Pediatr Nephrol (1991) 5: 690–695

Pediatric Nephrology

Original article

Spatial association of renin-containing cells and nerve fibers in developing rat kidney

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Received September 4, 1990; received in revised form January 10, 1991; accepted January 31, 1991

Abstract. The development of renin-containing cells and nerve fibers was studied in Sprague-Dawley rat kidneys during the last third of gestation and the first 15 days of postnatal life. Kidney tissue sections were stained for nerve fibers or double stained employing an anti-rat renin polyclonal antibody and a monoclonal antibody (TUJ1) directed against a neuron-specific class III beta-tubulin isotype. Renin-containing cells and nerve fibers were detected at 17 days of gestation, in close spatial relationship along the main branches of the renal artery. During fetal life, renin-containing cells and nerve fibers were spatially associated along arcuate and interlobular arteries, renin-containing cells being also present throughout the entire length of afferent arterioles supplying juxtamedullary glomeruli. During postnatal life the distribution of renin-containing cells progressively shifted to a restricted juxtaglomerular position in afferent arterioles. Simultaneously, density and organization of nerve fibers increased with age along the arterial vascular tree. Our results suggest that innervation of renin-containing cells is present in fetal life and follows the centrifugal pattern of renin distribution and nephrovascular development.

Key words: Kidney – Renin – Innervation – Ontogeny – Double-label immunohistochemistry – Sprague-Dawley rat

Introduction

In the normal adult mammalian kidney, renin is localized in specialized smooth muscle cells of the juxtaglomerular apparatus [1, 2]. Recent immunohistochemical studies have demonstrated that renin distribution in developing metanephric kidney of mammals differs from the typical adult juxtaglomerular localization. During fetal life renin-containing cells are widely distributed along the walls of large renal arteries and afferent arterioles in the rat [3, 4] and in other species [5–7]. As maturation of the metanephric kidney progresses, renin distribution shifts to the classical juxtaglomerular position [3, 5].

Among several homeostatic mechanisms, the secretion of renin is directly regulated by the sympathetic nervous system through activation of beta-adrenergic-mediated events [8, 9]. In adult animals, the presence of nerve fibers innervating the afferent and efferent arterioles at the juxtaglomerular level has been documented by light [10, 11] and electron microscopy [12, 13]. Norepinephrine has been detected in rat fetal kidney at 21 days of gestation [14] and norepinephrine concentration has been shown to increase in rat kidney during the first weeks of postnatal life [14, 15]. Neural regulation of renin secretion and distribution in developing kidney has been suggested by in vitro [16, 17] and in vivo [18] studies. However, few morphological studies have investigated the innervation of the immature renal vasculature [14, 19, 20]. These studies yielded important information restricted to a particular stage of development in different animal species. However, a complete ontogenic study of changes in the distribution of nerve fibers in the developing kidney has not been performed.

The establishment and integrity of innervation of renin-containing cells and renal vasculature during development may be of fundamental importance in the regulation of renal hemodynamics. However, the ontogeny and temporal relationship between the appearance of nerve fibers and renin expression in the vasculature of the developing kidney is unknown. The present study was undertaken to investigate the chronological and morphological relationship between nerve fibers and renin-containing cells in developing fetal and postnatal rat kidney.

Materials and methods

Sprague-Dawley rats (Hilltop, Scottsdale, Pa., USA) at different fetal (15, 17, 19, 21 days of gestation) and postnatal (5 and 15 days of postnatal life) ages were studied. Each age group consisted of four to five animals from one to two different litters. Gestational age was determined as previously described [3]. Kidneys harvested from 17-, 19-, and 21-

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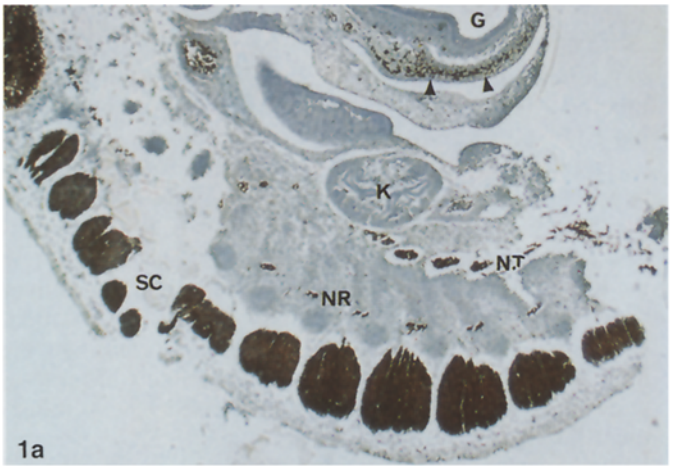

