

Analysis of cell turnover in the macula densa through the normal aging process

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Abstract. The kidney undergoes changes during the lifespan of an individual, resulting in increased susceptibility to renal diseases in the elderly. The macula densa (MD) controls basic kidney functions, which deteriorate with aging. The present study for the first time, to the best of our knowledge, analyzed the relationship between cell proliferation and apoptosis (cell turnover) in the MD of mice through the normal aging process. Kidney specimens from CD1 mice aged 2, 6, 12, 18 or 24 months were fixed in neutral-buffered formalin and embedded in paraffin. Tissue sections were immunostained to analyze cell proliferation, subjected to a TUNEL assay to evaluate apoptosis, or stained with hematoxylin and eosin to determine total cell number. A markedly dynamic cell turnover pattern was observed in the MD throughout the aging process, in which, when the number of proliferating cells increased, the number of apoptotic cells decreased and vice versa. However, there were no significant differences in the total cell number among the ages analyzed. Thus, other mechanisms may be involved in the deterioration of renal functions regulated by the MD in elderly people. Further research is required to determine these mechanisms, which could be the target of

therapeutic strategies against age-related kidney diseases in the future.

Introduction

Life expectancy is increasing worldwide. The global population over 65 years of age is expected to exceed 1.5 billion by 2050 (1,2). Aging is accompanied by structural and functional changes in the body, resulting in increased susceptibility to diseases (3). The kidney is one of the organs that most deteriorates with aging. At a functional level, this deterioration is characterized by a decreased glomerular filtration rate (GFR), alterations in renal blood flow (RBF), reduction in kidney size and increased fibrosis and inflammation (4). An age-related decline in renal functional reserve may increase susceptibility to acute kidney injury and chronic kidney disease in the elderly (5).

A complex interplay among genetic predisposition, environmental factors, and cellular dysfunction leads to the characteristic changes observed in the aging kidney (6). Most of the research on renal aging has focused on the glomerulus. The number of functioning glomeruli decreases during the life-time of an individual, while the proportion of sclerotic and hyalinized glomeruli increases. The number of podocytes also decreases with age (7,8). In comparison, research devoted to changes in renal tubules through the aging process has received less attention. Previous research has shown that with increasing age, in addition to tubular atrophy, there is a decrease in the number and volume of tubules (5).

The macula densa (MD) is a group of epithelial cells located at the end of the thick ascending limb (TAL) of the loop of Henle, where it transitions to the distal convoluted tubule. The MD detects changes in sodium chloride concentration in tubular fluid composition, generating and sending signals that control basic kidney functions, including GFR and RBF through the tubuloglomerular feedback mechanism and renin release (9,10). The MD, along with the extraglomerular mesangial cells and the juxtaglomerular cells of the afferent glomerular arteriole, constitute the juxtaglomerular apparatus (11).

Given the functional importance of the MD, the limited number of studies on the changes in its structure or function

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Abbreviations: BAX, BCL2-associated X protein; BCL-2, B-cell lymphoma 2 protein; BM, basement membrane; EGF, Epidermal growth factor; GFR, glomerular filtration rate; MD, macula densa; PCD, programmed cell death; PCNA, proliferating cell nuclear antigen; RBF, renal blood flow; RT, room temperature; TAL, thick ascending limb; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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through aging is noteworthy. Most of the existing studies are associated with changes in the expression of cyclooxygenase-2 and neuronal nitric oxide synthase, enzymes involved in the control of afferent vascular tone (12,13).

Cell turnover plays an important role in maintaining normal tissue function and morphology. Programmed cell death (PCD) is required for the normal turnover of cells in numerous tissues that are maintained by cell division, including the intestinal epithelium, blood, epidermis, kidney and lungs (14). However, apoptosis is the PCD that occurs most frequently during cell turnover (15). During cell turnover, older differentiated cells are eliminated, commonly by apoptosis, and replaced by progeny of dividing adult stem cells. Thus, apoptosis and cell proliferation represent the two key components of this homeostatic process (14-16). Age-specific changes in cell turnover may lead to cell loss, and compromise tissue homeostasis, structure and function (17,18). To the best of our knowledge, there have been no studies analyzing the relationship between apoptosis and cell proliferation (cell turnover) at different ages through the normal aging process in the MD.

In previous studies, the existence of cell turnover in adult lung cartilage was identified (19,20), and an age-related increase in apoptosis in the bronchiolar epithelium, accompanied by a decrease in cell proliferation was observed, changes that may contribute to the development of lung diseases (21). In the present study, the findings from the analysis of cell turnover in the MD of mice throughout the normal aging process are reported.

Materials and methods

Animals and experimental design. In the present study, the MD (Fig. 1A and B) was analyzed in naturally aging CD1 mice. These mice develop numerous phenotypes similar to those observed in normal human aging (22,23) and have been widely used in various related studies (24-26). Furthermore, CD1 is an outbred strain, meaning that the mice exhibit genetic variations similar to those observed in some human populations (27). To ensure that observed differences between groups were attributable solely to age, only male mice were used, thereby minimizing the influence of other factors, such as sex.

