Granulocyte Colony Stimulating Factor Prevents Kidney Infarction and Attenuates Renovascular Hypertension

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Key Words

Granulocyte colony stimulating factor (G-CSF) • Renin-angiotensin system • Renovascular hypertension • Kidney infarct • Mice

Abstract

Background: G-CSF is a critical regulator of hematopoietic cell proliferation, differentiation and survival. It has been reported that G-CSF attenuates renal injury during acute ischemia-reperfusion. In this study we evaluated the effects of G-CSF on the renal and cardiovascular systems of 2K1C hypertensive mice. **Methods:** Male C57BL/6 mice were subjected to left renal artery clipping (2K1C) or sham operation and were then administered G-CSF (100 µg/kg/day) or vehicle for 14 days. **Results:** Arterial pressure was higher in 2K1C + vehicle animals than in 2K1C + G-CSF (150±5 vs. 129±2 mmHg, $p<0.01$, $n=8$). Plasma angiotensin I, II and 1-7 concentrations were significantly increased in 2K1C + Vehicle when compared to the normotensive Sham group. G-CSF prevented the increase of these vasoactive peptides. The clipped kidney/contralateral kidney weight ratio showed a less atrophy of the ischemic kidney in the

treated group (0.50±0.02 vs. 0.66±0.01, $p<0.05$). The infarction area in the clipped kidney was completely prevented in 7 out of 8 2K1C + G-CSF mice. Administration of G-CSF protected the clipped kidney from apoptosis. **Conclusion:** Our data indicate that G-CSF prevents kidney infarction and markedly attenuates the increases in plasma angiotensin levels and hypertension in 2K1C mice, reinforcing the protective effect of G-CSF on kidney ischemia.

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Introduction

Granulocyte colony-stimulating factor (G-CSF) promotes the proliferation and differentiation of myeloid progenitor cells including the mobilization of bone marrow stem cells [1-4]. G-CSF exerts reparative and regenerative effects in various ischemic tissues, including heart [3, 5], brain [6] and kidney [7]. Additionally, pretreatment with G-CSF attenuates renal ischemia/reperfusion injury, and this cytoprotection seems to be associated with its anti-apoptotic action [8].

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Even though the kidney has a remarkable capacity to regenerate after injury, progressive and irreversible insults result in chronic renal disease [9]. However, little is known about the potential of G-CSF in protection against permanent kidney injury. The experimental murine model of renovascular hypertension, which is induced by partial clipping of the left kidney artery [10], seems suitable to test the protective actions of G-CSF in a form of persistent renal ischemia/infarction not yet studied. Moreover, in this model of hypertension, the reduced perfusion pressure in the artery of the clipped kidney stimulates the renin-angiotensin system.

Therefore, in the present study we tested the hypothesis that G-CSF has a cytoprotective effect on renal ischemia/infarction and that this could help prevent or attenuate the development of renovascular hypertension in the mouse.

Materials and Methods

Animals

The experiments were performed with 8- to 9-week-old male C57BL/6 mice (n=62), averaging 23 g, obtained from the breeding facilities of the Health Sciences Center at the Federal University of Espirito Santo. The animals were housed according to the institutional guidelines for animal research, including constant room temperature, 12-hour light/dark cycle, 50±5% humidity, standard mouse chow and water *ad libitum*. The procedures were previously approved by the Animal Use Ethics Committee of the Research Center of Emescam College of Health Sciences (Protocol # 006/2009).

Two-kidney, one-clip (2K1C) hypertension was produced using stainless steel clips (0.12 mm opening width, Exidel SA, Switzerland). The clip was placed around the left renal artery according to the procedure described by others [10-12]. Age-matched Sham mice were used as control animals. Immediately after surgery, groups of 2K1C and Sham mice were administered recombinant human G-CSF (Filgrastim®, Bio Sidus S.A., Argentina; 100 µg/kg/day, s.c.) for 14 days. An equal number of animals per group received vehicle (5% glucose solution).

Surgical Procedures for Hemodynamic Measurements

Mice were anesthetized with ketamine/xylazine (91.0/9.1 mg/kg, i.p.), and catheters (0.040 mm O.D. x 0.025 I.D., Micro-Renathane, Braintree Science Inc., MA, USA) were inserted into the right carotid artery for direct measurement of mean, systolic and diastolic arterial pressure and heart rate. Experiments were performed in conscious, freely moving mice 48 hours after catheter placement. For mean arterial pressure and heart rate recordings, the arterial catheter was plugged into a disposable blood pressure transducer (Cobe Laboratories Inc., Lakewood, CO, USA) and connected to a pressure processor amplifier and data acquisition system (MP100, Biopac Systems Inc., Goleta, CA, USA).

