

Gentamicin treatment induces simultaneous mesangial proliferation and apoptosis in rats

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Background. Gentamicin (G)-induced acute renal failure is characterized by an impairment of glomerular function without apparent changes in glomerular structure. However, G stimulates reactive oxygen species (ROS)-mediated mesangial cell proliferation *in vitro*. We studied whether G promotes mesangial cell apoptosis *in vitro*, and if apoptosis and proliferation in parallel may occur in glomerular cells *in vivo* after a renal damage induced by G treatment.

Methods. For *in vivo* studies, rats were treated with G (100 mg/kg body weight/day) for 6 days, and functional and histologic studies were performed. For *in vitro* studies, mesangial cell proliferation and apoptosis were evaluated after 24, 48, and 72 hours of 10^{-5} mol/L G incubation.

Results. After G injections, the number of nuclei per glomerulus did not change, whereas proliferating and apoptotic cell numbers increased. G increases DNA synthesis and cell number in cultured mesangial cells, and increases markedly the apoptotic cell number. ROS scavengers superoxide dismutase and catalase reduce G-induced mesangial cell apoptosis, whereas the incubation with the ROS donor system xanthine plus xanthine oxidase increases apoptosis to levels similar to G. G-induced cellular proliferation and apoptosis either *in vitro* or *in vivo* is associated to an early increase in the pro-apoptotic protein Bax and a delayed increase in the survival protein Bcl-2.

Conclusion. G simultaneously induces proliferation and apoptosis of mesangial cells *in vitro* and glomerular mesangial cells *in vivo*. ROS may mediate G-induced mesangial apoptosis *in vitro*. The equilibrium proliferation/apoptosis may maintain mesangial cell number within normal limits after a G-induced glomerular insult.

The main side effect of gentamicin (G) with clinical relevance is nephrotoxicity. The major effect of G in the kidney is tubular cell toxicity, but prolonged treat-

ments also modify glomerular hemodynamics [i.e., reduces renal blood flow (RBF) and glomerular filtration rate (GFR) without apparent glomerular damage] [1]. G-induced GFR reduction is attributed to a decline either in glomerular plasma flow, ultrafiltration coefficient (Kf), or both [2–4]. Kf regulation depends mainly on intraglomerular mesangial cells' activity because they possess the capacity to contract or relax, thus modifying the ultrafiltration surface, a dynamic phenomenon that is highly regulated by numerous vasoactive substances [5]. Kf reduction observed after G treatment is attributed to mesangial contraction [3, 4]. We reported that G induces dose-dependent mesangial cell contraction and proliferation *in vitro* [6–8]. Nevertheless, despite the fact that G activates cell proliferation, mesangial expansion is not a characteristic of G-induced renal damage either in clinical or experimental studies. We hypothesize that G might also induce apoptosis, returning mesangial cell number to control levels. Apoptosis is involved in cell deletion observed in various glomerular diseases leading to sclerosis [9, 10]; mesangial proliferation followed by apoptosis also occurs in anti-Thy1-induced proliferative glomerulonephritis [11, 12]. Thus, the purpose of this study was to assess if G-induced mesangial cell proliferation *in vitro* is accompanied by apoptosis. Moreover, we assessed whether G treatment *in vivo* simultaneously induces proliferation and apoptosis in glomerular cells. In agreement with our hypothesis, apoptosis has been recently observed in two renal cell lines and in proximal tubular cells after G treatment either *in vitro* or *in vivo*, respectively [13, 14].

Previous *in vivo* and *in vitro* studies strongly suggest the mediation of ROS in the renal effects of G. Administration of antioxidants such as superoxide dismutase (SOD) or dimethyl-thiourea prevented the G-induced GFR reduction [15–17]. Treatment with SOD in G-treated rats is associated to an increase in RBF, and, therefore, superoxide anion (O_2^-) must be responsible for renal vasoconstriction induced by G [15]. ROS scavengers are beneficial in arresting renal damage developed after an injection

Key words: gentamicin, mesangial cells, acute renal failure, reactive oxygen species, apoptosis, proliferation.

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of endotoxin and G [16]. Moreover, ROS are mediators responsible for proximal tubular necrosis and acute renal failure caused by G [18], and of the increased renal susceptibility to G observed in obstructive jaundice [19]. In summary, an enhanced production of ROS, mainly O_2^- , has consistently been involved in the development of G-induced acute renal failure. The sources of ROS enhanced by G are isolated renal cortical mitochondria [20]. Recently, we showed that G elicited O_2^- production in cultured mesangial cells via nitric oxide synthase (NOS) and NAD(P)H oxidase activations. This O_2^- mediate the proliferative response to G [21]. It is also well established that free radicals induce apoptosis. Oxidant-initiated apoptosis is described in mesangial cells [22], and this process is proposed as the mechanism responsible for the loss of such cells in glomerular diseases [23]. Thus, another purpose was to study the mediation of reactive oxygen species (ROS) in the possible apoptotic effect of G in vitro.

METHODS

Materials and reagents

Xanthine (X), xanthine oxidase (XO), catalase (CAT), SOD, propidium iodide, phenylmethylsulfonyl fluoride (PMSF), Nonidet-P40 (NP40), and sodium dodecyl sulfate (SDS) were purchased from Sigma Química (Madrid, Spain). [3 H]-methyl thymidine was from New England Nuclear (Bad Homburg, Germany). Crystal violet was obtained from Fluka (Buchs, Switzerland). Super Sensitive Immunodetection System and levamisol were purchased by Biogenex Laboratories (San Ramón, CA, USA). Mouse antihuman proliferative cell nuclear antigen (PCNA) was from Cormedica Corp. (Medac Diagnostika, Wedel, Germany). Rabbit antihuman Bcl-2, rabbit antimouse Bax, and biotinylated streptavidin-alkaline phosphatase conjugated antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat antirabbit IgG (H+L) and goat antimouse horseradish peroxidase (HRP)-conjugated antibodies were from Bio-Rad (Madrid, Spain). Annexin V-FITC was purchased from PharMingen, BD Biosciences (San Jose, CA, USA). Enhanced chemiluminescence (ECL) Western blotting system was obtained from Amersham (Buckinghamshire, UK). Terminal transferase, biotin dUTP, proteinase K, and RNase A were from Boehringer Mannheim (Barcelona, Spain). Culture medium RPMI 1640 was from Gibco Labs (Barcelona, Spain). Fetal calf serum (FCS) and trypsin solution were from Whittaker Labs (Barcelona, Spain). The sterile plastic material used in cell culture was obtained from Nunc (Roskilde, Denmark). G sulfate was a kind gift of Shering Plough SA (Madrid, Spain). All other reagents were of analytical grade and obtained from Sigma Química, Probus (Madrid, Spain), and Merck (Madrid, Spain).