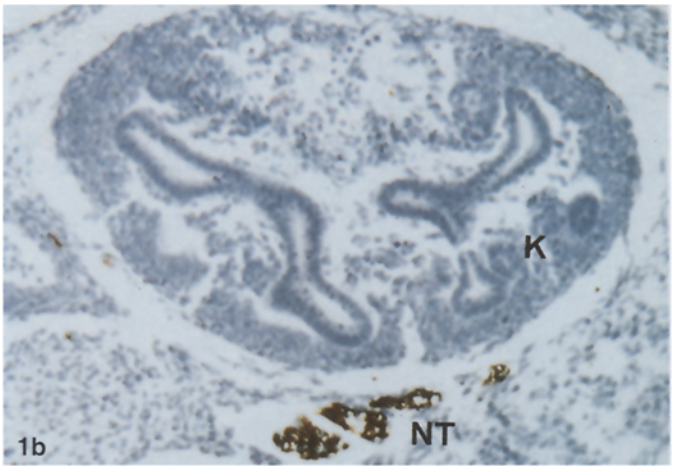

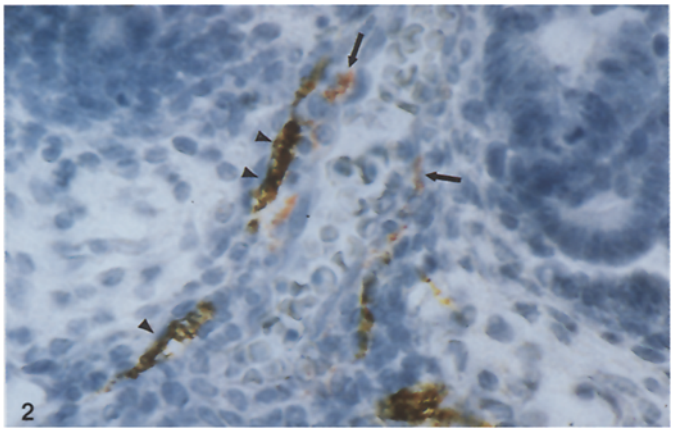

Fig. 1 a, b. Longitudinal section of 15-day-old fetus. Intense staining for beta-tubulin (brown color) in spinal cord (*SC*), nerve roots (*NR*) and nerve trunks (*NT*). *Arrowheads,* wide nerve plexus in wall of primitive gastrointestinal tract (*G*). The kidney anlage (*K*) is present. Double-label immunohistochemistry, ×43. **b** Higher magnification of the section in **a** showing lack of renin deposits or nerve fibers in the kidney. Double-label immunohistochemistry, ×170

Fig. 2. Fetal kidney section at 17 days' gestation. Renin (red, *arrows*) and bundle of nerve fibers (brown, *arrowheads*) are spatially associated in the main branch of the renal artery, ×360

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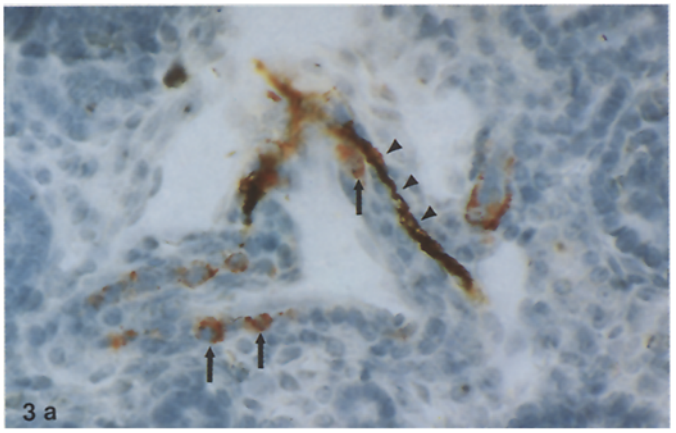

