Journal of Biological Research **6**: 131 – 138, 2006
J. Biol. Res. is available online at http://www.jbr.gr

Renal cell turnover results in a fine balance between apoptosis and cell proliferation

JUANJOSÉ BOLLAIN-Y-GOYTIA[1], ESTEBAN MEZA-LAMAS[1], ADRIÁN LÓPEZ[1], ESPERANZA AVALOS-DÍAZ[1], CRISTINA RODRÍGUEZ-PADILLA[2] and RAFAEL HERRERA-ESPARZA[1*]

[1] *Department of Molecular Biology, Institute for Experimental Biology, Universidad Autónoma de Zacatecas, Guadalupe, Zacatecas, México*
[2] *Department of Immunology, School of Biological Sciences, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, México*

Received: 2 January 2006 Accepted after revision: 27 June 2006

Renal cell turnover (RCT) results in a fine balance between apoptosis and cell proliferation. RCT starts early in developing mammalian kidneys and after birth it supports the structure and function of the kidney. After renal inflammation, the role of RTC is critical in repair. Current studies have been addressed to whether apoptosis participates in renal cell turnover at different stages of renal life. Kidneys were obtained from a group of newborn and adult normal Balb/c mice. Tissues were preserved in RNAlater, and sections were obtained and processed with the TUNEL method. The protein expression of fas, fasL and pcna was studied by immunofluorescence. The expression of the fas, fasL, caspase 3, dff40, pcna and g3pdh genes was determined by RT-PCR. Results showed a low apoptotic rate in kidneys of newborn mice. However, this rate progressively increased by elderly. On the opposite, a high cell proliferation and transcription of pcna was observed in newborn mice which declined by elderly. Our study demonstrated that apoptosis is a normal process in the kidney. Apoptosis is increased by elderly and is concurrent with a decline of cell proliferation. Both mechanisms contribute to maintain renal cell turnover. This work attempts to provide a rational of the renal development which can be applied for better comprehension of the molecular mechanisms on renal pathology and novel designs of therapeutic interventions.

Key words: apoptosis, cell proliferation, renal cell turnover, TUNEL, fas/fasL, caspase 3.

INTRODUCTION

The cell induction of metanephric mesenchyma transforms the neighbour ureteric bud into a differentiated epithelium which produces the nephron. The collecting system is formed later by the ureter branching under the signals of the MAP kinase pathway (Lechner & Dressler, 1997; Fisher *et al*., 2001).

Apoptosis normally occurs in developing vertebrate organs. In the mammalian kidney, less than 3% of the nephrogenic region and the medullar papilla exhibit an apoptotic morphology. Death cells become cleared rapidly by phagocytosis executed by the neighbouring parenchyma (Harriet *et al*., 1993). After birth, apoptosis of kidney cells is low and contributes to maintain the structure and function of the renal tissue. After renal injury, its role is critical in healing.

Studies on newborn animals and human infants have shown that the renal cell proliferation activity decreases after birth; in preterm neonates cell proliferation of the kidney continues 40 days after birth (Rodriguez *et al*., 2004). In the rat, the kidney is not fully developed until approximately 12 days after birth, and mechanisms involved in renal cell proliferation depend in part on the platelet-derived growth

* Corresponding author: fax: +52 492 922 6070, e-mail: herrerar@intranet.uaz.edu.mx, rafael.herreraesparza@gmail.com

factor (PDGF), that is a potent chemotactic and proliferation factor for mesenchyma-derived cells. PDGF plays a critical role in kidney development of neonatal mice, therefore the blockade of PDGFR-beta results in glomerular mesangial cell and/or pericyte apoptosis (Sano *et al*., 2002). Additional external factors such as sodium intake, modulate the expression of pcna and the renal cortex development (Balbi *et al*., 2004).

Because the issue of the renal cell turnover after birth is not fully defined, the present study explores the rate of apoptosis in different stages of life and addresses the relationship with cell proliferation. To clarify these important issues, the apoptotic rate was appraised in kidneys of normal Balb/c mice at different ages.

MATERIALS AND METHODS

Experimental animals

Balb/c mice were studied after birth, at the 7th and 52nd week of the extra-uterine life (n = 20 per group). Animal studies were approved by the ethics committee following the principles of laboratory animal care (NIH publication 86-23, revised 1985). Kidneys obtained by dissection were rinsed in phosphate buffer saline (PBS). The kidneys were transferred into a RNAlater' based buffer (Ambion Inc., Austin, TX) to avoid RNA degradation. They were then fixed with 10% formaldehyde, embedded in paraffin, cut in 4 mm sections and stained with hematoxylin and eosin. Tissue sections were also used for immunohistochemistry and TUNEL assays. Total RNA was extracted from tissue sections by TRIzol (Gibco BRL, Gaithersburg, MD) and the RNA was used for RT-PCR amplification.

