Kidney International, Vol. 52 (1997), pp. 387–392

Mesangial cell abnormalities in spontaneously hypertensive rats before the onset of hypertension

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Mesangial cell abnormalities in spontaneously hypertensive rats before the onset of hypertension. To identify kidney biosynthetic abnormalities that may precede the onset of hypertension, we studied the expression of fibronectin (FN) and collagen IV (Coll IV) in young SHR (4 weeks of age) whose systolic blood pressure was normal and similar to that of age-matched control WKY rats. In isolated glomeruli the level of FN protein assessed by immunoblotting tended to be lower in the SHR than in the WKY rats. By Northern analysis the FN/actin mRNA ratio was significantly lower in glomeruli from SHR (0.56 ± 0.47) than in glomeruli from WKY rats (2.0 ± 0.8). These abnormalities were maintained *in vitro* since the expression of FN was significantly lower in SHR than in WKY cultured mesangial cells (FN/actin mRNA ratio = 0.84 ± 0.46 vs. 1.9 ± 0.7, $P = 0.029$). No differences in Coll IV mRNA or protein levels were observed in SHR glomeruli and mesangial cells when compared with WKY rats. The levels of aortic FN and Coll IV mRNAs were not different in SHR and WKY rats. In addition, mesangial cells from SHR showed a significantly higher growth rate than those from WKY. The biosynthetic and proliferative abnormalities observed in the SHR mesangial cells appear to reflect genetic characteristics, and could provide novel insights into cellular mechanisms linking the genetics of hypertension with predisposition to glomerular pathology.

Several lines of evidence suggest that the kidney plays a primary role in the pathogenesis and maintenance of hypertension [1–7]. Patients with hypertensive nephrosclerosis had normalization of their blood pressure after renal transplantation from donors without a genetic background for hypertension [2]. Conversely, genetically normotensive rats develop hypertension when receiving a kidney transplant from hypertensive strains of rats [4–6], even when the donors are maintained normotensive throughout life with an angiotensin-converting enzyme inhibitor [4]. Although an impaired ability of the kidney to excrete sodium is likely to be the final functional pathway to hypertension, both in human essential hypertension and in hypertensive strains of rats [7], the nature of the responsible cellular and genetic mechanisms remain incompletely defined [7].

In patients with diabetes mellitus susceptibility to renal disease is variable, and it has been suggested that an important contributor to such susceptibility is predisposition to hypertension [8–10]. However, the mechanisms by which the genetics of hypertension magnify the risk of diabetic nephropathy in patients with diabetes mellitus is not clearly understood. An increase in mesangial cell volume or number and, especially, in mesangial matrix are histological hallmarks of diabetic nephropathy, and the degree of mesangial matrix expansion best predicts functional abnormalities [11]. It may thus be asked whether the genetics of hypertension specify an abnormal behavior of mesangial cells.

The identification of early abnormalities in the kidney of individuals who are destined to become, but are not as yet, hypertensive, could provide new insights relevant to the kidney pathology and susceptibility to diabetic renal disease that accompany the genetics of hypertension. Because such studies cannot readily be performed in humans, we used a widely studied model of essential hypertension, namely the spontaneously hypertensive rat (SHR), which exhibits a normotensive period (the first 4 weeks of life) prior to the genetically determined development of hypertension, which occurs in 100% of the animals [12]. The aim of our study was to investigate if the kidney of young (4 weeks of age) normotensive SHR displays biosynthetic and proliferative abnormalities when compared with the kidney of genetically normotensive controls, the Wistar Kyoto (WKY) rats.

METHODS

Animals

SHR and WKY rats four weeks of age were obtained from Taconic (Germantown, NY, USA). As noted by other investigators [13, 14], at this age SHR were smaller than WKY rats and had a slightly higher blood pressure. In the 13 groups of rats used for experiments the weight was 60 ± 9 g for the SHR ($N = 90$) and 100 ± 23 g for the WKY ($N = 70$; $P < 0.001$). Systolic blood pressure was measured by Taconic on the day of shipment. Blood pressure determinations were obtained by tail-cuff plethysmography on unanesthetized rats (5 determinations in each rat) at 26°C using a MK III physiograph (Narco Bio-System, Houston, TX, USA). Systolic blood pressure was 124 ± 12 mm Hg in the SHR and 114 ± 12 in the WKY ($P < 0.05$), documenting the absence of hypertension. Experimental procedures were conducted according to institutional and NIH guidelines and were approved by the institutional Animal Care and Use Committee.