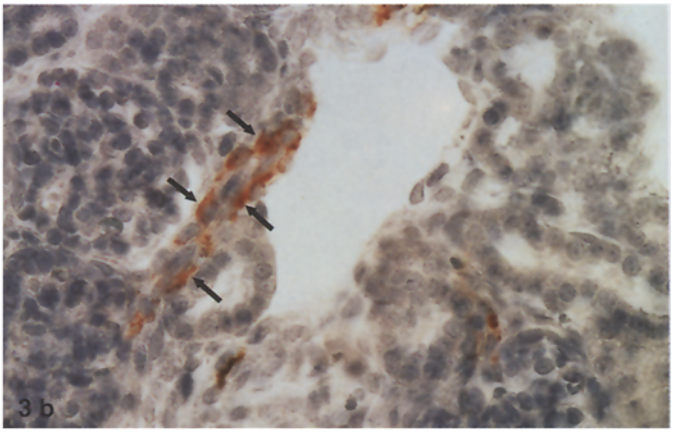

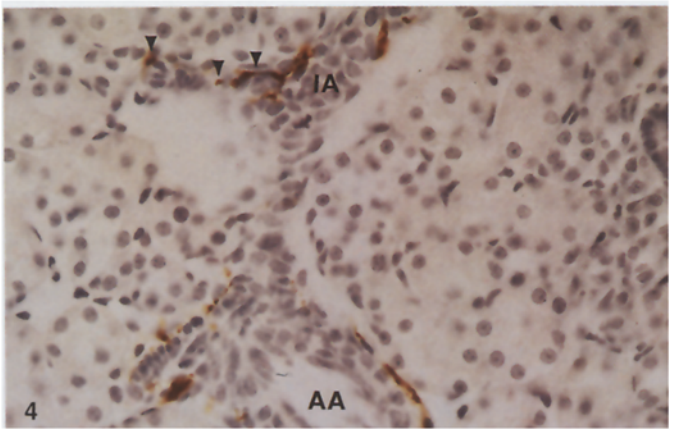

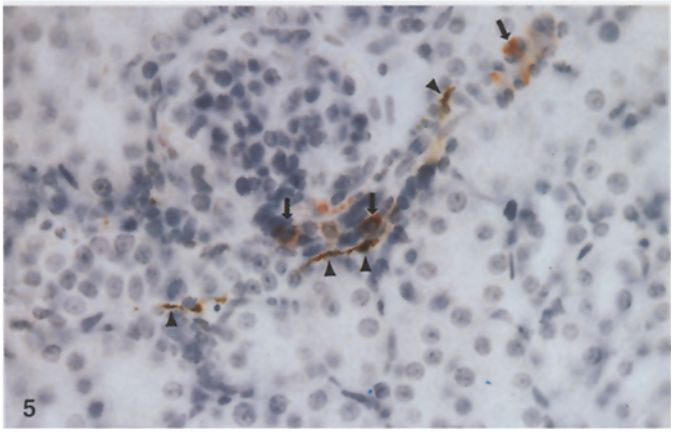

day-old fetuses and 5- and 15-day-old newborn rats were fixed in 10% buffered formalin for 24–72 h. The 15-day-old fetuses were processed as whole fetuses. After dehydration, tissues were embedded in paraffin and cut into 8-μm consecutive sections.