Biochemical Analyses

Mice were placed in metabolic cages for a 24-hour adaptation period followed by another 24-hour period for biochemical analysis of urine. After that, animals were euthanized, and their blood was collected for creatinine and urea measurements. Plasma and urinary creatinine were measured by Jaffe's method using a creatinine test kit (Bioclin®, Belo Horizonte, Brazil). Proteinuria was measured in urine samples by spectrophotometry (SP-220, Bioespectro, Sao Paulo, Brazil) after endpoint reaction with a colorimetric kit (Sensiprot, Labtest Diagnostica S.A., Sao Paulo, Brazil).

Blood and Tissue Samples

Animals were euthanized in a CO₂ chamber. Immediately after, blood samples were collected from the right ventricle to determine hematological features using a T890 Hematology Analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Mononuclear cells (MNCs) were isolated from both femur and tibia bone marrow and from the spleen using a gradient (Histopaque® - 1083, Sigma-Aldrich, Saint Louis, MO, USA) and then counted in a Neubauer chamber. Ventricles, kidneys and spleen were excised and dried for 48 h in an oven at 37°C for weight determination. Ventricular weight index was calculated as dry weight/body weight (mg/g). The left kidney weight/right kidney weight ratio was also calculated.

Measurements of Plasma Angiotensin Levels

Blood was collected in the presence of EDTA and protease inhibitor cocktail (Product # P2714, Sigma-Aldrich), and samples were centrifuged at 9.5 g for 15 min in a refrigerated centrifuge (4°C) to remove the plasma for later analysis. Angiotensins were extracted in Oasis C18 columns previously activated with methanol (5 mL), tetrahydrofuran (5 mL), hexane (5 mL), methanol (5 mL) and water (10 mL). After activation, the samples were applied to the columns, washed with water and eluted in ethanol/acetic acid/water in the proportions 90% - 4% - 6%. The eluates were dried, redissolved in 500 µL of mobile phase A (5% acetonitrile in 0.1% phosphoric acid) and filtered for analysis by high performance liquid chromatography (HPLC). The angiotensin in each sample was separated in a reversed-phase ODS Aquapore300 (250 x 4.6 mm) HPLC column, 7µm particle size (PerkinElmer's Brownlee Columns, Norwalk, USA), using the gradient 5–35% of mobile phase B: 95% acetonitrile in 0.1% phosphoric acid under a flow of 1.5 mL/min for 40 min. The angiotensins were identified by comparing them with the retention times of standard angiotensins.

Histological Kidney Processing and Morphometry

Kidneys were removed and fixed in 4% buffered formaldehyde solution for 48 hours, embedded in paraffin, and sectioned at 5 µm thicknesses, and slides were stained with hematoxylin-eosin (Sigma-Aldrich). Evident color differences in the captured images of kidney infarct areas stained with hematoxylin-eosin were quantified using Image J software (1.33u, Public Domain; National Institutes of Health, Bethesda, USA). Picrosirius red for collagen I-specific staining was performed to evaluate the effect of G-CSF on kidney glomerulosclerosis. A total of 20 glomeruli were randomly used

to calculate the percentage of stained area of each kidney at 400x magnification. Images of individual glomerular areas were captured with a color video camera (VKC150, Hitachi, Tokyo, Japan) connected to a microscope (AX70, Olympus, Center Valley, PA, USA) and quantified using Image J. In all histological and morphometric analyses, the examiner was blinded to the experimental groups.

TUNEL Assay

A separate group of 2K1C mice (n=10) was sacrificed 48 hours after renal artery clipping for evaluation of cell death by measuring DNA fragmentation using an *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) Cell Death Detection Kit, POD (Product # 11 684 817 910, Roche, Mannheim, Germany). TUNEL assay was performed on paraffin-embedded sections from cortex of ischemic kidney. Typical images were captured with a color video camera (Nikon Digital Sight DS - U2) connected to a fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) by using excitation wavelengths in the range of 450–500 nm and detection in the range of 515–565 nm (green). DNase-treated (Macherey-Nagel, Düren, Germany) sections were used as positive controls, according to the protocols of the manufacturer. TUNEL-positive tubular cells in proximal and distal tubules were counted from 5 different cortical fields and averaged in each section (4/animal), by using a high-power-field (hpf, x200). All tissue sections were viewed and labeled a blinded examiner.

