 12 weeks gestational age (not shown).

Discussion

Our study on the presence of all components of the RAS in the human embryo from 3 weeks to 40 weeks of gestational age extends the previous observations in rodent fetuses to the humans. Expression of the five major RAS components Ago, renin, ACE, and the two AngII receptor subtypes, AT_1 and AT_2, was assessed in the human embryo by *in situ* hybridization or immunohistochemistry, both macroscopically and microscopically. These techniques detect mRNA or protein with high sensitivity but can only demonstrate the presence, not the function, of the gene products at the different stages studied.

All components of the RAS were detected concomittantly at stage 14 of human embryonic development, i.e., when most tissues undergo organogenesis. Some components of the RAS were expressed even earlier in embryonic or extraembryonic tissues of the uteroplacental unit. Though AngII might be provided by the mother, the presence of AngII receptors is a prerequisite condition for target cells to respond to an AngII stimulus to allow a functional role for AngII in fetal development. In this study we demonstrate the early expression of the two AngII-

Figure 3. *Localization of RAS components by* in situ *hybridization* (A *to* D *and* a *to* d) *or immunohistochemistry* (e) *in the human fetal kidney. The Ago hybridization signal is weak in the kidney cortex* (A) *and, at high magnification of a kidney section* (a), *the Ago mRNA appears to be restricted to the proximal tubules* (arrow). *Renin expression is observed in the juxtaglomerular apparatuses* (B *and* b, arrows). *AT_1-receptor-mRNA expression is detected in the kidney cortex* (C), *in cells of the glomerular tuft* (c, arrow), *and in the proximal tubule. AT_1-receptor mRNA expression is also detected in the neocortex of the adrenal gland* (C). *AT_2-receptor mRNA is most abundantly expressed at the corticomedullary junction* (D) *and can be assigned to cortical* (d) *and medullary mesenchymes. ACE protein is detected by immunohistochemistry in the brush border of the proximal tubule* (e, arrow). *G: glomerulus; M: mesenchyme; T: distal tubule.* A, B, C, *and* D: *× 5; scale bars,* a, b, c, d, *10 μm.* a, b, c, *and* d: *stained with toluidine blue.*