The animals and procedures used were described in previous studies (19,21,28). Male CD1 mice were maintained under standard conditions in stainless steel cages, receiving food and water *ad libitum*, at a temperature of 18-21°C, a relative humidity of 55-60% and a 12:12 h day-night cycle. The animals were supplied by the Department of Embryology of the Autonomous University of Nuevo León (Monterrey, Mexico). Animal care was provided in accordance with the principles and procedures outlined in the National Research Council Guide for the Care and Use of Laboratory Animals (8th edition) (29) and in the Mexican Guidelines ZOO-062 (30). Experimental procedures were approved (approval no. PA19-00001) on December 9, 2019, by the Institutional Review Board and Ethics Committee of the School of Medicine of the Autonomous University of Nuevo León (Monterrey, Mexico). The humane endpoints considered in this study were as follows: i) Inability to stand; ii) agonal

breathing and cyanosis; iii) severe muscular atrophy; iv) severe skin lesions or self-trauma; v) uncontrolled bleeding; vi) diarrhea or dehydration for more than 48 h; vii) weight loss of >25%; viii) piloerection; ix) dilated pupils for >3 days; ix) sunken or squinted eyes; x) general lack of grooming. However, none of these criteria were exhibited by the mice during the study.

Three animals were sacrificed at the age of 2, 6, 12, 18 or 24 months by cervical dislocation. The weight of the mice at 2 months was 41.5±1.4 g, at 6 months 45.0±3.4 g, at 12 months 46.0±2.9 g, at 18 months 48.3±1.3 g and at 24 months 49.8±2.9 g. At the beginning of the study the mice were aged 2 months, and each group was sacrificed when they reached the aforementioned ages; therefore, 15 mice were sacrificed in total. Only the right-side kidneys were analyzed in order to have a more consistent control in the selection of samples, allowing age to be the only variable in our model. Furthermore, previous studies in different species indicate that the right and left kidneys do not present significant morphological differences. Differences are mainly anatomical and positional (31-35).

The kidneys were cut longitudinally into two equal halves, fixed in 10% neutral buffered formalin at room temperature (RT) for 24 h, and embedded in paraffin with the samples oriented with the flat surface parallel to the surface of the paraffin block to be sectioned. Serial 5-μm sections were cut, deparaffinized in xylene and hydrated in a graded series of alcohol. Sections were immunostained for proliferating cell nuclear antigen (PCNA) to analyze cell proliferation (Fig. 1C), subjected to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay to evaluate apoptosis (Fig. 1D), or stained with hematoxylin and eosin to assess total cell number (Fig. 1B). Results of the analysis of cell proliferation, apoptosis and total cell number were compared between mice of the different ages studied.

Analyses were conducted using three tissue sections per animal per assay. Analyzed sections were selected using the systematic random sampling method, which is the most efficient approach for obtaining a uniform sample (36). This design was previously used in related studies of the lung (21,37) and kidney (28), with excellent results. Generally, the cells that constitute the MD are easily distinguishable from the cells of the TAL in which they are immersed, since they are taller and their nuclei lie closer together ('dense') than in neighboring cells. However, in order to not exclude any cells from the MD when performing the analyses, only the cells from the MD were considered, and then separately, the cells from the MD were considered together with the cells from their corresponding TAL (MD-TAL). This approach has been used in previous research (13). All available MD/MD-TAL from each mouse were analyzed. All MD/MD-TAL were assigned consecutive numbers to avoid duplication of the analysis. On average, 14 MD/MD-TAL per slide (range, 3-28) were analyzed. Only transversely sectioned MD/MD-TAL were included in the analysis.

Sections were examined using a Primo Star light microscope, and high-resolution color images (x400) were captured using an Axio-Cam ICc1 camera linked to image analysis software Zen lite 2011 (microscope, camera and software were all obtained from Carl Zeiss AG). No corrections were made for tissue shrinkage due to processing, as kidneys were