In vivo studies

G-induced nephrotoxicity in rats. In vivo experiments were carried out in Wistar rats (250 g) placed into metabolic cages in a temperature (20°C)-, light-, and humidity-controlled animal house. Daily subcutaneous injections of G sulfate in saline solution (100 mg/kg body weight) were administered; studies were performed in rats treated with G during 2, 4, and 6 days, and 2 days after finishing the treatment with G during 6 days.

Animals were treated following the Recommendations from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals stated in the international regulations and in the following European and national institutions: Conseil de l'Europe (published in the Official Daily N. L358/1-358/6, 18th December 1986), Spanish Government (published in Boletín Oficial del Estado N. 67, pp. 8509-8512, 18th March 1988, and Boletín Oficial del Estado N. 256, pp. 31349-31362, 28th October 1990).

Functional studies

Urine free of food and feces was collected daily. Urine samples were used to determine creatinine concentration, urinary flow (UF), and urinary protein (UP), sodium (UNa), and potassium (UK) excretions. We also took daily blood samples (0.15 mL) from the caudal vein to determine plasma creatinine concentration (PCr). Plasma and urine creatinine concentrations were determined by a colorimetric method based on the Jaffé reaction. UP was measured by refractometry (Refractometer, American Optical Co., Buffalo, NY, USA). UNa and UK were measured by flame photometry.

Histologic and immunohistochemical techniques. Rats were anesthetized with ketamine (Ketolar, 50 mg/kg body weight, intramuscular); kidneys were perfused through the abdominal aorta with cold isotonic saline. Pieces of each kidney were fixed by immersion in 4% buffered formalin for 24 hours. After dehydration, pieces were embedded in paraffin. Sections (3- μ m thick) were mounted on glass slides and stained with hematoxylin-eosin; additional sections were processed for immunohistochemistry to detect PCNA as previously described [24]. Thirty glomerular sections were examined for each rat, and the number of positively stained nuclei per glomerulus was determined.

In vitro studies

Glomerular isolation and mesangial cell culture. Glomeruli were isolated by successive mechanical sieving of kidneys from 150 g Wistar rats, primary cultures of mesangial cells were obtained as previously described, and the identity of the cells was confirmed by morphologic and functional criteria [25]. A cellular quiescent

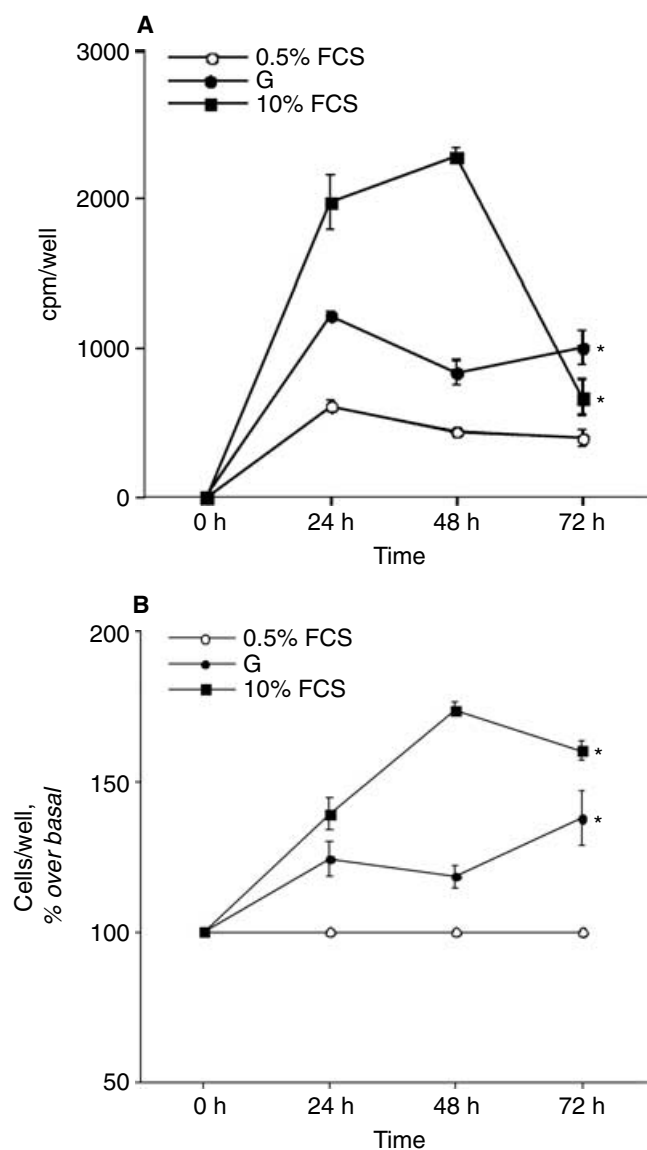


Fig. 1. Effect of gentamicin (G, 10^{-5} mol/L) on [3 H-methyl]thymidine incorporation into DNA (A) and on crystal violet nuclear staining (B) in cultured mesangial cells at different times. The effect of G is compared with the effect in nonstimulated cells incubated in the presence of 0.5% fetal calf serum (FCS). Positive controls are mesangial cells incubated in the presence of 10% FCS. Data are mean \pm SEM of 4 experiments performed by triplicate. *Statistically significant difference ($P < 0.01$) with respect to cells incubated in 0.5% FCS.

state was induced as described [21]. Then, cells were reactivated as described [21] in the presence of G (10^{-5} mol/L), and X (0.2 mmol/L) plus XO (2 mU/mL); in some plates, SOD (15 U/mL) plus CAT (80 U/mL) were added just before G. We previously showed that G stimulates mesangial proliferation, with 10^{-5} mol/L being the concentration that induces the maximum response without cytotoxic effects [6, 8].

Assessment of mesangial cell proliferation. Cell proliferation was measured by both [3 H-methyl]thymidine

