Renin-Expressing Cells Are Associated with Branching of the Developing Kidney Vasculature

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Abstract. To define the relationship between renal vascular development and renin distribution during kidney ontogeny, the complete renal arterial tree of Sprague Dawley rats during fetal (20 d) and postnatal (1 to 90 d) life was microdissected and immunostained for renin. A shift in renin distribution from interlobar and arcuate arteries in the fetus to the afferent arterioles in the adult was observed. In addition, seven types of renin distribution along the afferent arterioles were identified. In type I, renin was distributed continuously along the whole length of the afferent vessel. This pattern was most frequently observed in the fetus. In type II, renin extended upstream from the glomerulus but did not occupy the whole length of the arteriole. This type was relatively constant throughout postnatal life. In type III, renin was present as bands along the afferent vessel; it was most frequently observed in the fetal and early perinatal periods. In type IV, renin was restricted to the “classical” juxtaglomerular localization. It was the most frequent type observed in the adult rat. In addition, two “mixed” patterns, type III/IV and type III/II, were occasionally observed. The distribution of renin-expressing cells was spatially and temporally associated with the development of blood vessels. Development of a new arterial branch was preceded by the appearance of renin-expressing cells at the point of branching. This was followed by an outpouching of the arterial wall that progressively elongated to form a new arteriole. During this process, renin-expressing cells were distributed along the whole of the newly formed vessel. As the vessel matured, renin-expressing cells became restricted to the juxtaglomerular portion of the afferent arteriole. It is concluded that throughout life and within each individual arterial tree, expression of renin is heterogeneous, following patterns that are unique for each developmental stage. Furthermore, the association of renin-expressing cells with branching of renal arterioles suggests a role for these cells in the development of the kidney vasculature. (J Am Soc Nephrol 9: 63–71, 1998)

During development of the mammalian kidney, renin localization changes markedly from larger vessels in the fetus to the classical juxtaglomerular (JG) location in the adult (1–7). Similar changes have been observed along the phylogenetic scale during vertebrate evolution (8–10).

Most of the studies on renin localization have been performed in kidney sections. Although valuable information has been obtained with this approach, multiple consecutive sections encompassing several planes of cut are often required to get an idea of renin localization along the length of a particular vessel. The procedure is laborious and it is possible to miss valuable information, making statistical handling cumbersome and interpretation of results difficult. These problems could be circumvented if the whole population of renal vessels was available for analysis, providing an integrated picture of the complex anatomy of the renal vasculature. In addition, staining for renin would provide important information regarding the spatial distribution of renin-expressing cells throughout the entire kidney vasculature.

Recently, an acid digestion technique followed by immunostaining was developed by Casellas et al. (11). With this technique, the renal arterial tree can be dissected in its entirety and subjected to immunohistochemical staining for renin. The expression pattern of renin and its relationship with the anatomical development of the arterial vasculature have not been studied in a comprehensive manner. In the present study, we examined the relationship between renin distribution and the morphological development of the preglomerular vessels during ontogeny of the rat kidney.

Materials and Methods

Adult female and time-dated pregnant Sprague Dawley rats were purchased from HillTop Laboratory (Scottsdale, PA) and maintained on regular rat chow (Agway Brand Prolab Animal Diet Series 300, New York, NY) and tap water until the day of the study. Dating of pregnancy and staging of embryos was performed as described previously (5). Fetal (20 d of gestation), newborn (1, 3, and 6 d), young (10, 20, and 30 d), and adult (90 d) rats were killed with an overdose of intraperitoneal sodium pentobarbital (Abbott Laboratories, Chicago, IL), and their kidneys were harvested and processed for microvascular dissection as described below. Kidneys were harvested from three different animals per age group for dissection of vascular trees.
Dissection of Arterial Trees

Kidneys harvested from all ages were microdissected using the technique described by Casellas et al. (11). Briefly, the kidneys were incubated in hydrochloric acid (6 M, 42°C) for either 1 hr (postnatal ages, 20 to 90 d) or 30 min (fetuses and early postnatal ages, 1 to 10 d). After incubation, the kidneys were washed several times with acidified water (pH 2.5) and sonicated in a sonicator (Branson-sonic-12, Smith-Kline, Shelton, CT) for 3 to 5 min. The entire intrarenal arterial vasculature (arterial "tree") was then carefully dissected from each kidney under direct stereoscopic visualization (Sterezoom 7, Bausch & Lomb, Rochester, NY), using a pair of 1-μm tip tungsten needles (Fine Science Tools, Foster City, CA) attached to the eraser of a pencil at an angle of 120°.