Statistical Analyses

All data are expressed as means±SEM. The means of groups and the interaction of factors were compared by two-way ANOVA followed by the *post hoc* Fisher test or Student's *t*-test for independent samples when appropriate. The level of significance was set at $p < 0.05$.

Results

Body weight, cardiac weight index, and hemodynamic parameters

Initial body weight was statistically similar among groups. At the end of experiments, body weight was reduced ($p < 0.05$, two-way ANOVA) in hypertensive groups (Vehicle: 23.1 ± 0.4 g and G-CSF: 22.8 ± 0.2 g) when compared to their respective Sham groups (Vehicle: 24.2 ± 0.4 g and G-CSF: 23.5 ± 0.3 g). As shown in Fig. 1 (bottom panel), cardiac weight index was significantly higher in the 2K1C than in the Sham (1.31 ± 0.06 vs. 1.05 ± 0.02 mg/g, $p < 0.01$, two-way ANOVA) group, without significant effects of G-CSF in between the 2K1C and Sham groups (1.22 ± 0.03 vs. 1.31 ± 0.06 mg/g).

Figure 1 shows the effect of G-CSF administered for 14 days on hemodynamic parameters in hypertensive animals. As expected, 14 days after renal artery clipping, 2K1C mice showed arterial hypertension (systolic,

Fig. 1. Bar graphs showing the effects of G-CSF administration for 14 days on hemodynamic parameters in normotensive (Sham, n=8) and hypertensive (2K1C, n=8) mice. MAP: mean arterial pressure, HR: heart rate, VW/BW: ventricular weight/body weight. * $p < 0.01$ vs. 2K1C vehicle group; ## $p < 0.01$ vs. respective Sham group. Values are mean ± SEM.

diastolic and mean arterial pressure: 182 ± 7 , 119 ± 2 , 150 ± 5 mmHg, respectively) when compared to the Sham group (128 ± 2 , 82 ± 1 , 105 ± 1 mmHg, respectively; $p < 0.01$, two-way ANOVA). G-CSF did not modify arterial blood pressure in normotensive animals but caused a significant attenuation of hypertension (about -23 mmHg) in 2K1C animals ($p < 0.01$, for interaction). As expected, hypertension in 2K1C animals was accompanied by tachycardia, which was not significantly affected by the G-CSF.

Hematological features

The effects of G-CSF on hematological features, mononuclear cells (MNCs) number and spleen weight in Sham and 2K1C animals are summarized in Table 1. 2K1C hypertensive animals showed a significant increases in the numbers of leukocytes and the subpopulation of

Parameters	Sham		2K1C	
	Vehicle (8)	G-CSF (8)	Vehicle (8)	G-CSF (8)
Leukocytes/mm ³	2370±288	8057±1820**	4495±487 [#]	12413±2366* [#]
Neutrophils/mm ³	355±98	3142±815*	1438±272 [#]	5463±869
Lymphocytes/mm ³	1836±95	4673±807*	2877±315	6455±997
Erythrocytes/mm ³	7889±616	6800±347	9169±763	7188±494
Hemoglobin (g/dL)	11±1	11±0.4	14±1	12±1
Hematocrit (%)	37±2	32±1	41±3	34±2
Platelets/mm ³ ×10 ³	800±51	798±142	629±61	633±60
BM MNCs/mL ×10 ⁷	2.310±0.148	4.594±0.780**	2.118±0.162	3.803±0.437
Spleen MNCs/mL ×10 ⁷	1.526±0.206	4.568±1.021*	2.068±0.750	11.316±3.439* [#]
Spleen weight (mg)	17±1	26±3**	18±1	31±1* [#]

Table 1. Effects of G-CSF administration on hematological features, MNCs number and spleen weight in normotensive (Sham) and hypertensive (2K1C) mice. Values are mean ± SEM from the number of animals indicated in parentheses. BM, bone marrow; MNCs, mononuclear cells; *p< 0.05 and **p< 0.01 vs. respective Vehicle group; [#]p<0.05 vs. respective Sham group (two-way ANOVA).