TdT-mediated dUTP nick end labeling (TUNEL)

Tissue sections were de-waxed by immersion in xylene for 10 min. The nuclear stripping was performed on de-waxed tissue sections by incubation for 5 min in 10 mM Tris-HCl, pH 8.0. The slides were incubated for 15 min in 20 μg ml^{-1} proteinase K (dissolved in Tris-HCl buffer) and then washed in PBS. DNA fragment elongation was carried out by 60 min incubation at 37°C with 75 μl of reaction mixture (DDW, 10x TdT buffer: 30 mM Tris base, 140 mM sodium cacodylate, pH 7.2, 1 mM cobalt chloride, 1 mM DTT; 10% of the final volume) and fluorescein-11-dUTP (0.5 mg dissolved in 1 ml of 10 mM Tris-HCl, pH 7.0), and also TdT enzyme (0.3 enzyme units μl^{-1}). Termination of the reaction was performed with stop solution composed of 300 mM NaCl and 30 mM sodium citrate, pH 8.0 (Deng & Wu, 1983). Finally, the slides were washed in PBS and counterstained with 2% propidium iodide. To differentiate the true green tag of apoptotic cells from the background incorporation, cells were counterstained with 2% propidium iodide. By this procedure the non-apoptotic nuclei develop a red stain. The rate of apoptotic cells by TUNEL was the percent of cells exhibiting green tag in 100 fields. The total cellular amount resulted in the addition of red and green tagged cells.

Immunofluorescence

The expression of the fas, fasL and pcna was detected by incubation of the sections for 2 h in a 1:500 dilution of a fluorescein-labelled monoclonal anti-fasL and anti-fas (Research Diagnostics Inc, Flanders NJ) and anti-pcna antibodies (Santa Cruz Laboratories, Santa Cruz, CA). The slides were washed, mounted, and examined using a confocal scanning microscope LSM (Axiovert 200M, Carl Zeiss, Göttingen, Germany). Combination of fluorescein filters with excitations of 450-490 nm and rodamin filters with emissions of 515-565 nm were used. Objectives were LCI "Plan-Neofluar" and image processing was done with a Zeiss LSM Image examiner.

Oligonucleotides

The primers used for PCR were the following: fasL forward 5′-CAA GTC CAA CTC AAG GTC CAT GCC-3′ and backward 5′-CAG AGA GAG CTC AGA TAC GTT TGA C-3′ (Giordano *et al*., 1997); fas forward 5′-GGT GGG TTA CAC TGG TTT ACA-3′ and backward 5′-GTG CTA CTC CTA ACT GTG AC-3′ (Vaishnaw *et al*., 1999); caspase 3 forward 5′-TCC AGT CGG AGG CCA GAT CTG AG-3′ and backward 5′-CTG AAG CCT GCC TCC CGG GAT GA-3′ (SNP000005036); dff40 forward 5′-CTC TGG GGT ACT CGT TGG AT-3′ and backward 5′-ACT GCT GTT CAG ATC CGC GT-3′ (Iguchi *et al*., 2002); g3pdh forward 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′ and backward 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′ (Clontech, Palo Alto, CA).

Reverse-transcription/polymerase chain reaction (RT-PCR)

Total RNA was extracted from the kidney sections by acid guanidium thiocyanate/phenol/chloroform (TRIzol, Gibco BRL, Gaithersburg, MD). RNA was measured by OD at 260 nm. For cDNA synthesis, 250 ng of the total RNA was incubated with 200 mM dNTP, and 0.7 μM of the forward and backward primers were mixed with 0.5 U/20 μl of rTth/DNA polymerase (SuperScript™ One-Step, Invitrogen Life Technology, Carlsbad, CA). The reverse transcription was performed at 55˚C for 30 min. The amplification of fas, fasL, caspase 3, dff40 and g3pdh cDNAs was carried out by PCR. Reaction tubes containing 25 μl of sample mixture were amplified in a thermocycler (Perkin Elmer, GeneAmp PCR system 2400, Foster City, CA) with 30 cycles under the following conditions: 94˚C for 2 min, 55˚C for 2 min and 72˚C for 1.4 min. At the end of the PCR reaction, the samples were electrophoresed in 2% agarose containing 0.5 mg ml^{-1} ethidium bromide. PCR products were observed under UV light (Wang & Mark, 1990).

Band migration and density were documented using an image analysis system by BioRad. Pro-apoptotic transcript levels were determined by comparing with the g3pdh densitometry. Differences between samples were evaluated using the ANOVA test (Number Cruncher Statistical Systems, NCSS program).