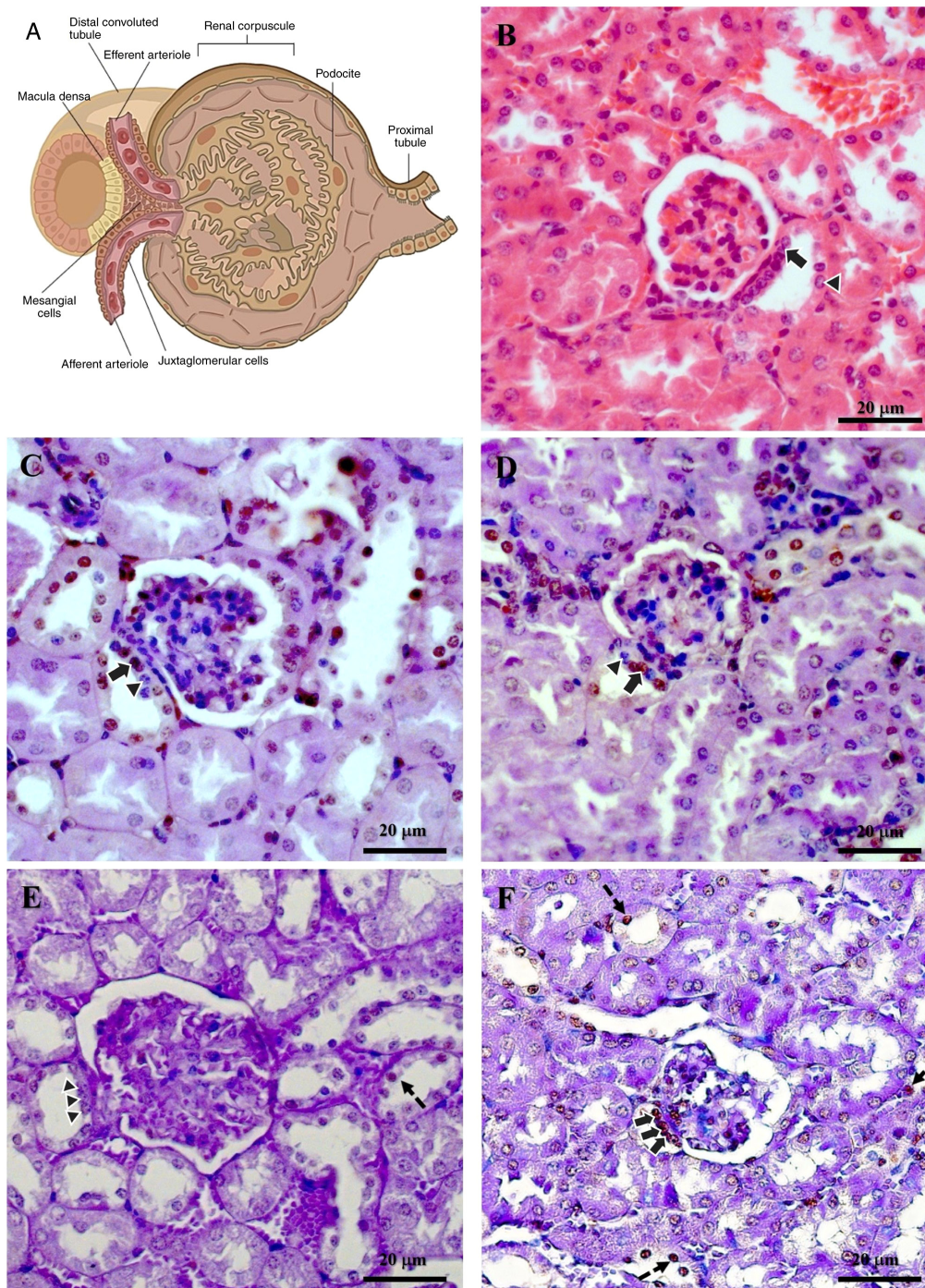


Figure 1. Analysis of cell turnover in the MD of 2-, 6-, 12-, 18- and 24-month-old mice. (A) Schematic of the MD and adjacent structures in the kidney. (B) Tissue sections were stained with hematoxylin and eosin to assess the total number of cells. The arrow indicates a cell of the MD, while the arrowhead indicates a cell of the thick ascending limb. (C) PCNA was detected in proliferation analysis by immunohistochemistry. The arrow indicates a proliferating cell (brown nucleus), while the arrowhead indicates a non-proliferating cell (blue nucleus). (D) Apoptosis was detected by *in situ* end-labeling of fragmented DNA using the TUNEL assay. The arrow indicates an apoptotic cell (brown nucleus), while the arrowhead indicates a non-apoptotic cell (blue nucleus). (E) The number of MD cells undergoing proliferation in mice at 24 months of age was scarce. The arrowheads indicate non-proliferating MD cells (blue nuclei). The dotted arrow indicates a proliferating tubular cell (brown nucleus). (F) The number of MD cells undergoing apoptosis in mice at 24 months of age was high. The arrows indicate apoptotic MD cells. Apoptosis was also high in other tubular cells (dotted arrows, brown nuclei). Mice in (B-D) belonged to the 6-month age group. Scale bar 20 μ m. MD, macula densa; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method.

processed identically and were therefore assumed to have experienced an equivalent degree of shrinkage in all age groups (38). Identifying information on each slide was temporarily masked, until after the analyses were completed by a single observer.