Isolation of kidney glomeruli and aortas

Rats were sacrificed by CO_2 asphyxiation and the kidneys and aortas were rapidly excised. Glomeruli were isolated by differential sieving technique [15, 16]. Briefly, finely minced decapsulated

Key words: hypertension, mesangial cell, genetics and hypertension, diabetes.

Received for publication August 22, 1996
and in revised form February 27, 1997
Accepted for publication March 3, 1997

kidney cortex was gently pressed and rinsed with Hank's balanced salt solution (HBSS) through stainless steel screens (W. Tyler Co., Mentor, OH, USA) of 60 (pore size 250 μ) and 150 (pore size 106 μ) mesh, and the glomeruli were collected on 200 mesh screen (pore size 53 μ). The purity of the preparation, as assessed by phase contrast microscopy, was approximately 95%. Glomeruli were used to isolate RNA, study protein levels, and culture mesangial cells. Aortas were removed taking care to avoid stretching or compressing the tissue. The region of the aorta extending from the arch to the iliac bifurcation was used in all studies. Aortas were carefully cleaned of periadventitial tissue, snap frozen in liquid nitrogen, and stored at −80°C until processed for RNA extraction.

Isolation and culture of mesangial cells

In each experiment the glomeruli isolated from 4 to 5 SHR and 3 to 4 WKY rats, respectively, were pooled and incubated in HBSS containing 750 U/ml collagenase (CLS4; Worthington, Freehold, NJ, USA) for 30 minutes at 37°C followed by a wash in HBSS to remove epithelial cells [17]. The glomerular remnants were plated in 60 mm tissue culture dishes (Falcon Primaria; Becton Dickinson, Lincoln Park, NJ, USA), and cultured in RPMI-1640 tissue culture medium (Gibco, Grand Island, NY), buffered with 15 mM Hepes (Sigma Chemical Co., St Louis, MO) and containing 20% decomplemented fetal bovine serum (Sigma), 10 mM glucose, penicillin (60 U/ml), streptomycin sulfate (60 μg/ml), and fungizone (50 μg/ml) (Gibco). Incubation was at 37°C in 5% CO_2 and 95% air; the media were changed every 48 to 72 hours. Mesangial cells outgrowths appeared after 2 to 3 days in culture and reached confluence after 10 days. The cells were passaged every 7 to 10 days using trypsin-EDTA (Gibco). Confluent dishes of cells from passage three to four, when contamination by other cell types such as endothelial or epithelial was virtually absent, were used for all experiments. Mesangial cells in culture were identified by phase-contrast microscopy on the basis of their characteristic stellate to fusiform morphology [18]; in addition the cells were negative for both uptake of acetylated-low density lipoprotein (Biomedical Technologies Inc., Stoughton, MA, USA) and staining with keratin antibodies (ICN Biomedicals, Costa Mesa, CA, USA), thus excluding contamination with endothelial and epithelial cells, respectively.

Northern blot analysis

Total RNA was extracted from the isolated glomeruli, aortic tissue, and cultured mesangial cells at confluence using the guanidine thiocyanate method [19], and the RNA samples (15 μg) were size-fractionated by electrophoresis on 1% agarose gel containing 2.2 M formaldehyde. Lack of degradation was verified by acridine orange staining of the 28S and 18S rRNA bands. Northern blot onto nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, NH, USA) was performed as described previously [20]. Blots were hybridized [20] for 12 to 48 hours at 42°C to a ^{32}P-labeled rat fibronectin cDNA consisting of a 582 bp fragment obtained by polymerase chain reaction [21], a mouse collagen α_1 (IV) cDNA (gift of Dr. M. Kurkinen) [22], and chicken β-actin cDNA (Oncor, Gaithersburg, MD, USA). After hybridization, the nylon membranes were washed twice in 6 × SSPE [20], 0.1% SDS at 37°C, and two more times in 1 × SSPE, 0.1% SDS at 55°C. The membranes were exposed to Kodak X-Omat film at −80°C for 4 to 6 hours (actin), 12 to 96 hours (fibronectin) and 24 to 48 hours (collagen IV). Laser densitometry, performed using the Model 330A computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA) was used to quantitate signal intensity. Results are expressed as fibronectin/actin and collagen IV/actin ratios.

