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Increased expression of apoptosis-associated proteins in puromycin aminonucleoside nephrosis.

Ninoska T. Viera[1], Adriana Pedreáñez[2], Maritza J. Romero[3], Jaimar C. Rincón[3] y Jesús A. Mosquera[3]

[1]Instituto de Investigaciones Odontológicas, Facultad de Odontología.
[2]Cátedra de Inmunología, Escuela de Bioanálisis e
[3]Instituto de Investigaciones Clínicas "Dr. Américo Negrette".
Facultad de Medicina, Universidad del Zulia. Maracaibo, Venezuela.
E mail: mosquera99ve@yahoo.com

Key words: Fas, Fas-L, p53, Bax, Bcl-2, puromycin, nephrosis.

Abstract. Increased apoptosis has been reported in acute puromycin aminonucleoside nephrosis (PAN). The aim of this study was to investigate if increased apoptosis is related to increased expression of apoptosis-associated proteins (AAP) in this model of nephrosis. Sprague-Dawley rats were made nephrotic by intraperitoneal injection of one dose of puromycin aminonucleoside. Renal tissues were obtained at 1, 2 and 7 weeks after injection and apoptosis was investigated by TUNEL and by electron microscopy. Fas, Fas ligand, p53, Bax and Bcl-2 expressions were analyzed by the respective monoclonal and polyclonal antibodies, using indirect immunofluorescence. In the glomerulus of nephrotic animals, increased apoptosis was accompanied with increased expression of p53, Fas and Bax. In the interstitium, high expression of apoptosis, Fas, Fas-L and Bax were observed and in tubules increased apoptosis was accompanied with increased expression of p53, Fas and Fas-L. Bcl-2 was increased in interstitium and tubules during PAN. The incidence of apoptosis during PAN was correlated with the expression of AAP in glomerulus (p53), interstitium (Fas, Fas-L and Bax) and tubules (Fas, Fas-L, p53 and Bcl-2). There was correlation between Fas and Fas-L expression in interstitium and tubules. About 4% of glomerular and 25% of tubular p53 positive cells were apoptotic cells. The data suggest that increased local expression of AAP could contribute to renal apoptosis in the glomerular, interstitial and tubular compartments during this experimental model of nephrosis.

Corresponding author: Jesús A Mosquera. Instituto de Investigaciones Clínicas, Facultad de Medicina, Universidad del Zulia. Apartado Postal 23, Maracaibo 4001-A, Zulia, Venezuela. Telephone number 58-261-7597247, Fax number: 58-261-7597247. E mail: mosquera99ve@yahoo.com

Aumento en la expresión de las proteínas asociadas
a la apoptosis en la nefrosis por aminonucleósido de puromicina.
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Palabras clave: Fas, Fas-L, Bax, Bcl-2, puromicina, nefrosis.

Resumen. Estudios previos han demostrado la presencia de apoptosis en el tejido renal de ratas con nefrosis por aminonucleósido de puromicina (NAP). Este estudio está orientado a determinar si la expresión de la apoptosis está relacionada con aumento en la expresión de proteínas asociadas a la apoptosis (PAP) durante el curso de NAP. Se utilizaron ratas Sprague. Dawley las cuales fueron hechas nefróticas con una inyección única intraperitoneal de aminonucleósido de puromicina. Los controles fueron ratas inyectadas sólo con el vehículo. Se obtuvieron tejidos renales a las 1, 2 y 7 semanas después de la inyección y se analizó la apoptosis por TUNEL y microscopia electrónica y Fas, Fas-L, p53, Bax y Bcl-2 mediante la inmunofluorescencia indirecta, usando anticuerpos policlónales y monoclonales. Se encontró incremento en la apoptosis en el glomérulo de los animales con NAP, acompañado con incremento en la expresión de p53, Fas y Bax. En el intersticio se incrementaron la apoptosis y la expresión de Fas, Fas-L y Bax y en los túbulos el aumento de la apoptosis se acompañó de aumento de p53, Fas, Fas-L. Bcl-2 se incrementó en intersticio y túbulos. La incidencia de apoptosis en este modelo estuvo correlacionada con la expresión de PAP en glomérulo (p53), intersticio (Fas, Fas-L y Bax) y en túbulos (Fas, Fas-L, p53 y Bcl-2). Hubo correlación entre las expresiones de Fas y Fas-L en intersticio y túbulos. Cerca del 4% en el glomérulo y el 25% en túbulos de las células p53 positivas estaban en apoptosis. Estos datos sugieren que una expresión aumentada de las PAP en glomérulo, intersticio y túbulo puede estar relacionada con el incremento de la apoptosis en los diferentes compartimientos renales durante este modelo experimental.

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INTRODUCTION

Apoptosis, one type of cell death, is characterized by morphological and biochemical distinct features (1). It is known to occur during the normal development of multicellular organisms. Apoptosis or its defect is also known to cause a variety of pathological conditions (2). Fas, Fas ligand, Bax and p53 may be expressed in cells undergoing apoptosis; in contrast, some other molecules are known to decrease levels of apoptosis. Regarding the latter, the anti-apoptotic effect of Bcl-2 is well known (3). Acute puromycin aminonucleoside nephrosis (PAN) in rats is characterized by heavy proteinuria associated with renal hypercellularity and it has served as an experimental model of minimal lesion nephrotic syndrome in humans (4-6). Increased occurrence of apoptosis has been documented in weeks 1 and 2 of the disease and a role in the resolution and restoration of normal renal cellularity by apoptosis has been suggested (7). The present study was designed to determine whether the inci-

dence of apoptosis is accompanied and correlated with the expression of apoptosis associated proteins (AAP) Fas, Fas ligand, Bax, p53 and Bcl-2, during the course of PAN.