Immunohistochemistry. The detection of PCNA was performed according to the standard protocol. Sections were subjected to a heat-induced epitope retrieval step performed by microwave treatment at 89°C for 10 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was

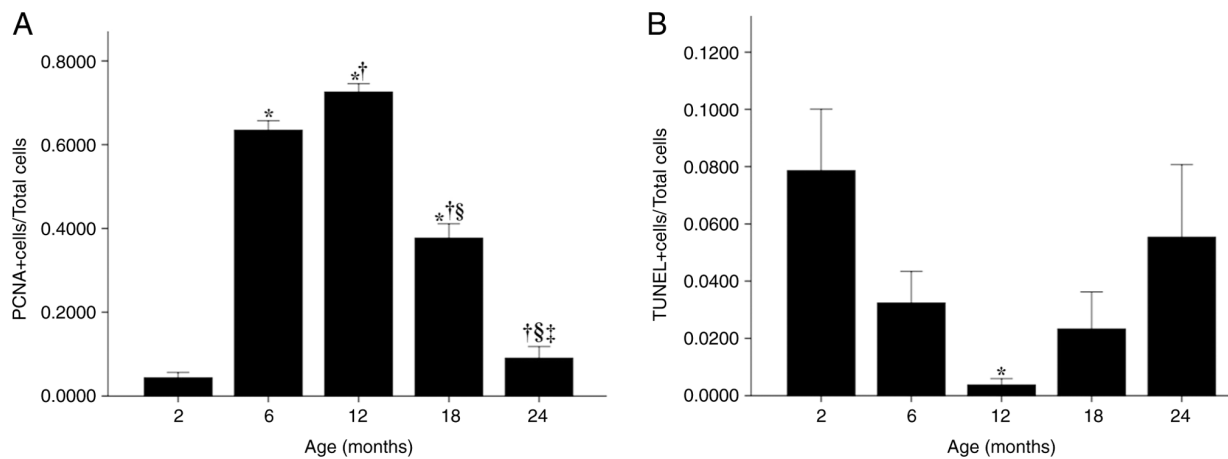


Figure 2. Analysis of cell proliferation and apoptosis in the macula densa of 2-, 6-, 12-, 18- and 24-month-old mice. (A) The number of cells undergoing proliferation in mice at 2 months of age was significantly lower than in mice at 6, 12 and 18 months of age ($^*P<0.001$). Mice at 6 months of age had a significantly higher number of proliferating cells than mice at 18 and 24 months of age (both $^{\dagger}P<0.001$), but this number was significantly lower than in mice at 12 months of age ($^{\dagger}P=0.043$). Mice at 12 months of age had a significantly higher number of proliferating cells than mice at 18 and 24 months of age (both $^{\dagger}P<0.001$). Finally, mice at 18 months of age had a significantly higher number of proliferating cells than mice at 24 months of age ($^{\dagger}P<0.001$). (B) The number of cells undergoing apoptosis in mice at 2 months of age was significantly higher than in mice at 12 months of age ($^*P=0.002$). Values are expressed as the means $\pm$ 1 standard error. Data were analyzed using ANOVA and the Bonferroni post hoc test. Results were considered statistically significant at $P<0.05$. PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method.

blocked with 3% hydrogen peroxide at RT for 15 min. The slides were incubated with a monoclonal antibody against PCNA (cat. no. ab29; dilution, 1:1,000; Abcam) overnight at RT in blocking solution. Detection was carried out using an anti-mouse Ig HRP Detection kit (cat. no. 551011; BD Pharmingen; BD Biosciences), according to the manufacturer's instructions; diaminobenzidine (DAB) was used as a chromogen. Slides were counterstained with 0.5% methyl green for 10 min at RT. For the negative control sections, an isotype control was used instead of the primary antibody or the primary antibody incubation step was omitted. PCNA-positive cells were counted and normalized by the total cell number of MD or MD-TAL.

TUNEL assay. Sections were treated with 20 μ g/ml proteinase K for 15 min at RT, and then with 3% hydrogen peroxide for 5 min at RT to quench endogenous peroxidase. DNA strand breaks of apoptotic cells were detected using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (EMD Millipore), according to the manufacturer's instructions. The sections were incubated with a mixture of TdT-enzyme and nucleotides linked with digoxigenin in a humidified chamber at 37°C for 1 h. The sections were further incubated with anti-digoxigenin-peroxidase for 30 min at RT. This system is based on the labeling of the DNA strand breaks with digoxigenin-nucleotides, to which an anti-digoxigenin antibody conjugated to a peroxidase reporter molecule is then attached. DAB was used as a chromogen. Finally, the slides were counterstained for 10 min at RT with 0.5% methyl green. Tissue sections were mounted with Entellan (Sigma-Aldrich; Merck KGaA). In the controls, the digoxigenin-nucleotides incubation step was omitted. TUNEL-positive cells were counted and normalized by the total cell number of MD or MD-TAL.

Total cell number. Sections were stained with hematoxylin and eosin to assess total cell number. Hematoxylin staining was

performed for 10 min at RT and eosin staining was performed for 1 min at RT according to the standard technique (39). The cell number was normalized by the basement membrane (BM) length for MD or by the BM perimeter for MD-TAL. BM length and BM perimeter were obtained using the software aforementioned.

Statistical analysis. The results are presented as means $\pm$ 1 standard error (SE). One-way ANOVA test was used to determine statistical significance, followed by post hoc analysis with the Bonferroni test when significant differences were found between the age groups analyzed. $P<0.05$ was considered to indicate a statistically significant difference. The data were analyzed using SPSS for Windows 24 (IBM Corp.).