Sections were stained with a modification of the avidin-biotin-peroxidase complex [21] as previously described [3, 22]. Renin and nerve fiber localization in the same section was accomplished by a modification of the double-label immunostaining method of Barajas et al. [2]. After deparaffinization and hydration, the sections were washed in H_2O for 5 min, exposed to 1% hydrogen peroxide (H_2O_2) in methanol for 30 min to quench endogenous peroxidase activity, and rinsed in phosphate-buffered saline (PBS), pH 7.2, for 20 min. After a 40-min pre-incubation with normal horse serum containing 3% bovine serum albumin (BSA, type V Sigma Chemical, St. Louis, Mo. USA) in PBS, the sections were exposed to a monoclonal antibody (TUJ1, gift of Dr. A. Frankfurter, University of Virginia) directed against a neuron-specific class III beta-tubulin isotype [23–25]. The TUJ1 antibody was characterized previously by Frankfurter et al. and Lee et al. [23–25], and has been also employed to study the peripheral axon outgrowth in the trigeminal system [26]. Purified TUJ1 antibody was applied to the sections for 2 h at a dilution of 1 : 200 in PBS containing 3% BSA. After a rinse in PBS, the sections were incubated for 30 min with anti-mouse IgG biotinylated antibody (Vectastain ABC kit, Vector, Burlingame, Calif., USA), rinsed in PBS, and exposed for 45 min to the avidin-biotinylated-peroxidase complex (Vectastain ABC kit, Vector). Sections were rinsed in PBS and exposed for 7 min to 0.1% diaminobenzidine tetrahydrochloride (Polyscience, Warrington, Pa., USA) added prior to use to 0.02% H_2O_2 in 50 mM Tris buffer, pH 8. Thereafter, the sections were rinsed for 20 min in PBS, pre-incubated for 40 min with normal goat serum and incubated for 90 min with anti-rat renin polyclonal antibody (gift of Dr. T. Inagami, Vanderbilt University, Nashville, TN) diluted 1 : 2500 in PBS containing 3% BSA. The specificity and characterization of this antibody have been documented previously [27, 28]. The anti-rat renin polyclonal antibody reacts with both active renin and prorenin. After a rinse in PBS, anti-rabbit IgG biotinylated antibody and the avidin-biotinylated horseradish peroxidase complex were applied as described above and the reaction was visualized by incubation of the sections for 20 min with 3-amino-9-ethylcarbazole (AEC, Sigma Chemical). AEC was prepared as described by Childs et al. [29] with a minor modification: 10 mg AEC dissolved in 1 ml dimethylformamide were added to 15 ml acetate buffer and 15 μl H_2O_2 were added prior to use. All the incubations were performed at room temperature. Finally, the sections were rinsed in tap H_2O, counterstained with Mayer's hematoxylin and mounted in glycerol. With the two different peroxidase substrates, beta-tubulin was stained brown whereas renin appeared red. From each animal, three double-labelled sections and one section stained with TUJ1 antibody alone were analyzed under a Leitz Dialux 20 EB microscope (Leitz, Wetzlar, FRG).

For negative controls, all staining steps were omitted individually and the primary renin antibody was replaced by normal rabbit serum at the same dilution. Nerve structures present in fetuses of 15 days' gestation were employed as positive controls for beta-tubulin. In order to exclude possible interference of the renin immunostaining with the immunostaining of delicate nerve fibers, one section from each animal was stained only with the TUJ1 antibody.

Results

No significant staining was observed for renin or beta-tubulin when primary antibody either was omitted or replaced by normal rabbit serum.

In 15-day-old fetuses, intense staining for beta-tubulin was observed in the primitive brain as well as the spinal cord and ganglia (Fig. 1 a). Staining for beta-tubulin was also evident in nerve roots and in nerves (Fig. 1 a). Among the thoracic and abdominal organs, neither heart nor liver showed evidence of nerve fibers at 15 days of gestation. Additionally, no significant staining was present in the lung primordium, whereas in the gastrointestinal tract a widespread plexus of fibers was observed in the primitive muscular layer (Fig. 1 a).

At 15 days' gestation the metanephric kidney consisted of tubules and vesicles dispersed in a loose matrix of connective tissue. Renal tissues contained no renin or beta-tubulin immunoreactivity (Fig. 1 b).

At 17 days' gestation, the renal cortex contained glomeruli and tubules at different stages of maturation, including S-shaped bodies, the most mature glomeruli being located in the inner cortex. Renin-positive cells and large nerve bundles were present along the main branches of the renal artery, which crossed the renal parenchyma perpendicular to the kidney surface (Fig. 2). Nerve fibers were localized in the outer aspect of the vessel walls. Renin-containing cells were observed in the developing tunica media of the renal arteries.