Western blot analysis

Freshly isolated glomeruli were homogenized in a buffer containing 30 mM Tris-HCl, pH 7.5, 10 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol, and 250 mM sucrose at a 10:1 ratio (vol/vol) of buffer to tissue wet wt. The homogenate was sonicated three times for five seconds. An aliquot of 10 μl was separated for protein quantitation performed by the Bradford procedure [23] with bovine serum albumin as the standard. The remaining homogenate was mixed in Laemmli sample buffer [24], heated at 100°C for five minutes, and stored at −80°C.

Five, ten and fifteen micrograms of total protein from glomeruli of SHR and WKY rats, respectively, were analyzed by SDS-polyacrylamide gel electrophoresis as described by Laemmli [24] on linear 4 to 20% acrylamide gradient separating gels, using Mini-Protean II Dual Slab Cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfer onto nitrocellulose membranes was done at room temperature for two hours at constant current (200 mA). The membranes were blocked overnight at 4°C in 5% dried milk in buffer containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween-20 (TBST), and then incubated with the primary antibody for one hour at room temperature. A mouse anti-rat fibronectin monoclonal antibody (BR 5.3; gift of Dr. R. Hynes) [25] was used diluted 1:100 in TBST, and a rabbit antiserum against rat type IV collagen (gift of Dr. R.G. Spiro) [26] was used diluted 1:200 in TBST. The membranes were washed three times in TBST and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. The anti-mouse secondary antibody was diluted 1:2000 in TBST, and the anti-rabbit 1:5000. Immunoreactive bands were visualized using the enhanced chemiluminescence method (SuperSignal® CL-HPR Substrate System; Pierce, Rockford, IL, USA) according to manufacturer's protocol.

Densitometric analysis of the autoradiographs was done through the Model 300 A computing densitometer. The values for signals generated by the increasing amounts of sample lysate (5, 10, and 15 μg of total protein) were averaged to yield the fibronectin and collagen IV values for each sample (densitometric U/μg total protein). For quantitation of type IV collagen, the absorbance of the α1-chain (≈ 200,000 Mr) was measured. Fibronectin was determined on the basis of its 230,000-Mr component.

Cell proliferation

To estimate the rate of growth of mesangial cells of SHR and WKY rats, cells companion to those destined to mRNA and protein experiments, were plated onto 24-well plates at 10,000 cells/well. The cell numbers were determined [27] in triplicate on days 2, 7 and 12 after plating.

Statistical analysis

Data are presented as mean ± SD. The weight and blood pressure values of SHR and WKY rats, as well as the results