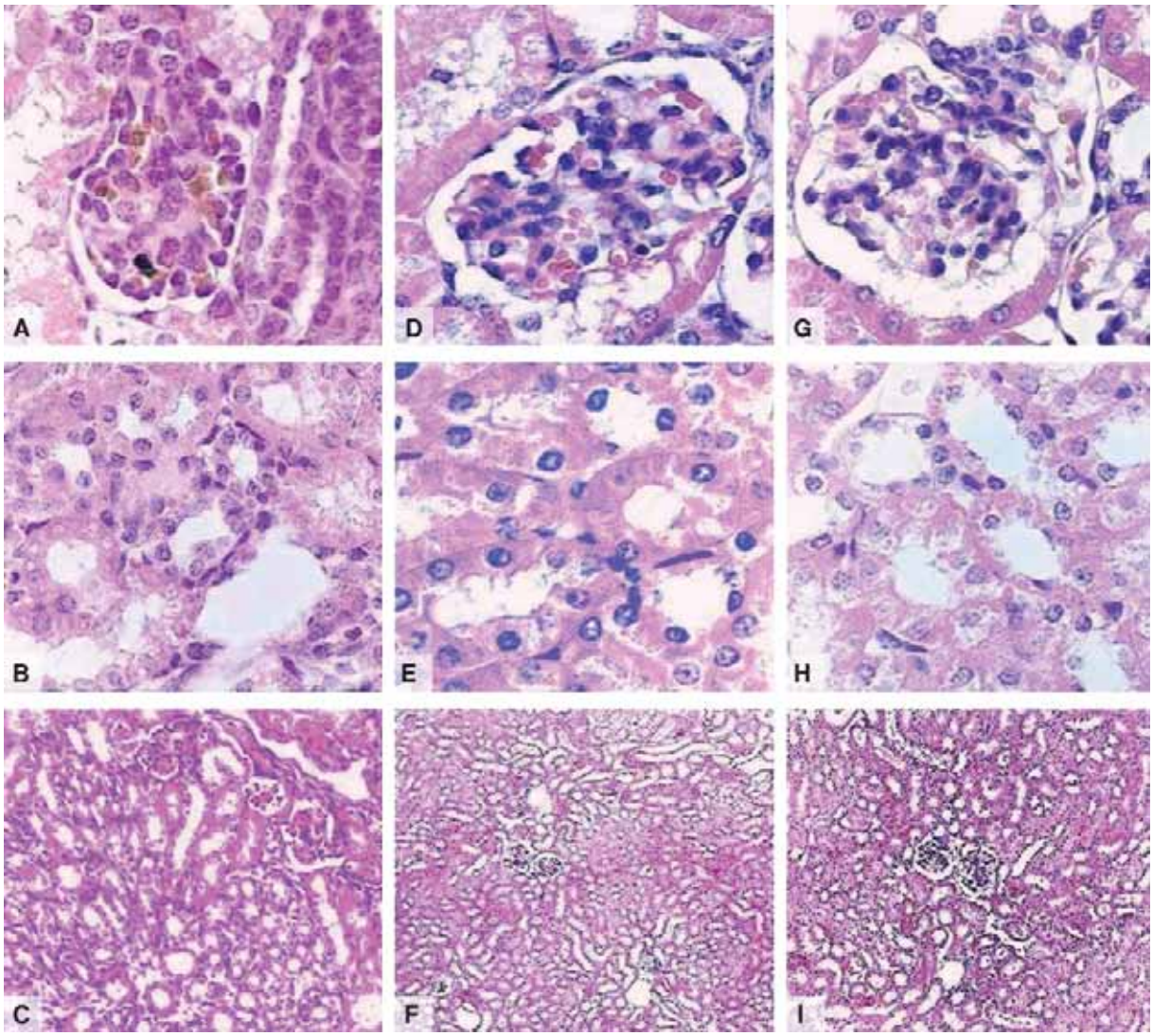

FIG. 1. Balb/c mice kidney histology. A, B, C: newborn mouse kidney; D, E, F: middle-age mouse kidney; G, H, I: elderly mouse kidney. Main differences are: the glomerulus of newborn is ovoid, in contrast to the bouquet of the fully developed glomerulus of an elderly mouse.

