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ORIGINAL ARTICLE

A Role for galectin-3 in renal tissue damage triggered by ischemia and reperfusion injury

Ana Paula Fernandes Bertocchi,[1] Gabriela Campanhole,[1] Pamella Huey Mei Wang,[1] Giselle Martins Gonçalves,[1] Márcio José Damião,[1] Marcos Antônio Cenedeze,[1] Felipe Caetano Beraldo,[2] Vicente de Paula Antunes Teixeira,[3] Marlene Antônia dos Reis,[3] Marilda Mazzali,[2] Alvaro Pacheco-Silva[1] and Niels Olsen Saraiva Câmara[1,4]

1 Laboratory of Clinical and Experimental Immunology, Division of Nephrology, Universidade Federal de São Paulo, Escola Paulista de Medicina, São Paulo, Brazil
2 Division of Nephrology, Universidade Estudal de Campinas, Campinas, Brazil
3 Department of Pathology, Universidade Federal de Uberaba, Minas Gerais, Brazil
4 Laboratory for Transplantation Immunology, Department of Immunology, Universidade de São Paulo, São Paulo, Brazil

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Correspondence
Niels Olsen Saraiva Câmara, MD, PhD, Department of Immunology, Institute of Biomedical Science IV, Universidade de São Paulo, Rua Prof Lineu Prestes, 1730, 05508-900 São Paulo, SP, Brazil. Tel.: 5511 3091 7388; fax: 5511 3091 7224; e-mail: niels@icb.usp.br

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Summary

Ischemic-reperfusion injury (IRI) triggers an inflammatory response involving neutrophils/macrophages, lymphocytes and endothelial cells. Galectin-3 is a multi-functional lectin with a broad range of action such as promotion of neutrophil adhesion, induction of oxidative stress, mastocyte migration and degranulation, and production of pro-inflammatory cytokines. The aim of this study was evaluate the role of galectin-3 in the inflammation triggered by IRI. Galectin-3 knockout (KO) and wild type (wt) mice were subjected to 45 min of renal pedicle occlusion. Blood and kidney samples were collected at 6, 24, 48 and 120 h. Blood urea was analyzed enzymatically, while MCP-1, IL-6 and IL-1β were studied by real-time PCR. Reactive oxygen species (ROS) was investigated by flow cytometry. Morphometric analyses were performed at 6, 24, 48 and 120 h after reperfusion. Urea peaked at 24 h, being significantly lower in knockout animals (wt = 264.4 ± 85.21 mg/dl vs. gal-3 KO = 123.74 ± 29.64 mg/dl, $P = 0.001$). Galectin-3 knockout animals presented less acute tubular necrosis and a more prominent tubular regeneration when compared with controls concurrently with lower expression of MCP-1, IL-6, IL-1β, less macrophage infiltration and lower ROS production at early time points. Galectin-3 seems to play a role in renal IRI involving the secretion of macrophage-related chemokine, pro-inflammatory cytokines and ROS production.

Introduction

Galectins belong to a family of lectins, having specific affinity for an oligosaccharide called β-galactoside. Galectin-3 (gal-3) is the most studied of these lectins, because of its important multifunctional biological role [1]. Within cells, gal-3 regulates gene expression by influencing pre-mRNA splicing and also inhibits apoptosis because of its molecular homology with bcl-2. In extracellular milieu, gal-3 has a role in organogenesis, tumorigenesis, as well as cell-cell and cell-matrix adhesion. Concerning the immunologic system, gal-3 acts on autoimmune and allergic diseases. It has been demonstrated that gal-3 is expressed in endothelial and epithelial cells and activated macrophages. Specifically in macrophages, gal-3 is linked to their ability to phagocyte in response to noninfectious inflammatory agents [2,3]. Furthermore, gal-3 is able to induce secretion of IL-1β, thus enhancing the adhesion between leukocytes and endothelial cells by promoting the expression of ICAM, ELAM and VCAM [4]. In mast cells, gal-3 is associated with IL-4 secretion and histamine release [5]. Finally, gal-3 can also be found

in $CD4^+$ and $CD8^+$ T cells activated by anti-CD3 or concanavalin A, but is absent in naïve cells [6].