METHODS

Animals

Male Sprague-Dawley rats, weighing 250 to 300g and obtained from the Instituto Venezolano de Investigaciones Científicas (Venezuela) were used for this study. They had free access to food and water throughout the experiments.

Reagents

Apoptosis was evaluated using an apoptosis detection kit. utilizing fluorescein (FITC)-labeled nucleotides (Promega Corporation, WI, USA). Monoclonal antibodies (mAb), anti- rat p53 tumor suppressor protein, anti- rat Bcl-2 protein and anti- rat Bax protein were purchased from Biosource International Inc. (Camarillo, CA, USA). FITC-conjugated $F(ab')_2$ fragment rat anti-mouse IgG and TRITC (tetramethylrhodamine isothiocyanate) -labeled $F(ab')_2$ fragment rabbit anti-mouse IgG antibodies were obtained from Accurate Chemical & Scientific Corporation (Westbury, NY, USA). Rabbit anti- rat Fas, rabbit anti-rat Fas-L and FITC-labeled $F(ab')_2$ fragment sheep anti-rabbit IgG antibodies were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Puromycin aminonucleoside was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Puromycin aminonucleoside nephrosis model

Rats were made nephrotic by a single intraperitoneal injection of 15 mg/100 g body weight of puromycin aminonucleoside (PA) dissolved in 0.9% saline. Animals injected with 0.9% saline were used as controls. Experimental and control rat groups (n = 5 to 6 per group) were sacrificed on weeks 1, 2 and 7 after the PA injection. In order to determine proteinuria, 24 hour urine samples were collected from all rats before the sacrifice and proteinuria was determined by the sulphosalicilic acid method. At the time of sacrifice, kidneys were removed after perfusion with 20 mL of 0.9% saline solution through the abdominal aorta. For immunofluorescence studies, pieces of perfused kidneys were included in OCT compound (Tissue Tek, Miles Inc. Diagnostics Division, Kankakee, IL, USA), frozen in dry ice and acetone and stored at –70°C until use. For light and electron microscopy studies pieces of renal cortex from control and experimental rats were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer.

Identification of apoptosis

To determine apoptosis a histologic assay that allows for selective labeling of cells with degraded DNA in tissue sections was utilized (8). This assay takes advantage of the ability of Klenow DNA polymerase to fill gaps of degraded DNA in apoptotic cell nuclei with FITC modified-nucleotides, easily detected by epifluorescence microscope. The frozen sections (4 µm) from normal and nephrotic animals were fixed in 10% neutral-buffered formalin and post-fixed in ethanol:acetic acid for 5 minutes at –20°C. Fragmented DNA was nick end-labeled using an in situ apoptosis detection kit following the indications of the manufacturer. The embryonic rat forelimb bud was used as a positive control to detect developmental cell deletion. Negative controls were represented by renal and embryonic rat forelimb bud tissues incubated with distilled water instead of the terminal deoxynucleotidyl transferase in the reaction buffer. Slides were mounted with p-phenylenediamine to delay fluorescence quenching and examined

with an epifluorescence microscope. Apoptotic morphological features were also confirmed by light and electron microscopy procedures (see below).

Determination of apoptosis-associated protein expression in PAN

Renal frozen sections (4 µm) from control and nephrotic animals were divided into two groups: One group was fixed in acetone at –20ºC for 15 minutes, washed and incubated with rabbit anti-rat Fas or rabbit anti-rat Fas-L for 1 hour at room temperature. The other group was fixed in 10% of neutral buffered formalin for 15 minutes and treated with anti-rat p53 mAb for 24 hours at 4ºC or with anti-rat Bax or anti-rat Bcl-2 mAbs, for 1 hour at room temperature. Monoclonal antibodies were used at a concentration of 5 µg/mL. Indirect immunofluorescence was performed using an FITC-conjugated $F(ab')_2$ fragment rat anti-mouse IgG for 1 hour at room temperature to determine the presence of monoclonal antibodies and an FITC- conjugated $F(ab')_2$ fragment goat anti-rabbit IgG to determine rabbit antibodies on the renal tissue. Negative controls were represented by tissues obtained under the same conditions but incubated with antibodies against nonrelevant proteins. Sections were mounted in a solution of p-phenylenediamine in PBS-glycerol and observed in a fluorescence microscope (Axioskop, Zeiss, Germany).

Light and electron microscopy studies

For electron microscopy, renal pieces were fixed for 5 hours at room temperature in 2.5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (pH 7.2), rinsed in cacodylate buffer, postfixed for 1 hour in 2% OsO4, dehydrated in a graded ethanol series and embedded in EPON 812 (Fluka Chemie, Buchs, Switzerland). Thin sections were cut with a diamond knife, collected on copper grids and double stained with uranyl acetate and lead citrate before examination in a Jeol 1010 electron microscope (Tokyo, Japan). Semithin sections on glass slides were stained with toluidine blue for light microscopy studies.