Immunohistochemistry for Renin

The vascular trees of all age groups were permeabilized with 5% Triton X-100 in distilled water for 20 min. Subsequently, the trees were fixed in 10% neutral-buffered formaldehyde for 30 min and washed in phosphate-buffered saline (pH 7.4) twice for 10 min. The arterial trees were then transferred to a multi-well dish (Nunc Intermed, Naperville, IL), and immunostaining for renin was done as described previously (5,11,12). Briefly, the primary antibody was a polyclonal rabbit anti-rat renin antibody (diluted 1:2000; gift from Dr. T. Inagami, Vanderbilt University, Nashville, TN). The high specificity of this antibody has been documented previously (4,13,14). The secondary antibody was a biotin-conjugated goat anti-rabbit IgG. After treatment with the secondary antibody, the vascular trees were incubated in avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kits, Vector Laboratories, Burlingame, CA) and exposed to Sigma Fast diaminobenzidine tetrahydrochloride peroxide substrate (Sigma Chemical Co., St. Louis, Mo). Counterstaining was deliberately avoided to obtain a better contrast between the immunopositive regions and the rest of the vascular tree. With this technique, renin-containing cells were stained dark brown. Negative controls included omission of primary or secondary antibody from the staining reaction. The trees were then transferred onto a glass microscope slide, air-dried, cleared with xylene, and mounted with Permount (Fisher Scientific, Springfield, NJ). Photomicrographs were made with an Olympus AH-2 compound microscope (Olympus Vanox-S AHBS, Tokyo, Japan). Three arterial trees obtained from three different animals per age group were examined by light microscopy, and the pattern of arterial branching and distribution of renin was assessed.

Classification and Quantification of Renin Distribution

On the basis of the extent and pattern of renin immunoreactivity within the afferent arterioles, seven types of renin distribution were identified:

Type I: Renin is distributed continuously along the whole length of the afferent arteriole (see Figure 6a).

Type II: Renin immunoreactivity extends upstream from the glomerulus but does not occupy the whole length of the arteriole (Figure 6b, lower right).

Type III: Renin immunoreactivity appears in the forms of bands across the width of the afferent arteriole (Figure 6c).

Type IV: Renin is restricted to the JG area (Figure 6b, upper left).

Type V: No renin immunoreactivity is observed along the afferent arteriole (Figure 6f).

Type III/II: Type III and II patterns of renin immunodistribution are seen along the vessel (Figure 6d).

Type III/IV: Renin is seen both at the JG area and as bands along the afferent arteriole (Figure 6e).

The relative frequency of the various patterns of renin distribution along the afferent arterioles was studied as a function of age from fetal to 90 d of postnatal life. All afferent arterioles from each individual tree were examined, and the number of afferent arterioles corresponding to each type of renin distribution (see above) was counted (n = 3 per age group). The relative frequency of each type was calculated by dividing the number of afferent arterioles belonging to a particular type by the total number of afferent arterioles and multiplying by 100.

Twenty-five afferent arterioles of each type were also randomly chosen from three different 90-d-old “trees,” and the length of immunostaining was measured. To obtain the percentage length of renin immunostaining within the afferent arterioles, the extent of renin immunoreactivity (defined as the distance from the tip of afferent arteriole to the most proximal renin-positive area) was measured, and the percentage length of stained afferent arteriole was calculated (15):

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\% \text{ LAA} = \frac{\text{Length of renin immunostaining}}{\text{Total length of afferent arteriole}} \times 100
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Statistical Analyses

The values obtained after measuring the length of afferent arterioles (n = 25), the extent of renin immunoreactivity within the arterioles (n = 25), and the ratios obtained from the application of the above formula (n = 25) were all pooled individually and averaged. The data are presented as mean ± SEM, and comparisons were made by ANOVA followed by unpaired t test.