The ischemia-reperfusion injury (IRI) is currently accepted as an important inflammation process involving endothelium injury, leukocyte infiltration, reactive oxygen (ROS)/nitrogen species production, and generation of lipid mediators by sublethally injured tubular end endothelial cells [7–9]. During the early stages of IRI, cell infiltration and activation lead to the production of chemokines such as monocyte chemoattractant protein 1 (MCP-1) and cytokines such as IL-6 and IL-1β, ultimately amplifying the inflammatory response [10–14]. In renal IRI, immunohistochemistry studies have revealed higher gal-3 expression in proximal and distal tubular cells, thick ascending loop and collecting ducts as well as in resident macrophages at late stages of regeneration [15].

On the basis of the above-mentioned data, we hypothesize that gal-3 knockout mice will show less tissue damage following renal IRI, in part because of lower chemokine expression and less prominent inflammatory response.

Material and methods

Animals

Isogenic male gal-3 knockout and wild type C57Bl/6 mice ($H2\text{-}A^b$), age 5–9 weeks (25–28 g), were purchased from the Animal Facilities Center of CEDEME at Universidade Federal de São Paulo. All animals were housed in individual and standard cages and had free access to water and food. All procedures were previously reviewed and approved by the Ethics Committee of the Institution.

IRI model

Surgery was performed as previously described [16]. The mice were briefly anesthetized with intraperitoneal Ketamine-Xylazine injection (Agribrands do Brazil, São Paulo, Brazil). A midline incision was made and both renal pedicles were clamped. During this procedure, animals were kept well hydrated with saline and kept at a constant temperature (~37 °C) through a heating pad device. Forty-five minutes later, the microsurgical clamps were removed. The abdomen was closed in two layers and the animals were placed in single cages then warmed by indirect light until completely recovered from anesthesia.

Study design

Animals were assigned to the following groups: (i) group 1: gal-3 wild type animals ($n = 10$, at all time points) subjected to IRI and (ii) group 2: gal-3 knockout animals ($n = 10$) subjected to IRI. Additionally, animals were submitted to the surgical procedure without renal vessel occlusion (sham) ($n = 5$). For technical and scientific purposes, the surgeries were performed on the same day for each group, namely, animals subjected to IRI and their controls. All animals were sacrificed at days 1, 2 and 5 days after reperfusion, except in specific experiments described later in the text, where animals were sacrificed at different times after surgery. Gal-3 knockout animals and wild type animals were kindly donated by Prof. Liu-Fu Tong through Prof. Roger Chamas from the University of São Paulo.

Renal function outcome

Blood was collected for urea measurements and kidneys were harvested for histological and mRNA analyses at the end-points. Blood urea was measured by the urease methodology. The extension of acute tubular necrosis (ATN) morphology and patterns of regeneration in the outer medulla were examined by blinded morphometric assessment using a computer-assisted image system with an Olympus BX40F-3 microscope (Olympus Optical Company, Tokyo, Japan) and the software program KS300 (Zeiss, Jena, Germany). The results were expressed as a ratio of the injured area versus the total area (1 458 467 μm^2). Twenty-five random fields were counted at an original magnification ×835, almost covering the entire outer medulla and corresponding to an area of 36 461 675 μm^2, for each animal, per slide. For morphometric analyses, acute tubular necrosis and regeneration were analyzed at 6, 24, 48 and 120 h after reperfusion. Tubular necrosis patterns included: loss of cytoplasmatic and nuclear membrane integrity, loss of brush border, vacuolization of tubular epithelial cells, and presence of intratubular debris. Tubular regeneration or repair was characterized by attenuated epithelium enclosing dilated lumens filled with proteinaceous fluid or granular material. Mitotic figures were sometimes present or tubules lined by basophilic epithelium, with basophilic cytoplasm and hyperchromatic nuclei or vesicular nuclei with prominent nucleoli.