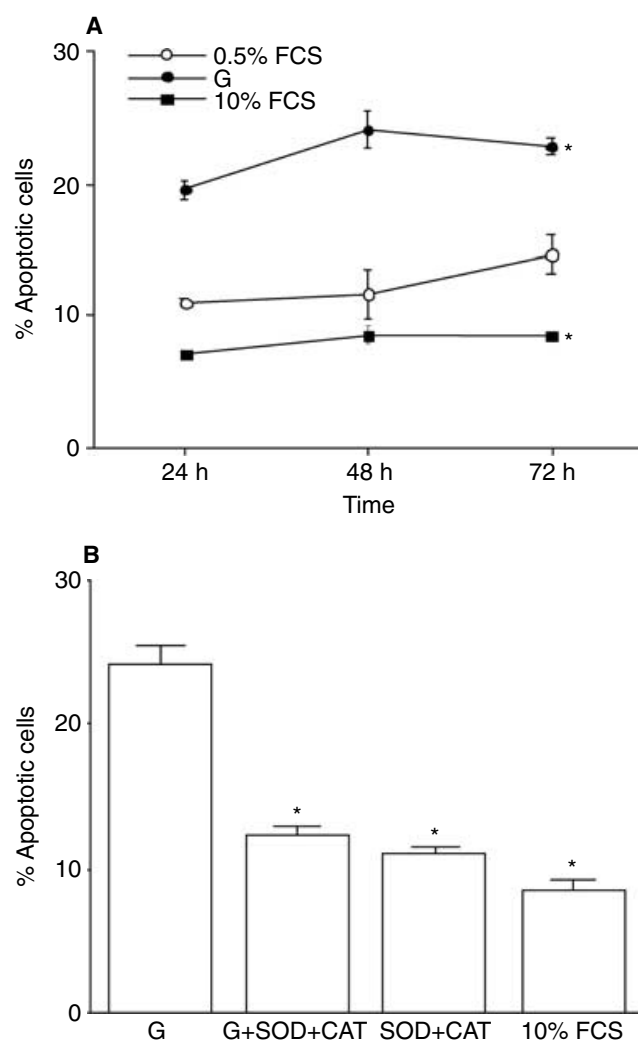


Fig. 2. Effect of gentamicin (G, 10^{-5} mol/L) on DNA fragmentation detected by propidium iodide nuclear staining in cultured mesangial cells at different times (A) or incubated (48 hours) in the presence of reactive oxygen species (ROS) scavengers (B). The effect of G is compared with the effect in nonstimulated cells incubated in the presence of 0.5% fetal calf serum (FCS). Negative controls are mesangial cells incubated in the presence of 10% FCS. Data are mean \pm SEM (expressed as % of stained nuclei respect to the total number of nuclei) of 3 experiments, 8 areas of 30 cells counted in each one. *Statistically significant difference ($P < 0.01$) with respect to cells incubated in 0.5% FCS (A) or with respect to cells incubated with G (B). SOD+CAT: 15 U/mL superoxide dismutase + 80 U/mL catalase.

incorporation into DNA and counting the number of viable cells using the crystal violet method [21]. For this purpose, cells were subcultured by treatment with 0.05% trypsin and 0.02% EDTA, and plated in 6×4 well plates. Experiments were performed on cells approaching confluence from the first passage in order to avoid cell de-differentiation.

Techniques to assess apoptosis. DNA fragmentation associated with apoptosis was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP

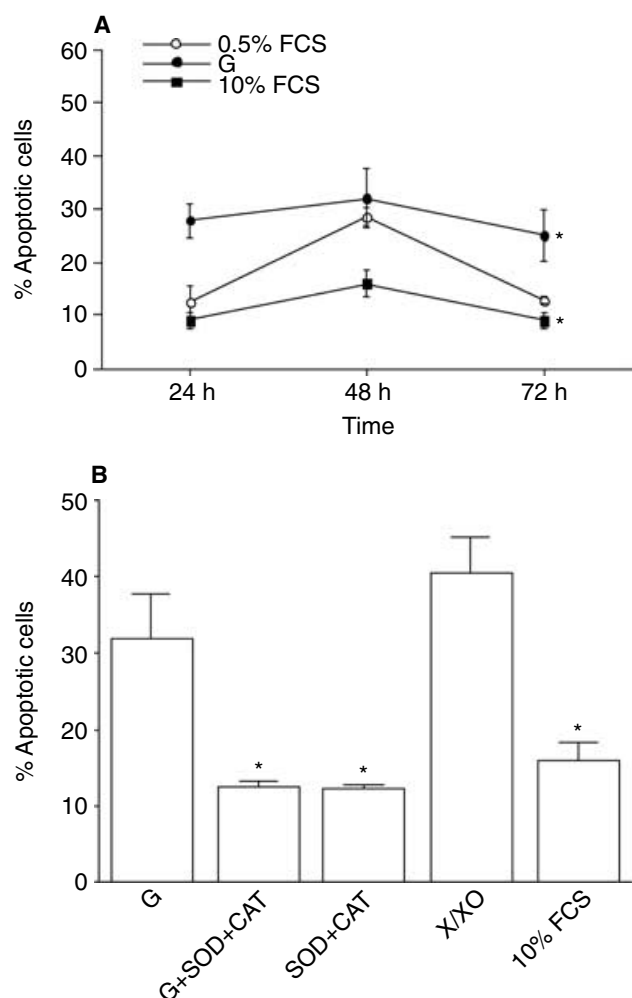


Fig. 3. Effect of gentamicin (G, 10^{-5} mol/L) on DNA fragmentation detected by the TUNEL method in cultured mesangial cells at different times (A) or incubated (48 hours) in the presence of reactive oxygen species (ROS) scavengers and ROS donors (B). The effect of G is compared with the effect in nonstimulated cells incubated in the presence of 0.5% fetal calf serum (FCS). Negative controls are mesangial cells incubated in the presence of 10% FCS. Data are mean \pm SEM (expressed as % of stained cells with respect to the total number of cells) of 3 experiments, 8 areas of 30 cells counted in each one. *Statistically significant difference ($P < 0.01$) with respect to cells incubated in 0.5% FCS (A) or with respect to cells incubated with G (B). SOD+CAT: 15 U/mL superoxide dismutase + 80 U/mL catalase; XIXO: 0.2 mmol/L xantine + 2 mU/mL xantine oxidase.

nick end-labeling (TUNEL) method and by nuclear staining with propidium iodide. For propidium-iodide staining, renal slices (fixed as previously described for histologic techniques) or monolayers of mesangial cells (fixed in 4% buffered formalin) were incubated for 45 minutes at 37°C in phosphate-buffered saline (PBS) (pH 7.4) containing propidium iodide (0.1 μ g/mL) plus RNase A (1 μ g/mL). TUNEL labeling was performed as previously described [10, 26]. Quantification of apoptotic nuclei in the glomeruli of renal slices was performed as previously described for the PCNA.

Previous papers have shown that the enzymatic methods of labeling DNA fragmentation associated with apoptosis can also label necrotic cells with nonspecific DNA degradation, and sometimes transient DNA strand breaks that occurs in mitotic cells [27–29]. In the present study, care was taken to exclude TUNEL-labeled nuclei that had the cellular appearance of mitosis or necrosis. When counting propidium-iodide labeled nuclei, only those with obviously condensed chromatin were counted.

Early apoptosis was assessed using annexin V-fluorescein isothiocyanate (FITC), which binds to negatively charged phospholipid surfaces with a higher specificity for phosphatidylserine than most other phospholipids; translocation of phosphatidylserine to the external cell surface occurs early in the apoptotic process [30]. Annexin V-FITC binding was detected by flow cytometry as previously described [30].

Western blot analysis. Immunoblots of mesangial cells or glomeruli were carried out as previously described [31]. Proteins were separated in 15% SDS-polyacrylamide gel. Primary antibodies (anti-Bcl-2, anti-Bax, and anti-PCNA) and secondary antirabbit IgG or antimouse HRP-conjugated antibodies were used at final concentrations between 0.1 to 1 μ g/mL and 0.01 to 1 μ g/mL, respectively. For quantification, films were digitalized with Adobe Photoshop 3.0, and relative optical densities for each lane were measured using an image analysis program (MacBAS 2.2; Fuji Film, Rochester, NY, USA).