Results

Cell proliferation

MD. The results of the ANOVA test showed a significant difference in the number of cells undergoing proliferation among the analyzed ages ($F=149$; $P<0.001$). The Bonferroni test revealed that the number of cells undergoing proliferation in mice at 2 months of age (0.0434 ± 0.0126 PCNA + cells/total cells) was significantly lower than in mice at 6, 12, and 18 months of age (0.6345 ± 0.0232 , 0.7259 ± 0.0197 and 0.3769 ± 0.0343 PCNA + cells/total cells, respectively; all $P<0.001$). Mice at 6 months of age had a significantly higher number of proliferating cells than mice at 18 ($P<0.001$) and 24 months of age (0.0909 ± 0.0272 PCNA + cells/total cells; $P<0.001$), but this number was significantly lower than in mice at 12 months of age ($P=0.043$). In addition, mice at 12 months of age had a significantly higher number of proliferating cells than mice at 18 and 24 months of age (both $P<0.001$). Finally, mice at 18 months of age had a significantly higher number of proliferating cells than mice at 24 months of age ($P<0.001$; Fig. 2A).

Table I. Cell turnover parameters in the macula densa of mice through the normal aging process.

Parameter	Age (months)				
	2	6	12	18	24
MD					
Proliferating cells (PCNA + cells/total cells)	0.0434±0.0126	0.6345±0.0232 ^a	0.7259±0.0197 ^{a,b}	0.3769±0.0343 ^{a-c}	0.0909±0.0272 ^{b-d}
Apoptotic cells (TUNEL + cells/total cells)	0.0786±0.0214	0.0323±0.0110	0.0037±0.0021 ^a	0.0233±0.0129	0.0553±0.0253
Total cells (Total cells/μm BM)	0.3180±0.0167	0.3180±0.0080	0.3111±0.0090	0.3407±0.0228	0.3114±0.0189
MD-TAL					
Proliferating cells (PCNA + cells/total cells)	0.0677±0.0136	0.6852±0.0193 ^a	0.7890±0.0273 ^{a,b}	0.3780±0.0328 ^{a-c}	0.0854±0.0262 ^{b-d}
Apoptotic cells (TUNEL + cells/total cells)	0.0728±0.0203	0.0338±0.0115	0.0029±0.0015 ^a	0.0185±0.0109	0.0524±0.0229
Total cells (Total cells/μm BM)	0.1577±0.0107	0.1636±0.0045	0.1584±0.0053	0.1648±0.0054	0.1634±0.0089

Data are reported as the mean ± standard error. Data were analyzed using ANOVA and the Bonferroni post hoc test. Results were considered statistically significant at $P < 0.05$. ^aStatistically significant compared with 2-month-old mice. ^bStatistically significant compared with 6-month-old mice. ^cStatistically significant compared with 12-month-old mice. ^dStatistically significant compared with 18-month-old mice. MD, macula densa; MD-TAL, macula densa-thick ascending limb; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method; BM, basement membrane.