Gene profiles

Kidney samples were fast frozen in liquid nitrogen. Total mRNA was isolated from kidney tissue using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) methodology while mRNA concentration was determined by spectrophotometer readings at 260 nm absorbance. First-strand cDNAs were synthesized using the MML-V reverse transcriptase (Promega, Madison, WI, USA). RT-PCR was performed using the SYBR Green real-time PCR assay (Applied Biosystem, Foster City, CA, USA). Sequences of primers used were: MCP-1 (sense) 5′-ACT GCA TCT

GCC CTA AGG TCT TCA-3′, (anti-sense) 5′-AGA AGT GCT TGA GGT GGT TGT GGA-3′, IL-6 (sense) 5′-CAC AAA GCC AGA GTC CTT CAG AGA-3′, (anti-sense) 5′-CTA GGT TTG CCG AGT AGA TCT-3′, IL-1β (sense) 5′-TGA TGA GAA TGA CCT GTT CT-3′, (anti-sense) 5′-CTT CTT CAA AGA TGA AGG-3′, and hypoxanthine guanine phosphoribosyltransferase (HPRT) (sense) 5′-CTC ATG GAC TGA TTA TGG ACA GGA C-3′ and (anti-sense) 5′-GCA GGT CAG CAA AGA ACT TAT AGC C-3′. Cycling conditions were as follows: 10 min at 95 °C followed by 40 cycles of 20 s at 95 °C, 20 s at 53.5 °C and 20 s at 72 °C. The samples were run in triplicate and relative quantification of mRNA levels was performed using the Comparative threshold cycle method, with the amplification of the target genes and HPRT in separated tubes (described in detail in User Bulletin 2; Perkin-Elmer/Applied Biosystems, Branchburg, NJ, USA 1997). Briefly, the amount of the target gene was normalized first to an endogenous reference (HPRT), and then relative to a calibrator (the sample with the lower expression, sham-operated animals), using the formula 2-ΔΔCt. Hence, steady-state mRNA levels were expressed as an n-fold difference relative to the calibrator. Analysis used Sequence Detection Software 1.9 (SDS; Applied Biosystem, Foster City, CA, USA).

Reactive oxygen species

To study ROS production, kidney tissue extracts were incubated with 2′7′dichlorofluorescein diacetate (DCFH-DA 0.3 mM, Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS; Sigma), with agitation for 30 min at 37 °C in a shaking water bath. Then 2 ml of ethylene diamine tetraacetic acid (EDTA; Sigma) was added in order to terminate the reaction. After centrifugation, the erythrocytes were removed by hypotonic lyses, and the cell pellet was resuspended in 1.0 ml of 3 mM EDTA in PBS. Intracellular DCFH was studied by flow cytometry [17]. Histograms of the fluorescence intensity were constructed for each tube and the geometric mean of the fluorescence intensity (mean fluorescence intensity—MFI) of DCFH in the population of cells was determined. Samples were acquired in a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA).

Immunohistochemistry

Slides were dewaxed, endogenous peroxidase blocked in 3% H_2O_2, antigen retrieved by incubation with 0.1% tripsin or exposition to microwave in citrate buffer. After unspecific blocking, the slides were incubated overnight at 4 °C with primary antibody (F4/80 MF48000 Caltag, monoclonal rat anti-mouse, 1:40, Invitrogen). In the next morning the procedure was completed by incubations with specific biotinylated secondary antibody, ABC detection kit (Dako, Glostrup, Denmark) and diaminobenzidine tetrahydrochloride color developer (Dako, Carpinteria, CA, USA). Macrophages were counted in 15 random cortical and ten medullar tubulointerstitial HPF (×400). Using the KS 300 Imaging System (Carl Zeiss Microimaging, Jena, Germany), the HPFs (×400) were grid lined (10 × 10) and the number of stained grid fields was counted in each HPF. The results are expressed as percentage of positive fields.