neutrophils when compared to Sham normotensive animals (p<0.05, two-way ANOVA). G-CSF caused a significant increase in the number of white blood cells in both normotensive (p<0.01, two-way ANOVA) and hypertensive (p<0.05) animals when compared to their respective vehicle-treated groups. The number of neutrophils was significantly augmented by both hypertension (about 4-fold) and G-CSF administration (about 9-fold) when compared to Sham normotensive animals. The number of lymphocytes was significantly increased (about 2.5-fold) only in normotensive animals treated with G-CSF. The numbers of other white blood cells, such as monocytes, eosinophils and basophils, were influenced neither by hypertension nor by G-CSF administration (data not shown). Neither hypertension nor G-CSF administration caused a significant change in erythrocytes number, platelet number, hemoglobin, or hematocrit. MNCs were elevated in tibia and femur bone marrow from normotensive animals treated with G-CSF (about 2-fold). MNCs from the spleen were significantly augmented by G-CSF administration (about 3-fold) and additively by hypertension (about 5.5-fold). G-CSF administration caused a significant increase in the spleen dry weight in both normotensive (p<0.01, two-way ANOVA) and hypertensive (p<0.05) animals.

Plasma angiotensin

Figure 2 shows average values of plasma angiotensin. As expected, 2K1C hypertensive animals

Fig. 2. Bar graphs showing the effects of G-CSF administration for 14 days on plasma angiotensin I (Ang I), II (Ang II) and 1-7 (Ang 1-7) in normotensive (Sham, n=8) and hypertensive (2K1C, n=13) mice. Values are mean ± SEM. **p<0.01 vs. 2K1C vehicle group; ##p< 0.01 vs. the respective Sham groups (two-way ANOVA).

Fig. 3. Left panel: typical photomicrography of the clipped (white arrows) and the contralateral kidney showing the attenuation of atrophy by G-CSF administration in a 2K1C hypertensive mouse compared with a mouse receiving vehicle. Right panel: bar graph showing left kidney/right kidney weight ratio (LKW/RKW) in normotensive Sham, $n=8$) and hypertensive (2K1C, $n=8$) mice treated with G-CSF. * $p<0.05$ vs. 2K1C Vehicle group; ## $p<0.01$ vs. the respective Sham group (two-way ANOVA).

Fig. 4. Left top panel shows typical histological micrographs from clipped kidneys stained with hematoxylin-eosin. A: image from a 2K1C+Vehicle mouse showing a large infarcted area containing no nuclei (pink staining; coagulative necrosis). a: higher magnification showing a renal corpuscle with a “ghost glomerulus” containing erythrocytes. B and b are images of a 2K1C mouse treated with G-CSF, showing its protective effect against the advent of renal infarction. Right bar graph shows the effect of G-CSF administration on kidney infarct area in 2K1C mice ($n=8$) compared with the 2K1C+Vehicle group ($n=8$); * $p<0.05$ vs. Vehicle group (Student’s t test). Bottom panel: representative images from TUNEL assay showing that the process of apoptosis (green fluorescent tubular cells, indicated by arrows) in an ischemic kidney from a 2K1C+Vehicle mouse (C) was prevented in a 2K1C mouse treated with G-CSF for 48 hours (D). Bottom bar graph shows a significant difference in the number of TUNEL-positive tubular cells between 2K1C+ Vehicle ($n=5$) and 2K1C + G-CSF groups ($n=5$); * $p<0.01$ vs. Vehicle group (Student’s t test), suggesting a protective effect of G-CSF on renal tubular apoptosis.

showed significant increases ($p<0.01$, two-way ANOVA) in angiotensin I (164 ± 11 pmol/mL), angiotensin II (142 ± 11 pmol/mL) and angiotensin 1-7 (177 ± 12 pmol/mL) when compared to Sham animals (85 ± 6 , 30 ± 5 and 88 ± 7 pmol/mL, respectively). G-CSF administration did not affect angiotensin levels in Sham animals. In contrast, this G-

CSF did prevent the increase of plasma angiotensin I ($p<0.01$, two-way ANOVA) and significantly attenuated the increase of plasma angiotensin II ($p<0.01$) in 2K1C hypertensive mice. The increase in plasma angiotensin 1-7 also was prevented by G-CSF administration ($p<0.01$, two-way ANOVA) in these hypertensive animals. The

Fig. 5. Right panel shows typical histological micrographs from clipped kidneys stained with Picrosirius red. Left bar graph shows the effect of G-CSF administration on kidney glomerular collagen in 2K1C mice (n=7) compared with the 2K1C+Vehicle group (n=5). ** $p<0.01$ vs. Vehicle group (Student's *t* test).

two-way ANOVA showed a significant interaction ($p<0.05$) between factors (drug treatment and hypertension model) in all tested variables.