Statistical methods

The Kolmogorov-Smirnov test was used to assess normality of the data distribution. One-way analysis of variances (ANOVA) and Scheffé's test were used for normally distributed data. Kruskal-Wallis multiple comparison Z-value test was used for non-Gaussian data. A P value less than 0.05 was considered statistically significant.

RESULTS

Effect of G on cultured mesangial cells apoptosis and proliferation

The first purpose of our study was to assess the effect of G on proliferation and apoptosis in mesangial cells. 10^{-5} mol/L G induced a significant increase in DNA synthesis and viable cell number, with a maximal increase after 24-hour incubation and a further increase after 72-hour incubation (Fig. 1). G also induced a marked increase in the number of cells showing features of apoptosis assessed by propidium iodide nuclear staining (Fig. 2A), by TUNEL (Fig. 3A), and by flow cytometry using annexin V-FITC (Fig. 4). Thus, these experiments demonstrate that G simultaneously induces proliferation and apoptosis in mesangial cells.

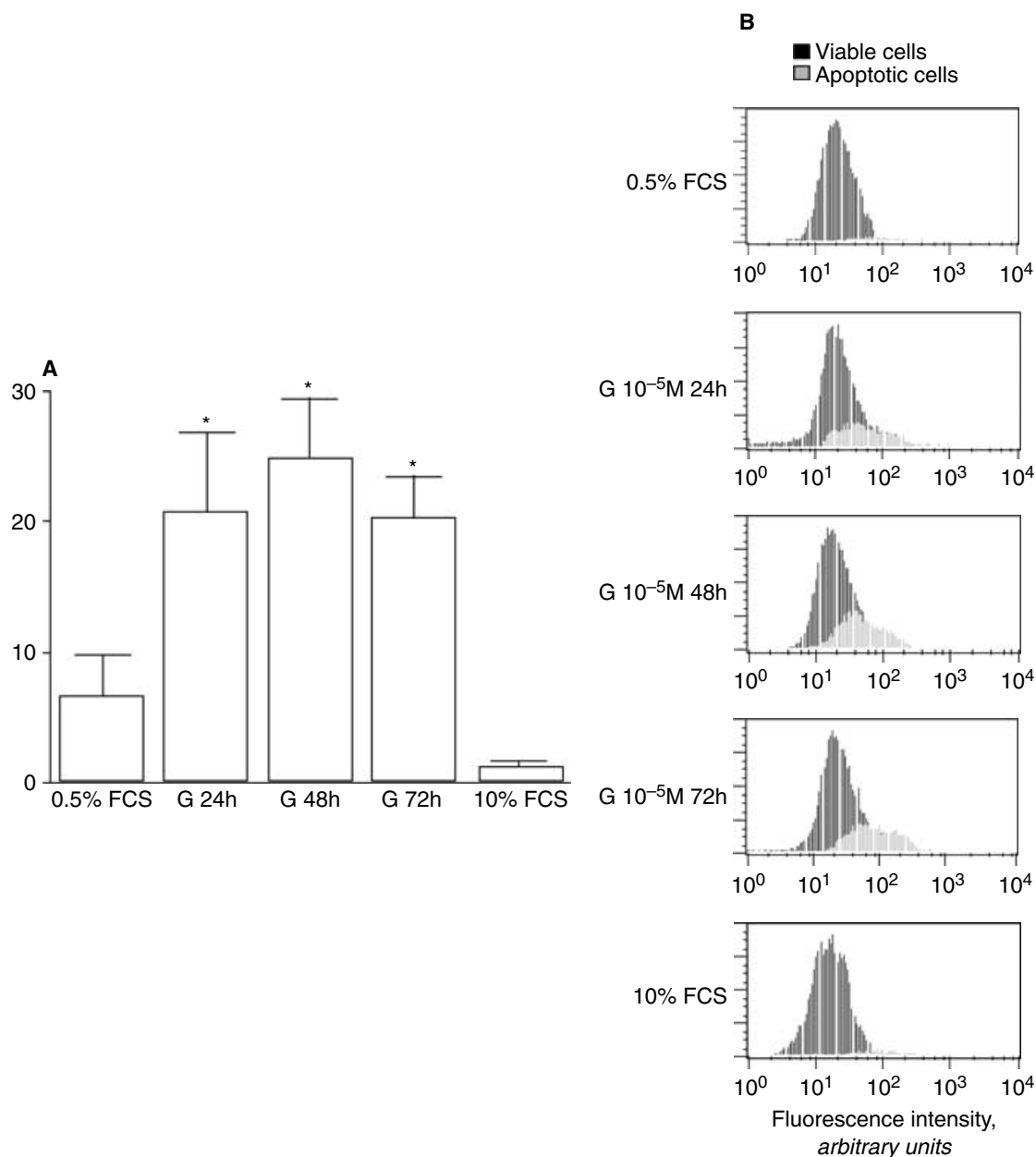


Fig. 4. Effect of gentamicin (G, 10^{-5} mol/L) on apoptosis detected by flow cytometry with annexin V-FITC in cultured mesangial cells at different times. (A) The effect of G is compared with the effect in nonstimulated cells incubated in the presence of 0.5% fetal calf serum (FCS). Negative controls are mesangial cells incubated in the presence of 10% FCS. Data are mean \pm SEM (expressed as % of apoptotic cells with respect to the total number of cells) of 4 experiments performed by triplicate. *Statistically significant difference ($P < 0.01$) with respect to cells incubated in 0.5% FCS. (B) Panels show a single experiment representative of the data shown in (A). Cell number (y axis) and fluorescence intensity of annexin V-FITC (x axis, in logarithmic scale) is expressed in arbitrary units.

To assess if ROS production is involved in the proliferative and apoptotic effect of G, we analyzed the effects of ROS scavengers in mesangial cells. Incubation with SOD (15 U/mL) + CAT (80 U/mL) significantly reduced G-induced mesangial cell apoptosis assessed either by propidium iodide nuclear staining (Fig. 2B) or by TUNEL (Fig. 3B). The ROS donor system X (0.2 mmol/L) + XO (2 mU/mL) induced an increase in the apoptotic rate similar to that induced by G 10^{-5} mol/L (Fig. 3B).