Statistics

All data were described as mean ± SD. Parametric (*t*-test) and nonparametric (Mann–Whitney *U*-) tests were performed when the sample distribution were considered normal or otherwise. The null hypothesis was rejected when $P < 0.05$. All statistical analyses were performed with aid of Stata statistical software 5.0 (Stata Corporation, College Station, TX, USA) and Prism 4 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

The absence of galectin-3 was related to better renal outcomes

Blood urea levels were used to estimate renal function. Six and 24 h after IRI, wild-type mice presented higher urea levels than gal-3 knockout animals (6 h wt = 130.92 ± 13.64 mg/dl vs. gal-3 KO = 81.45 ± 21.38 mg/dl, $P = 0.05$; 24 h wt = 264.4 ± 85.21 mg/dl vs. gal-3 KO = 123.74 ± 29.64 mg/dl, $P = 0.001$), indicating poorer renal function (Fig. 1). Forty-eight and 120 h following reperfusion, the gal-3 knockout mice continued to present

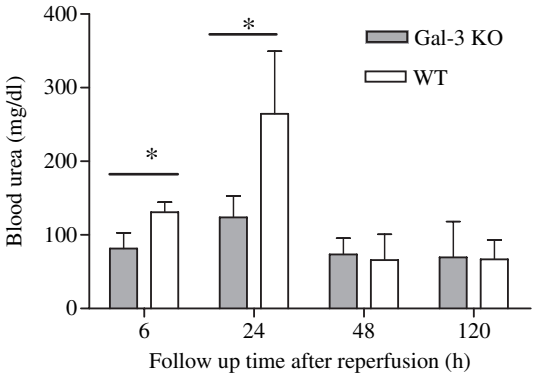

Figure 1 Urea levels in wild type and gal-3 knockout animals subjected to the surgical procedure or otherwise. The knockout animals presented lower urea levels in comparison with wild type counterparts. *$P < 0.05$.

better renal function, however, there was no statistical difference when compared with wild-type. Sham-operated animals presented urea levels similar to normal animals (Basal levels: wt: 67.1 ± 2.4 vs. gal-3 KO: 68.6 ± 7.7, $P = 0.45$; 6 h: wt: 64.4 ± 6.3 vs. gal-3 KO: 62.5 ± 12.6, $P = 0.8$; 24 h: wt: 67.5 ± 14.3 vs. 68.3 ± 13.4, $P = 0.64$; 48 h: wt: 50.6 ± 11.5 vs. gal-3 KO: 47.0 ± 5.3, $P = 0.83$; and 120 h: wt: 65.6 ± 7.4 vs. gal-3 KO: 61.7 ± 5.9, $P = 0.45$).

Morphometric analyses confirmed renal protection presented in knockout animals. Twenty-four hours from reperfusion, wild type animals had the highest values of ATN (wt = 48.60 ± 12.84% vs. gal-3 knockout = 11.15 ± 11.64%, $P < 0.0001$) (Fig. 2a). Simultaneously, gal-3 knockout animals presented more tubular regeneration at all time points studied (24h wt = 1.39 ± 1.59 vs. gal-3 knockout = 5.62 ± 4.09, $P < 0.0001$; 120 h wt = 26.09 ± 18.27 vs. gal-3 knockout = 40.06 ± 18.19, $P = 0.0049$) (Fig. 2b). No significant ATN or tubular regeneration was found in sham-operated animals (data not shown).