Double staining for apoptosis/p53

Renal frozen sections (4 µm) from 1 and 2 weeks PAN rats were fixed in 10% neutral-buffered formalin, post-fixed in ethanol-acetic acid and treated as described in the apoptosis identification section. Thereafter, sections were incubated with a monoclonal antibody against p53 for 24 hours at 4°C. Tissues were washed and incubated with TRITC-labeled rabbit anti-mouse IgG antibody as described above. Renal sections were mounted in a solution of p-phenylenediamine in PBS-glycerol and observed under a fluorescence microscope.

Calculations and statistical analysis

Positive cells for TUNEL, Fas, Fas-L, Bcl2, Bax and p53 were counted in at least 20 glomerular cross sections per renal tissue and in their respective periglomerular areas. Positive interstitial and tubular cells were counted in at least 20 tubulointerstitial areas of $0.0625 mm^2$ in each renal sample, except for tubular Fas and Fas-L, when the number of positive tubular cross section per $0.0625 mm^2$ was counted. These observations were performed with the help of an ocular piece fitted with a grid. Results in the groups are shown as mean ± SD. Multiple comparisons were done using the analysis of variance (ANOVA) test, followed by Dunnet's post-test. For correlation between two variables, the Pearson's correlation was used. Two tailed $p < 0.05$ was considered statistically significant.

RESULTS

Renal apoptosis and apoptosis-associated protein expression in PAN

Basal levels of apoptosis expression were found in controls. In the glomerulus of nephrotic animals, increased apoptosis was observed at weeks 1 and 2, accompanied with increased expression of p53 (week 1), Fas and Bax (week 2) (Figs. 1A and 2A, Table I). Apoptosis was also increased in the periglomerular area at weeks 1 and 2, but only p53 and Bax were found increased at week 2 (Table I and Fig. 2B). In the interstitium, a significant increase of apoptotic nuclei and expression of Fas, Fas-L and Bax were observed at week 2 (Table I and Figs. 1B and 2C). The number of apoptotic tubular cells was found increased at weeks 1 and 2 of nephrosis, accompanied with increased expression of p53; in addition, Fas and Fas-L tubular expressions were found increased at week 2 (Table I and Figs. 1B and 2D). Bcl-2 was found increased in interstitium and tubules at weeks 1 and 2, respectively (Fig. 2C and 2D). Apoptosis and AAP expression values returned to the normal levels by week 7 of nephrosis.

Renal apoptosis was also observed by electron microscopy. Glomerular apoptosis was observed mainly in endothelial and epithelial cells. Fig. 1C shows a glomerular en-