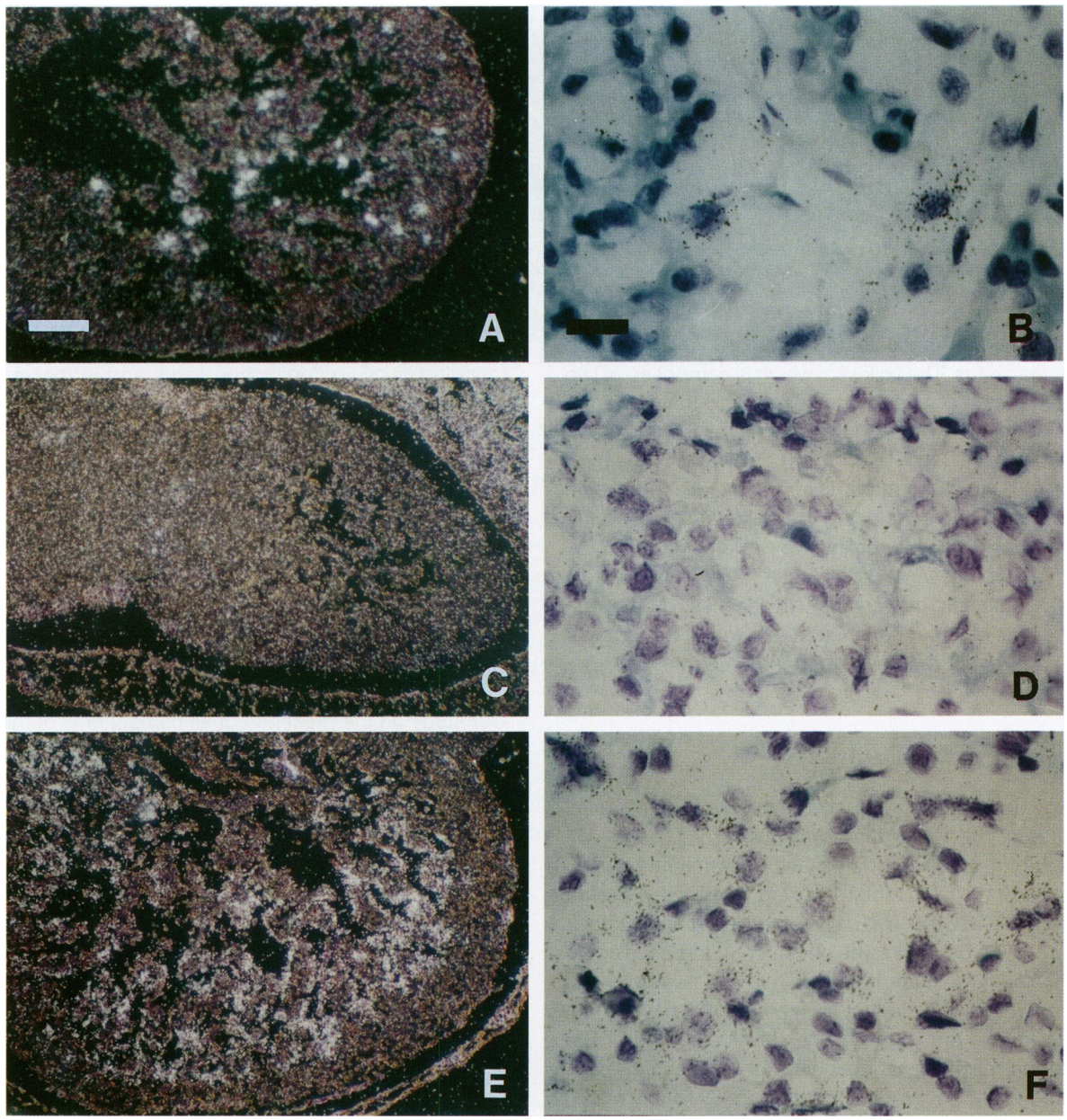


Figure 5. *Localization of RAS components by* in situ *hybridization in the human fetal heart (stage 16). Renin mRNA expression is restricted to a few cells of the left ventricle* (**A**). *At higher magnification* (**B**) *the labeling appears located in the innermost layers of cells lining the ventricular cavity. Labeling with the AT_1 probe* (**C**) *is diffuse and dispersed over the whole thickness of the myocardial wall of left and right ventricles. A higher magnification* (**D**) *shows a weak and diffuse signal in all cells. AT_2-receptor mRNA* (**E**) in situ *hybridization shows a strong labeling in the subendocardial and innermost layers of the myocardial wall and trabecules. A higher magnification* (**F**) *confirms the localization of the AT_2-receptor mRNA in the endothelial and subendothelial layers of the heart wall.* **A**, **C**, *and* **E**: *dark field illumination;* **B**, **D**, *and* **F**: *bright field illumination; Scale bars:* **A**, **C**, *and* **E**, *100 μm;* **B**, **D**, *and* **F**, *20 μm.*

receptor subtypes, AT_1 and AT_2, in different tissues and organs of the human fetus, starting at stage 11 (3 weeks of gestation) and throughout fetal life. The AT_1 receptor is predominantly expressed in the major adult target organs for the RAS, including the kidney, adrenal gland, heart, and liver. In contrast, expression of the AT_2 receptor is restricted to the mesenchymal component of developing organs such as the kidney, adrenal gland, and heart, as well as derivatives of neural crest tissues, and progressively becomes undetectable as functional differentiation proceeds. The AT_2 receptor was the first component to be expressed in the embryo itself and was located in the mesenchyme of the mesonephros. AT_2 was expressed in a variety of tissues not classically implicated in RAS functions that are, in part, deriva-