Results

At 20 d of gestation (Figure 1), the renal vasculature contained all of the major arterial branches, appearing like a miniature version of the adult renal vasculature. Briefly, the renal artery on entering the renal hilum divides into two major branches, the hilar arteries. These arteries divide into interlobar (IL) arteries, which give rise to arcuate arteries (AA). Corticoradial arteries (CR) arise usually from the cortical aspect of the arcuate arteries and occasionally directly from interlobar arteries. The corticoradial arteries in turn give rise to the afferent arterioles (Figure 1, aa), which terminate in the glomeruli. In the 20-d-old fetus, it is often difficult to distinguish the transition from the corticoradial arteries to the afferent arterioles.

Shift in Renin Distribution with Maturation

The distribution of renin within the renal arterial tree of the rat changes markedly with the maturation of the renal vasculature. In 20-d-old fetuses, renin is distributed throughout the interlobar and arcuate arteries and developing corticoradial arteries (Figure 1), with the extent of immunostaining gradually increasing in a direction distal to the hilum (Figure 1).

By day 1 of postnatal life, renin was observed in arcuate arteries beyond the point of their bifurcation, and in the corticoradial arteries (Figure 2, arrowheads). At this stage, renin was distributed in discrete, ringlike bands surrounding the arcuate and corticoradial arteries (Figure 2, arrows in the inset). By 3 d of postnatal life, renin distribution was similar to the pattern on day 1, except it was confined to the more distal regions of the arcuate arteries where new branches of cortico-
radial arteries had appeared (not shown). By 6 d of postnatal life, most of the renin was confined to the distal region of the arcuate arteries, the corticoradial arteries, and afferent arterioles; however, renin immunoreactivity was less intense than in the previous stage, appearing scanty and dotlike in distribution (Figure 3, arrow). By 10 d of postnatal life, most of the renin appeared as wide rings surrounding the afferent arterioles (Figure 4, arrow); no renin was seen in the larger vessels. Similarly, at 20 d of postnatal life, renin was seen in the afferent arterioles (Figure 5, arrowheads and inset).

**Heterogeneous Expression of Renin in Afferent Arterioles**

On the basis of the pattern of renin localization within the afferent arterioles, seven types of renin distribution were identified. In type I, renin was distributed along the whole length of the afferent arteriole (Figure 6a). Arteries with the type I pattern exhibited great variation in their lengths. By definition, the percentage length of renin immunoreactivity was 100%. The number of arterioles with type I renin distribution was highest in the fetal stage, reaching 74% of the total number of afferent vessels. Their number gradually decreased with age, and by 10 d of postnatal life their percent contribution to the pool of afferent arterioles was zero (Table 1).

In the type II pattern, renin immunoreactivity extended continuously upstream of the typical JG location but not occupying the more proximal region of the afferent arteriole (Figure 6b). In adult rats, afferent arterioles measured up to $109.5 \pm 4 \mu m$ in length, with the percentage length of renin staining at $42.7 \pm 1.6%$. In the fetus and the 1-d-old newborn, type II pattern arterioles were not seen. However, by 6 d of postnatal life, 13% of the afferent arterioles had the type II pattern. Afterward, their number declined but continued to persist in later stages of life, even up to 90 d of postnatal life (Table 1).

In type III, renin occurred in the form of bands surrounding the afferent arterioles. These bands varied in number from 2 to 25, arranged either close to one another or widely separated by immunonegative areas (Figure 6c). In adult rats, type III afferent arterioles exhibited some variation in length, measuring

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*Figure 1.* Renin immunolocalization in the renal arterial vasculature of fetal rat kidney (20 d of gestation). Inset shows enlarged view of the interlobar artery with renin localized in round or oval areas (arrows). aa, afferent arteriole; CR, corticoradial artery; AA, arcuate artery; IL, interlobar artery.