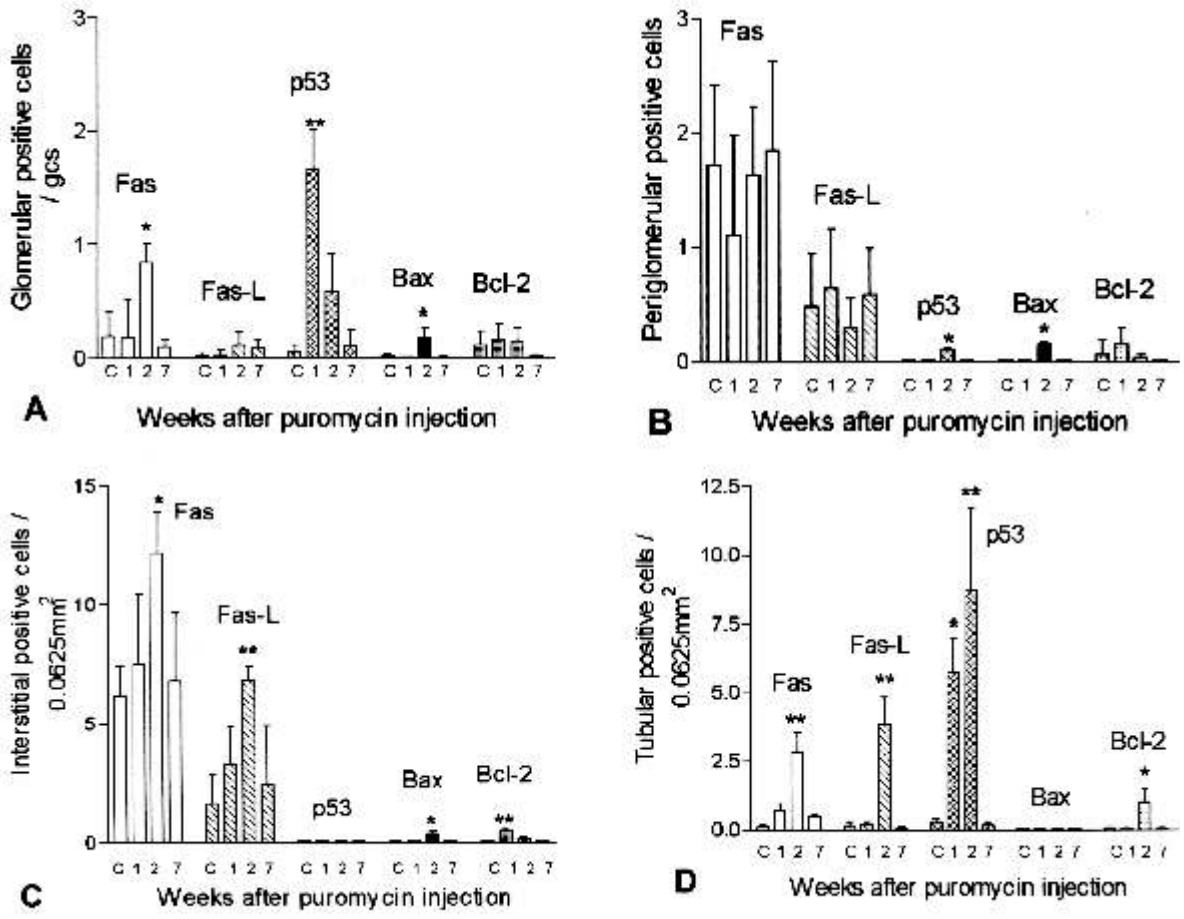

Fig. 1. Apoptosis-associated protein expression in the different renal compartments. In D, values from tubular Fas and Fas-L expressions represent number of positive tubular cross sections. C: Controls. 1, 2 and 7 represent weeks after puromycin injection. * $p < 0.05$; ** $p < 0.01$.

TABLE I
APOPTOSIS IN PUROMYCIN AMINONUCLEOSIDE NEPHROSIS

Area	Control	PAN 1	PAN 2	PAN 7
Glomerular	0.09 ± 0.12	0.48 ± 0.15*	0.27 ± 0.07**	0.09 ± 0.03
Periglomerular	0.07 ± 0.05	0.43 ± 0.18*	0.28 ± 0.14*	0.11 ± 0.07
Interstitial	0.51 ± 0.27	1.27 ± 0.17	4.86 ± 1.45*	0.77 ± 0.20
Tubular	2.42 ± 1.95	5.38 ± 1.61**	11.7 ± 2.88*	0.88 ± 0.41

* $p < 0.01$. ** $p < 0.05$.

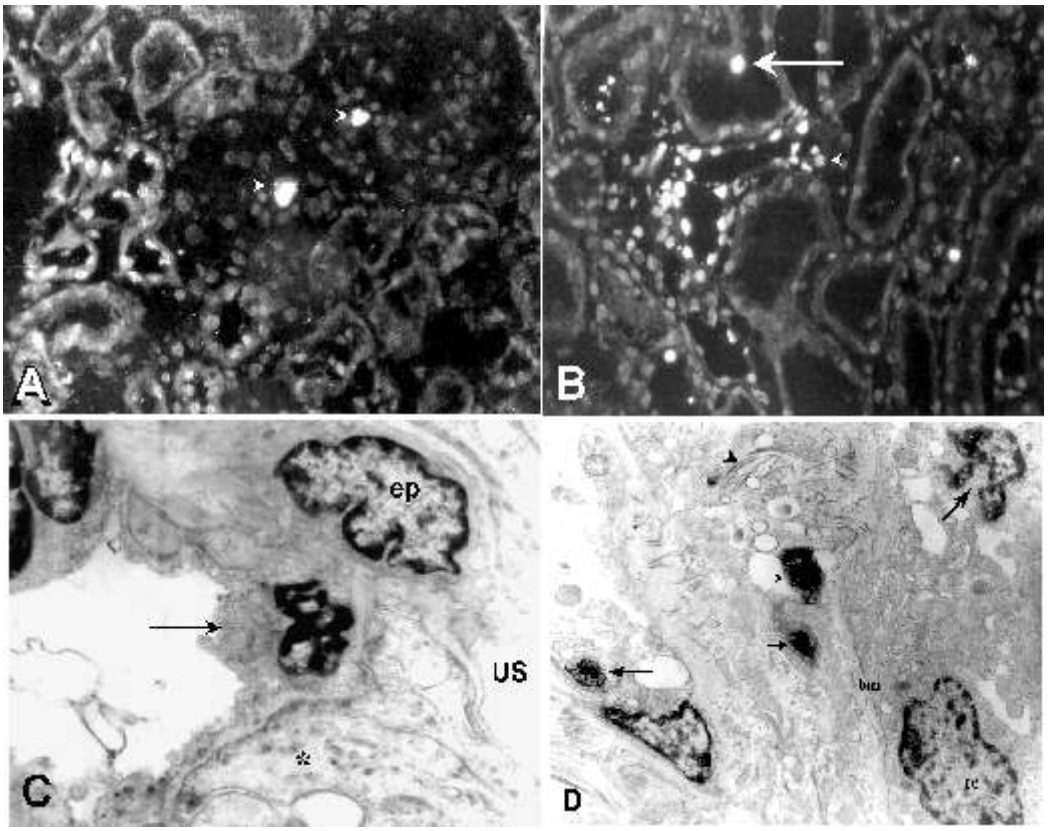

Fig. 2. Immunofluorescence localization of apoptosis in glomerulus (A: arrows), interstitium (B: small arrow) and tubule (B: thick arrow) by TUNEL. Morphological evidence of apoptosis was also observed by electron microscopy. (C) Apoptotic endothelial cell (arrow) in a nephrotic rat. Note the extensive fusion of foot processes (asterisk). ep: epithelial cell; US: urinary space. X 15,000. (D) Interstitial (arrows) and tubular (thick arrow) apoptotic cells. Collagen fibers (arrowhead). tc: normal tubular cell; bm: tubular basement membrane. X 10,000.

dothelial cell with apoptotic morphology. Apoptotic cells were also observed in the different tubular segments, as well as in the interstitium (Fig. 1D). Alterations of glomerular epithelial cells, as loss of foot processes were observed in nephrotic animals (Fig. 1C). Superficial, cytoplasmic and nuclear patterns of immunofluorescence were observed when AAP expression was studied (Fig. 3).