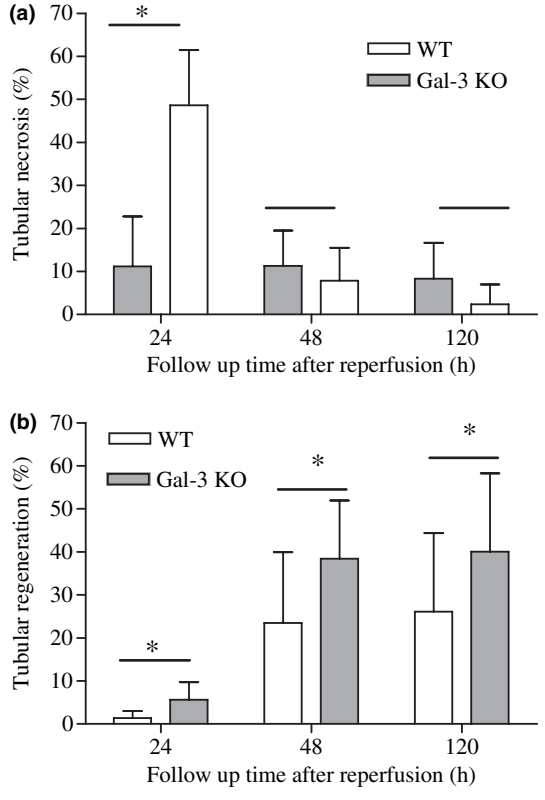

Figure 2 Acute tubular necrosis and tubular regeneration percentages in wild type and gal-3 knockout animals subjected to the surgical procedure. Kidneys were harvested at different times from reperfusion, and ATN was evaluated by hematoxylin-eosin staining. *$P < 0.05$.

Reduced ROS production in galectin-3 knockout animals subjected to renal IRI

Reactive oxygen species production was measured by flow cytometry. ROS peaked 30 min after reperfusion, returning to basal levels thereafter. Interestingly, gal-3 knockout mice presented lower hydrogen peroxide (H_2O_2) production at 30 min measured by DCFH fluorescence when compared with wild type animals ($P = 0.003$) (Fig. 3). Three hours after reperfusion, the values were similar among the groups.

Reduced expression of MCP-1 and IL-6 and IL-1β in galectin-3 knockout animals subjected to renal IRI

The expression of the chemokine MCP-1 was investigated as a surrogate of macrophage infiltration and activation. The highest levels of MCP-1 mRNA expression were obtained at 120 h after the insult. Although the wild type mice presented higher MCP-1 expression at 48 and 120 h of reperfusion, there was no statistically significant difference in comparison with gal-3 knockout animals. However, at 24 h of reperfusion, wild type animals had a significantly higher expression of mRNA of MCP-1 when compared with gal-3 knockout mice (Fig. 4a).

IL-6 and IL-1β are two important pro-inflammatory cytokines participating in renal IRI. We studied the expression of both the cytokines at 24 h after surgery, a time point of maximal injury. Gal-3 knockout animals had a lower expression of both the molecules than the wild type mice, indicating less inflammation (Fig. 4b).

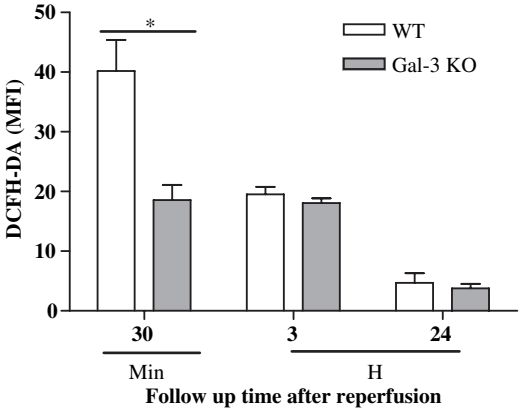

Figure 3 Reactive oxygen species production in wild type and gal-3 knockout animals subjected to the surgical procedure or otherwise. Hydrogen peroxide production was assessed by flow cytometry in wt and knockout animals subjected to renal IRI. *$P < 0.05$.