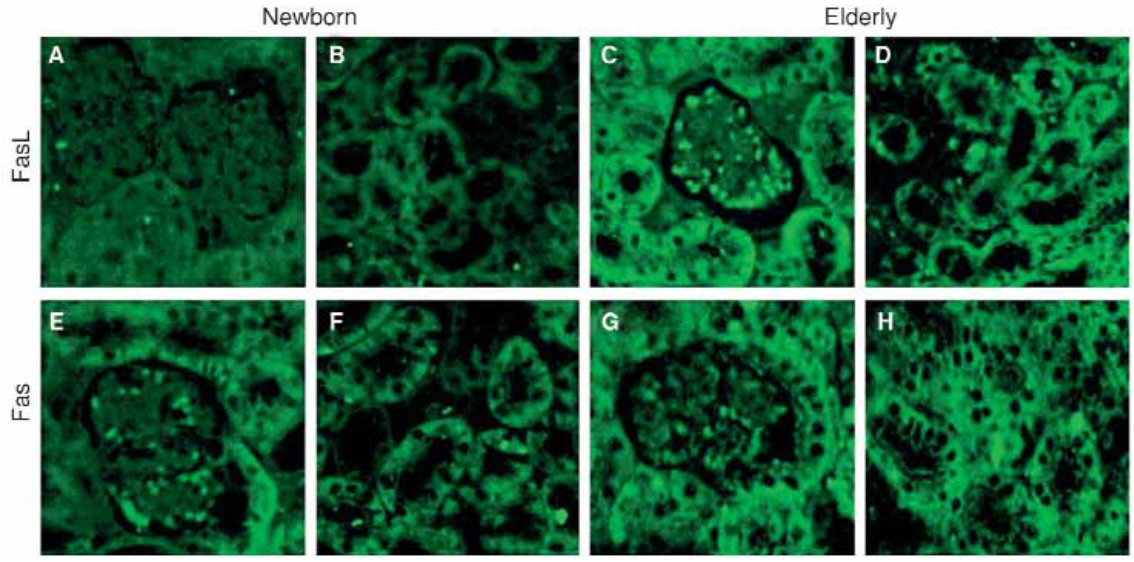

FIG. 2. The fasL and fas protein expression by direct immunofluorescence in kidneys of Balb/c mice. FasL is distributed in mesangial and endothelial glomerular cells and also in epithelial tubular cells. Fas is broadly distributed whiting glomerulus and tubules.

RESULTS

Microscopic differences between kidneys of newborn and elderly animals were detected in renal cortex. Morphological differences were observed in the glomerulus of new born mice which had an ovoid form, rather than the full developed bouquets of adult glomeruli. The tubular morphology was similar in both groups (Fig. 1).

Fas and fasL were expressed differently in the kidney. Both fas and fasL proteins were present in the kidneys of newborn mice. FasL was faintly expressed on the surface of mesangial cells and tubules. By contrast, the fas receptor was widely distributed in the mesangial cells and the tubular epithelium. The expression of the fasL and the fas proteins was superior in the adult mice (Fig. 2).

Elderly enhances the fas pathway gene transcription. The gene expression of the fas receptor was similar in all studied groups. By contrast, the fasL, caspase 3 and dff40 transcripts were barely detected

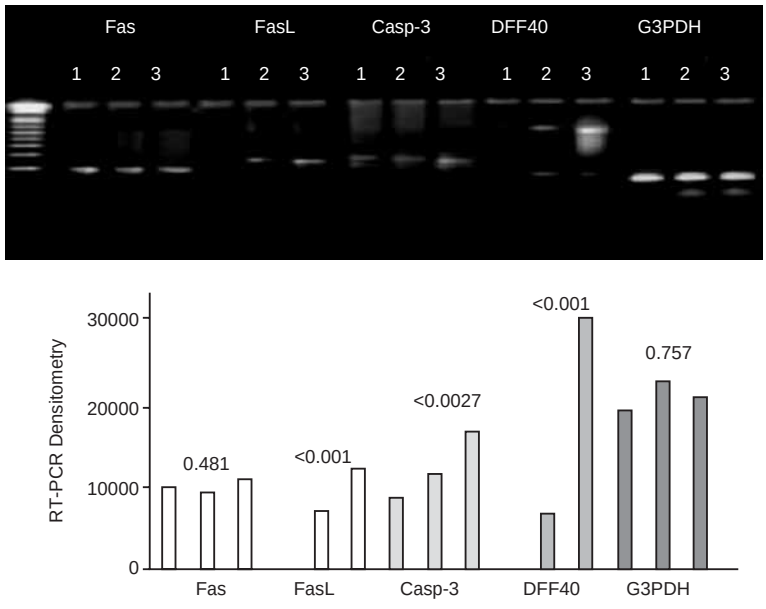

FIG. 3. A representative panel of RT-PCR amplification showing in the upper part a gel electrophoresis with RT-PCR products from fas, fasL, caspase 3 and dff40. The g3pdh correspond to the housekeeping gene. The graph of the lower part matches up with the gel electrophoresis, and shows the mean densitometry values. Group 1: newborn, 2: seven weeks old, 3: elderly mice. The *p* value was obtained by ANOVA.