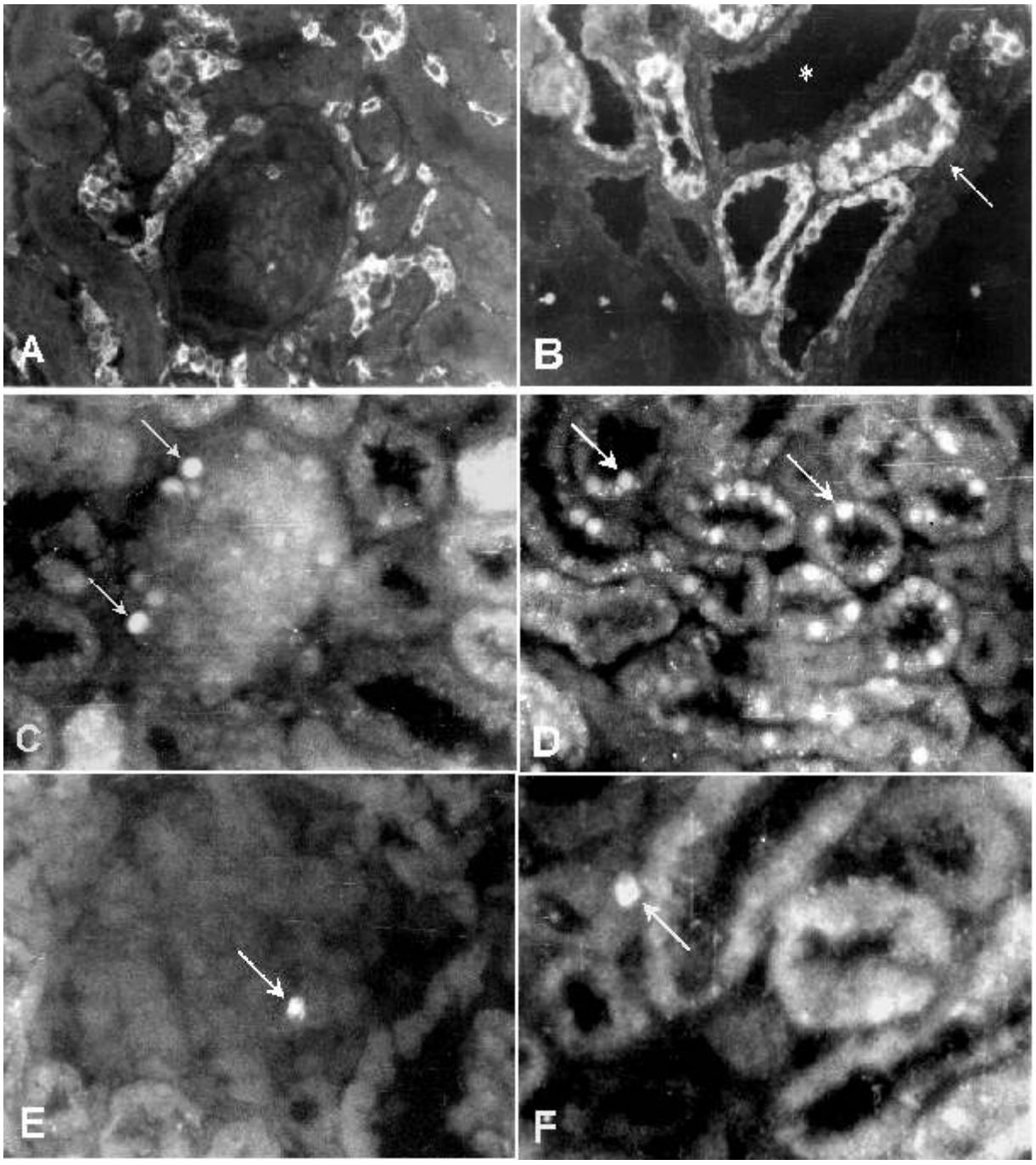

Fig. 3. Immunofluorescence of renal pro-apoptotic proteins in PAN. (A) Interstitial Fas-positive cells in a nephrotic rat. (B) Increased expression of Fas positive tubules was observed in nephrotic rats (arrow), note negative tubules (asterisk) beside positive tubules. Increased p53 positive nuclei were observed in glomeruli (C: arrows) and in tubules (D: arrows). Bax-positive cells were observed in glomeruli (E: arrow) and interstitium (F: arrow).

Proteinuria

All animals injected with PA showed a significant increment of proteinuria at weeks 1 and 2 of nephrosis, returning to normal values by week 7 (control: 3.56 ± 2.54; PAN 1 week: 192 ± 81.52 ; PAN 2 week: 201.30 ± 57.18; PAN 7 week: 3.79 ± 2.08 mg/24 hours).