At 19 days' gestation, nerve bundles surrounded the main intrarenal arterial branches and arcuate arteries (Fig. 3 a). Renin immunoreactivity was associated with nerve fibers along large vessels such as arcuate arteries (Fig. 3 a). Nevertheless, renin-containing cells were present also in developing interlobular arteries and in several afferent arterioles of more differentiated glomeruli located in the juxtamedullary region. When afferent arterioles to mature glomeruli were cut longitudinally, renin-containing cells appeared to be distributed throughout the entire length of these vessels (Fig. 3 b).

At 21 days' gestation, renin immunostaining was still present in arcuate and interlobular arteries. In the juxtamedullary region, the number of afferent arterioles showing renin immunoreactivity was increased when compared with earlier fetal ages. Bundles of nerve fibers surrounding the main arterial branches and arcuate arteries gave rise to smaller bundles innervating the interlobular arteries and occasional afferent arterioles in the juxtamedullary region (not shown).

During postnatal life the distribution of renin-containing cells and nerve fibers progressively approached that seen in the adult. At 5 days of postnatal life, the nephrogenic zone was still present, but mature glomeruli were distributed in all cortical regions. Renin immunostaining was still detected in some interlobular arteries; however, renin distribution was mainly confined to the afferent arterioles. Nevertheless, the number of mature glomeruli whose afferent arterioles demonstrated immunostaining for renin was increased when compared with that seen at earlier ages. Nerve bundles following arcuate arteries branched off in plexuses along the interlobular arteries, and

Fig. 3 a,b. Fetal kidney section at 19 days gestation. **a** Renin (red, *arrows*) and nerve fibers (brown, *arrowheads*) close together in large arteries. Double-label immunohistochemistry, ×460. **b** Renin immunostaining (red, *arrows*) throughout the length of afferent arteriole supplying juxtamedullary glomerulus. Double-label immunohistochemistry, ×360

Fig. 4. Kidney section at 5 days of postnatal life. Nerve bundles (brown) around arcuate (*AA*) and interlobular (*IA*) arteries. From interlobular artery nerve fibers distribute to afferent arteriole (*arrowheads*). Section stained with TUJ1 antibody alone, ×360

Fig. 5. Kidney section at 15 days of postnatal life. Renin-containing cells (red, *arrows*) in afferent arteriole. Afferent arteriole appears innervated by nerve fibers (brown, *arrowheads*) located in outer aspect of vessel. Double-label immunohistochemistry, ×360

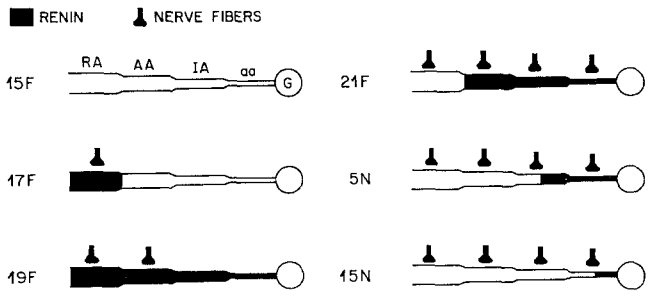

Fig. 6. Schematic representation of the developmental changes in renin and nerve fiber distribution in the developing kidney. *RA*, Intrarenal branches of renal artery; *AA*, arcuate artery; *IA*, interlobular artery; *aa*, afferent arteriole. *15F, 17F, 19F* and *21F* indicate a 15-, 17-, 19- and 21-day-old fetus, respectively. *5N* and *15N* indicate 5- and 15-day-old newborns, respectively

more delicate fibers surrounded afferent (Fig. 4) and efferent arterioles. Few nerve fibers were observed in close proximity to renal tubules (not shown).

At 15 days of postnatal life, the nephrogenic zone was not present. When compared with animals under 15 days, the density of nerve fibers had increased further. Occasionally, nerve fibers were observed between afferent and efferent arterioles in close approximation to the macula densa. In the outer medullary region, delicate fibers also were detected where vasa recta were developing (not shown). Although an immature pattern of renin distribution was still present in several afferent arterioles, renin-containing cells were found mainly in the juxtaglomerular position. Thus, at this age the juxtaglomerular apparatus represented the only area where renin-containing cells and nerve fibers were spatially associated (Fig. 5). In summary, as schematically represented in Fig. 6, renin-containing cells and nerve fibers were detected contemporaneously in the intrarenal branches of renal artery of the 17-day-old rat fetus. As kidney maturation progresses, renin distribution shifted from large arteries to the adult juxtaglomerular position. Simultaneously, density and organization of nerve fibers increased with age along the vasculature.