*Figure 2.* Renin immunolocalization in the arterial tree of 1-d-old newborn rat kidney. Inset shows enlarged view of arcuate artery and corticoradial artery with ringlike distribution of renin (inset, arrows). Arrowheads indicate the point of bifurcation of IL arteries. Abbreviations as in Figure 1.
260 ± 25 μm. The percentage length of renin immunoreactivity was 68.5 ± 3.4%. Type III was seen throughout fetal and postnatal life. In the fetus, their numbers were low, followed by a marked increase in the early postnatal stages and then a gradual decline throughout postnatal life (Table 1).

The type III pattern was occasionally observed in combination with other types. In type III/II, the afferent arterioles have a combination of type II and type III patterns (Figure 6d). This

Figure 3. Renin immunolocalization within the arterial tree of a 6-d-old newborn rat kidney. Renin immunoreactivity is seen in the form of dots across the arterial branches (arrow). Abbreviations as in Figure 1.

Figure 4. Renin immunolocalization within the afferent arterioles after 10 d of postnatal life (arrow). Abbreviations as in Figure 1.

Figure 5. Renin immunolocalization at the juxtaglomerular (JG) areas of the afferent arterioles in 20-d-old rat kidney (arrows). Inset shows renin at the JG area of an afferent arteriole.
Figure 6. Heterogeneity of renin distribution in the afferent arteriole. (a) Type I afferent arteriole with renin distributed throughout its length. (b) Type II and type IV renin distribution in a single arterial tree. (c) Type III afferent arteriole with renin immunolocalization in the form of bands encircling the width of the artery (arrow). (d) Type III/II afferent arteriole. (e) Type III/IV afferent arteriole. (f) Type V afferent arteriole with no renin staining. Note the glomerulus still attached to the arteriole.
type was not seen in fetal and early perinatal periods. It was observed by 6 d of postnatal life and was present in a small percentage of arterioles throughout postnatal life (Table 1).

In type III/IV, renin was seen both at the classical JG area and along the length of the afferent arteriole in the form of bands (Figure 6e). This type was found frequently in the early perinatal period. After 6 d of postnatal life, the number of afferent arterioles with type III/IV declined markedly to less than 1% in adult life (Table 1).

In type IV, renin was confined to the classical JG location (Figures 5 and 6b). In the adult rat, these afferent arterioles measured 72.5 ± 6 μm, and the percent length of renin staining was 32.5 ± 2%. Afferent arterioles with this type of distribution increased in number with age, reaching up to 47% of the total number of afferent arterioles by 90 d of postnatal life (Table 1). When adding types II, III/II, III/IV, and IV, JG localization was found in the adult animal with a frequency of 56.3% (Table 1).

Type V exhibited no immunoreactivity for renin (Figure 6, b and f). In adult rats, arterioles belonging to this type measured 131.2 ± 7.5 μm. This type was not seen in the fetus and 1-d-old newborn rat. It became evident by 6 d of postnatal life and constituted 34% of the afferent arterioles by 90 d of postnatal life (Table 1).

Figure 7 conceptualizes the shift in renin distribution from large arteries in the fetus to the afferent arterioles in the adult. In addition, the diagram illustrates the heterogeneity of renin distribution within afferent arterioles during fetal and adult life. The staining pattern of each afferent arteriole represents approximately 10% contribution to the overall distribution of types (described in Materials and Methods and Table 1).

Relationship Between Vessel Formation and Renin-Expressing Cells
In the present study, we also observed a spatiotemporal relationship between the appearance of renin-containing cells and the development of new branches. On the basis of this relationship, five stages were discerned (Figure 8, a through c):

Stage 1: This stage was characterized by a small, button-like concentration of renin on the walls of the arcuate and corticomedullary arteries (Figure 8a,1).

Stage 2: The area of renin staining is larger with a slight protrusion of the arterial wall (Figure 8a,2).

Stage 3: This stage was characterized by a finger-like protrusion of the arterial wall containing renin distributed along its length, with a greater concentration at the tip (Figure 8a,3) and 8b,3).