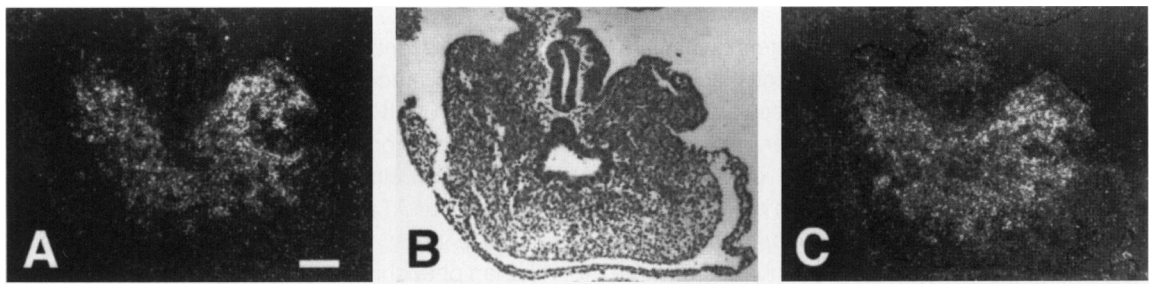


Figure 6. *Expression of Ago* (**A**) *and AT_1-receptor mRNA* (**C**) *by* in situ *hybridization in the first hepatocytes which differentiate in the liver of a stage 11 human embryo.* **A** *and* **C**: *dark field illumination;* **B**: *bright field illumination; Scale bar: 100 μm; stained with toluidine blue.*

tives of the neural crest. Another interesting observation was the presence of renin, ACE, AT_1, and AT_2 in the human fetal heart. Their different distribution and developmental regulation is evocative of a role for AngII in fetal cardiac growth as suggested by studies on cultured cells.[1,5]

Organs expressing one, several, or all RAS components were:

Mesonephros

A local RAS is present in the mesonephros as early as stage 14. The expression of the RAS in this preliminary excretory organ is of interest as similar mechanisms are involved in meso- and metanephric development. Furthermore remnants of the mesonephros form parts of the genital organs such as the ductus deferens, rete testis, and para- and epioophoron of the ovary, and continue to express AngII receptors later in life.

Metanephros

Renin could be detected in the fetal kidney in afferent and efferent arterioles much further away from the glomerulus than in the adult, where it is restricted to the close vicinity of glomeruli. This may suggest a lack of feedback control by AngII, because AT_1-receptor mRNA is not detected in renin producing cells of the young fetus, contrary to what is observed in the adult human (J.M. Gasc, unpublished observation) and rat.[32] In the course of gestation, renin expression becomes more and more restricted until it is localized uniquely to the juxtaglomerular apparatus. Interestingly, during renovascular hypertension fetal expression patterns of RAS are observed. In the ischemic kidney, renin is found in the juxtaglomerular apparatus of the deep cortex, i.e., in a localization observed before birth, whereas in the mature kidney its expression is confined to the juxtaglomerular apparatus of superficial glomeruli only. This change might be explained by the similarity of the fetal and the pathological ischemic state of the diseased kidneys, as there is low oxygen tension in both cases. ACE inhibition also leads to a recruitment of renin-expressing cells along afferent arterioles in the direction of interlobular arteries, a distribution that bears striking resemblance to a more immature state.[16] A second important observation is the implication of the RAS in renal tubular dysgenesis.[33] The study of developmental phenomena may therefore help to understand complex physiological and pathophysiological mechanisms in the adult.

A role for the RAS in kidney development is strongly supported by reports of homozygous disruption of the genes for Ago and ACE. Surviving Ago −/− animals showed structural abnormalities of the kidney: the renal cortex was thinned and there were

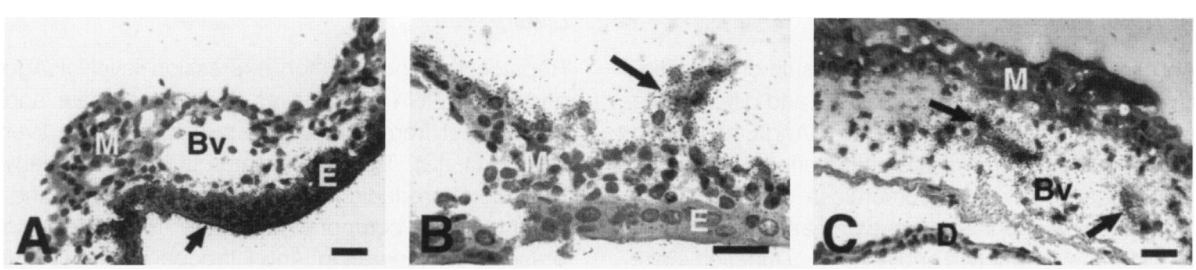


Figure 7. *Detection of RAS components in the yolk sac* (**A** *and* **B**)*, extra-embryonic mesoderm* (**B**)*, and chorionic mesenchyme* (**C**). In situ *hybridization shows high levels of expression of Ago mRNA in the inner endodermic layer of the yolk sac* (**A, arrow**) *in contrast to the AT_1-receptor mRNA detected in the outer mesodermal layer* (**B, arrow**). *Isolated cells of the chorionic mesenchyme expressed renin mRNA* (**C, arrow**). *Bv: blood vessel; E: endodermal layer of yolk sac; M: mesodermal layer of yolk sac. Scale bar: 30 μm.*