TABLE 1. Renal pro-apoptotic gene expression at different stages of life (mean ± SD)

RT-PCR	Newborn (n = 20)	7th week (n = 20)	52nd week (n = 20)	*p* value
Fas-CD95 mRNA	10100 ± 3053	9402 ± 2874	10970 ± 3739	0.418
FasL mRNA	9.00 ± 3.74	7351 ± 2148	12240 ± 4692	< 0.001*
Caspase 3 mRNA	8878 ± 3325	11760 ± 6169	16430 ± 7692	< 0.0027*
DFF40 mRNA	14.0 ± 7.5	6999 ± 3659	29600 ± 10360	< 0.001*
PCNA mRNA	30060 ± 33.7	29430 ± 487.6	3.90 ± 4.5	< 0.001*
G3PDH mRNA	19140 ± 6312	22330 ± 7506	20480 ± 6873	0.757

* = statistically significant by ANOVA

after birth. These genes were over-regulated by elderly and transcripts were significantly increased. G3PDH housekeeping transcripts were similar in all samples (Fig. 3 and Table 1).

Apoptotic rates increase by elderly in tubular epithelium and mesangium. The rate of apoptotic cells in the control kidney of newborn mice was less than 0.0023 in 100 fields. The kidney of adult mice, exhibited a rate of 0.0576 apoptotic cells. The differences between both groups were statistically significant. Interestingly, no apoptotic tag was detected in podocytes. The tubular epithelium of adult mice exhibited a superior apoptotic ratio, as seen in Table 2 and Fig. 4 that shows representative fields of apoptosis. However, most of the renal tissue did not show apoptotic features.

Cell proliferation is higher in the newborn. The pcna protein was used as a cell proliferation marker and was widely distributed throughout the mesangial, the glomerular epithelium and the tubuli of newborn mice. The pcna expression was declined during elderly and was almost absent in kidney samples of elderly animals. This finding was concordant with the pcna mRNA transcription which was detected only in new-

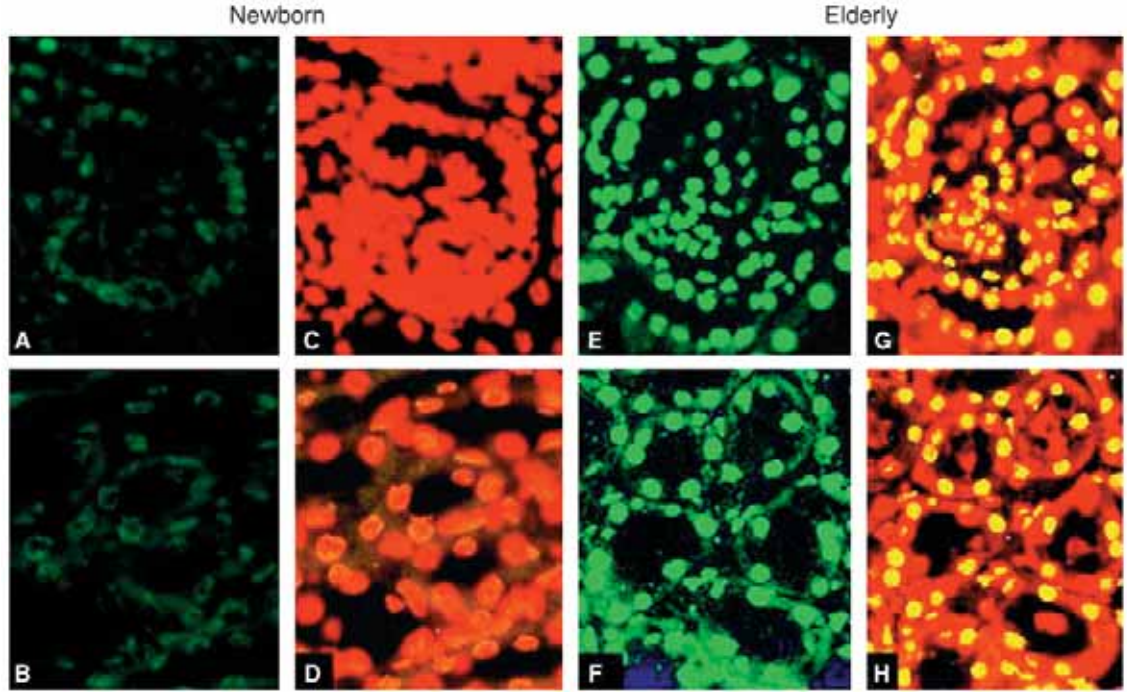

FIG. 4. Apoptotic cell features in kidneys of Balb/c mice studied by TUNEL. A, C, E and G are glomerulus; B, D, F and H are tubules. Apoptotic cells are green tagged indicating the incorporation of the fluorescent nick end labelling into apoptotic nuclear DNA fragments. Cells counterstained with 2% propidium iodide (C, D, G and H). The non-apoptotic nuclei develop a red staining and the apoptotic nuclei a yellow staining.

