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Induction of apoptosis during development of hypertensive nephrosclerosis[1]

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Induction of apoptosis during development of hypertensive nephrosclerosis.

Background. As the biology of programmed cell death, or apoptosis, is clarified, a role for this process in the pathophysiology of organ dysfunction and fibrosis has been hypothesized. Hypertensive nephrosclerosis represents an important cause of end-stage renal disease. One model of the progressive, noninflammatory, sclerotic renal lesion of hypertension is the Dahl/Rapp salt-sensitive rat, which was examined in this study.

Methods. Male, Dahl/Rapp salt-sensitive (SS) and Sprague-Dawley rats were placed on either 0.3 or 8.0% NaCl diets for three weeks. Blood pressure was determined, and the kidneys were harvested for histochemical analysis and to obtain total RNA for RNase protection assays and total protein for Western blotting.

Results. An increase in apoptosis in the glomerular and tubular compartments was observed only in kidneys of SS rats on the high-salt diet. These findings occurred at a time when renal function was markedly impaired and irreversible changes in renal morphology developed. Temporally associated with this increase in apoptosis was augmented expression of pro-apoptotic molecules that included Fas, Bax, and Bcl-X_S.

Conclusions. The inappropriate shift in expression of proteins that facilitate apoptosis in the nephron, along with ongoing cell death that manifested at a time when renal function was deteriorating, supported an important role for this process in development of hypertensive nephrosclerosis.

As many as 43 million individuals in the United States suffer from high blood pressure [1]. Hypertensive nephrosclerosis is relatively less common, developing in approximately 1 in 2500 hypertensive patients [2], but represents an important cause of end-stage renal failure. The pathophysiologic processes that result in progressive renal failure from hypertension are not yet clarified. Apoptosis, or programmed cell death, is typically a subtle ongoing process in vivo in the healthy kidney, but several studies have now suggested that in certain pathologic conditions, alteration of the apoptotic pathways may result in a loss of otherwise healthy cells and may contribute to the decrease in kidney function [3–5]. The role of apoptosis in development of hypertensive nephrosclerosis has not been determined.

The Dahl/Rapp salt-sensitive (SS) rat has served not only as a model of salt-sensitive hypertension but also hypertensive nephrosclerosis [6]. These animals developed a progressive hypertension-induced renal failure; over a four-week period, inulin clearance fell to levels incompatible with life. Renal dysfunction was completely reversible if blood pressure was corrected before three weeks of hypertension. After three weeks on the high-salt diet, an apparent fixed reduction in inulin clearance was observed, despite attempts to return the blood pressure toward normal. Detailed pathologic examination of the kidneys demonstrated a noninflammatory, sclerotic disease process in the arterioles, glomeruli, and tubulointerstitium [6]. Because fibrotic lesions in both kidney [3, 4] and lung [7] have been associated with an increase in apoptotic rates, we hypothesized that apoptosis contributed to the loss of functional renal cells in the salt-sensitive kidney during the development of hypertensive nephrosclerosis. In the present study, kidneys of SS rats given a standard high-salt diet were examined. The findings supported an important role for apoptosis during development of renal failure in the SS rat.

METHODS

Animal preparation

Studies were conducted using 24 male Dahl/Rapp salt-sensitive (SS) and 16 male Sprague-Dawley (SD) rats that were 28 days of age and obtained from Charles River Laboratories (Wilmington, MA, USA). The protocol that

[1] **See Editorial by Ortiz, p. 2235**

Key words: salt-sensitive hypertension, Fas, Bcl-2, chronic renal failure, Dahl/Rapp rat, programmed cell death.

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Table 1. Mean arterial pressure (MAP) of rats on days 7 and 21 of the study

	Day 7	Day 21
Group	MAP, *mm Hg*	
SS 0.3% NaCl	107 ± 4	114 ± 3
SS 8.0% NaCl	126 ± 1[a]	169 ± 7[a]
SD 0.3% NaCl	105 ± 3	115 ± 4
SD 8.0% NaCl	112 ± 4	118 ± 4