The involvement of Bcl-2/Bax balance in G-induced mesangial apoptosis was studied measuring the expression of both proteins in mesangial cells. The anti-apoptotic protein Bcl-2 is expressed in cells cultured in a serum-deprived medium (0.5% FCS); G induced a significant increase of Bcl-2 expression in quiescent cells only after 72 hours of incubation (Fig. 5A). The proapoptotic protein Bax is expressed in mesangial cells maintained in 0.5% FCS, and this expression increased after 24 hours

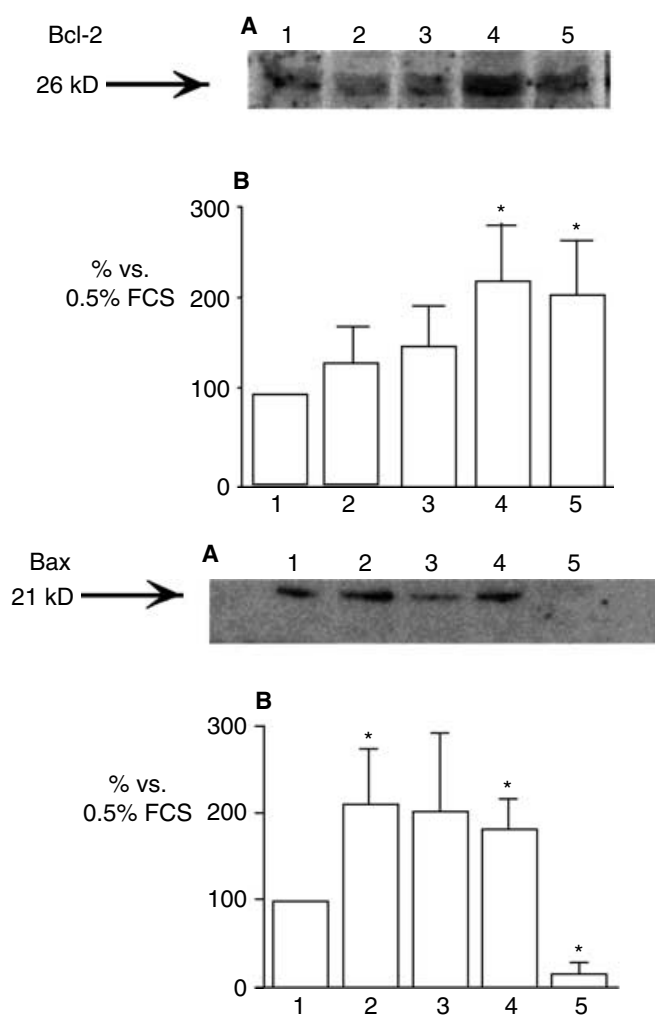


Fig. 5. (A) Expression of the proteins Bcl-2 (upper panel) and Bax in mesangial cells incubated with gentamicin (G, 10^{-5} mol/L) at different times (lane 2: 24 h; lane 3: 48 h; lane 4: 72 h). The effect of G is compared with the effect in nonstimulated cells incubated in the presence of 0.5% fetal calf serum (FCS, lane 1). Positive controls are mesangial cells incubated in the presence of 10% FCS (lane 5). Protein fractions (80 μ g) were size fractionated in a 15% polyacrylamide gel. The figure shows a representative blot of 3 different immunoblots performed under similar conditions. (B) Bars represent optical density (mean \pm SEM) of the bands from 3 different immunoblots, expressed as % of the band obtained in nonstimulated cells incubated with 0.5% fetal calf serum (lane 1).

of incubation with G; Bax expression is minimal in cells maintained in 10% FCS-containing medium (Fig. 5B).

Kidney alterations induced by G treatment in vivo

Treatment with G (100 mg/kg body weight/day) during 6 days induced a polyuric acute renal failure as demonstrated by the increased UF, UNa excretion, and PCr, and by the decreased creatinine clearance (CrCl). These changes persisted 2 days after the last administration of G (Table 1).

Light microscopy examination of renal tissue revealed mild lesions after 4 days of G treatment; patchy cell necrosis appeared in more than 80% of the proximal tubules in rats injected with G during 6 days (Fig. 6B). The tubular lumen was frequently filled with hyaline casts or heterogeneous cellular debris. The same pattern of lesions was observed 2 days after finishing the treatment with G. No apparent structural glomerular alterations were observed.

Glomerular cell proliferation in vivo was assessed by counting the number of glomerular PCNA-positive cells, and by measuring the relative expression of PCNA in glomerular lysates. PCNA-positive cell number increased constantly along the study (Table 2). Cells expressing PCNA seem to be mainly mesangial (Fig. 6D). Glomerular PCNA protein expression increased after 6 days of G treatment and remained elevated 2 days after withdrawal from the treatment (Fig. 7). The number of nuclei per glomerulus did not change with G treatment (Table 2).

Glomerular apoptotic cell number was assessed with two techniques, TUNEL and nuclear staining with propidium iodide (Fig. 8); apoptotic cell death increased after 2 days of G administration and remained constant 2 days after withdrawal of G injections (Fig. 9). Apoptosis seemed to be mainly restricted to mesangial cells (Fig. 8).

The expression of Bcl-2 and Bax was checked by Western blot in glomerular protein fractions. Bcl-2 was not detectable in control rats. A later enhancement in Bcl-2 expression (up to 10 times) appeared in glomeruli after 6 days of G treatment (Fig. 10A). Bax expression increased after 4 days of G treatment (Fig. 10B). The expression of both Bcl-2 and Bax reached maximum values at the end of the study (2 days after G withdrawal).

DISCUSSION

The aims of the present study were to elucidate whether G promotes mesangial apoptosis (parallel with proliferation) in vitro, and then correlate the effect of G in vitro with its effect in vivo by studying cellular proliferation and apoptosis in glomeruli of rats subjected to an acute systemic treatment with G up to 6 days. This study emerged from the fact that a G insult modifies glomerular physiology by targeting the mesangium as part of the cortical alterations that this antibiotic exerts on the kidney [2-4]. Besides, it is widely demonstrated that G activates mesangial cell proliferation in vitro [21]. However, nobody has reported an alteration of glomerular structure, including mesangial cell proliferation, in patients or animals treated with G. Therefore, we hypothesized that the effect of G on glomerular structure might be characterized by mesangial cell proliferation followed by cell apoptosis, thus maintaining glomerular cell number within normal limits. The coincidence of both processes

Table 1. Renal function studies in control rats and in rats treated with gentamicin (G, 100 mg/kg body weight/day) during 2, 4, 6 days, and 2 days after finishing the treatment with G during 6 days (G 6+2 days)

	UF $\mu\text{L}/\text{min}$	PCr mg/mL	CrCl mL/min	UP mg/day	Una mEq/day	UK mEq/day
Control (8 days)	3.54 ± 0.55	10.9 ± 1.6	0.56 ± 0.06	9.0 ± 2.3	0.20 ± 0.05	0.98 ± 0.15
G 2 days	2.73 ± 0.38	15.6 ± 1.5	0.39 ± 0.07	14.7 ± 1.5	0.14 ± 0.02	1.06 ± 0.14
G 4 days	4.36 ± 1.10	12.7 ± 0.8	0.35 ± 0.11	14.0 ± 3.0	0.29 ± 0.07	0.92 ± 0.24
G 6 days	8.77 ± 1.83 ^a	18.5 ± 3.0 ^a	0.27 ± 0.08 ^a	30.6 ± 4.6 ^a	0.53 ± 0.14 ^{a,b,c}	1.39 ± 0.23
G 6+2 days	9.82 ± 1.76 ^a	31.7 ± 3.3 ^{a,d}	0.22 ± 0.05 ^a	71.0 ± 10.8 ^{a,d}	0.48 ± 0.10 ^{a,b}	1.38 ± 0.19

Renal functional parameters measured were: urinary flow (UF), plasma creatinine (PCr), creatinine clearance (CrCl), proteinuria (UP), urinary sodium (UNa), and urinary potassium (UK) excretions. Control rats received a similar volume of saline solution. Basal values of all functional parameters were obtained from each rat before starting the treatment ($N = 50$, data not shown), and there were no differences with respect to control data after 8 days of saline solution injections. Data are shown as mean ± SEM, $N = 10$ rats in each group. Statistical significant differences ($P < 0.05$) were: ^a vs. control; ^b vs. group treated with G 2 days; ^c vs. group treated with G 4 days; ^d vs. groups treated with G 2, 4, and 6 days.