Correlation studies

The expression of apoptosis in the kidney during PAN was correlated with pro-apoptotic protein expressions. Glomerular apoptosis was correlated with p53 expression ($p = 0.0004$). In the interstitium, apoptosis was correlated with the expression of Fas ($p < 0.01$), Fas-L ($p < 0.0001$) and Bax ($p < 0.0002$). Tubular apoptosis was correlated with Fas ($p < 0.0001$), Fas-L ($p < 0.0001$) and p53 ($p = 0.0004$) expressions (Table II). Tubular Bcl-2 expression was also correlated with apoptosis ($r = 0.5542$; $p < 0.003$). There was correlation between Fas and Fas-L expressions in the periglomerular area ($p = 0.0003$), interstitium ($p < 0.01$) and tubules ($p < 0.0001$) (Table III). There was not correlation between Bax and Bcl-2 expressions or between Bax and p53 expressions. Apoptosis in the different renal compartments was correlated with intensity of proteinuria (glomerular: $p < 0.0001$; periglomerular: $p < 0.0001$; interstitial: $p = 0.001$ and tubular: $p = 0.001$). In this regard, Fas tubular ($p = 0.0004$), Fas-L interstitial ($p = 0.003$) and Fas-L tubular ($p = 0.002$) expressions were correlated with proteinuria. Glomerular ($p = 0.002$) and tubular ($p = 0.0006$) expressions were also correlated with proteinuria. There was

TABLE II
CORRELATION BETWEEN APOPTOSIS AND PRO-APOPTOTIC PROTEIN EXPRESSION IN ACUTE PAN

Protein expression	Area	Pearson r	P values
Fas	Glom	0.0591	NS
Fas-L	Glom	0.0468	NS
p53	Glom	0.6394	0.0004
Bax	Glom	0.0223	NS
Fas	Per	–0.0536	NS
Fas-L	Per	0.2449	NS
p53	Per	0.1371	NS
Bax	Per	–0.0118	NS
Fas	Int	0.4846	< 0.01
Fas-L	Int	0.6959	< 0.0001
p53	Int	0.0929	NS
Bax	Int	0.6593	< 0.0002
Fas	Tub	0.6967	< 0.0001
Fas-L	Tub	0.7547	< 0.0001
p53	Tub	0.6427	0.0004

Glom: Glomerular. Per: periglomerular. Int: interstitial. Tub: tubular.

TABLE III
CORRELATION BETWEEN FAS EXPRESSION AND FAS-L EXPRESSION IN ACUTE PAN

Area	Pearson r	P values
Glomerular	0.1333	NS
Periglomerular	0.6494	0.0003
Interstitial	0.4628	< 0.01
Tubular	0.7055	< 0.0001

not correlation between Bax expression and proteinuria (Table IV). In general, the expression of apoptosis in the different compartments was accompanied and correlated with apoptosis-associated proteins and proteinuria.

Double staining studies

To determine the number of p53 positive cells undergoing apoptosis a double staining for TUNEL and p53 was performed.

TABLE IV
CORRELATION BETWEEN APOPTOSIS AND APOPTOSIS-ASSOCIATED PROTEINS WITH PROTEINURIA

Tissue expression	Pearson r	P values
Apoptosis		
Glomerular	0.7768	< 0.0001
Periglomerular	0.7891	< 0.0001
Interstitial	0.6065	0.001
Tubular	0.6053	0.001
Fas		
Glomerular	0.4547	NS
Periglomerular	-0.1818	NS
Interstitial	0.2579	NS
Tubular	0.6418	0.0004
Fas-L		
Glomerular	0.0798	NS
Periglomerular	-0.0312	NS
Interstitial	0.5481	0.003
Tubular	0.5617	0.002
p53		
Glomerular	0.5599	0.002
Periglomerular	0.3251	NS
Interstitial	0.2435	NS
Tubular	0.6248	0.0006
Bax		
Glomerular	0.1072	NS
Periglomerular	0.0447	NS
Interstitial	0.1533	NS
Bcl-2		
Glomerular	0.0856	NS
Periglomerular	0.2034	NS
Interstitial	0.3951	0.04
Tubular	0.1520	NS

About 4% of glomerular p53 positive cells (week 1) and 25% of tubular p53 positive cells (weeks 1 and 2) were apoptotic cells (Table V and Fig. 4).

DISCUSSION

Increased expression of apoptosis and its possible participation in the resolution of infiltrative and proliferative events during acute PAN in rats have been reported (7). In this study, we showed that the incidence of apoptosis in glomerulus, interstitium and tubules was accompanied and correlated with increased expression of apoptosis promoting (Fas, Fas-L, p53 and Bax) and blocking (Bcl-2) proteins. The Fas death receptor is a member of the tumor necrosis factor family and may be expressed by renal tubular cells, interstitial fibroblasts and infiltrating leukocytes, all of which may also express Fas ligand. Binding of Fas ligand to cell surface Fas leads to activation of the caspase protease cascade with subsequent cleavage of numerous intracellular proteins resulting in apoptosis (9-11). A body of experimental data indicates a role for Fas/FasL system in tubulointerstitial disease (12). The parallel expression of apoptosis and Fas or Fas-L in the glomerular, interstitial and tubular compartments during PAN suggests that the Fas/FasL system could be involved in the apoptotic events in PAN. The high correlation between apoptosis and the expression of Fas/FasL system in interstitium and tubules suggests that proposal. Fas and FasL could be co-expressed during PAN as suggested by the correlation between both protein expressions in the tubulointerstitial area and can involve interstitial leukocytes and myofibroblasts (13).