Discussion

The present study demonstrates that nerve fibers and renin-containing cells appear contemporaneously as early as 17 days of gestation along the developing vascular tree in the rat fetal kidney. The distribution of renin and innervation of the kidney progresses during fetal and postnatal life following the development of nephrovascular structures from the inner to the outer regions of the kidney cortex.

In the rat metanephric kidney innervation occurs during the last third of gestation and follows along developing arteries. Innervation of renal developing vasculature has been documented also in the human fetus. Ultrastructural studies of the human fetus demonstrated the presence of nerve terminals along renal vasculature and distal tubules at 13–16 weeks of gestation [20]. These data suggest that maturation of renal innervation occurs relatively earlier in humans than in the rat (normal gestation period in the rat is 22 days).

To detect nerve fibers, we employed an antibody that reacts with a structural protein of neurons. We were not able, therefore, to provide information regarding either neurotransmitter maturation or its type. Nevertheless, tyrosine hydroxylase-positive nerve fibers have been reported in rat kidney at 21 days of gestation [14]. Further, norepinephrine was detected in the rat kidney at 21 days of gestation [14], and norepinephrine concentration was observed to increase during the first weeks of postnatal life [14, 15]. The age-dependent increase in renal norepinephrine concentration in the developing rat kidney supports our observation concerning progressive maturation of renal innervation throughout fetal and postnatal life. A similar pattern of development of renal sympathetic innervation has been described in the dog kidney during postnatal life [19]. However, at variance with these findings we did not find nerve fibers in the nephrogenic zone of the rat kidney. In agreement with our present observation, physiological studies of unanesthetized fetal lambs have demonstrated that the renal nerves were functional in the fetus and modulated renal hemodynamics in response to stressful conditions such as hypoxemia [31]. Furthermore, renal nerve stimulation produced a decrease in renal blood flow velocity and a rise in renal vascular resistance [32]. Interestingly, these hemodynamic changes were less pronounced in the fetus than in the adult animal [32]. The ontogenic increase in the density of nerve fibers along the intrarenal vessels observed in the present study supports the hypothesis that the decreased hemodynamic response in early life may be due to reduced sympathetic innervation as well as adrenoceptor immaturity [14, 33, 34].

Simultaneously with the appearance of nerve fibers along the vascular cells, the smooth muscle cells of these vessels demonstrated renin immunoreactivity. The finding of renin in myoepithelial cells of large arterial vessels during fetal life confirms previous observations in rat [3, 4, 30] and other species including mouse [5], pig [6] and human [7]. Renin distribution progressively changes to reach the classical juxtaglomerular localization during postnatal life. In the rat, this process was not fully completed at 15 days of postnatal life as previously reported [3], while in the mouse the adult pattern of renin distribution was evident 2 days after birth [5].

Physiological studies have demonstrated that the fetal kidney is capable of releasing renin in response to diverse stimuli [35, 36]. Pharmacological stimulation of beta-adrenoceptors causes renin release from renal cortical slices in sheep fetuses and lambs [16]. The results of the present study provide morphological evidence supporting the feasibility of a role of nerve fibers (perhaps through a beta-adrenoceptor-mediated mechanism) in the control of renin release during fetal and postnatal life.

In addition to modulating renal hemodynamics and renin release, the renal nerves may play other roles during kidney ontogeny. Results from an in vitro study have suggested that neural elements are involved in renal differentiation [37]. When rat kidneys are transplanted into the anterior eye chamber of adult animals, providing a rich neuronal environment, the number of renin-containing

cells is increased [17]. Recently, it has been reported that sympathetic nerves play an important role in regulating renin gene expression and distribution in developing rat kidney subjected to chronical ureteral obstruction [18]. Further studies are necessary to define whether renal nerves influence the morphological development and endocrine differentiation of the juxtaglomerular cells.

