Nephrogenesis and angiotensin II receptor subtypes gene expression in the fetal lamb

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Gimonet, Valérie, Laurence Bussieres, Anissa A. Medjebeur, Bernard Gasser, Brigitte Leongt, and Kathleen Laborde. Nephrogenesis and angiotensin II receptor subtypes gene expression in the fetal lamb. Am. J. Physiol. 274 (Renal Physiol. 43): F1062–F1069, 1998.—To investigate the role of angiotensin II (ANG II) in nephrogenesis, a developmental study of renal AT₁ and AT₂ receptor mRNA expression was performed in parallel with the quantitative and qualitative analysis of metanephros development in fetal lamb from 60 to 140 days of gestation. Both ANG II receptor subtypes were expressed early during nephrogenesis but displayed specific spatial and temporal distribution during gestation. High-AT₂ mRNA expression took place in the outermost nephrogenic area and in the undifferentiated mesenchymal cells surrounding the ampulla, level of AT₂ expression in this localization followed closely glomeruli proliferation rate and disappeared after nephrogenesis completion (>120 days). AT₂ mRNA was also detected in the differentiated epithelial cells of macula densa of maturing glomeruli. Although most of AT₁ mRNA labeling was found in the mesangial cells of maturing glomeruli, where it persisted after nephrogenesis completion, additional labeling was found in undifferentiated cells, in cells invading the inferior cleft of S-shaped bodies (80 days), and in medullar cells between tubules (120 days). Our results suggest that each receptor subtype has a specific role in renal morphogenesis, i.e., AT₂ in mesenchymal proliferation or apoptosis and AT₁ in vascular smooth muscle cells differentiation.

angiotensin II receptor expression; nephrogenesis; in situ hybridization; sheep

ANGIOTENSIN II (ANG II) exerts multiple renal effects, including regulation of blood flow, glomerular filtration, tubular reabsorption of electrolytes, and feedback inhibition of renin secretion in the juxtaglomerular apparatus (1, 4, 9, 18). In addition, ANG II stimulates cell growth, expression of growth factors, and growth-related protooncogenes (16, 20, 22). The biological effects of ANG II are mediated by specific receptors located in the plasma membrane of the target tissues. Two major ANG II receptors subtypes, AT₁ and AT₂, have been pharmacologically characterized using peptide and nonpeptide ANG II receptor antagonists (2). Molecular cloning techniques have made possible the elucidation of the molecular structure of the receptor subtypes (14, 15). In mature animals, AT₁ receptors coupled to calcium-phospholipid signaling systems are predominant in the kidney and appear to mediate most of the known effects of ANG II; the mechanisms of action and physiological effects of the AT₂ receptor remain to be elucidated.

In the developing animal, abundant ANG II receptors are widely distributed in various organs, but their distribution differs from that of the adult. In the fetal kidney, both AT₁ and AT₂ receptors have been detected (7, 8), but the AT₂ receptor subtype predominates, whereas, in the mature kidney, only AT₁ subtype receptors are present. The role and significance of the specific spatiotemporal pattern of ANG II receptor distribution during renal ontogenesis is unknown. Because all the components of the renin-angiotensin system are present within the fetal/placental unit and ANG II plays a role in cell growth (5, 22), it has been suggested that ANG II contributes to the growth and development of the immature kidney (5). This hypothesis is supported by observations that treatment of fetal or immature rats with either an angiotensin-converting enzyme inhibitor or an AT₁ receptor antagonist leads to abnormalities of renal growth and function (31); recent studies in AT₁ (21) or AT₂ (19) gene-deleted mice have yielded similar observations.

Most studies of ANG II receptors ontogeny (7, 26, 27, 30) have been performed in neonatal rat, a species in which nephrogenesis is not achieved until the second postnatal week, as opposed to humans, in which kidney maturation is completed at birth. Although, in these species, kidneys continue to undergo nephrogenesis postnatally, these are not fetal kidneys: patterns of renal homeostasis and function in postnatal animal may not be relevant to the fetus and may influence the postnatal distribution of ANG II receptors. To correlate ANG II receptor subtypes development with kidney organogenesis independently of neonatal environmental factors, we undertook a developmental study of renal AT₁ and AT₂ receptor mRNA expression in the fetal lamb, at macroscopic and microscopic levels.