Stage 4: This stage was similar to stage 3. However, the length of the branch was greater than in stage 2 (Figure 8b4).

Stage 5: In this stage, a new branch arising from a newly formed artery was apparent (Figure 8c). The branch contained renin throughout its length. As a new artery developed, the five stages described above were repeated in association with renin immunoreactivity.

Discussion
The results of the present study indicate that during development there is a gradual shift in renin localization from the proximal hilar regions of interlobar and arcuate arteries in the fetus to the afferent arterioles in the adult. Such a centrifugal shift in renin distribution within the renal arterial branches has been reported by several researchers (1–4,7,16–20). A similar pattern of renin distribution has also been reported along the
whether the above mechanism is responsible for the wide arterial expression of renin in early life. It is more likely that the unique expression of renin during ontogeny is part of a developmental program that also coordinates the development of the kidney vasculature. It is likely that renin, directly or indirectly (through angiotensin generation), controls vascular growth (22). Corresponding with this hypothesis, in the early postnatal stages, the centrifugal shift in renin immunoreactivity within large arteries is coincident with a shift in the regions of active sprouting of new branches to the more distal regions of the arterial tree. In the distal regions where renin immunoreactivity is present, new branches can be seen arising, whereas in the proximal renin-negative regions, no new branches are detected. In addition, we observed a spatiotemporal coexistence of renin with the initial appearance, growth, and development of new corticoradial arteries and afferent arterioles. It is tempting to speculate that either renin or renin-producing cells have a role in the initiation, branching, and elongation of the renal arterial tree. This hypothesis, however, remains to be tested.

An additional interesting result of the present study is that there is considerable heterogeneity in renin immunodistribution within the afferent arteriole. On the basis of these observations, we classified the afferent arterioles into seven types, according to the location of renin within the vessel: renin throughout (type I), upstream-continuous (type II), upstream, bandlike (type III), JG (type IV), no staining (type V), combination of type III and II (type III/II), and combination of type III and type IV (type III/IV).

Type I distribution has been reported previously by us (4,5). It is seen predominantly in the fetus and early perinatal period and disappears after 6 d of postnatal life. This type also corresponds to stages 3 through 5 found in the evolution of arterial branching described above. This pattern seems to be the predecessor of all other types. As arterioles mature, the type I pattern is replaced by intermediate types, such as types II and III, and, eventually, by the “classical” JG localization (type IV), suggesting that these types are derived from and are the consequence of repression of renin expression along afferent arterioles with type I distribution.

Arterioles with type II distribution represented a small fraction of the total number of arterioles at every stage of development examined. We and others have observed that in adult animals, the fraction of arterioles bearing this type of renin distribution increases in conditions that induce the recruitment of renin-producing cells (15,23–27). It remains to be determined whether the process of recruitment is more frequent in the type II arterioles observed in normal ontogeny or whether all other arteriolar types are capable of increasing the number of renin-synthesizing cells.

The type III afferent arteriole described in the present study deserves further comment. Taugner et al. (28), who studied renin immunodistribution in kidney sections, reported the presence of afferent arterioles with scattered renin activity over a distance of 200 µm from the JG region. Such a pattern appears identical to the bands in type III afferent arterioles observed in our study. Those authors suggested that the afferent arterioles

phylogenetic scale (9,10). In primitive fish, renin is found in large arteries upstream from the glomerulus, whereas in adult mammals renin is found near the glomerulus (9,10). It has been suggested that in lower species, renin functions in an endocrine manner to control systemic arterial pressure. In mammals, the JG apparatus contains the macula densa and renin is close to the glomerulus, an anatomical arrangement that favors a dual paracrine-endocrine function, such as the regulation of systemic pressure, glomerular hemodynamics, and tubular function. Because renin ontogeny recapitulates its phylogeny, it has been postulated that during mammalian development the endocrine functions of renin precede its paracrine functions. However, as we discuss below, the wide expression of renin in embryonic vessels may serve other functions, such as the control of arteriolar branching and elongation.