TABLE 2. Apoptosis of renal cells at different stages of life (mean ± SD)

TUNEL	Newborn (n = 20)	7th week (n = 20)	52nd week (n = 20)	*p* value
Glomerular apoptotic rate	0.0023 ± 0.0008	0.0118 ± 0.0032	0.0576 ± 0.0063	< 0.0001*
Tubular apoptotic rate	0.0150 ± 0.0206	0.0550 ± 0.0243	0.262 ± 0.30	< 0.0001*

* = statistically significant by ANOVA

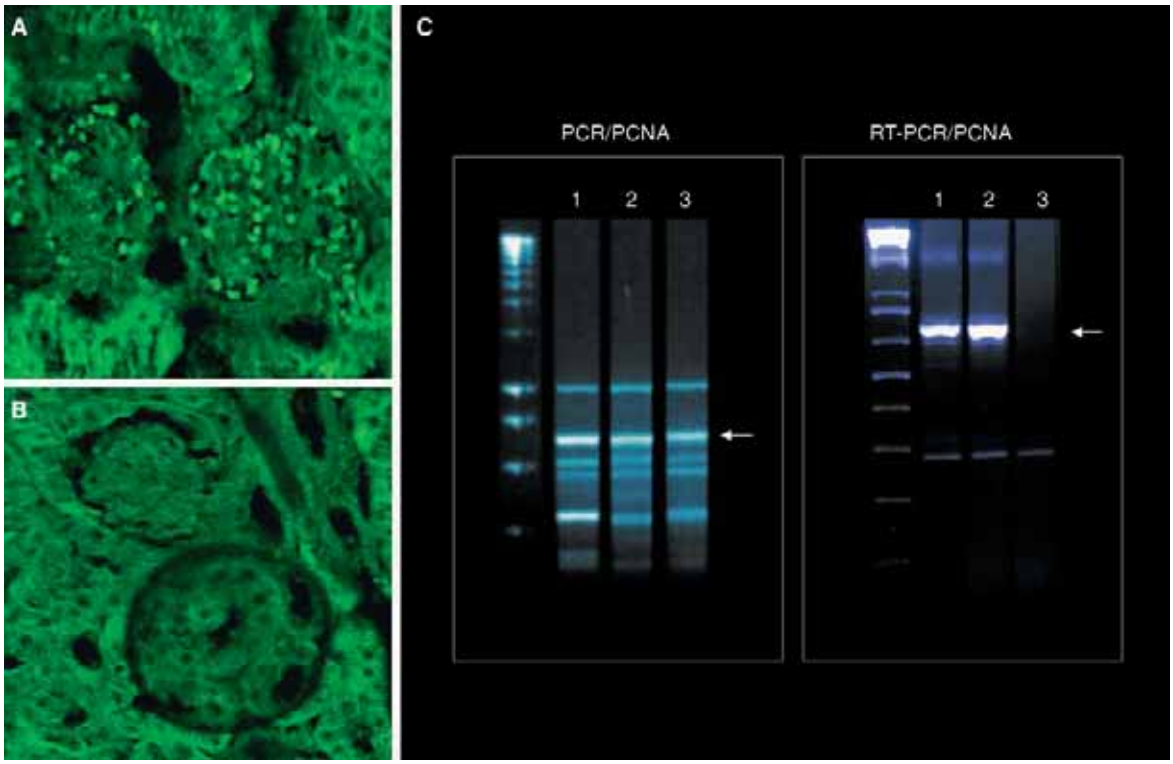

FIG. 5. The pcna expression determined by immunofluorescence in newborn (A) and aged mouse kidney (B). The constitutive pcna (C) was determined by PCR amplification using DNA extracted from kidney. The synthesized pcna was determined by RT-PCR amplification using the total renal RNA extracted from newborn (1), seven weeks old (2) and aged animals (3).

born and young animals, in contrast to the pcna transcription which was shut down in the kidneys of elderly mice (Fig. 5).

DISCUSSION

Apoptosis was studied during kidney development within the course of diverse inflammatory processes. It was demonstrated that apoptosis normally occurs in a low proportion after birth and this process progressively increases by elderly. Apoptotic cells are replaced by cell proliferation (renal cell turnover).