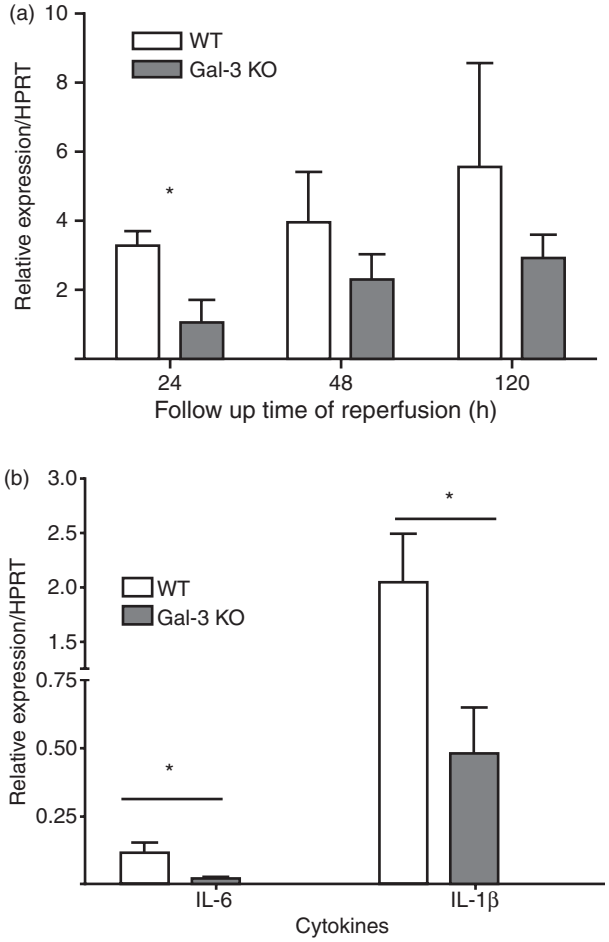

Figure 4 (a) MCP-1 mRNA expression in wild type and gal-3 knockout animals subjected to renal IRI. There was a statistical difference between wild type and knockout mice only at 24 h after reperfusion. $*P < 0.05$. (b) IL-6 and IL-1β mRNA expression in wild type and gal-3 knockout animals subjected to 24 h renal IRI. Knockout mice had less expression of both cytokines. $*P < 0.05$.

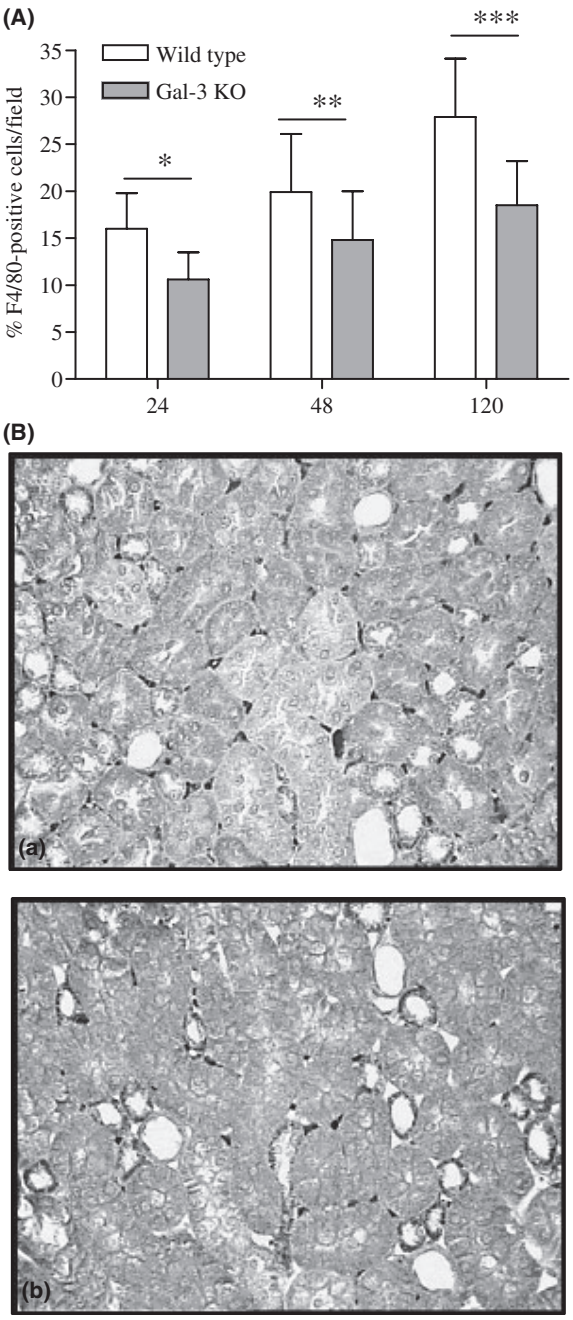

Figure 5 (A) Percentages of macrophage infiltration (F4/80 positive cells/field) in wild type and gal-3 knockout animals subjected to renal IRI. There was a statistical difference between wild type and knockout mice at all time points after reperfusion (24 h. $P < 0.001*$, 48 h: $**P = 0.014$ and 120 h: $***P < 0.001$). (B) Illustrative photo of immunohistochemistry for F4/80 in wild type (a) and gal-3 knockout (b) mice subjected to IRI after 24 h of reperfusion (400×).