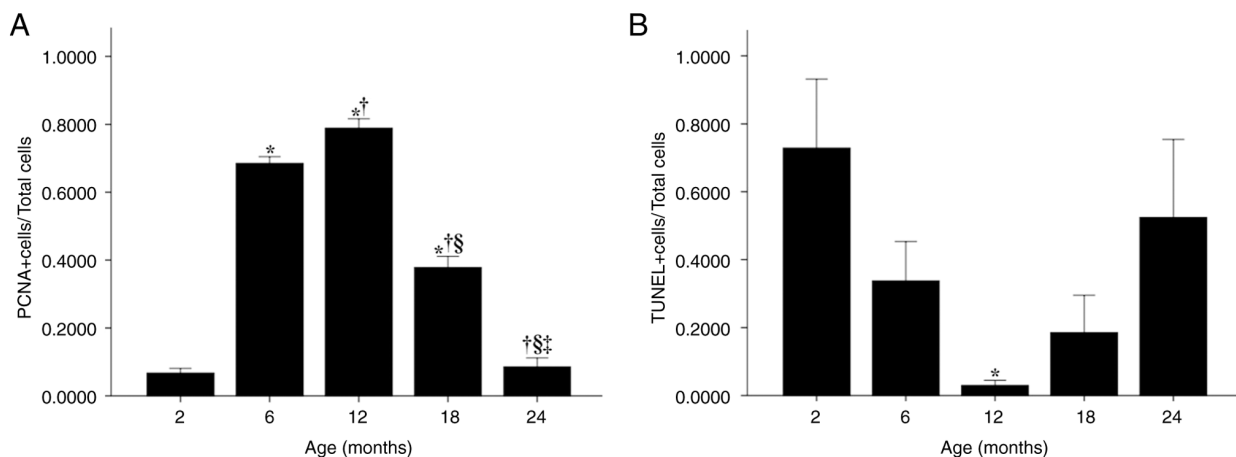


Figure 3. Analysis of cell proliferation and apoptosis in the macula densa-thick ascending limb of 2-, 6-, 12-, 18- and 24-month-old mice. (A) The number of cells undergoing proliferation in mice at 2 months of age was significantly lower than in mice at 6, 12 and 18 months of age ($P < 0.001$). Mice at 6 months of age had a significantly higher number of proliferating cells than mice at 18 and 24 months of age (both $P < 0.001$), but this number was significantly lower than in mice at 12 months of age ($P = 0.018$). Mice at 12 months of age had a significantly higher number of proliferating cells than mice at 18 and 24 months of age (both $P < 0.001$). Finally, mice at 18 months of age had a significantly higher number of proliferating cells than mice at 24 months of age ($P < 0.001$). (B) The number of cells undergoing apoptosis in mice at 2 months of age was significantly higher than in mice at 12 months of age ($P = 0.002$). Values are expressed as the means ± 1 standard error. Data were analyzed using ANOVA and the Bonferroni post hoc test. Results were considered statistically significant at $P < 0.05$. PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method.

MD-TAL. According to the ANOVA test, there was a significant difference in the number of proliferating cells among the analyzed ages ($F = 163$; $P < 0.001$). The results of the Bonferroni test showed that the number of cells undergoing proliferation in mice at 2 months of age (0.0677 ± 0.0136 PCNA + cells/total cells) was significantly lower than in mice at 6, 12, and 18 months of age (0.6852 ± 0.0193 , 0.7890 ± 0.0273 and 0.3780 ± 0.0328 PCNA

+ cells/total cells, respectively; all $P < 0.001$). Mice at 6 months of age had a significantly higher number of proliferating cells than mice at 18 ($P < 0.001$) and 24 (0.0854 ± 0.0262 PCNA + cells/total cells; $P < 0.001$) months of age, but this number was significantly lower than in mice at 12 months of age ($P = 0.018$). In addition, mice at 12 months of age had a significantly higher number of proliferating cells than mice at 18 and 24 months

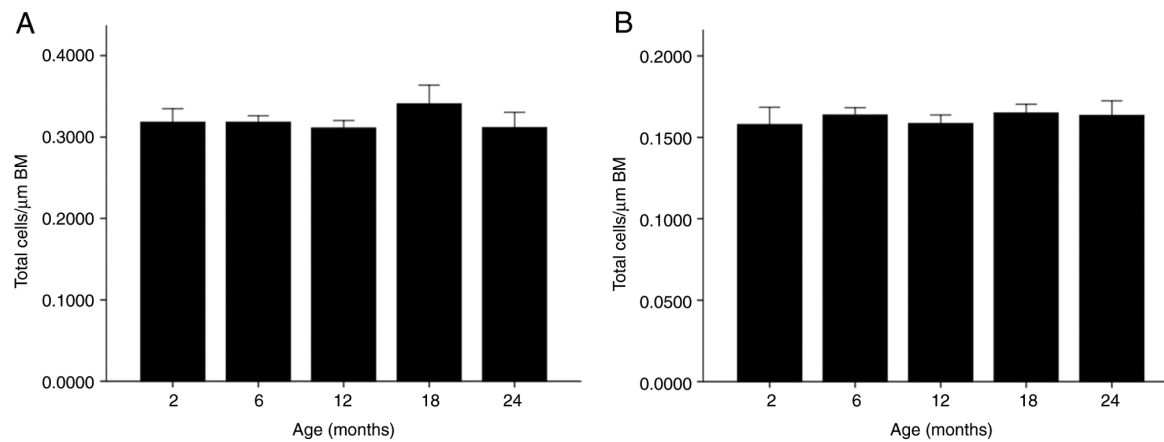


Figure 4. Analysis of total cell number in the MD and MD-TAL of 2-, 6-, 12-, 18- and 24-month-old mice. There was no significant difference in the number of total cells among the analyzed ages in the (A) MD and in the (B) MD-TAL. Values are expressed as the means $\pm$ 1 standard error. Data were analyzed using ANOVA test. Results were considered statistically significant at $P < 0.05$. MD, macula densa; MD-TAL, macula densa-thick ascending limb; BM, basement membrane.

of age (both $P < 0.001$). Finally, mice at 18 months of age had a significantly higher number of proliferating cells than mice at 24 months of age ($P < 0.001$) (Fig. 3A).

Apoptosis

MD. The results of the ANOVA test showed a significant difference in the number of cells undergoing apoptosis among the analyzed ages ($F = 4.1$; $P = 0.003$). The Bonferroni test revealed that the number of cells undergoing apoptosis in mice at 2 months of age (0.0786 ± 0.0214 TUNEL + cells/total cells) was significantly higher than in mice at 12 months of age (0.0037 ± 0.0021 TUNEL + cells/total cells; $P = 0.002$) (Fig. 2B).

MD-TAL. According to the ANOVA test, there was a significant difference in the number of apoptotic cells among the analyzed ages ($F = 4.1$; $P = 0.003$). The results of the Bonferroni test showed that the number of cells undergoing apoptosis in mice at 2 months of age (0.0728 ± 0.0203 TUNEL + cells/total cells) was significantly higher than in mice at 12 months of age (0.0029 ± 0.0015 TUNEL + cells/total cells; $P = 0.002$) (Fig. 3B).