MATERIALS AND METHODS

Animals and surgical preparation. Studies were performed in fetal sheep (Prealpes) at different periods of gestation: 50 (n = 6), 60 (n = 5), 80 (n = 5), 100 (n = 4), 120 (n = 5), and 140 (n = 3) days of gestation (gestation duration, 145 days). The gestation age of fetuses was based on the induced ovulation technique (11). Anesthesia was induced with 1 g Nedsotal (Centravet Laboratories, Paris, France). The pregnant ewe was placed under general endotracheal (1% halothane and 50% nitrous oxide in 50% oxygen) anesthesia, and maternal respiratory rate and volume were adjusted to maintain normal maternal arterial pH, PaCO₂, and PaO₂ values. Under sterile conditions, the uterus was exteriorized to gain access to the fetus, which was immediately removed and killed. Kidneys were dissected, cut longitudinally through the hilus in two halves, and processed for morphological analysis and in situ hybridization studies.

Morphological analysis. For morphological analysis of kidney during fetal development, tissue was fixed in 10% formaldehyde, and longitudinal frontal renal cross sections were
taken through the hilus and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin. The state of nephrogenic blastema and the number of glomeruli on whole kidney sections were determined, as well as the date of nephrogenesis arrest. The initial stages of glomerular development were excluded from this count; glomeruli were considered as primitive or mature when they clearly demonstrated both distinct Bowman's spaces and vascularized floculi, regardless of the glomerular width. The average number of glomeruli was then determined per square millimeter, which corresponds to the mean number of glomeruli counted on seven microscopic fields, then calculated for 1 mm². We used a Reichert-Jung Microstar 110 microscope, fitted with ×10 W.F. oculars and a plane 10/0.25 lens (corresponding microscopic field surface, 3.14116 mm²). After determination of cortical surface, the glomerular count was given by the product glomerular mean × cortical surface. This procedure was used for kidney of fetal lamb older than 80 days but was not relevant to younger ones because of the lack of clearly delineated medullocortical differentiation. In those cases, glomeruli were counted one by one by scanning the entire kidney sections (6).

Hybridization probes. Two different CDNA probes (gift from J. E. Robillard, University of Iowa) were used in this study (23). A 670-bp BamHI/NotI fragment in the coding region of ovine ANG II receptor type 1 was subcloned into pT7/T3 18U plasmid vector, allowing the generation of both sense and antisense probes. For AT1 probe, the 780-bp Hind III/EcoR I fragment was subcloned into the same vector. After linearization, single-strand sense and antisense RNA probes were synthesized by in vitro transcription by incorporation of [35S]UTP (Amersham, Les Ulis, France), using either T7 or T3 RNA polymerase (Boehringer, Mannheim, Germany).