Macrophage infiltration in gal-3 knockout animals after renal IRI

To investigate the cellular infiltration after renal IRI and whether gal-3 would have an impact, we performed immunostaining for F4/80, a marker of macrophages. At all time points, gal-3 knockout mice subjected to IRI presented a reduced extent of macrophage infiltration, with statistical significance. Interestingly, macrophages were more prominent at later time-points in wild type animals (Fig. 5).

Mortality

The mortality observed was 30% in wild type versus 20% in gal-3 knockout group at 24 h after reperfusion. Forty-eight hours later, wild type and gal-3 knockout mice presented the same rate (10%). There was no statistical difference between wild type and knockout animals.

Discussion

Although the pathogenesis of acute renal failure has been the subject matter of much investigation through different models of IRI, there are currently no clinical approaches that can be used in clinical practice to improve patient outcome. Acute renal failure is related to a high rate of mortality mainly when renal replacement therapy is required. In transplantation, the negative impact of delayed graft function, representing one of the clinical presentations of IRI on late graft outcome, is well established [18].

Ischemic-reperfusion injury is an inflammatory syndrome where innate and adaptive immune responses participate [8,19,20]. Macrophages are well recognized cells involved in tissue aggression and repair following IRI, secreting IL-6 and IL-1β, some of their important pro-inflammatory products [21–23]. IL-6 is one of the most important mediators of the acute phase of inflammation response. In the muscle and fatty tissue, IL-6 stimulates energy mobilization which leads to increased body temperature. IL-1β is produced by activated macrophages as a pro-protein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). Its important role in inflammatory response includes involvement in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. Domanski *et al.* quantified these serum cytokine concentrations during the first 5 min of reperfusion and observed increased levels of IL-6 and IL-1β in recipients of deceased renal grafts [24]. Therefore, enhanced cytokine production during reperfusion might have an influence on early graft function.

In this work, we investigated the additional role of gal-3 in renal IRI. We made use of a model that elicits substantial impairment of renal function and the existence of a genetically modified gal-3-deficient animal. Gal-3 knockout animals present normal development and phenotype [3]. Nevertheless, they present decreased phagocyte infiltration following a nonpathogenic inflammation [2,3]. Nishyama *et al.* have previously demonstrated that gal-3 is up regulated 2 h after an ischemic insult in rats, decreasing 28 days thereafter [15]. Many kinds of cells expressed gal-3 at different times, pointing to a specific role at different times.

In this study, we took advantage of these genetically modified animals to address the role of gal-3 in renal IRI. We understand that this acute renal injury model does not fit what the patients present in clinical practice. Particularly with respect to renal transplantation, this acute renal injury model fails to incorporate the feature of cold ischemia. However, animals presented a severe impairment in renal function and a high rate of tubular necrosis, mainly at early time point that allowed us to answer properly on the issues involved.

Our initial data showed that gal-3 knockout animals presented less severe renal dysfunction through urea levels and less tubular necrosis. At 24 h after reperfusion, we found more tubular necrosis in wild type animals ($P < 0.0001$). It reinforces the participation of gal-3 in inflammatory tissue injury. By analyzing 48 and 120 h of reperfusion, we found high levels of tubular necrosis in knockout animals, albeit to a lesser extent and without statistical significance. We know that gal-3 is important both in early and late stages of inflammation. Nishiyama *et al.* also showed gal-3 is over-expressed at late phase of tissue recovery, emphasizing its role at this stage. We believe that its role on macrophage chemotaxis could justify these results. Gal-3 knockout mice had a lesser extent of macrophage infiltration in all time points after reperfusion with statistical significance. The role of macrophages in renal IRI is already established [22,25–29] and in this study, we demonstrated the impact of gal-3 on the macrophages. Furthermore, we can imply that as the knockout mice presented less acute aggression, the injured tissue potentially has the ability to regenerate more quickly, probably because of a better cytoprotective response.