Table 2. Total number of cells and number of proliferating cells (PCNA positive) per glomerular cross section in control rats and in rats treated with gentamicin (G, 100 mg/kg body weight/day) during 2, 4, 6 days, and 2 days after finishing the treatment with G during 6 days (G 6+2 days)

	Number of proliferating cells	Total number of cells
Control	0.58 ± 0.09	48.8 ± 6.2
G 2 days	1.57 ± 0.12 ^a	59.4 ± 8.9
G 4 days	1.99 ± 0.22 ^a	54.4 ± 7.9
G 6 days	2.19 ± 0.34 ^a	54.6 ± 7.5
G 6+2 days	2.69 ± 0.56 ^a	44.6 ± 6.8

Data are expressed as mean ± SEM of the number of nuclei counted in 50 glomerular cross-sections obtained per rat in 10 rats sacrificed at each of the indicated times. ^a $P < 0.001$ vs. control.

was proposed as the mechanism involved in the glomerular cell loss observed in various glomerular diseases [9–12].

Gentamicin induces ROS-mediated mesangial cell apoptosis in vitro

An oxidative stress has been linked to the renal dysfunction caused by systemic treatment with G [15–20]. We demonstrated that G and ROS, individually, can stimulate glomerular and mesangial cell contraction and proliferation in vitro [6, 8, 32]. More recently we described that mesangial cells exposed to G produced O_2^- through NAD(P)H oxidase and NOS activation; this O_2^- mediates the proliferative response to G [21]. Thus, we assessed a possible apoptotic effect of G on glomerular mesangial cells and the role of ROS as mediators of this process.

The results of this study demonstrate that the maximal noncytotoxic concentration of G (10^{-5} mol/L) induces an increase in proliferation and apoptosis in mesangial cells. An apoptotic effect of G in vitro was also recently recognized in embryonic fibroblasts and in two renal epithelial cell lines (LLC and MDCK cells) exposed during 4 days to concentrations up to 3×10^{-6} mol/L [14] or for 15 days to concentrations of 10^{-5} mol/L [33].

This study suggests that O_2^- are involved in G-induced mesangial apoptosis because the O_2^- donor system X

plus XO enhanced the number of apoptotic cells in a degree quantitatively similar to that induced by G. Moreover, G-induced apoptosis is inhibited by incubation with SOD plus CAT, a ROS scavenger system. There is increasing evidence showing that pro-oxidant stimuli elicit an apoptotic response in mesangial cells [22, 23, 34–37]. In addition, glomerular and mesangial apoptosis induced by tumor necrosis factor (TNF)- α and interleukin (IL)-1 α is mediated by ROS [36]. In agreement with our hypothesis, O_2^- has been recognized as a selective mediator for TNF- α -induced apoptosis of rat mesangial cells [38].

A balance between Bax and Bcl-2 proteins seems to play a major role in the regulation of cellular apoptosis and survival [39]. We observed that Bax is expressed in mesangial cells maintained in a serum-deprived medium, and that its expression increased early after G addition. These results agree with the known proapoptotic role of Bax. The mesangial content of Bcl-2, which is known to block programmed cell death in quiescent mesangial cells, was enhanced after 72 hours of incubation with G. Therefore, G induced an oxidant-mediated apoptosis in mesangial cells characterized by an early elevation in Bax and a delayed increase in Bcl-2. An up-regulation of Bcl-2 expression has been also observed in an ovary cell line in response to geneticin (G418), an aminoglycoside rather similar to G [40]. Bcl-2 localizes at the sites of ROS generation, and is recognized to prevent oxidative damage. Thus, a similar Bcl-2 overexpression was described to have a protective role against oxidant-induced apoptosis in a neural cell line [41] and in mesangial cells [42]. In addition, Bcl-2 itself can function as an antioxidant to prevent apoptosis [43, 44]. The delay in Bcl-2 increase in G-stimulated mesangial cells is compatible with the hypothesis, suggesting that it can be overexpressed in later phases of the apoptotic process to protect cells against further apoptosis [45].

Gentamicin induces glomerular cell proliferation and apoptosis in vivo

The present study confirms that a daily injection of G at the dose of 100 mg/kg body weight induced a polyuric