We recently demonstrated that coincidentally with the centrifugal maturation of nephrons, renin mRNA distribution shifts from arcuate and interlobular arteries in the rat fetus to a classical juxtaglomerular position in the adult kidney [38]. Similar developmental changes in the expression of smooth muscle isoactin have also been demonstrated [22]. The aforementioned studies, together with the present, demonstrate that as the vessel wall is being assembled, the expression of contractile proteins, renin-containing myoepithelioid cells and neural elements are temporarily associated. These studies suggest that during differentiation and further development of the renal vasculature, there is a coincident expression of structural and functional genes responsible for vessel wall function.

Acknowledgements. The findings of the present study were presented at the annual meeting of the American Federation for Clinical Research, January 1990, and have appeared in abstract form [Clin Res (1990) 38: 69 A]. This work was supported by the University of Virginia Vascular Smooth Muscle Program Project (NIH grant POI-HL-19 242) (R. M. C.) and AHA, Virginia affiliate (J. B. T.). Dr. Pupilli is the recipient of a research fellowship issued by the Italian Ministry of Public Instruction. R. A. G. is a Syntex scholar; R. A. G. and J. B. T. are recipients of NIH Research Career Development Awards. We thank Dr. T. Inagami (Vanderbilt University) for his generous supply of the renin antibody and Dr. A. Frankfurter (University of Virginia) for his kind gift of TUJ1 antibody and his suggestions for its use. We also thank Mrs. D. R. Hannum for her technical assistance in processing tissues.

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Literature abstracts

Am J Med (1990) 89: 432–435

Treatment of the anemia of chronic renal failure with subcutaneous recombinant human erythropoietin

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Purpose: The purpose of this study was to determine the efficacy of recombinant human erythropoietin (rHuEPO) given subcutaneously three times/week in patients with chronic renal failure and anemia (predialysis).

Patients and methods: Eleven patients with predialysis chronic renal failure participated in a double-blind, placebo-controlled study of subcutaneously administered erythropoietin.

For 12 weeks, patients received either rHuEPO 100 μ/kg body weight three times/week subcutaneously or a placebo. After 12 weeks of placebo, patients now also received rHuEPO in a dose up to 150 μ/kg three times/week until target hematocrit was achieved. Throughout the study, blood pressure was monitored closely and blood work was obtained regularly for hemoglobin, hematocrit, reticulocyte count, and iron profile determinations.

Results: At 12 weeks, the hematocrit of the treated group had risen from 29% ± 2% to 35% ± 2% ($p < 0.001$). The placebo group baseline hematocrit was 28% ± 2% and at 12 weeks 26% ± 2%. After 12 weeks of rHuEPO therapy, the hematocrit of the prior placebo group was 32% ± 2% ($p < 0.001$ versus baseline). No significant change in biochemical parameters was noted. Mean blood pressure values were comparable before and after treatment. All patients ultimately required iron supplementation. In two patients, the rate of progression of renal failure appeared to increase as their hematocrit rose and rHuEPO was discontinued.

Conclusions: It is concluded that rHuEPO given subcutaneously is an effective and safe therapy for patients with chronic renal failure who are anemic and who are not receiving dialysis.

Arch Dis Child (1991) 66: 501–503

Urinary oxalate and glycolate excretion and plasma oxalate concentration

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The diagnosis of primary hyperoxaluria in young children is hampered by the lack of a reliable reference range for urinary oxalate excretion, especially in infants. We present data on urinary oxalate and glycolate excretion in 137 normal children, on the plasma oxalate concentration in 33 normal children and 53 with chronic renal failure, and on amniotic fluid oxalate concentration in 63 uncomplicated pregnancies. The urinary oxalate : creatinine molar ratios were log normally distributed: mean (range) values were <1 year 0.061 (0.015–0.26), 1–5 years 0.036 (0.011–0.12), 5–12 years 0.030 (0.0059–0.15), and >12 years 0.013 (0.0021–0.083). Geometric mean (range) plasma oxalate concentration in the normal children was 1.53 (0.78–3.02) μmol/l and was independent of age. The mean (SD) plasma oxalate : creatinine molar ratio in these normal children and 50 with chronic renal failure was 0.033 (0.013), and was independent of age and renal function. Mean (SD) amniotic fluid oxalate concentration was 19.0 (4.3) μmol/l.