The parallel changes between proliferation detected by the expression of the proliferating cell nuclear antigen (PCNA) and those related to apoptosis are intriguing. The proliferation stimulus can lead to the production of death signals that make cells more sensitive to undergo apoptosis (14, 15). Apoptosis can be accompanied by the expression of early cell-cycle genes followed by the transition of the cell to the G1/S border when it is expressed PCNA (16). One of the most important links between the proliferation and cell death machinery is the tumor suppressor p53 protein, which promotes cell cycle arrest or apoptosis in response to DNA damage or a strong stimu-

TABLE V
GLOMERULAR AND TUBULAR DOUBLE STAINING FOR APOPTOSIS AND P53 IN ACUTE PAN

Animal	Week 1		Animal	Week 2	
	Glom	Tub		Glom	Tub
R1	0	36.36	R7	0	19.40
R2	10.53	31.80	R8	0	30
R3	0	25	R9	0	35
R4	15.4	18.50	R10	0	20
R5	0	24.14	R11	0	28.2
R6	0	16.67			
Total	4.36 ± 6.27	25.41 ± 6.91		0	26.52 ± 6.0

Data represent percentage of p53 positive cells also positive for TUNEL staining.
Glom: glomerulus. Tub: tubule.

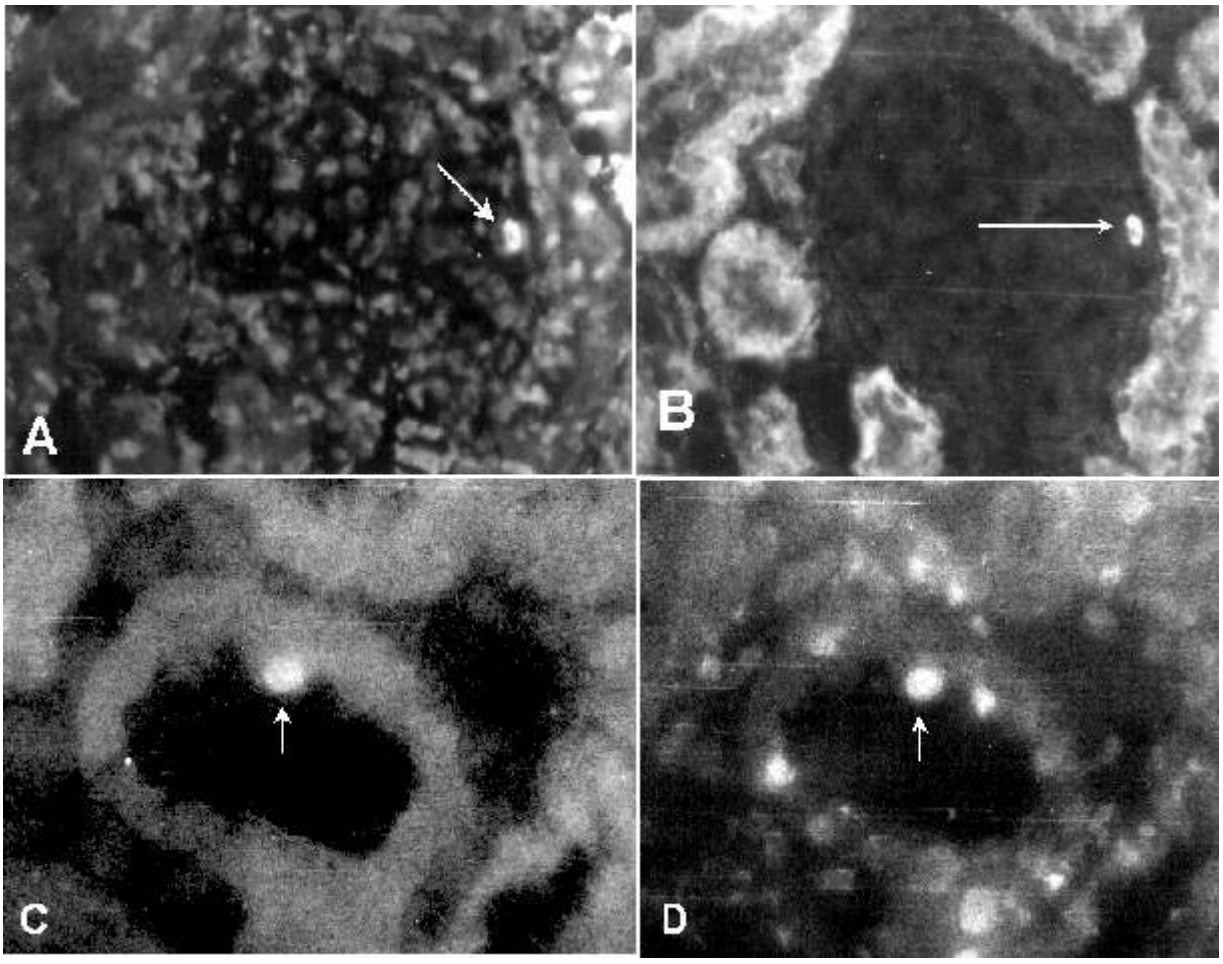

Fig. 4. Double stained frozen sections from a nephrotic rat kidney for TUNEL (A and C) and p53 (B and D). A glomerular TUNEL positive cell in A (arrow) corresponds with a p53 positive cell in B (arrow). In C, a tubular TUNEL positive cell (arrow) correspond with a p53 positive cells in D (arrow). Renal tissues in A and C were treated with FITC-labeled nucleotides and tissues in B and D with a TRITC-labeled antibody against mouse IgG.