During embryogenesis, a TGF-beta1-dependent apoptosis in endothelial areas contributes to the development of the glomerular loop. By this mechanism, the capillary lumens of the vascular bed become opened (Fierlbeck *et al*., 2003). The mesangium is composed of a matrix with two main cell types: a bone marrow-derived phagocyte and a smooth muscle-like cell, the latter being more abundant. The mesangium maintains the structure and function of the glomerular capillary bouquet. During embryogenesis, the mesangial tissue suffers apoptosis induced by the VEGF specific endothelial cell mitogen (Kreisberg *et al*., 1985; Yamagishi *et al*., 2002). After birth, diverse inflammatory conditions may increase the nitric oxide (NO) and free radical concentration, a fact resulting in an abnormal increase of the mesangial apoptosis (Keil *et al*., 2002).

By the end of the embryonic development, the renal epithelium is differentiated into tubules. A down-regulation of the bcl-X (L), bcl-w and bcl-2 genes induces tubular maturation by triggering massive apoptosis of the collecting ducts and the distal tubular epithelia. Signaling is promoted by the TGF-beta1 (Moser *et al*., 1997; Schaefer *et al*., 2003). By this process, tubules achieve their definitive shape.

The glomerular cell turnover is below 1% per day. This reflects a long life span of the endothelial and mesangial structures. Concurrently, cell differentiation and trans-differentiation support this process. By cell trans-differentiation, the glomerular epithelium acquires a mesenchymal reminiscent embryonic phenotype (like-mesangioblast/myofibroblasts) and produces huge amounts of extra cellular matrix. Under the effect of IL-1, TGF-beta1, and EGF, the renal cells with an adult-mature-phenotype of the proximal tubules may regress to an embryonic stage (Pabst & Sterzel, 1983; El Nahas, 2003). Renal plasticity principally occurs during embryogenesis. After birth, it supports the structure and function of the kidney. This process can be activated by renal insults (Dai *et al*., 2003).

Renal apoptosis depends in part on the fas receptor, which is broadly expressed on the cell surface of the mesangial cells, the tubular epithelium and the fibroblasts (Strutz & Müller, 2000). The activation of the fas receptor requires stimulation by its ligand. FasL is expressed in the renal infiltrating leukocytes and faintly in the mesangial, tubular epithelial cells, and the renal interstitial fibroblasts. Glomerular cells damaged by the immune-complexes, also express fasL. Consequently, fas receptor and fasL ligands are both present in certain areas of the kidney (French *et al*., 1996; Ortiz *et al*., 1999; Lorz *et al*., 2000). However, under homeostatic conditions the autocrine or paracrine apoptosis is unusual, because fas and fasL remain segregated in different cell compartments. The mechanisms involved in the renal autocrine expression of fasL under physiological conditions, remain largely unknown. Our results demonstrated that apoptosis of renal cells is low and is increased by elderly. Tubules display more apoptosis, probably due to the broad distribution of the fasL/ fas pair along the tubular epithelium.

Cell proliferation is superior in the kidneys of new born animals than in the kidneys of elderly animals. We infer that genes involved in cell proliferation including pcna, are down-regulated in senescent kidneys (Rodriguez *et al*., 2004). Cellular senescence describes a phenotype with permanent and irreversible growth arrest. Such a *turn down* mechanism was described in mammalian cells in culture. This mechanism may be similar to that of senescent mammalian kidneys (Wright & Shay, 2002; Melk, 2003).

In conclusion, our results suggest that renal apoptosis is a physiological process executed by the fas/ fasL pathway, and is important to support the structure and function of the kidney. Our results provide a rational about renal development that in clinics can be applied in comprehension of the molecular mechanisms of renal pathology and the novel designs of therapeutic interventions.

ACKNOWLEDGEMENTS

This research was supported by PROMEP/103.5/04/2310. The Ph. D. study of JJ Bollain y Goytia was supported by CONACYT grant 114642.

REFERENCES

Balbi AP, Costa RS, Coimbra TM, 2004. Postnatal renal development of rats from mothers that received increased sodium intake. *Pediatric nephrol*ogy, 19: 1212-1218.

Dai C, Yang J, Liu Y, 2003. Transforming growth factor-beta1 potentiates renal tubular epithelial cell death by a mechanism independent of Smad signaling. *Journal of biological chemistry*, 278: 12537-12545.

Deng GR, Wu R, 1983. Terminal transferase: use in the tailing of DNA and for in vitro mutagenesis. *Methods in enzymology*, 100: 96-116.

El Nahas M, 2003. Kidney remodelling and scarring: the plasticity of cells. *Nephrology dialysis transplantation*, 18: 1959-1962.

Fierlbeck W, Liu A, Coyle R, Ballermann BJ, 2003. Endothelial cell apoptosis during glomerular capillary lumen formation in vivo. *Journal of the american society of nephrology*, 14: 1349-1354.