Abbreviations are: SS, salt-sensitive Dahl/Rapp rats; SD, Sprague-Dawley rats.
[a] $P < 0.002$ compared with MAP of the other three groups

was followed has been standardized in our laboratory [6, 8, 9]. Rats were housed under standard conditions and given a formulated diet (AIN-76A; Dyets, Inc., Bethlehem, PA, USA) that contained either 0.3 or 8.0% NaCl. These diets were prepared specifically to be identical in protein and electrolyte composition and differed only in NaCl and sucrose content. On days 7 and 21 of study, rats were anesthetized with ethyl,1-methylpropylthiobarbiturate (Inactin; BYK Gulden, Hamburg, Germany), 100 mg/kg, by intraperitoneal injection. Tracheostomy was performed using PE-240 tubing, followed by catheterization of the right femoral artery with PE-50 tubing. Mean arterial pressure (MAP) was monitored for 15 minutes using a computerized system (MacLab; Analog Digital Instruments, Dunedin, New Zealand). Laparotomy was then performed, and the kidneys were perfused in situ through the aorta with cold isotonic heparinized saline until blanched (50 to 60 mL saline over 2 minutes). Both kidneys were harvested under sterile conditions to obtain protein for Western blotting and enzyme-linked immunosorbent assay (ELISA) and total RNA for RNase protection assay. Some kidney tissue was also used for the isolation of glomeruli and tubule segments, as described later in this article. In some experiments, the right kidney was harvested and frozen for immunofluorescence studies. Kidney tissue was also placed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Sections 5 μm in thickness were stained in routine fashion (hematoxylin and eosin and periodic acid-Schiff).

Proliferating cell nuclear antigen staining and semiquantitative analysis

Paraffin-embedded sections were deparaffinized by immersion twice into xylene for five minutes each, followed by immersion twice for three minutes each in 100% ethanol and then 95% ethanol. Slides were rinsed for 30 seconds using deionized water and then immersed twice in deionized water for five minutes. Slides were covered in 0.1% H_2O_2 for five minutes at room temperature and then were incubated in 50 μmol/L Tris-HCl, pH 7.2, containing 10% goat serum and a mouse monoclonal antibody directed against proliferating cell nuclear antigen (PCNA; Dako Corporation, Carpinteria, CA, USA), 1:1600 dilution, for 30 minutes at room temperature. Slides were rinsed and covered with peroxidase-labeled polymer conjugated to goat anti-mouse IgG (Dako Envision System; Dako Corporation) containing 10% rat serum for 30 minutes at room temperature, and color was developed using 3,3′-diaminobenzidine (DAB) chromogen solution (Dako). Cells were counterstained using hematoxylin, and the slides were mounted in standard fashion. As a negative control, the primary antibody was omitted from the reaction.

To semiquantitate interstitial cellular proliferation, cells with nuclear staining for PCNA were counted manually in six fields observed at ×20 magnification; counts from the six views were then averaged to produce an interstitial proliferation index for each kidney. To determine the number of proliferating cells in the glomerular compartment, PCNA-positive nuclei were counted in 25 glomeruli. Four rats were examined in each group.

In situ detection of DNA fragmentation using TUNEL

In situ detection of DNA fragmentation was performed on tissue sections of SS and SD rats by incorporation of fluorescein-12-dUTP at the 3′-OH ends of DNA using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL assay; Apoptosis Detection System, Fluorescein; Promega, Madison, WI, USA). Frozen sections, 5 μm in thickness, were cut using a cryostat, mounted on poly L-lysine–coated slides, and fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in phosphate buffer for 30 minutes at 4°C. The slides were washed twice by immersion into fresh PBS for five minutes at room temperature and then permeabilized in 0.2% Triton X-100 (Sigma Chemical Co.) in PBS for five minutes on ice and rinsed in PBS for five minutes at room temperature. Following preequilibration in 100 μL of buffer containing 200 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl, 0.2 mmol/L dithiothreitol (DTT), 0.25 mg/mL bovine serum albumin (BSA), and 2.5 mmol/L cobalt chloride, strands of DNA were end labeled by incubation at 37°C for one hour in 50 μmol/L fluorescein-12-dUTP, 100 μmol/L dATP, 10 mmol/L Tris-HCl (pH 7.6), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and TdT; the reaction was stopped by immersing the slides in 2 × sodium chloride/sodium citrate hybridization solution for 15 minutes at room temperature. The slides were then stained by immersion in propidium iodide (Sigma Chemical Co.), 1 μg/mL in PBS, for 15 minutes in the dark. After washing, the samples were mounted and examined and photographed at ×40 magnification using a fluorescence microscope (Leica, Heidelberg, Germany) equipped with a digital camera (Model C5810; Hamamatsu Photonics KK, Hamamatsu City, Japan). For fluorescein, the excitation and barrier filters were set at 450 to 490 and 515