lus to proliferate (17, 18). In this study, increased incidence of apoptosis in glomerulus, periglomerular area and tubule was accompanied with increased expression of p53. Both events were highly correlated in glomerular and tubular compartments and double staining studies address the possibility that apoptosis and p53 protein can be co-expressed. These data suggest that proliferating cells during PAN could overexpress p53 leading to apoptosis. In this regard, we previously showed that proliferation and apoptosis may be a common cellular event in PAN, supported by the observation that cells expressing PCNA were also positive for TUNEL staining (7). In addition, increased renal apoptosis correlated with increased proliferative activity (PCNA+ cells), has been reported in several experimental and human renal pathologies (19-21). Since p53 holds a damaged cell in G1 while the damage is repaired and cells which try to oppose the G1 block may end up by activating the suicide pathway (3),

the relative low percentage of TUNEL/p53 positive cells (about 4% in glomeruli and 25% in tubules) suggests that a high percentage of cells after the induction of p53 accumulation, repaired the DNA damage and escaped from apoptosis during the course of PAN. p53 also functions as a transcription factor for Bax and in some cells Bax can be expressed in a p53-dependent manner (22, 23). We could not find co-expression or correlation in the expression of both proteins during this study, suggesting that expression of Bax was not p53-dependent.

Bcl-2 was the first gene shown to be specifically involved in the process of apoptosis. It can inhibit apoptosis of many cells triggered by diverse etiology. Bax, having a structural similarity to Bcl-2, is able to antagonize the protection offered by Bcl-2 (24). The balance of Bcl-2/Bax expression may be critical in the course of human glomerular disease (25). Since there was not co-expression or correlation between Bcl-2 and Bax during this study, the Bax antagonic effect on Bcl-2 could not be present. As an unexpected finding, Bcl-2 was highly correlated with tubular apoptosis ($r = 0.5542$; $p < 0.003$). The expression of this anti-apoptotic protein in tubules could indicate that renal cells exposed to apoptotic stimuli during PAN may over express Bcl-2 to prevent from entering to apoptosis. A similar correlation has been reported in the glomerulus during the course of an experimental model of focal segmental glomerulosclerosis by puromycin (26). Bcl-2 has also been shown to prevent cells from apoptosis induced by reactive oxygen species apparently by an antioxidative mechanism (27). The up regulation of Bcl-2 in this study could be protective during the generation of reactive oxygen species reported in PAN (28-30).

It is important to note that the expression of apoptosis and AAP varied in the different renal compartments during this study. We have no clear explanation for this finding, but the nature of the renal injury could be involved. In the glomerulus, the toxic effect of PA on epithelial cells could induce typical morphological alterations and apoptosis (7, 26, 31-35). Our findings agree with those observations, since ultrastructural features of apoptosis and vacuolization, loss of foot processes and podocyte effacement of epithelial cells were observed in the glomerulus. In addition to the PA effect, glomerular apoptosis can be induced by cytokines, granenzymes and radical oxygen species from leukocyte infiltration during PAN (5, 29, 30, 32). In the interstitium, cellular infiltration can be involved in the apoptotic events. Previous studies have shown an increased number of T-cytotoxic/suppressor cells, natural killer cells and monocyte/macrophages in rats during the course of PAN (35, 36). In the tubular compartment, the interaction between interstitial and tubular cells could be a source of apoptotic stimuli for both types of cells (35-37). Alternatively, damage to tubular epithelial cells by filtered proteins may represent another apoptotic stimulus. Native serum albumin with its lipidic components elicited apoptosis of tubular epithelial cells (38, 39). In this regard, increased activity of two cysteine proteases in the proximal tubules in rats with PAN has been reported (40). In addition to apoptotic stimuli, glomerular, interstitial and tubular compartments have different types of cells and the response to apoptotic stimulation could be also mediated by cellular type and cellular phenotype (proliferation) (14, 15).

Apoptosis and AAP expression values returned to the normal levels by week 7 of nephrosis. In the glomerular recovery could be involved the proliferation of podocytes in order to reestablish the normal number of cells after the apoptotic changes. Detachment of degenerated podocytes from the

outside of the glomerular basement membrane is the first step, thereafter, podocytes in glomeruli of nephrotic rats can proliferate (41). Proliferation could have an important role in the recovery since insufficient proliferation in podocytes may be involved in the progression of focal segmental glomerulosclerosis (26). Glomerular PCNA positive cells have been reported during PAN (7) and they have been related to epithelial cell proliferation (42). Recovery from the change in podocyte configuration begins with the formation of new foot processes (43) and cytoskeletal proteins very likely play a central role in these changes since they are primarily responsible for the maintenance of cell structure in almost all cells (44). In this regard, membrane-bound form microtubule-associated protein 1 light chain 3 increases in podocytes during the differentiation and recovery from puromycin aminonucleoside-induced nephrosis (45).

Our study demonstrates that increased apoptosis in PAN is in close association with the up-regulation of the cell death-promoting proteins Fas, Fas ligand, p53 and Bax. The increased expression of Bcl-2 could be a protection against apoptotic stimuli during PAN. Further investigations are required to determine the apoptotic stimuli capable of inducing the expression of apoptosis and apoptosis regulatory genes in the different renal compartments.

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