It has been demonstrated that gal-3 knockout animals have less amount of glomeruli (11%) than wild type with kidney hypertrophy [30]. The knockout animals had mild renal chloride loss and reduced blood pressure but similar GFR to controls (356 ± 42 vs. 342 ± 52 μl/min in WT). Our controls, normal and sham-operated knockout and control animals, had the same blood urea levels. Therefore, our baseline data were comparable among the groups, although we could not rule out the impact of this phenomenon in ischemic injury.

To further characterize the model, we quantified the ROS production in gal-3 knockout and wild type animals that had been subjected to IRI. ROS is produced during ischemia and released after reperfusion in part because of the cleavage of arachidonic acid by cyclo-oxygenases [31]. In our model, gal-3 knockout mice produced less ROS after IRI when compared with wild type controls. Our data clearly showed that ROS production was more prominent at early reperfusion time. Basically, this production is a result of endothelial cells dysfunction because of their inability to use the oxygen and later on by the oxidative burst from leukocytes. In short ischemic time, cells are more susceptible to oxidative stress and overproduce ROS [32]. ROS promote over-expression of pro-inflammatory genes, cytokines, adhesion molecules and chemokines from macrophages. Early data have demonstrated that gal-3 could stimulate superoxide production by neutrophils [33] and by monocytes [34]. Almkvist and Karlsson described that gal-3 can be secreted from activated macrophages and

induces activation of the NADPH oxidase [35,36]. Moreover, the same investigators demonstrated that gal-3 could induce NADPH oxidase activation, measured as superoxide production, in lipopolysaccharide (LPS)-primed neutrophils [36]. Matarrese *et al.* showed that over-expression of gal-3 protected cells from death through inhibition of ROS formation by promoting mitochondrial homeostasis [37]. It is conceivable that in the absence of gal-3, less ROS was induced in neutrophils and macrophages, both critically involved in IRI, thus decreasing tissue damage.

Subsequently, we decided to quantify the expression of MCP-1, a chemokine related to the function of gal-3 and known to be involved in migration and activation of macrophages. Up regulating of MCP-1 has been previously demonstrated in renal IRI and associated with worse dysfunction. Macrophages can migrate into inflamed tissue through MCP-1 gradient, or by action of molecules such as gal-3 [38–41]. Our data demonstrated gal-3 knockout animals presented lower levels of MCP-1 at early time points after reperfusion, suggesting the presence of this lectin might indeed be implicated in chemoattraction of phagocytes into the tissue.

Early data from Tilney's group have established that renal IRI involved up regulation of pro-inflammatory cytokines, such IFN gamma, IL-1β, IL-6 and TNF-α [42–44]. Over recent years during which period, T cells have been implicated in renal IRI pathogenesis, the actual role of these cytokines has become apparent. In animals suffering from IRI, there is a consensus in the literature that the cytokine profile was deviated toward a Th1 [13]. Conversely, STAT-6 and IL-4 deficient animals presented a marked Th1 immune response [45]. We investigated the presence of two cytokines, IL-1β and IL-6. Gal-3 knockout animals presented a lower expression of both pro-inflammatory molecules when subjected to IRI. Gal-3 is up regulated in macrophages, nerve [46] and islets [47] in response to IL-1β. Moreover, gal-3 over-expression can counteract the pro-apoptotic effects of IL-1 [47]. Finally, gal-3 can potentiate IL-1 production by human macrophages [4]. The lesser tissue damage observed in knockout animals could reflect reduced inflammation because of the decreased levels of ROS and pro-inflammatory cytokines and chemokines.

Pooling all data, we demonstrated that gal-3 plays a role in renal IRI, while its absence is associated with reduced extent of renal injury through diminished macrophage function, indirectly demonstrated by lower expression of mRNA of MCP-1, IL-6, IL-1β and ROS production. These data could lead to new insights into the complex pathogenesis of IRI.

Authorship

APFB: acquired, did the experiments, analysed and interpreted the data; revised and made the final approval of the version to be published. NOSC: contributed to the conception and the design of the study in addition to having done some experiments together. GC, PHMW, GMG, MJD, MAC, FCB, VPAT, MAR, MM and AP-S: participated in the work, each one contributing in one or other experiment.

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