In situ hybridization. Tissues were fixed overnight in 4% paraformaldehyde phosphate-buffered saline (PBS), processed routinely, embedded in paraffin, and stored at 4°C until used for hybridization studies. Three-micrometer tissue sections of kidney were cut and mounted on Silane-treated slides. They were then processed through deparaffinization and rehydration, following a routine histological procedure. They were rinsed for 5 min in 0.85% NaCl and for 5 min in PBS and pretreated in a boiling solution of 0.1 M citrate-buffer, pH 6, for 12 min in microwave oven. After this treatment, slides were left at room temperature for 20 min and fixed in 4% paraformaldehyde for 20 min. They were then treated for 20 min by 20 µg/ml proteinase K, again postfixed for 20 min in 4% paraformaldehyde, rinsed in PBS for 5 min, passed in NaCl 0.85% for 5 min, successively dehydrated in 30% to 100% ethanol, and finally air dried. Sections were covered with 30–50 µl of hybridization mixture containing 35S-labeled riboprobe (concentration adjusted at 0.4 × 10⁶ cpm/section), 50% deionized formamide, 10% dextran sulfate, 1 µg/ml salmon sperm DNA, 70 mM dithiothreitol (DTT), and 2× SSC (1× SSC is 0.15 mol/l sodium chloride and 0.015 mol/l sodium citrate), and hybridization was carried out overnight (16 h) at 50°C. The slides were washed in the following solutions: 5× SSC with 10 mmol/l DTT at room temperature for 30 min and with the same solution at 50°C for 30 min; 2× SSC, 50% formamide, and 10 mmol/l DTT at 55°C for 30 min; twice in NaCl TE (0.5 mol/l NaCl, 10 mmol/l Tris-HCl at pH 7.6, and 5 mmol/l EDTA) at 37°C for 10 min; RNase A (20 µg/ml) in 0.5 mol/l NaCl TE at 37°C for 20 min; NaCl TE at 37°C for 90 min; and 0.1× SSC at room temperature for 15 min. Sections were then dehydrated with successively decreasing concentrations of ethanol with 0.3 M ammonium acetate and air dried (28). The results were analyzed both macroscopically and microscopically. For macroscopic observation, the probed sections were exposed on Biomax film (Kodak, Rochester, NY) for 24–90 h, and the hybridization signal was analyzed directly by film autoradiography. For microscopic analysis, the slides were dipped in Kodak NTB2 liquid emulsion, counterstained with toluidin blue, and photographically processed after 5 wk of exposure. Each experiment was performed in triplicate.

RESULTS

Morphological analysis. Kidney morphology was studied through development from 50 to 140 days of gestation (Fig. 1). At 50 days (Fig. 1A), nephrogenesis is already started, as fetal lamb kidney shows divisions of the ureteric bud within the mesenchyme. Condensate cells around the buds and comma- and S-shaped bodies are present, as well as differentiated glomeruli. In the subcapsular area, only undifferentiated glomeruli (vesicles and comma- and S-shaped bodies) are found, whereas, in the deeper zone of the metanephros, glomeruli in a more developed stage are observed. Morphological structure shows little changes at 60 days (Fig. 1B) compared with 50 days. At 80 days (Fig. 1C), the cortex and medulla are well defined; however, the medulla is not yet well organized. Mature glomeruli are not found in the outer cortex, whereas the inner cortex contains mature and developing glomeruli. At 100 days (Fig. 1D), the subcapsular area shows a higher density of developing glomeruli compared with previous developmental stages. The metanephrogenic blastema disappears totally by day 105 (data not shown). At 120 days (Fig. 1E), no more comma- or S-shaped bodies are detected, and only maturing glomeruli are present. The outer and inner medulla are well defined. Only mature glomeruli are observed in kidney at 140 days of gestation (Fig. 1F). The number of glomeruli, used as a time-dependent marker of renal development, shows a consistent increase from 50 to 120 days of gestation (day 50, 98 ± 12; day 60, 194 ± 18; day 80, 555 ± 130; day 100, 1,675 ± 468; day 120, 1,792 ± 178; and day 140, 1,985 ± 73 glomeruli/frontal section).