focal areas of atrophy with crowding of glomeruli, shrinkage of tubules, and chronic interstitial inflammation and fibrosis. All these changes suggest a lack of a growth stimulus. Renovascular changes were also observed with mural hypercellularity and narrowing of the lumen, especially of interlobular arteries.[11] These changes were more marked for the Ago −/− than for the ACE −/− mice. In this regard the few histological investigations of human fetal kidneys from pregnancies with ACE inhibitor use are of great interest because they bear striking resemblence to the above described changes.[12]

Mice −/− for the AT_{1A}-receptor gene are not affected anatomically and only exhibit a substantial reduction in blood pressure.[34] It may be argued that at least some of the effects of AngII in development are mediated through the AT_{1B}-receptor isoform, which is expressed in some tissues that also express AT_{1A}, including the zona glomerulosa of the adrenal gland and the mesangial cells of glomeruli in the kidney.[18,35] It is possible that most effects of the AT_{1A}-receptor deficiency are prevented by compensation through AT_{1B}. Mice −/− for AT_{1B} have not yet been described, so mice lacking both receptor types at the genetic level have not yet been studied. However, AT_1-receptor antagonism of pregnant rat leads to renal abnormalities, hypotension, reversible renal failure and a differential expression of the RAS in the neonates.[36,37] A high mortality rate among these rats and in primate fetuses[38] underlines the importance of AngII for normal development. The reduction in blood pressure observed in Ago, ACE, and AT_{1A}-receptor −/− mice is again congruent with the prolonged hypotension reported in infants born to ACE-inhibitor-treated mothers. Interestingly, mice −/− for the AT_2 receptor gene show an increase in blood pressure and increased sensitivity to the pressor action of AngII, suggesting that effects of AngII through its different receptor types could be indeed opposite for the regulation of blood pressure and cell proliferation.

Adrenal Gland

AngII might contribute to the expansion of the definitive cortex, which continues to form and differentiate after birth in humans, given that AngII has been reported to have growth-promoting effects of AngII in cultured adrenal zona glomerulosa cells.[3,39] AngII could also be important for the primary stimulation of aldosterone production and release, a function that it maintains later in life.[40] In contrast to observations in the rat fetus,[20,21] the adrenal medulla of the human fetus does not express AT_2-receptor mRNA. Furthermore, because cells around the neural tube that might represent migrating neural crest cells are positive for AT_2-receptor mRNA in the human but not in the rat fetus. It therefore seems that expression of AT_2 receptor occurs earlier in the differentiation of neural crest cells in the human than in the rat fetus. In addition, rat AT_2 receptor is known to be expressed in PC12 cells, a rat pheochromocytoma cell line. Human pheochromocytomas investigated were negative for AT_2-receptor mRNA (unpublished observations of the authors), consistent with its absence in the adrenal medulla of the human fetus. The function of the AT_2 receptor in the neural crest and organs derived from it remains unknown.

Heart

Ventricular hypertrophy develops in response to a mechanical stretch of the heart wall, as occurs after myocardial infarction. AngII has been shown to link the mechanical stimulus to the resulting hypertrophy.[1] In addition, AngII produces hypertrophy of fetal cardiomyocytes and hyperplasia of fibroblasts in culture via the AT_1 receptor.[2] A critical role for the RAS during ventricular hypertrophy is further supported by reports describing the beneficial effects of ACE inhibitors or AngII receptor antagonists in mediating the regression of ventricular hypertrophy.[41] This appears to be a direct effect on growth because it occurs even at concentrations that are ineffective for lowering blood pressure.[1] The role(s) of AngII in the pathogenesis of common and often fatal cardiac diseases such as ventricular hypertrophy, myocardial scarring,[42] and dilative cardiomyopathy[31,43,44] may result, at least in part, from reactivation of fetal programs. A process similar to pathological hypertrophy is the rapid growth of pig hearts during the first three days of life. The increase in heart weight can be prevented by ACE inhibitors and AT_1 antagonists, demonstrating the importance of the RAS for physiological and pathological growth of the heart.[45]

Liver

Hepatocytes show a high expression level of Ago and AT_1 mRNA as soon as they differentiate and migrate out from the hepatic bud to form the liver (stage 11–12). The human fetus is thus very early capable of producing Ago at a very early stage and, as the other components of the RAS are also present, of generating AngII independently of the mother. The Ago production might underlie a feedback control through AngII itself, because AT_1 is expressed in the same cells that produce Ago.