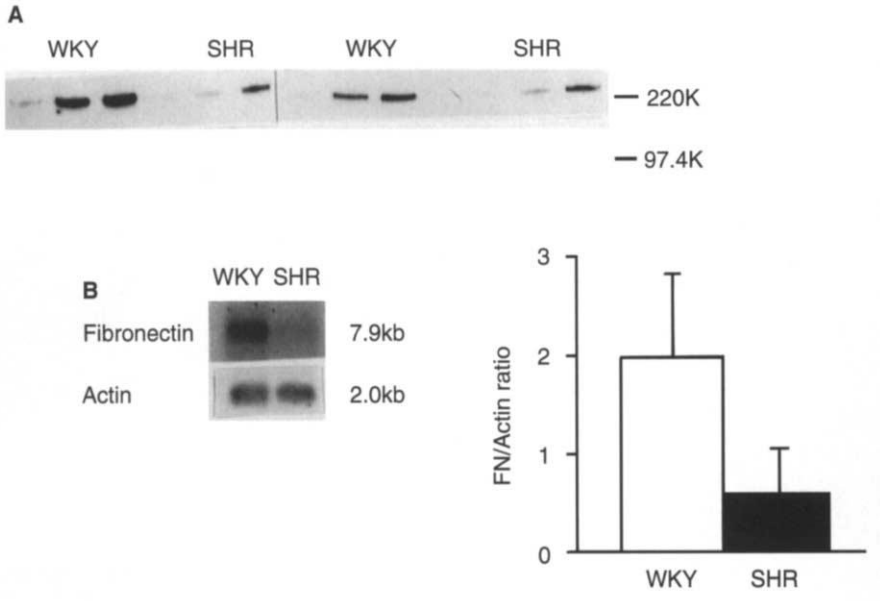

Fig. 1. Fibronectin protein and mRNA levels in isolated glomeruli of WKY and SHR. (*A*) Immunological detection (Western blot) of glomerular fibronectin. Two experiments are depicted; in each experiment glomeruli were pooled from 3 WKY rats and 4 SHR, respectively, and the glomerular lysates were subjected to PAGE (5, 10, and 15 μg of total protein per lane) and immunoblotted with anti-rat fibronectin antibodies as described in Methods. Designated molecular weight markers are myosin (220 K) and phosphorylase b (97.4 K). K is kiloDaltons. (*B, left*) Northern blot of glomerular fibronectin and β-actin. Each lane contains 15 μg of total RNA extracted from glomeruli pooled from 3 WKY and 4 SHR. Blots were hybridized with rat fibronectin cDNA and a chicken β-actin cDNA as described in the **Methods** section. kb is kilobases. (*B, right*) Fibronectin/actin mRNA ratio as determined by Northern blot analysis in isolated glomeruli of WKY rats (□) and SHR (■). Each bar represents the mean ± SD of the results obtained in three experiments.

obtained in the Northern and Western blots and in the cell proliferation studies were compared using the two-tailed unpaired Student's *t*-test.

RESULTS

In freshly isolated glomeruli the level of immunoreactive fibronectin as detected by Western blots (Fig. 1A) tended to be lower in the SHR than in the WKY rats (mean ± SD, 29 ± 8 and 48 ± 30 densitometric U/μg total protein, $P = 0.2$, $N = 4$). Although differences could not be accounted for by differences in plasma fibronectin because Western blot analysis showed similar levels of plasma fibronectin in SHR (2464 ± 318 densitometric units/10 μl of plasma) and WKY (2230 ± 331), we could not exclude confounding influences by plasma trapped in the tissue. We thus examined the level of fibronectin mRNA. The fibronectin/actin mRNA ratio was significantly lower ($P = 0.05$) in glomeruli from SHR (0.56 ± 0.47) than in glomeruli from WKY rats (2.0 ± 0.8; Fig. 1B). Actin could appropriately be used as control for RNA loading because the levels of actin mRNA were similar in the glomeruli of SHR (2352 ± 2965 densitometric units) and WKY rats (1941 ± 1719).

At variance with fibronectin, the levels of collagen IV were similar in the SHR and WKY glomeruli. In four experiments, collagen IV protein was 136 ± 62 densitometric U/μg total protein in the SHR and 142 ± 47 in the WKY rats; in two experiments the collagen IV/actin mRNA ratio was 1.23 ± 0.3 in SHR and 1.74 ± 0.7 in WKY rats.

To examine whether the lower level of fibronectin expression observed in the SHR glomeruli reflected an intrinsic biosynthetic characteristic of mesangial cells, these cells were cultured from glomeruli of SHR and WKY rats, and tested at the third or fourth passage. The fibronectin/actin mRNA ratio was significantly lower ($P = 0.03$) in mesangial cells of SHR (0.84 ± 0.46) than in mesangial cells of WKY rats (1.9 ± 0.7; Fig. 2). The magnitude of the difference (2.2-fold less fibronectin mRNA in SHR mesangial cells) was similar to that observed in freshly isolated glomeruli (3.5-fold). In four experiments, the collagen IV/actin mRNA ratio was similar in the mesangial cells of SHR (2.4 ± 1.0) and WKY rats (2.4 ± 1.2), as was the level of actin mRNA (2203 ± 906 densitometric units in SHR and 2542 ± 1550 in WKY rats).