Fisher CE, Michael L, Barnett MW, Davies JA, 2001. Erk MAP kinase regulates branching morphogenesis in the developing mouse kidney. *Development*, 128: 4329-4338.

French LE, Hahne M, Viard I, Radlguber G, Zanone R, Becker K, Müller C, Tschopp J, 1996. Fas and Fas ligand in embryos and adult mice: Ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. *Journal of cell biology*, 133: 335-343.

Giordano C, Stassi G, De Maria R, Todaro M, Richiusa P, Papoff G, Ruberti G, Bagnasco M, Testi R, Galluzzo A, 1997. Potential involvement of Fas and its ligand in pathogenesis of Hashimoto's thyroiditis. *Science*,

275: 960-963.

Harriet S, Coles R, Burne JF, Raff MC, 1993. Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development*, 118: 777-784.

Iguchi K, Hirano K, Ishida R, 2002. Activation of caspase-3, proteolytic cleavage of DFF and no oligonucleosomal DNA fragmentation in apoptotic Molt-4 cells. *Journal of biochemistry*, 131: 469-475.

Keil A, Blom IE, Goldschmeding R, Rupprecht HD, 2002. Nitric oxide down-regulates connective tissue growth factor in rat mesangial cells. *Kidney international*, 62: 401-411.

Kreisberg JI, Venkatachalam M, Troyer D, 1985. Contractile properties of cultured glomerular mesangial cells. *American journal of physiology*, 249: F457-463.

Lechner MS, Dressler GR, 1997. The molecular basis of embryonic kidney development. *Mechanisms of development*, 62: 105-120.

Lorz C, Ortiz A, Justo P, González-Cuadrado S, Duque N, Gómez-Guerrero C, Egido J, 2000. Proapoptotic Fas ligand is expressed by normal kidney tubular epithelium and injured glomeruli. *Journal of the american society of nephrology*, 11: 1266-1277.

Melk A, 2003. Senescence of renal cells: molecular basis and clinical implications. *Nephrology dialysis transplantation*, 18: 2474-2478.

Moser M, Pscherer A, Roth C, Becker J, Mucher G, Zerres K, Dixkens C, Weis J, Guay-Woodford L, Buettner R, Fassler R, 1997. Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2b. *Genes and development*, 11: 1938-1948.

Ortiz A, Lorz C, Egido J, 1999. New kids in the block: the role of FasL and Fas in kidney damage. *Journal of nephrology*, 12: 150-158.

Pabst R, Sterzel RB, 1983. Cell renewal of glomerular cell types in normal rats. An autoradiographic analysis. *Kidney international*, 24: 626-631.

Rodriguez MM, Gomez AH, Abitbol CL, Chandar JJ, Duara S, Zilleruelo GE, 2004. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatric and developmental pathology*, 7: 17-25.

Sano H, Ueda Y, Takakura N, Takemura G, Doi T, Kataoka H, Murayama T, Xu Y, Sudo T, Nishikawa S, Nishikawa S, Fujiwara H, Kita T, Yokode M, 2002. Blockade of platelet-derived growth factor receptor-beta pathway induces apoptosis of vascular endothelial cells and disrupts glomerular capillary formation in neonatal mice. *American journal of pathology*, 161: 135-143.

Schaefer L, Beck KF, Raslik I, Walpen S, Mihalik D, Micegova M, Macakova K, Schonherr E, Seidler DG, Varga G, Schaefer RM, Kresse H, Pfeilschifter J, 2003. Biglycan, a nitric oxide-regulated gene, affects adhesion, growth, and survival of mesangial cells. *Journal of biological chemistry*, 278: 26227-26237.

Strutz F, Müller GA, 2000. Transdifferentiation comes of age. *Nephrology dialysis transplantation*, 15: 1729-1731.

Vaishnaw AK, Orlinick JR, Chu JL, Krammer PH, Chao MV, Elkon KB, 1999. The molecular basis for apoptotic defects in patients with CD95 (Fas/Apo-1) mutations. *Journal of clinical investigation*, 103: 355-363.

Wang AM, Mark DF, 1990. Quantitative PCR. In: Innis MA, Gelfand DH, Snisky JJ, Withe TJ, eds. *PCR Protocols*. *A guide to methods and applications*. Academic Press, San Diego, USA: 70-75.

Wright WE, Shay JW, 2002. Historical claims and current interpretations of replicative aging. *Nature biotechnology*, 20: 682-688.

Yamagishi S, Inagaki Y, Okamoto T, Amano S, Koga K, Takeuchi M, Makita Z, 2002. Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells. *Journal of biological chemistry*, 277: 20309-20315.