Whether AngII plays a role in the differentiation of the hepatocytes has not been investigated so far. The expression of Ago or AT_1 mRNA could serve as an early specific marker for differentiating hepatocytes.

Brain

The AT_2 receptor is known to be expressed in the adult[46] and fetal (J.M. Gasc, personal communication) rat brain. This expression is consistent with observations in AT_2-receptor-deficient mice that have an impaired drinking response to water deprivation possibly related to the lack of AT_2 receptors in brain regions implicated in thirst regulation such as the paraventricular and supraoptic nuclei. Reduced spontaneous movements and an attenuated exploratory behavior were also observed in the AT_2 −/− mice and support a role for AngII in central nervous system function.[47,48] In addition, ACE protein could be detected in the developing nervous system and AT_1 receptor in parts of the developing brain. The function of RAS components at these distinct sites remains unknown.

Conclusions

In conclusion, the components of the RAS are present at a very early stage of development in the human fetus. Their distribution varies from that in the adult with regard to the structures/organs that express the RAS components as well as the cellular components within the same organ. An independent fetal production of AngII might create a local functional tissue RAS in addition to the systemic, circulating RAS provided by the fetus and/or mother. It is therefore of note that the other components of the RAS are expressed at approximately the same gestational age as the AngII receptors. If AngII, and its receptors are present in the human fetus at a time when organogenesis is taking place, it is conceivable that AngII plays an important role in this process.

Acknowledgments

The authors thank M.-T. Morin and F. Mongiat for excellent technical assistance, Dr. Z. Lenkei for scientific advice, Dr. E. Aubeny for help in obtaining the human embryos, Drs. A.-L. Delezoide and M.-C. Labastie for providing samples of human fetal kidney, adrenal gland, heart, and gonads, and Drs D. Schlöndorff, G. Schütz, and K.C. Curnow for critical reading of the manuscript.

References

1. Sadoshima J, Izumo S: Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Circ Res 1993, 73:413–423
2. Ray PE, Bruggeman LA, Horikoshi S, Aguilera G, Klotman PE: Angiotensin II stimulates human fetal mesangial cell proliferation and fibronectin biosynthesis by binding to ATI receptors. Kidney Int 1994, 45:177–184
3. Tian Y, Balla T, Baukal AJ, Catt KJ: Growth responses to angiotensin II in bovine adrenal glomerulosa cells. Am J Physiol (Endocrinol Metab 31) 1995, 268:E135–E144
4. Huckle WR, Earp HS: Regulation of cell proliferation and growth by angiotensin II. Prog Growth Factor Res 1994, 5:177–194
5. Miyata S, Haneda T: Hypertrophic growth of cultured neonatal rat heart cells mediated by type 1 angiotensin II receptor. Am J Physiol (Heart Circ Physiol 35) 1994, 266:H2443–H2451
6. Nakajima M, Hutchinson HG, Fujinaga M, Hayashida W, Morishita R, Zhang L, Horiuchi M, Pratt RE, Dzau VJ: The angiotensin II type 2 (AT(2)) receptor antagonizes the growth effects of the AT(1) receptor: gain-of-function study using gene transfer. Proc Natl Acad Sci USA 1995, 92:10663–10667
7. Stoll M, Steckelings M, Paul M, Bottari SP, Metzger R, Unger T: The angiotensin AT2 receptor mediates inhibition of cell proliferation in coronary endothelial cells. J Clin Invest 1995, 95:651–657
8. Tufro-McReddie A, Romano LM, Harris JM, Ferder M, Gomez RA: Angiotensin II regulates nephrogenesis and renal vascular development. Am J Physiol (Renal Fluid Electrolyte Physiol 38) 1995, 269:F110–F115
9. Kim H-S, Krege JH, Kluckman KD, Hagaman JR, Hodgin JB, Best CF, Jennette JC, Coffman TM, Maeda N, Smithies O: Genetic control of blood pressure and the angiotensinogen locus. Proc Natl Acad Sci USA 1995, 92:2735–2739
10. Tanimoto K, Sujiyama F, Goto Y, Ishida J, Takimoto E, Yagami K-IF, A., Murakami K: Angiotensinogen-deficient mice with hypotension. J Biol Chem 1994, 269: 31334–31337
11. Krege JH, John SWM, Langenbach LL, Hodgin JB, Hagaman JR, Bachman ES, Jennette JC, O'Brien DA, Smithies O: Male-female differences in fertility and blood pressure in ACE-deficient mice. Nature 1995, 375:146–148
12. Shotan A, Widerhorn J, Hurst A, Elkayam U: Risks of angiotensin-converting enzyme inhibition during pregnancy: experimental and clinical evidence, potential mechanisms, and recommendations for use. Am J Med 1994, 96:451–456
13. Kingdom JP, McQueen J, Connell JMC, Whittle MJ: Fetal angiotensin II levels and vascular (type 1) angiotensin receptors in pregnancies complicated by intra-