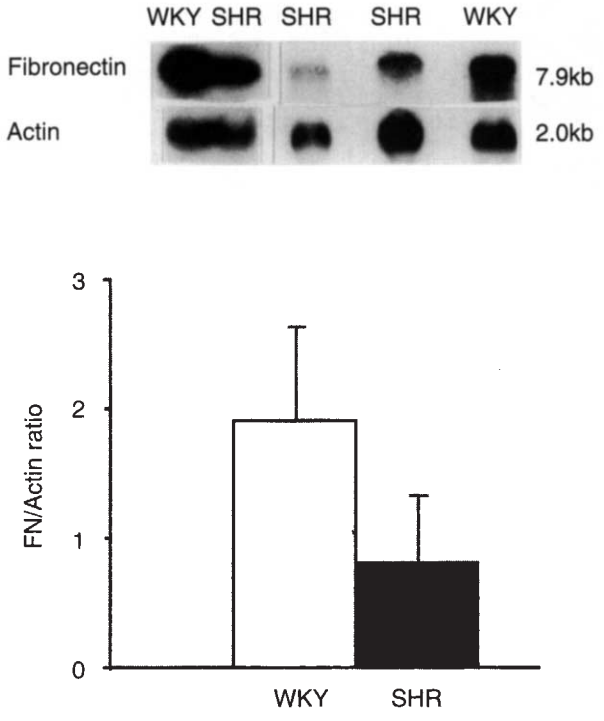

Fig. 2. Fibronectin and β-actin mRNA levels in cultured mesangial cells of WKY and SHR. (Top) Northern blots (15 μg total RNA/lane). Each lane represents a different mesangial cell isolate, cultured from a pool of glomeruli isolated from WKY rats and SHR, respectively. Two experiments are depicted. (Bottom) Fibronectin/actin mRNA ratios as determined by Northern analysis in mesangial cells of WKY rats (□) and SHR (■). Each bar represents the mean ± SD of the results obtained in three experiments.

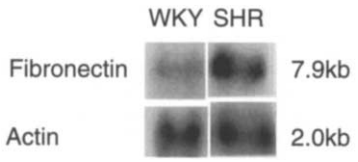

Fig. 3. Fibronectin and β-actin mRNAs in aortas of WKY and SHR. RNA was extracted from a pool of 3 to 4 aortas of WKY and SHR, respectively, and subjected to Northern blot analysis (15 μg of total RNA/lane).

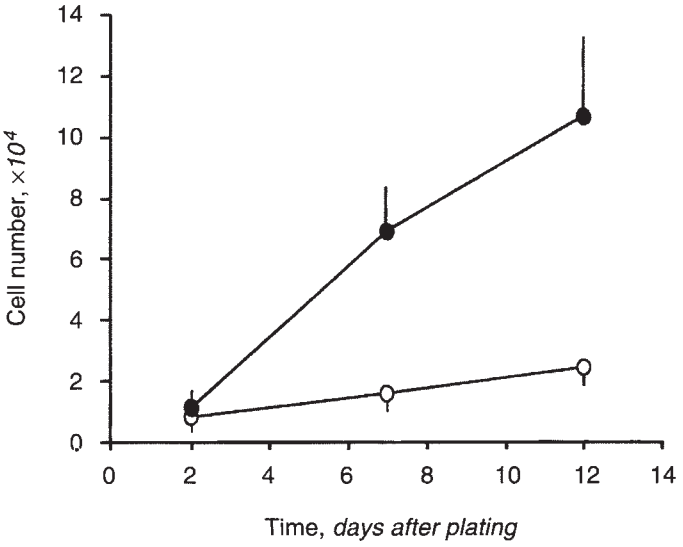

Fig. 4. Proliferation of cultured mesangial cells of WKY and SHR. Mesangial cells from WKY (○) and SHR (●) were concomitantly plated, in triplicate, at 10,000 cells/well of a 24-well plate. The cells were harvested and counted on days 2, 7 and 12 after plating. Each point represents the mean ± SD of the results obtained in 4 independent experiments.

To ascertain if other tissues in the SHR exhibited fibronectin mRNA levels lower than in the WKY rats, the aortas of the same animals from which kidneys were obtained, were also studied. The fibronectin/actin mRNA ratio of aortic tissue was found in four experiments to be similar in the SHR (0.47 ± 0.30) and WKY rats (0.64 ± 0.41, $P = 0.5$; Fig. 3), as were collagen IV/actin mRNA ratio (0.24 ± 0.23 in the SHR and 0.27 ± 0.12 in the WKY rats) and β-actin mRNA levels (3648 ± 1460 densitometric units in the SHR and 4335 ± 2393 in the WKY).

We observed that the mesangial cells obtained from the SHR proliferated in culture at a faster rate than the cells obtained from the WKY rats. The impression was confirmed by the results of formal proliferation experiments (Fig. 4). At day 2 the number of SHR mesangial cells was similar to that of mesangial cells from the WKY rats excluding differences in plating efficiency, but it was 4.4-fold greater at days 7 and 12 ($P = 0.001$).