Developmental changes in AT1 and AT2 mRNA expression. To investigate AT1 and AT2 mRNA distribution during kidney development, sections of kidney at different gestational stages were hybridized with respective antisense cDNA probes. Hybridization with the sense AT1 and AT2 cDNA, under experimental conditions identical to those used for the antisense probes, showed extremely low background in all experiments (Figs. 2, 3D, and 4D). The distribution of AT1 and AT2 receptors mRNAs in fetal lamb kidneys was different for each receptor type (Fig. 2). AT1 mRNA was detected at all studied stages. At day 60, AT1 mRNA was widely distributed throughout the cortex and outer medulla with a clear punctate hybridization in the cortex. At day 80, a similar punctate pattern was seen in the cortex with a notable hybridization of medullary rays. After 80 days of gestation, hybridization signal increased markedly in the juxtamedullary area and exhibited an intense extension to the outer medulla. In contrast, AT2 mRNA decreased progressively during development. At day 60 and 80, AT2 mRNA was clearly
highly expressed in the subcapsular region; lower mRNA expression was found in cortex and outer medulla. At day 120, the staining extended through the cortex, and, at day 140, AT₂ mRNA expression had almost totally disappeared. Microscopic analysis indicated that localization of AT₁ and AT₂ mRNA expression differed: AT₂ mRNA was present in undifferentiated mesenchymal cells surrounding the ureteric bud until the disappearance of the nephrogenic area (Fig. 3A) in the distal tubule or ascending limb of loop of Henle of developing glomeruli (Fig. 3B) and in the macula densa (Fig. 3C). The interstitial cells in medullary rays showed labeling from 60 to 120 days. Neither the glomeruli nor tubular structures, except that of juxtaglomerular apparatus, exhibited any evidence of AT₂ mRNAs. AT₁ mRNA was found in glomeruli, probably in mesangial or endothelial cells of mature or maturing glomeruli located in the cortical and juxtamedullary areas (Fig. 4B). Abundant labeling was present in the medullary rays in interstitial tissue (Fig. 4C). Faint labeling was present in proximal tubules. To a lesser extent, AT₁ mRNA was found in the nephrogenic area, in the mesenchymal cells invading the inferior cleft of S-shaped bodies (Fig. 4A).

DISCUSSION

Morphological analysis of metanephros development in sheep is necessary to correlate kidney ANG II receptor subtype mRNA expression with kidney organogenesis and understand ANG II role in normal and abnormal renal development. Although the sheep has been extensively used as an experimental model in renal fetal function studies, few data (24) are available concerning the different morphological stages of kidney development in this species; therefore, we performed a
systematic and quantitative description of fetal morphological renal development in our model. In mammals, during nephrogenesis, undifferentiated cortical mesenchymal cells are locally induced by branching ureter bud epithelium to aggregate and transform into epithelial comma- and S-shaped bodies through the acquisition of condensations. Later during development, the S-shaped bodies give rise to nephrons consisting of glomeruli and proximal and distal tubules. The complete functional glomerulus, however, includes capillary bundles originated from direct in-growth of vessels initially external to the kidney rudiment during the tubule elongation. Because we did not analyze kidneys of fetal lambs younger than 50 days of gestation, we did not determine the time of occurrence of the first generation of nephrons. It is nevertheless likely that an initial slow nephron formation takes place before the 50th day, as in sheep metanephros induction begins around the 30th day of gestation, when the first divisions of the ureteral bud appear (32). As in other species, fetal kidney lamb has a centrifugal pattern of glomeruli development. At 50 days of gestation, a majority of immature glomeruli, vesicle or S-shaped, which, at this stage of development, are avascular, are found in the subcapsular area of the cortex or nephrogenic zone; they can be detected until 100 days of gestation. Maturing glomeruli, the first stage where glomerular filtration has been demonstrated (32), are found at 50 days of gestation; accordingly, urine is formed at this stage of gestation. At 140 days of gestation, there is no morphological evidence of nephrogenesis; most glomeruli are fully developed, and only a few maturing glomeruli are still observed in the outer cortex. The quantitative analysis performed in this study demonstrates that no significant nephrogenesis occurs after 120 days of gestation and that, in this species, unlike mouse or rat but like human, nephrogenesis is completed before birth. These results are in agreement

Fig. 2. In situ hybridization of fetal kidneys sections with AT₂ and AT₁ receptor probes at different stages of gestation (day 60, n = 5; day 80, n = 5; day 120, n = 5; day 140, n = 3). Controls were performed using sense probes for AT₂ and AT₁. Figure is representative of the results in 3 different experiments.
with the pioneer study of Robillard et al. (24), who found no histological evidence of active nephrogenesis in fetuses over 130 days of gestation and no significant differences in total glomeruli number among fetuses over 130 days of gestation and newborn lambs. Analysis of glomeruli number throughout gestation shows that, in sheep, as in humans, nephrogenesis is not a continuous phenomenon and that a critical step of the renal development is observed around the 80th day of gestation, when the nephrogenetic process reaches its highest level.