uterine growth retardation. Br J Obstet Gynaecol 1993, 100:476–482
14. Gershon MD: Do developing enteric neurons need endothelins? Curr Biol 1995, 5:601–604
15. Gomez RA, Cassis L, Lynch KR, Chevalier RL, Wilfong N, Carey RM, Peach MJ: Fetal expression of the angiotensinogen gene. Endocrinology 1988, 123:2298–2302
16. Gomez RA, Pupilli C, Everett AD: Molecular and cellular aspects of renin during kidney ontogeny. Pediatr Nephrol 1991, 5:80–87
17. Gomez RA, Norwood VF: Developmental consequences of the renin-angiotensin system. Am J Kidney Dis 1995, 26:409–431
18. Shanmugam S, Monnot C, Corvol P, Gasc J-M: Distribution of type 1 angiotensin II receptor subtype messenger RNAs in the rat fetus. Hypertension 1994, 23: 137–141
19. Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE: Expression of AT2 receptors in the developing rat fetus. J Clin Invest 1991, 88:921–933
20. Shanmugam S, Llorens-Cortez C, Clauser E, Corvol P, Gasc J-M: Expression of angiotensin II AT2 receptor mRNA during development in the rat kidney and adrenal gland. Am J Physiol (Renal Fluid Electrolyte Physiol. 37) 1995, 268:F922–F930
21. Shanmugam S, Lenkei ZG, Gasc JMR, Corvol PL, Llorens-Cortes C: Ontogeny of angiotensin II type 2 (AT2) receptor mRNA in the rat. Kidney Int 1995, 47: 1095–1100
22. Celio MR: Ontogeny of renin immunoreactive cells in the human kidney. Anat Embryol 1985, 173:149–155
23. Graham PC, Kingdom JCP, Raweily EA, Gibson AAM, Lindop GBM: Distribution of renin-containing cells in the developing human kidney: an immunohistochemical study. Br J Obstet Gynecol 1992, 99:765–769
24. Mounier FN, Hinglais N, Sich M, Gros F, Lacoste M, Deris Y, Alhenc-Gelas F, Gubler M-C: Ontogenesis of angiotensin I-converting-enzyme in human kidney. Kidney Int 1987, 32:684–690
25. Zemel S, Millan MA, Feuillan P, Aguilera G: Characterization and distribution of angiotensin II receptors in the primate fetus. J Clin Endocrinol Metab 1990, 71:1003–1007
26. Sibony M, Commo F, Callard P, Gasc J-M: Enhancement of mRNA in situ hybridization signal by microwave heating. Lab Invest 1995, 73:586–591
27. O'Rahilly R, Müller F: Developmental Stages in Human Embryos, Publication 637. Washington, Carnegie Institution of Washington Publication 1987
28. Tsuzuki S, Ichiki T, Nakakubo H, Kitami Y, Guo D-F, Shirai H, Inagami T: Molecular cloning and expression of the gene encoding human angiotensin II type 2 receptor. Biochem Biophys Res Commun 1994, 200: 1449–1454
29. Gaillard I, Clauser E, Corvol P: Structure of human angiotensinogen gene. DNA 1989, 8:87–99
30. Soubrier F, Panthier JJ, Corvol P, Rougeon F: Molecular cloning and nucleotide sequence of a human renin cDNA fragment. Nucleic Acids Res 1983, 11:7181–7190
31. Falkenhahn M, Franke F, Bohle RM, Zhu Y-C, Stauss HM, Bachmann S, Danilov S, Unger T: Cellular distribution of angiotensin-converting enzyme after myocardial infarction. Hypertension 1995, 25:219–226
32. Gasc JM, Monnot C, Clauser E, Corvol P: Co-expression of type 1 angiotensin II receptor (ATI R) and renin mRNAs in juxtaglomerular cells of the rat kidney. Endocrinology 1993, 132:2723–2725