Morphological analysis of the kidneys

The effects of G-CSF administration on kidney morphology in normotensive and hypertensive mice are shown in Fig. 3. The left panel contains typical photomicrographs showing atrophy and a coagulative necrosis area in the clipped kidney and hypertrophy of the nonclipped kidney in a hypertensive mouse compared to the kidneys of a normotensive Sham mouse. As illustrated, G-CSF administration had a preventive effect on these developments in the clipped kidney of the hypertensive mouse. As summarized in the bar graph, G-CSF administration did not affect the left kidney weight/right kidney weight ratio in normotensive Sham animals ($p>0.05$, two-way ANOVA). In contrast, it was observed the characteristic decrease in this ratio in hypertensive 2K1C animals when compared to Sham groups ($p<0.01$, two-way ANOVA) and that it was significantly attenuated ($p<0.05$, two-way ANOVA) by G-CSF administration.

Figure 4 shows the effects of G-CSF administration on the infarction of the clipped kidney of 2K1C hypertensive animals. Panel A shows a typical infarcted tissue in a section of clipped kidney from a 2K1C+Vehicle mouse (Fig. 4, panel A), which was characterized by light staining (hematoxylin-eosin) with no nuclei and very little structural damage, giving the appearance of “ghost cells” (Fig. 4, panel a). In contrast, the sections from a 2K1C mouse treated with G-CSF for 14 days (panels B and b)

do not show such characteristics. The average kidney infarcted area in the bar graph (Fig. 4, right panel) shows that G-CSF administration prevented ($p<0.05$, Student's *t* test) kidney infarction of the ischemic kidney in 2K1C animals compared to 2K1C non-treated animals: G-CSF administration prevented clipped kidney infarction in 7 out of 8 2K1C animals. In addition, Fig. 4 shows typical sections from cortex of an ischemic kidney, in which it can be observed TUNEL-positive tubular cells. As shown in those sections, the protective effect of G-CSF administration on apoptosis was already evident at 48 hours after kidney clipping, as indicated by the large number of green fluorescent cells in the clipped kidney of a 2K1C non-treated mouse compared to the small number in the 2K1C treated mouse (Fig. 4, panels C and D). On average, the number of TUNEL-positive tubular cells was significantly decreased ($p<0.01$, Student's *t* test) in 2K1C mice administered with G-CSF when compared with the Vehicle group (12 ± 3 vs. 46 ± 7 cells/hpf) (Fig. 4, bottom bar graph).

Typical photomicrographs of the accumulation of collagen I in kidney glomeruli of a 2K1C hypertensive mouse in contrast with a reduced collagen I content in a 2K1C mouse administered with G-CSF are shown in Fig. 5. On average, glomerular collagen I in 2K1C hypertensive mice was significantly increase when compared with Sham mice (69 ± 4 vs. $35\pm1\%$, $p<0.01$, Student's *t* test). In 2K1C animals administered with G-CSF, glomerular deposition of collagen I was similar ($44\pm5\%$, $p>0.05$, Student's *t* test) to that observed in Sham mice (Fig. 5, bar graph).

Parameters	Sham		2K1C	
	Vehicle (8)	G-CSF (7)	Vehicle (8)	G-CSF (7)
Urinary excretion rate (mL/24h)	1.59±0.31	1.59±0.17	3.94±0.77 [#]	3.67±1.27
Urinary creatinine (mg/dL)	24.02±3.23	29.94±1.02	21.03±1.92	27.52±5.52
Plasma creatinine (mg/dL)	0.42±0.10	0.66±0.09	0.54±0.10	0.65±0.01
Plasma urea (mg/dL)	64±9	63±4	68±10	65±7
Proteinuria (mg/dL)	100±12	85±9	74±12	84±8

Table 2. Markers of renal function in normotensive and 2K1C hypertensive mice treated with G-CSF. Values are mean ± SEM from the number of animals indicated in parentheses. [#]p< 0.05 vs. Sham group.