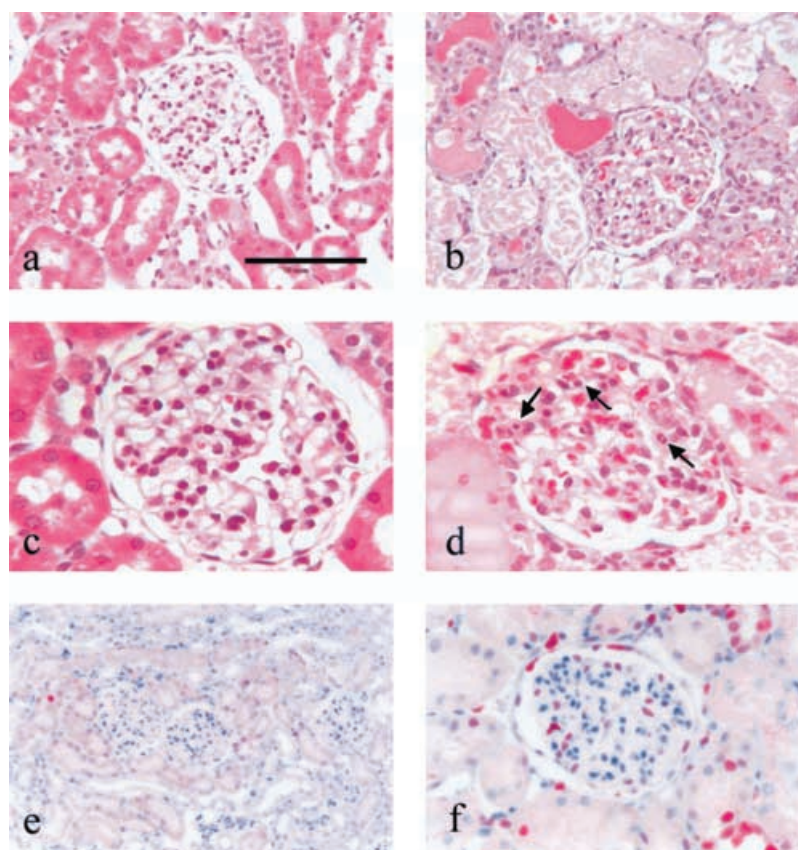


Fig. 6. Light micrographs of sections from kidneys stained with hematoxylin-eosin (A-D) and immunohistochemistry for PCNA (E and F). (A, C) Normal histology of kidney tissue in control rats. (B) Massive tubular necrosis with hyaline casts is observed in rats treated with gentamicin (G, 100 mg/kg body weight/day) during 6 days, and also several mesangial cells apoptotic nuclei (D, arrows). (E) Only occasional nuclei expressing PCNA are observed in some tubules from control animals. (F) Numerous tubular and renal corpuscular cells are expressing the antibody in rats treated during 6 days with G. Bar is 100 microns in A, B, and F; 50 microns in C and D, and 200 microns in E.

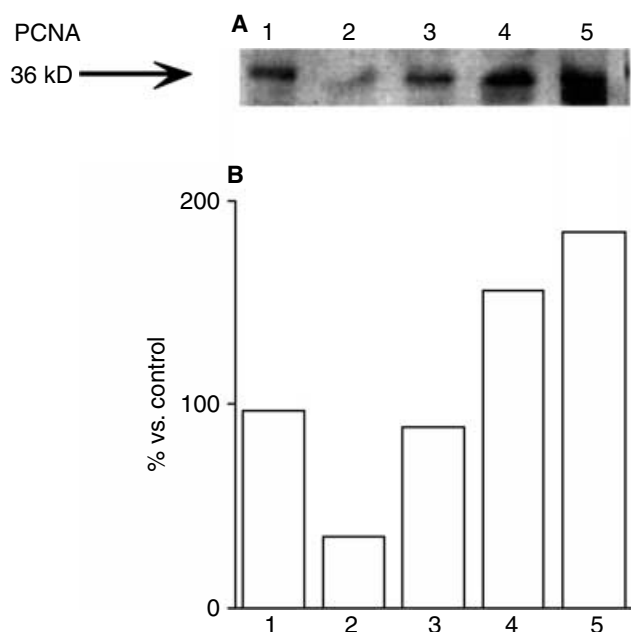


Fig. 7. (A) Expression of the protein PCNA in glomeruli of rats treated with gentamicin (100 mg/kg body weight/day) (lane 2: 2 days; lane 3: 4 days; lane 4: 6 days; lane 5: 6 days plus 2 days maintained without treatment). Protein fractions (80 μ g) were size fractionated in a 15% polyacrylamide gel. The figure shows a representative blot of 3 different immunoblots performed under similar conditions. (B) Bars represent optical density of the bands expressed as % of the band obtained in untreated rats (lane 1).

acute renal failure evident after 6 days that persists, and even aggravates, 2 days after. Our study also describes that G treatment in vivo induces a simultaneous increase in glomerular cell proliferation and apoptosis without changes in cell number. G apoptotic effect has been widely described in ciliated cells of vestibular sensory epithelium, where apoptosis seems to be the fate of the majority of hair cells following a G insult [46]. The few studies dealing with the apoptotic effect of G in the kidney are restricted to the tubular epithelium, mainly to proximal cells. Nouwen et al [27] demonstrated that after G treatment, the strongly increased cell proliferation in regenerating necrotic proximal cells was preceded by an equally important proliferation in the cortex distal tubules and outer stripe of the outer medulla, in the absence of necrosis, but displaying enhanced apoptosis. More recently, El-Moueden et al [13] showed apoptosis in the proximal epithelium of rats treated with low therapeutic doses of G (up to 20 mg/kg/day) during 10 days, whereas other aminoglycosides, even at higher doses, appeared safer in this respect. They also observed tubular and peritubular cell proliferation. Therefore, this is the first description of glomerular apoptosis induced by G in vivo.

Glomerular apoptosis induced by other pharmacologic agents in vivo remains to be studied. Cha et al [47] demonstrated that mesangial cells exposed in vitro to cytotoxics

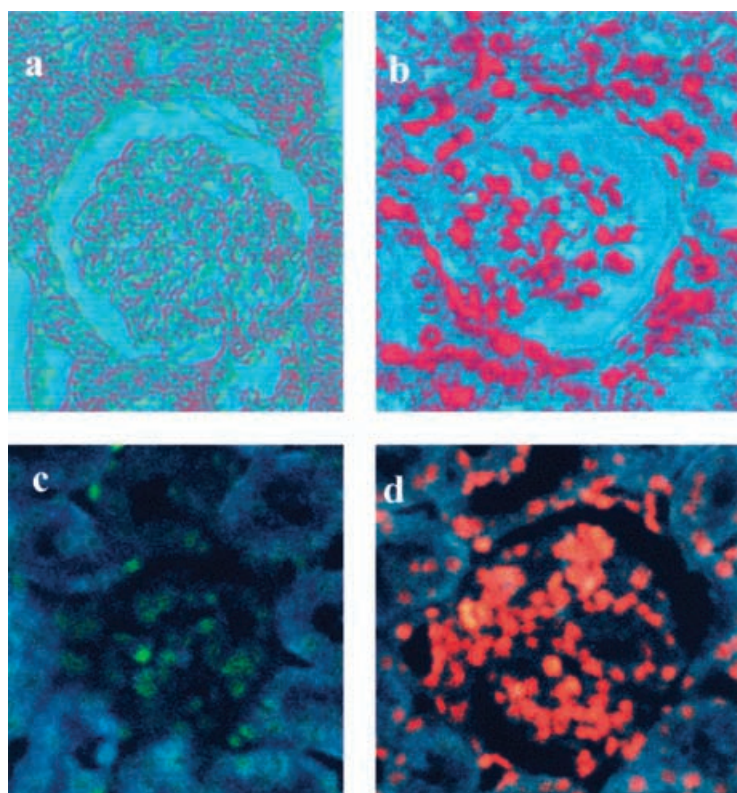


Fig. 8. Representative images of renal cortical slices of rats treated with gentamicin (100 mg/kg body weight/day during 6 days) after nuclear staining with the TUNEL method (a and b) or with propidium iodide (c and d). In (b), nuclei with DNA fragmentation show a more intensive coloration; in (d), apoptotic cells were readily identifiable in an inverted fluorescence microscope because they show a striking nuclear staining with propidium iodide. Controls are shown in (a) and (c). Images were color-enhanced using the Microsoft Photoeditor, and in the case of TUNEL staining, color was inverted to show a better contrast and improve the nuclei counting.