Total cell number

MD. The number of total cells in mice at 2, 6, 12, 18, and 24 months of age was 0.3180 ± 0.0167 , 0.3180 ± 0.0080 , 0.3111 ± 0.0090 , 0.3407 ± 0.0228 , and 0.3114 ± 0.0189 total cells/ μm BM, respectively. The ANOVA results showed that there was no significant difference in the number of total cells among the ages analyzed ($F = 0.594$; $P = 0.669$) (Fig. 4A).

MD-TAL. The number of total cells in mice at 2, 6, 12, 18, and 24 months of age was 0.1577 ± 0.0107 , 0.1636 ± 0.0045 , 0.1584 ± 0.0053 , 0.1648 ± 0.0054 , and 0.1634 ± 0.0089 total cells/ μm BM, respectively. According to the ANOVA test, there was no significant difference in the number of total cells among the analyzed ages ($F = 0.214$; $P = 0.929$) (Fig. 4B).

Discussion

The kidney undergoes complex changes during aging, which predispose it to the development of pathological processes.

The present study analyzed the relationship between cell proliferation and apoptosis (cell turnover) in the MD of mice through the normal aging process.

Due to the reasons previously aforementioned, when performing the analyses, on the one hand, only the cells from the MD were considered, and then separately, the cells from the MD were considered together with the cells from their corresponding TAL (MD-TAL). As can be observed when comparing Fig. 2 with Fig. 3, Fig. 4A with Fig. 4B, and the MD data with the MD-TAL data in Table I, the results obtained from both analyses were very similar. Furthermore, cell turnover was previously evaluated in all tubular structures of the kidney, and it was found that both the number of proliferating and apoptotic cells was different with respect to the MD and each other (40). Thus, the results obtained can be attributed to the cells of the MD.

To date, there is no consensus about the existence of cell turnover in the MD. Most studies have concluded that MD cells may be a stable population in the kidney. Romen *et al* (41) found that cells of the MD are able to proliferate only under extreme conditions, such as in a subtotal nephrectomy, and to a much lesser extent than other tubular cells. Razga and Nyengaard reported that candesartan treatment significantly elevated the total number of MD cells in rats, but through trans-differentiation from normal tubular cells to MD cells (42). Lorenzi *et al* (43) observed that immunohistochemical markers for cell proliferation and apoptosis were negative in MD cells. However, they analyzed only four samples of normal human renal tissue. When these authors investigated the tissue by electron microscopy, they found the presence of degenerating, as well as undifferentiated cells, which led them to hypothesize that the MD could undergo cell turnover. The findings obtained in the present study indicate that cell turnover does indeed occur in the MD.

The pattern of cell turnover associated with MD over the course of aging is revealed in Fig. 2. The number of proliferating cells increased from the first age analyzed (2 months) until reaching its maximum level at 12 months of age. Thereafter, the number of proliferating cells decreased until almost reaching its minimum level again at 24 months of age (Fig. 1E). Notably, apoptosis followed a 'complementary'

pattern to that of cell proliferation. The number of apoptotic cells decreased from the first age analyzed (2 months) until reaching its minimum level at 12 months of age. Thereafter, the number of apoptotic cells increased until approaching its maximum level at 24 months of age (Fig. 1F).

Despite the results described above, there were no significant differences in the total cell number among the ages analyzed in the MD (Fig. 4A). In the older mice investigated (18 and 24 months of age), apoptosis increased while cell proliferation decreased. Conversely, Majumdar *et al* (44) and Vazquez-Padron *et al* (45) found that aging is associated with increased cell proliferation and decreased apoptosis in the colonic mucosa and in vascular smooth muscle cells, respectively. However, in both studies only animals from two age groups (young vs. old) were analyzed, thus it was not possible to establish a pattern of interaction between cell proliferation and apoptosis in the complete aging process.

Robinson *et al* (46) found that aging is accompanied with increased apoptosis in the olfactory epithelium, but they did not assess cell proliferation. In our previous study, an age-related increase in apoptosis accompanied by a decrease in cell proliferation in the mouse bronchiolar epithelium was demonstrated. The change in cell proliferation and apoptosis was almost linear over time, with the number of proliferating cells decreasing continuously from 2 to 24 months of age, and the number of apoptotic cells increasing continuously from 2 to 24 months of age (21). This finding is in contrast to the results revealed in the present study, where changes in cell proliferation and apoptosis were in a discontinuous manner over time.

Thus, the relationship between cell proliferation and apoptosis at a given time in the aging process could be specific, although not exclusive, to the tissue analyzed. In turn, this relationship could depend on molecular factors characteristic of each tissue.