In situ hybridization study with the specific AT$_1$ and AT$_2$ probes allowed detection of ANG II receptors mRNA at all studied stages; given the excellent resolution of labeling, it was possible to assign their precise localization. In sheep, AT$_2$ receptor expression is present at the onset of kidney development and decreases during gestation, similar to results obtained in rat (27) or mouse (12); however, in sheep, AT$_2$ receptor mRNA were almost undetectable after 120 days of gestation, whereas, in species (12, 27) with postnatal nephrogen-

Fig. 3. Photomicrographs of in situ hybridization for AT$_2$ receptor mRNA in the kidney of fetal sheep: AT$_2$ mRNA labeling is present in the nephrogenic mesenchymal tissue around renal vesicular structures (A, day 80), in the ascending limb of loop of Henle (B, day 80), and in the macula densa (C, day 120). D: no labeling is observed with AT$_2$ sense probe (day 120). Exposure time, 5 wk; magnification, ×330. Figure is representative of the results in 3 different experiments.
esis, AT2 mRNA disappears only some time after birth. The specific localization of AT2 mRNA in undifferentiated cells of mesenchyma surrounding the ampulla has already been reported in other species (12, 27); in addition, in the sheep, presence of AT2 mRNA expression was demonstrated in the differentiated epithelial cells of the macula densa of mature glomeruli. This unusual localization of AT2 receptor has been reported only in the

Fig. 4. Photomicrographs of in situ hybridization for AT1 receptor mRNA in the kidney of fetal sheep: AT1 mRNA is observed in the mesenchymal cells (arrow) invading inferior cleft of S-shaped bodies in the nephrogenic area (A, day 80), in cells of the glomerular tuft (arrow) of maturing glomerulus (B, day 120), and in interstitial tissue lying between the tubules in medulla (C, day 120). D: no labeling is observed with AT1 sense probe (day 120). Exposure time, 5 wk; magnification, ×330. Figure is representative of the results in 3 different experiments.
human fetus (in mesangial cells, Ref. 25) and, recently, in the sheep (in tubular epithelial cells, Ref. 3).

Consistent with experiments in rat and in mouse, which indicate the presence of AT 1 messenger early during fetal life (12, 26, 30), AT 1 receptor mRNA was present early during nephrogenesis in sheep. Expression remained clearly present during gestation at the same level; identical results have been reported in the fetal lamb with Northern blot technique, using the same probe during the last trimester of gestation (23). As previously observed in mature (12, 26, 30) as well as in immature animals (12, 26, 30), the messengers of AT 1 receptors were mainly localized in the mesangial or endothelial cells of immature and mature glomeruli; in addition, lower but clear mRNA expression was observed in the nephrogenic area in the mesenchymal cells invading the inferior cleft of S-shaped bodies, probably contributing to the formation of the glomerular mesangium. This localization has been reported in the rat by some (12, 26, 30) but not all authors. In another study performed on sheep, no labeling for AT 1 mRNA was found in the mesenchyme; since the probe used was the same, difference in the sensitivity or specificity of the technique used may explain these contradictory results. In the present study, the use of microwaves improved sensitivity, so that specific labeling could be evidenced easily, as background labeling was very low.