Renal function

Table 2 summarizes the values of parameters of renal function. 2K1C animals showed an increased 24-hour urine volume compared to Sham animals (p<0.05, two-way ANOVA), which was not modified by G-CSF administration. However, neither renovascular hypertension nor G-CSF had a significant effect on urine or plasma creatinine, plasma urea and proteinuria, indicating similar renal function between groups.

Discussion

The main findings of this study were a marked protective effect of G-CSF against kidney infarction and hypertension development in the murine model of angiotensin II-dependent renovascular hypertension.

Jia et al. [13] reported that administration of G-CSF in mice infused with angiotensin II by using an osmotic minipump reduced cardiac hypertrophy but did not affect blood pressure. In contrast, in our study we used a classical model of renovascular hypertension in which angiotensin II is endogenously increased due to the stenosis of the kidney artery. As previously observed by others [10, 14, 15], the 2K1C procedure in the mouse resulted in the development of hypertension accompanied by activation of the renin-angiotensin system. We observed that G-CSF administration significantly attenuated the development of hypertension by about 23 mmHg. Ventricular hypertrophy and tachycardia have been previously reported in this model of hypertension 4 weeks after renal artery clipping [10, 12] and we obtained similar findings at two weeks after the procedure. G-CSF administration did not have a significant preventive effect on the development of these two parameters. In a model

of exogenous angiotensin-dependent hypertension, others have reported a partial attenuation of ventricular hypertrophy by G-CSF administration for 4 weeks even without effects on hypertension [13].

We chose to use the 2K1C hypertensive mice because the goal of the present study was to investigate the protective effect of G-CSF on a model of persistent kidney ischemia. G-CSF ameliorates toxic renal injury caused by cisplatin [16] and ischemic renal injury [17] in animal models of ischemia/reperfusion [7]. In the present study we observed that G-CSF reduced the atrophy of the clipped kidney in hypertensive mice, as evidenced by the clipped kidney/contralateral kidney weight ratio, and prevented kidney infarction. Based on the study of Stokman et al. [17] the underlying mechanism by which G-CSF protects the kidney against ischemia is probably not due to stem cell transdifferentiation but to altered inflammatory kinetics. Recently Li et al. [8] demonstrated that the anti-apoptotic effect of G-CSF on ischemic kidney of C57 mice is via PI3K/Akt activation. This idea is corroborated by our observation of a protective effect of G-CSF against apoptosis in the ischemic kidney of 2K1C hypertensive mice, as indicated by increased DNA fragmentation in the TUNEL assay.

A notable consequence of prevention of kidney ischemia promoted by G-CSF was the marked reductions in plasma angiotensin I and II in hypertensive mice. The decrease in plasma angiotensin II in these animals seems to be the reason 2K1C animals treated with G-CSF showed a significant attenuation of hypertension. Recently, Jia et al. [13] showed that mice administered with angiotensin II or angiotensin II plus G-CSF have similar levels of arterial hypertension. This finding favors our hypothesis that the reduced angiotensin II we observed in 2K1C mice administered with G-CSF may be a

consequence of a protective effect of this cytokine on the kidney. This is evidenced by the observation that the action of G-CSF was restricted to hypertensive mice and supported by the significant interaction between factors (two-way ANOVA). Considering that G-CSF facilitates vascular regeneration in a mouse model of ischemia/reperfusion [18], we speculate that the improvement of renal blood perfusion in the clipped kidney in our study, in addition to a direct effect of G-CSF in juxtaglomerular cells, resulted in reduced liberation of renin. Although angiotensin 1-7 acts in an important endogenous counterbalancing mechanism that attenuates the hypertensive actions of angiotensin II [19], the decreased plasma level of this peptide in G-CSF 2K1C mice was expected because it follows the plasma concentration of angiotensin I and II [20].