33. Bernstein J, Barajas L: Renal tubular dysgenesis: evidence of abnormality in the renin-angiotensin system. J Am Soc Nephrol 1994, 5:224–227
34. Sugaya T, Nishimatsu S-I, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, Goto S, Imaizumi K, Hisada Y, Otsuka A, Uchida H, Sugiura M, Fukuta K, Fukamisu A, Murakami K: Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. J Biol Chem 1995, 270:18719–18722
35. Shanmugam S, Corvol P, Gasc J-M: Ontogeny of the two angiotensin II type 1 receptor subtypes in rats. Am J Physiol (Endocrinol. Metab. 30) 1994, 267:E828–E836
36. Friberg P, Sundelin B, Bohman S-O, Bobik A, Nilsson H, Wickman A, Gustafsson H, Petersen J, Adams MA: Renin-angiotensin system in neonatal rats: induction of a renal abnormality in response to ACE inhibition or angiotensin II antagonism. Kidney Int 1994, 45:485–492
37. Charbit M, Dechaux M, Blazy I, Vargas R, Laouari D, Brocart D, Lacoste M, Gubler M-C, Sachs C: Deleterious effects of inhibition of the renin-angiotensin system in neonatal rats. Pediatr Nephrol 1995, 9:303–308
38. Harewood WJ, Phippard AF, Duggin GG, Horvath JS, Tiller DJ: Fetotoxicity of angiotensin-converting enzyme inhibition in primate pregnancy: a prospective, placebo-controlled study in baboons (*Papio hamadryas*). Am J Obstet Gynecol 1994, 171:633–642
39. Balla T, Baukel AJ, Eng S, Catt KJ: Angiotensin II receptor subtypes and biological responses in the adrenal cortex and medulla. Mol Pharmacol 1991, 40: 401–406
40. Gupta P, Franco-Saenz R, Mulrow PJ: Locally generated angiotensin II in the adrenal gland regulates basal, corticotropin-, and potassium-stimulated aldosterone secretion. Hypertension 1995, 25:443–448
41. Gilbert BW: ACE inhibitors and regression of left ventricular hypertrophy. Clin Cardiol 1992, 15:711–714
42. Nio Y, Matsubara H, Murasawa S, Kanasaki M, Inada M: Regulation of gene transcription of angiotensin II receptor subtypes in myocardial Infarction. J Clin Invest 1995, 95:46–54
43. Hirsch AT, Talsness CE, Schunkert H, Paul M, Dzau VJ: Tissue-specific activation of cardiac angiotensin converting enzyme in experimental heart failure. Circ Res 1991, 69:475–482
44. Suzuki J, Matsubara H, Urakami M, Inada M: Rat angiotensin II (Type 1 A) receptor mRNA regulation and

subtype expression in myocardial growth and hypertrophy. Circ Res 1993, 73:439–447
45. Beinlich CJ, White GJ, Baker KM, Morgan HE: Angiotensin II and left ventricular growth in newborn pig heart. J Mol Cell Cardiol 1991, 23:1031–1038
46. Lenkei Z, Palkovits M, Corvol P, Llorens-Cortes C: Distribution of angiotensin II type II receptor (AT2) mRNA expression in the adult rat brain. J Comp Neurol 1996 (in press)
47. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK: Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor gene in mice. Nature 1995, 377:744–747
48. Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, Niimura F, Ichikawa I, Hogan BLM, Inagami T: Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. Nature 1995, 377:748–750