The existence of both AT 1 and AT 2 receptors mRNA in different structures during the nephrogenesis indicates that the receptors may play different roles during renal development. In the sheep, the early presence of AT 2 receptors mRNA in undifferentiated mesenchymal cells, the parallel between the degree of expression, and nephron formation rate suggest a role in the process of renal organogenesis. The pattern of ANG II receptor expression in species displaying a difference in kidney maturation (pre- or postnatal) shows a disappearance of AT 2 expression when nephrogenesis ends, independently of neonatal events, and seems therefore not involved in neonatal functional renal adaptation. Given its demonstrated trophic and mitogenic actions on renal cells, it is reasonable to speculate that ANG II plays an important role in mesenchymal differentiation and growth. However, although the abundant AT 2 receptor mRNA expressed during early development may have a role in these processes, all the recognized effects of ANG II on cell growth in kidney and other systems appear to be mediated primarily by AT 1 and not by AT 2 receptors. Moreover, blocking of AT 2 receptors in newborn rats does not alter kidney postnatal development (31); first studies performed in AT 2 knock-out mice (10) failed to report any dysfunction or modification of tissue during embryonic development. Alternatively, studies have reported that the AT 2 receptor mediates anti-growth effects on vascular smooth muscle (17) and endothelial cells (29), inhibits growth factor-induced cell proliferation (33), and, in an extreme way of cell growth inhibition, might even direct cells into programmed cell death or apoptosis. Because the pattern of distribution of AT 2 in undifferentiated cells adjacent to the stalk of the ureter epithelium overlaps that of cells undergoing apoptosis (13), it has been suggested that AT 2-expressing cells may be programmed to undergo apoptosis, which is a concomitant process of kidney morphogenesis (13). Supporting the relationship between AT 2 expression and apoptosis of noninduced mesenchymal cells, mice carrying a targeted null mutation of the AT 2 gene were recently reported to display renal congenital abnormalities characterized by impaired or delayed apoptosis of mesenchymal cells surrounding the developing ureter (19). The presence of AT 1 receptors in the differentiated epithelial tubular cells of juxtaglomerular apparatus must have a different signification, since this structure is definitive. In this localization, AT 1 receptors expression may be transient and participate to the final differentiation of the structure as proposed by Butkus et al. (3).

Because nitric oxide synthase is highly expressed in these cells and also plays a role in apoptosis, AT 2 expression could be in some way related to the enzyme activity. Alternatively, expression may be definitive and represent a feature of AT 1 localization in this species. In favor of this hypothesis, a low level of AT 2 was detected in this localization in adult sheep (unpublished data); although unusual, presence of AT 2 receptor in juxtaglomerular apparatus has been reported in mature monkey (34).

Although AT 1 receptor mRNA is present during nephrogenesis, the role of the ANG II receptor subtype in this process is still to be determined. Unlike AT 2, AT 1 receptor mRNA expression persists after the end of nephrogenesis and is localized in differentiated structures, suggesting that it may be the consequence rather than an effector of renal differentiation. In rat, the increase in AT 1 mRNA expression during postnatal development suggests a role related to functional aspects rather than to differentiation and growth. In sheep, it has been shown that, in the late gestation, ANG II is necessary for the maintenance of glomerular filtration rate; these effects could be mediated through the AT 1 subtype. However, in the present study, the early presence of AT 1 mRNA in undifferentiated cells invading the S-shaped bodies and more especially the abundant expression in medullary mesenchymal cells, possibly contributing to the formation of vasa recta, suggest a possible role in mesangial or smooth cell differentiation. Recent in vitro studies indicate that ANG II exerts its mitogenic action on a variety of cell types in the kidney, including mesangial and renal arteriolar smooth muscle cells through AT 1 receptor. Blockade of AT 1 receptor by Dup 753 in newborn rats induced an arrest in nephrovascular maturation and renal growth, resulting in altered kidney architecture characterized by fewer, thicker, and shorter afferent arterioles, reduced glomerular size and number, and tubular dilatation (17), suggesting that AT 1 receptor could have a role in the postnatal nephrovascular development of the kidney. AT 1 gene knock out resulted in abnormal intracellular vacuolization within vascular smooth muscle cells of outer medulla (21). This role may not be exclusive to the other well-known actions of AT 1 that could participate to the control of fetal or neonatal renal function.
In summary, this study describes a selective mRNA expression of ANG II receptor subtypes during kidney development in sheep. Comparing the receptor pattern of development in species with different pattern of maturation, it appears that AT2 receptor expression is tightly related to the process of nephrogenesis and has little or no role in the control of pre- or postnatal renal function that seems mediated through the AT1 receptor. In addition, expression of AT1 in undifferentiated cortical and medullary structure suggests a role for AT1 receptors in the development of renal structures, possibly vascular smooth cells. The significance of the unexpected presence of AT2 mRNA in the macula densa in the fetal lamb needs to be further investigated.

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