Glomerulosclerosis has been reported in 2K1C hypertensive rats [21]. In the present study we observed a high glomerular collagen I content in a murine model of renovascular hypertension. G-CSF administration prevented collagen I deposition in the clipped kidney of hypertensive mice. This beneficial effect of G-CSF seems to be more related to angiotensin II-induced renal pathologies. However, Stokman et al. [22] reported that G-CSF did not ameliorate the renal fibrosis in a mouse model of chronic obstructive nephropathy. Others have observed in C57 mice with angiotensin II-induced cardiac remodeling a cardioprotective effect of G-CSF, which was characterized by reduced fibrosis through inhibition of osteopontin and modulation of the expression of angiotensin-converting enzyme isoforms [13]. Moreover, others have reported that tumor necrosis factor-induced angiotensin II AT₁ receptor upregulation enhances cardiac fibrosis process [23] and that tumor necrosis factor- α , AT₁ receptor and transforming growth factor- β 1 are reduced by G-CSF in a chronic heart failure model [24]. Further studies are necessary to elucidate if the above mechanisms of cardioprotection by G-CSF could also be involved in the renoprotective effect of this cytokine in 2K1C hypertensive mice. A limitation in the morphological analysis is the lack of an accurate randomization to include specific kidney regions into the analysis. This was not done because the infarcted area had a diffuse occurrence and covered most of the kidney, i.e., most of the glomeruli were located in infarct zones of collagen deposition. Importantly, likewise in a study in the murine model of 2K1C hypertension by others [25], our results are based on a morphometric analysis done in a blinded fashion.

Despite a reduced blood flow to the clipped kidney with a consequent reduction in its glomerular filtrations

rate, the 2K1C model of hypertension does not increase total body fluid volume because pressure diuresis in the unclipped kidney prevents hypervolemia. In our analysis of kidney function, we observed increased urinary volume in both 2K1C + Vehicle and 2K1C + G-CSF mice, in agreement with findings in 2K1C hypertensive rats showing increased urinary volume without significant changes in fractional sodium excretion [26]. This polyuria is correlated with the polydipsia reported by Santos et al. [27] in the same murine model and could be due to the dipsogenic actions of angiotensin on the brain [28]. Although the clipped kidney showed morphological damage, plasma urea level, plasma and urine creatinine levels and proteinuria, which are predictors of renal function, were similar to those observed in Sham mice. This finding is not surprising because the contralateral kidney is hypertrophic and compensates for the dysfunction of the clipped kidney. G-CSF administration did affect renal function in Sham normotensive or 2K1C hypertensive mice. This finding is in agreement with many others [see review 7]. However, Tögel et al. [29] observed that G-CSF administration increased plasma creatinine, associated with granulocytosis, in a murine model of acute renal failure, and therefore, the therapeutic use of G-CSF should be approached with caution in this disease.

Neutrophils are among the first cells to be recruited to defend the body against invading pathogens and injury, but they are a major cause of pathology when uncontrolled [30]. Bone marrow, liver and spleen are each responsible for the clearance of approximately 30% of neutrophils from the circulation under homeostatic conditions [31]. We observed an increase in circulating neutrophils in 2K1C hypertensive mice compared with Sham normotensive mice. Administration of G-CSF, which is widely used in the treatment of neutropenic conditions [32], increased both the number of neutrophils and spleen weight in the Sham group, confirming the effectiveness of this drug. In 2K1C hypertensive mice administration of G-CSF increased spleen weight and had an additive effect on neutrophilia. The stimulatory effect of G-CSF on the spleen is in agreement with other findings that this drug promotes an enlargement of spleen in humans [33].

As expected, we observed a stimulatory effect of G-CSF on mononuclear cells in the spleen and bone marrow in Sham normotensive mice, in agreement with others [2, 34]. In 2K1C hypertensive mice, this effect of G-CSF was intensified, indicating a synergic effect of angiotensin II on the mobilization/production of MNCs from the spleen. This observation is supported by the findings that angiotensin II may stimulate the proliferation

of hematopoietic progenitors [35, 36]. The lack of an angiotensin II-mediated increase in MNCs number in the bone marrow of 2K1C mice does not necessarily mean that this effect does not occur in this murine model because, at least in part, mononuclear cells produced in the bone marrow are mobilized to the circulation and incorporated by the spleen.

In conclusion, our data indicate that G-CSF administration attenuates the high arterial blood pressure and prevents kidney infarction in 2K1C hypertensive mice, reinforcing the protective role of G-CSF against kidney ischemia. These findings support an expansion of the use of G-CSF, which is widely available for the treatment of neutropenia, to a therapeutic strategy aiming to regenerate

injured organs such as kidney and prevent their further injury.

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