The physiologic signals that govern the redistribution of renin during kidney maturation remain to be defined. It has been hypothesized that the continuous deposition of sodium into the growing bone creates a negative sodium balance responsible for the high activity of the renin-angiotensin system during early development (21). It remains to be determined

Figure 7. Diagram illustrating the shift in renin distribution (black) from large arteries in the fetus to the afferent arterioles in the adult. The diagram also illustrates the heterogeneity of renin distribution within the afferent arterioles. The staining type of each afferent arteriole represents approximately 10% of the contribution to the total pattern. All type III arterioles are combined in the adult (for description of types, see Table 1 and text). CR, corticoradial artery; aa, afferent arteriole; G, glomerulus.
with renin outside of the JGA are the type that come under the control of mechanoreceptors and β-adrenoreceptors, but not under the control of macula densa. This interesting hypothesis remains to be tested. Casellas et al. (11) also noted the presence of bandlike immunoreactivity in the whole mounts of the renal arterial trees of adult rats. Our study showed that the type III afferent arterioles are predominantly present in the early perinatal period. They then gradually decrease in number with age, although they are retained throughout maturity. This suggests that the banding pattern is a developmental feature. Why this feature persists in the adult stage is not clear. It is possible that renin thus localized subserves an entirely different function in the adult, exhibiting a mere morphological similarity to the developmental stages.

Type IV is the “classical” renin distribution restricted to the JG region (29). This type makes its appearance after 6 d of postnatal life and continues to increase in number up to 90 d of postnatal life. This agrees with previous observations using kidney sections. The increase in the number of type IV arterioles with age supports the notion that restriction of renin to the JG area is accompanied by arteriolar maturation (1,4-6,16,28).

We also observed type V afferent arterioles that exhibited no renin immunoreactivity. This lack of immunoreactivity could be an artifactual result of the dissection procedure, whereby the renin-positive tip is lost; or, this type could be a normal feature existing in nature. In support of the latter possibility, earlier researchers using kidney sections observed a similar type of afferent arteriole in normal and enalapril-treated rats (15,16). They reported an increased percentage of JGA staining for renin in enalapril-treated rats, as well as the presence of a group of JGA that retained nonimmunoreactive. In addition, we observed this renin-negative type V pattern not just in isolated arterioles, but also in some that had retained the glomerulus during dissection of the arterial trees (Figure 6f).

Both of these results suggest that certain JGA contain either undetectable amounts of renin or no renin at all. In the present study, we observed that the type V afferent arterioles make their first appearance by 6 d of postnatal life and then increase in number and continue to persist throughout maturation. The presence of 34% of type V arterioles in adult animals is in agreement with previous findings in kidney sections (29). From the results above, it may be inferred that the occurrence of JGA exhibiting no renin immunoreactivity is indeed a normal feature and not a result of the dissection procedure. The physiological significance of this type of afferent arteriole remains to be elucidated. The presence of afferent arterioles in type III/II and type III/IV combinations reported in the present study has been observed for the first time. Their functional significance is not clear.

Overall, the presence of arterioles with the above-mentioned types of renin distribution is a normal, although intriguing, feature throughout development and in adult life. It remains to be determined whether these different types of renin distribution are responsible for or are the result of functional heterogeneity among nephrons.

In summary, maturation of the kidney vasculature is accompanied by a shift in renin distribution from large arteries in the fetus to the afferent arterioles in the adult. In addition, distribution of renin within afferent arterioles is heterogeneous, following identifiable patterns that are unique for each developmental stage. Furthermore, the association of renin-containing cells with branching of renal arterioles suggests a role for these cells in the development of the kidney vasculature.
Acknowledgments

This study was supported by the Center of Excellence in Pediatric Nephrology and Urology (Grant DK 52612), the Child Health Research Center (Grant HD 22910), and the Center for Developmental Biology of the Kidney of the University of Virginia. We are grateful to Dr. Oliver Smithies of the University of North Carolina for reviewing the manuscript. The skillful secretarial assistance of Karen Trentham is greatly appreciated.

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