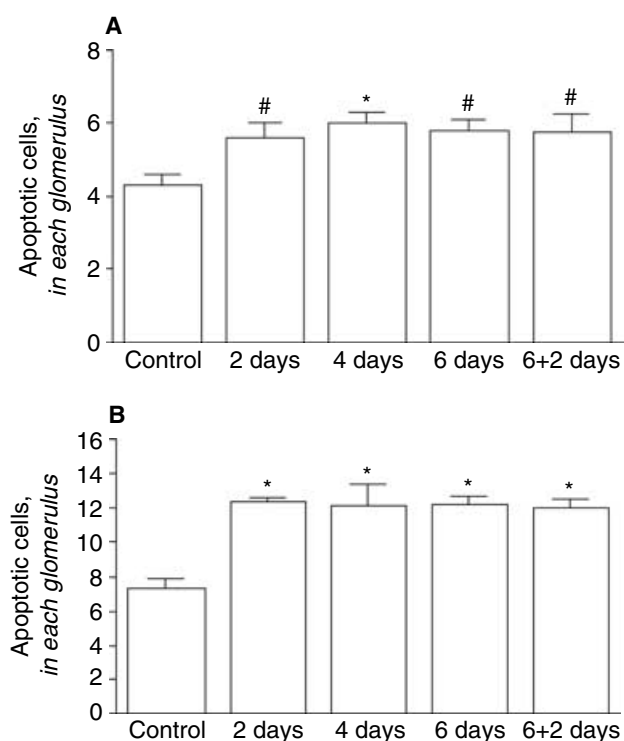


Fig. 9. DNA fragmentation detected by propidium iodide nuclear staining (A) and by the TUNEL method (B) in glomeruli of rats treated with gentamicin (100 mg/kg body weight/day) during 2, 4, and 6 days, and 2 days after 6 days of treatment (6+2 days). Data are mean \pm SEM (expressed as % of stained nuclei with respect to the total number of nuclei) of 200 glomeruli analyzed per group. Statistically significant differences were * $P < 0.01$ and $\dagger P < 0.05$ with respect to untreated control rats.

or irradiation showed apoptosis identified by light microscopy; these authors suggest that cytotoxic drugs used to treat glomerular disease can induce apoptotic mesangial death in part via this mechanism. However, they have studied the effect of these drugs only in vitro.

Glomerular cell apoptosis appears to be associated with a delayed increase in Bcl-2 expression in the last days of treatment that tends to be higher after withdrawal of G. Recent reports are in agreement with our observations because they show renal apoptosis associated with increased Bcl-2 in ischemic acute renal failure [29]. During kidney tubular damage, DNA damage may manifest itself as reparable single- and double-strand breaks [48, 49]. It can be hypothesized that cell protection via Bcl-2 up-regulation may allow time for DNA strand repair to occur. The cells in which DNA repair is not completed continue on to apoptosis.

Interestingly, we found that G induces glomerular proliferation and apoptosis simultaneously. Proliferation and apoptosis also coincide in tubular, interstitial, and glomerular cells in a model of chronic obstructive uropathy [50], and in a subtotal-nephrectomy model of chronic renal failure correlating with the progression of renal fibrosis [51]. Glomerular proliferation and apoptosis have been shown in several glomerulosclerotic lesions [9, 11, 12] and in glomeruli of uninephrectomized spontaneously hypertensive rats [10].

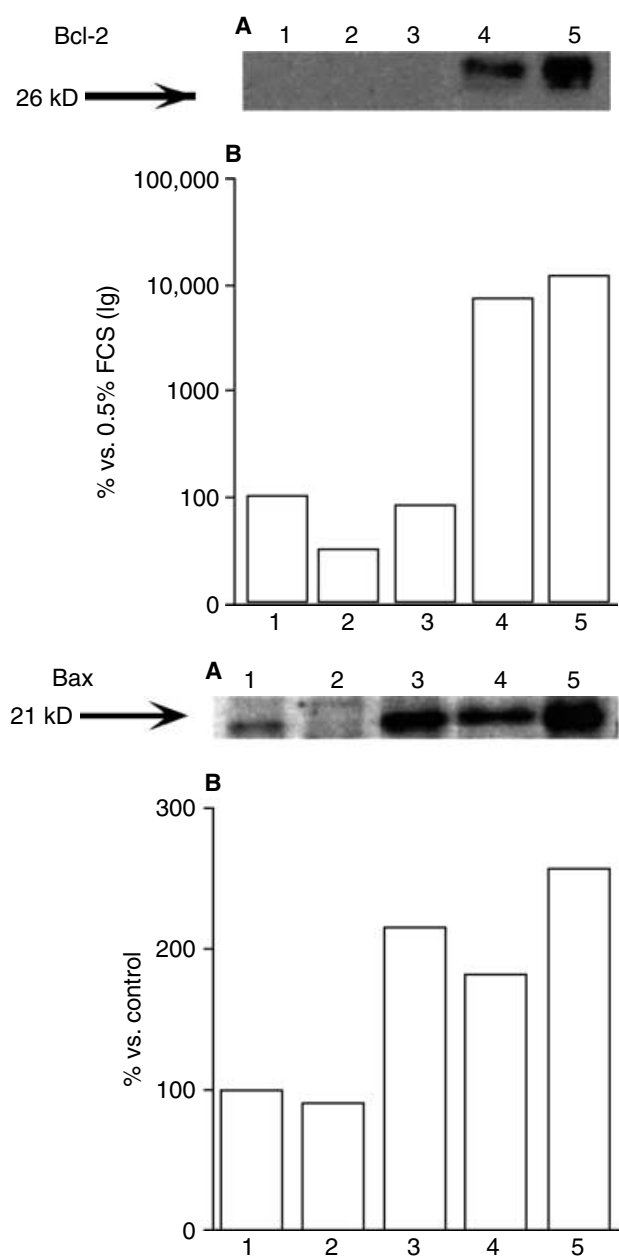


Fig. 10. (A) Expression of the proteins Bcl-2 (upper panel) and (B) Bax (lower panel) in glomeruli of rats treated with gentamicin (100 mg/kg body weight/day) (lane 2: 2 days; lane 3: 4 days; lane 4: 6 days; lane 5: 6 days plus 2 days maintained without treatment). Protein fractions (80 μ g) were size fractionated in a 15% polyacrylamide gel. The figure shows a representative blot of 3 different immunoblots performed under similar conditions. (B) Bars represent optical density of the bands expressed as % of the band obtained in untreated rats (lane 1).

CONCLUSION

This is the first observation of an apoptotic effect induced by G in mesangial cells in vitro; this apoptosis seems to be mediated by an elevation in ROS production (at least, O_2^-). Simultaneously, G induces mesangial cell proliferation. In addition, G treatment in vivo also induces glomerular cell proliferation and apoptosis

(mainly in the mesangium). G-induced proliferation and apoptosis either in vitro or in vivo are associated with an early increase in the proapoptotic protein Bax and a delayed increase in the survival-promoting protein Bcl-2. We propose that the simultaneous occurrence of cellular proliferation and apoptosis may be a mechanism regulating glomerular cell number after acute treatment with G.

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