Epidermal growth factor (EGF) plays important roles in normal development and in regenerative and neoplastic growth. One of its specific functions is the promotion of cell proliferation. The kidney expresses markedly high levels of EGF (47). Chou *et al* (48) found that EGF expression decreases in an age-dependent manner in the human kidney. In line with these observations, Shurin *et al* (49) found that the serum levels of EGF and the EGF receptor decrease with age in healthy human donors. Conversely, findings obtained in several studies (50-54) indicate that there is an age-associated increase in renal cell apoptosis, both under basal and stress conditions, that may be due, at least in part, to the mitochondrial pathway. The BCL-2-associated X protein (BAX) plays a crucial role as the executioner protein of mitochondrial-regulated apoptosis (50), while the B-cell lymphoma 2 (BCL-2) protein prevents the initiation of such a process by inhibiting BAX (51). Cytosolic cytochrome *c*, released by mitochondria, triggers apoptosome formation, which in turn activates caspases (52). Caspases are a family of cysteine-dependent aspartate-specific proteases that mediate the cleavage of a broad range of cellular proteins, and thereby induce apoptotic cell death (53). Lee *et al* (54) investigated the expression of the markers aforementioned in the kidneys of 12- and 24-month-old rats. They found that the expression levels of BAX were significantly increased, while those of BCL-2 were significantly decreased in the

24-month-old rats. In addition, the level of cytosolic cytochrome *c* and caspase-3 activation were markedly increased in the aged kidney.

The results from the aforementioned studies (47-54) are in line with those obtained in the present study; an increase in apoptosis and a decrease in cell proliferation in aged mice. However, the total number of cells in the MD of the older mice was not different from that of the other ages analyzed (Fig. 4A). This finding could be due to the fact that the number of proliferating cells per unit of surface was greater than the number of apoptotic cells in the older mice (Table I), or it indicates the possibility of the existence of a source other than proliferation to replace the cells eliminated by apoptosis in the MD. In certain studies that are not related to aging, the existence of trans-differentiation to form MD cells from neighboring tubular cells has been suggested (42,55). More research is required to determine whether this phenomenon actually occurs.

In our previous study, a decrease in the total number of bronchiolar epithelial cells in aged mice, which could predispose them to chronic respiratory diseases, was identified (21). Conversely, an age-related increase in the number of cells has been associated with the development of cancer in tissues such as the stomach and colon (44,56). Since the total number of MD cells did not differ between younger and older mice, other mechanisms may be involved in the deterioration of functions regulated by this structure in aging.

Specific cellular and molecular processes that have been implicated in the development and progression of renal aging include inflammation, autophagy, cellular senescence, and oxidative stress (4-8,57,58). More research is necessary to determine whether these mechanisms occur in the cells of the MD of aged populations, in the same way as has been proven in other kidney cells.

Some limitations of this study must be mentioned. First, since there is a sex-dependent influence on age-related decline in renal function (7,8,12,59), analyses similar to those presented herein should be performed in females. Second, although the sample size ($n=3$) was adequate for the analyses, it could be increased to support and expand the findings in subsequent studies.

Finally, it would be interesting to analyze how the pattern of cell turnover observed at 2 months of age affects postnatal kidney development. In mice, kidney development spans from the embryonic period to approximately the third week of postnatal life (60). During renal development, large numbers of proliferating cells are present throughout the kidney, persisting until after birth. Subsequently, the number of proliferating cells decreases as the last nephrons are formed (61). Toward the end of renal development, cell proliferation is regulated by increased apoptosis (62). Therefore, low levels of cell proliferation and high levels of apoptosis are expected in the mouse kidney at early ages. In the present study, the lowest levels of cell proliferation and the highest levels of apoptosis were identified in the 2-month-old mice (the youngest age analyzed). It is probable that if cell proliferation and apoptosis were evaluated in younger mice, their levels would be even lower and higher, respectively. Given the complexity of kidney structure and function, this topic would be best addressed in a separate study.

In conclusion, in the present study for the first time to the best of our knowledge, the relationship between cell proliferation and apoptosis (cell turnover) in the MD of mice through the normal aging process was analyzed. First of all, the existence of cell turnover in the MD remains a subject of ongoing debate. The present findings indicate the presence of cell turnover in the MD throughout the lifespan of mice. The relationship between cell proliferation and apoptosis appeared highly dynamic. In the oldest mice investigated, apoptosis predominated over cell proliferation, although there were no significant differences in the total number of MD cells across the age groups analyzed. Therefore, mechanisms other than cell turnover could be involved in the deterioration of functions regulated by the MD in aging. Further research is necessary to determine the mechanisms underlying the findings of the present study, as these mechanisms may be the target of therapeutic strategies against age-related kidney diseases in the future.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MOM, GJR, IMM and MdLCB conceived and designed the study. MOM, YGC and KGA acquired the data. AAA, JAR, GJR and JGJ analyzed and interpreted the data. MOM, YGC, KGA and IMM wrote the manuscript. AAA, JGJ, JAR and GJR revised and edited the manuscript. MOM and GJR confirm the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The animal study protocol was approved (approval no. PA19-00001) by the Institutional Review Board and Ethics Committee of the School of Medicine of the Autonomous University of Nuevo León (Monterrey, Mexico).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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