DISCUSSION

We observed that, when compared with the genetically normotensive WKY rats, SHR four weeks of age and still normotensive exhibit lower expression of fibronectin in the kidney glomeruli and cultured mesangial cells, and an accelerated proliferation of mesangial cells *in vitro*. Insofar as the glomerular collagen IV and β-actin expression did not differ between the SHR and WKY rats, the biosynthetic abnormality appears to be product-specific; and because aortic fibronectin mRNA levels were similar in the SHR and WKY rats, it appears to be organ-specific. The fact that the reduced basal expression of glomerular fibronectin occurring *in vivo* in the SHR was maintained in their cultured mesangial cells indicates that the abnormality detected *in vivo* is contributed, at least in part, by the mesangial cell component of the glomeruli, and that it does not reflect levels of microenvironmental regulation possibly lost *in vitro*, but rather genetic characteristics intrinsic to the mesangial cells.

These observations may have relevance to the mechanisms underlying the severe glomerular sclerosis that develops progressively in SHR, even when the rats are maintained normotensive with drug treatment [28]. Gene targeting experiments have recently demonstrated a critical role for fibronectin in vascular development [29], and an abnormally low expression of fibronectin in the SHR glomeruli might, if present during embryogenesis, compromise in some subtle way the development of the glomeruli and render them prone to pathology later in life. Additionally, a contribution may be made by accelerated mesangial cell proliferation. The level of fibronectin synthesis and cellular proliferation are, in some systems, inversely related. For example, overexpression of fibronectin during *in vivo* and *in vitro* aging of vascular endothelial cells and fibroblasts is coincidental with a decreased labeling index [30], and similar observations have been made in endothelial cells exposed to exogenous perturbations that increase fibronectin synthesis [31]. The reduced fibronectin synthesis and greater proliferative potential of SHR mesangial cells may thus represent two facets of the same cellular abnormality, and because dysregulation of mesangial cell mitogenesis is viewed as an early prerequisite for the development of glomerulosclerosis [32], such abnormality may have relevance to the kidney pathology of SHR.

The finding of greater proliferative potential of the mesangial cells in a genetic model of hypertension may also be relevant to the mechanisms underlying the predisposition to diabetic nephropathy of individuals with a family history of hypertension [8–10]. In fact, it has been recently reported that fibroblasts explanted from such individuals and cultured *in vitro* for several generations manifest accelerated growth when compared with fibroblasts from diabetic patients without nephropathy and nondiabetic control subjects [33–35]. It could be envisioned that, if the abnormal growth phenotype is also manifested in the mesangial cells of individuals prone to hypertension as it is in the prehypertensive SHR, and translates into accelerated turnover *in vivo*, it may play a critical role in the mesangial expansion that eventually leads to clinical nephropathy. The contribution would be not only through cellular hyperplasia, but also through a deposition of extracellular matrix made overabundant by the greater number of contributing cell generations, especially under the stimulatory effect of the high glucose levels [36]. Of interest, SHR made diabetic with streptozotocin manifest features of accelerated nephropathy when compared with diabetic WKY rats or control SHR, but fractional mesangial volume not greater than

the other two groups [37]. However, mesangial volume was measured at 32 weeks, after a prolonged course of both diabetes and hypertension, and the influence of hemodynamic abnormalities and possible incipient glomerular sclerosis occurring in the SHR [28] represent confounding features. Hamaguchi et al [38] reported that the glomeruli and other kidney structures of hypertensive SHR exhibit a greater number of replicating cells than the kidneys of WKY rats, and that the number is reduced by antihypertensive treatment. However, the investigators failed to indicate if treatment fully corrected the increased replication observed in the SHR.

It appears that to establish whether there is an *in vivo* counterpart to the abnormal growth phenotype of SHR mesangial cells that is solely attributable to the genetics of hypertension will require studies in the pre-hypertensive SHR. Such studies are made especially desirable by the findings in fibroblasts explanted from diabetic patients with nephropathy [33–35], which indirectly propose that genetically determined abnormalities in growth regulation may be a mechanism for the tendency to develop glomerular pathology.

ACKNOWLEDGMENTS

The work was supported by NIH grant EY-09122, Juvenile Diabetes Foundation International grant 195069, and the George and Frances Levin Endowment. Dr. Lopes de Faria was a Postdoctoral Fellow from the Nephrology Unit, University of Campinas, SP, Brazil in receipt of a scholarship from Fundação de Amparo a Pesquisa do Estado de São Paulo, SP, Brazil. The authors thank Drs. M. Kurkinen, R.O. Hynes, and R.G. Spiro for the gift of the rat collagen IV cDNA, fibronectin antibodies, and collagen IV antibodies, respectively. The authors also thank Drs. J.M. Lopes de Faria and S. Roy for technical assistance.

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