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Immunohistochemical localization of galectins-1 and -3 and monitoring of tissue galectin-binding sites during tubular regeneration after renal ischemia reperfusion in the rat

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Summary. Endogenous lectins act as effectors of cellular activities such as growth regulation, migration and adhesion. In this study, we report the histochemical detection of galectins and their binding sites in rat kidneys after ischemic injury (35 min) with regard to renal regeneration. In this context, we have shown in a previous publication (Vansthertem et al., 2008) that extrarenal cells (CD44+, vimentin +) could be involved in this process of tubular restoration. In controls, galectin-1 is expressed by fusiform-shaped cells within cortical and medullar interstitium. Two days after ischemia, the number of positive interstitial cells increased temporarily within OSOM in the vicinity of altered tubules to later reach control level. After ischemia, we identified a population of galectin-3 (+), CD44 (+), and vimentin (+) interstitial round cells located in the outer stripe of outer medulla (OSOM) in the vicinity of necrotic tubules, but also in the lumen of adjacent blood vessels. The immunocytochemical characteristics of theses cells, along with their distribution within OSOM, suggest the involvement of a unique cell population during kidney regeneration. On the other hand, the distribution and density of binding sites for galectins within OSOM were not modified after ischemia and remained similar to controls. Altogether, our observations suggest that galectin-3 may be involved in the complex process of kidney regeneration following ischemia/reperfusion injury.

Key words: Acute renal failure, Kidney regeneration, Renal interstitium, Tubular necrosis

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Introduction

One of the most frequent causes of renal pathology remains ischemia-reperfusion injury both for native or transplanted organs. This pathology manifests itself as an acute tubular necrosis mainly affecting S3 segments of the nephrons. In this context, the recovery of a normal renal function after ischemia requires a rapid restoration of altered tubular epithelium by regenerating cells. However, the source of these regenerating cells and the processes of tubular regeneration remain controversial and still need to be firmly established (Al Awqati and Oliver, 2002; Bonventre, 2003; Humphreys et al., 2006; Romagnani, 2009). In this context, we have shown in a previous publication (Vansthertem et al., 2008), that extrarenal cells could be involved in tubular regeneration after moderate ischemia. These cells seem able to migrate from the blood to injured tubules where they differentiate into mature epithelial cells. In a second step we have demonstrated that this population of extrarenal cells involved in tubular repair expressed transiently two markers of undifferentiated cells, vimentin and CD44, before differentiation into mature epithelial cells (Vansthertem et al., 2008). Our observations also

disclosed a consistent spatial organization of various cell populations within interstitium, along with changes in the phenotype of these cells during the process of regeneration. This was associated with the expression of embryonic markers (vimentin, nestin or NCAM) by interstitial cells, but also with extensive changes of the extracellular matrix (ECM), such as the distribution of hyaluronan, the main ligand of CD44 (Vansthertem et al., 2008). Altogether, these observations point to intricate interactions between migrating cells and ECM (Zuk et al., 1998, 2006) and suggest the involvement of other molecules known to function in modulating cell-cell and cell-matrix interactions. Due to their reactivity with matrix components and cell receptors such as laminin, fibronectin, CD44 or integrins members of the galectin family, potent effectors of cell adhesion, growth and migration, deserve attention in this respect (Barondes et al., 1994; Gabius, 2006, 2009).

Galectins are members of a phylogenetically conserved family of lectins sharing a consensus sequence of about 130 amino acids and a carbohydrate recognition domain (CRD) responsible for ß-galactoside- binding. Orchestration of glycan and galectin expression has been delineated as a means toward growth regulations, and galectins can sensitively react to changes in glycosylation, hereby linking this parameter to ensuing cellular responses (Ahmad et al., 2004a; André et al., 2007a,b). Of note, the presence of galectins in the cytoplasm and nucleus has been documented as an indication of functional versatility, together with distinct proteins such as ligands, as the non-classical pathway for secretion to exert extracellular functionality (Hughes, 2004; Smetana et al., 2006). This background explains the interest in this area to study galectins.

More specifically, evidence is given that members of the galectin family are involved in renal embryogenesis, during which galectin-1 is expressed by mesodermally derived cells. These cells are at the basis of the development of mesodermal mesenchyme that later gives rise to non-ureteric tubular epithelia (Hughes, 2004). Galectin-3 expression was also reported during later stages of nephrogenesis, during which its expression is confined to derivatives of the ureteric bud, such as collecting ducts and connecting segments of distal tubules (Bullock et al., 2001; Ochieng et al., 2004). In normal adult kidney, galectin-3 expression is restricted to collecting tubules and primary cilium, in addition to being temporarily expressed by altered proximal tubules during regeneration (Nishiyama et al., 2000; Chiu et al., 2006; Lohr et al., 2008). Whereas the expression for these two galectins has thus been documented in kidney, other members of the family have so far received less attention. In addition to addressing this issue, we also apply labeled human galectins to localize histochemically accessible binding sites, hereby reporting both the profiles of lectin presence and of tissue reactivity.

The questions that we address in this study are: 1) Using a panel of non-crossreactive antibodies, what is the distribution of galectins within kidney during tubular regeneration consecutive to an episode of moderate ischemia/reperfusion? 2) Using biotinylated galectins what is the pattern of galectin-reactive sites in the same model? 3) Are these staining patterns similar or different during the process of regeneration? 4) Are galectins expressed by extrarenal cells involved in tubular regeneration?

Materials and methods

Animals and treatment

All experiments were performed on two-month old male Wistar rats weighing 200 to 250 g. They were originally obtained from Iffa Credo (Belgium) and bred in our animal facility. The animals were maintained and treated in compliance with the guidelines specified by the Belgian Ministry of Trade and Agriculture (agreement n° LA1500021). Throughout the study, the rats were housed in a room with a regular 12L : 12D cycle and had free access to standard rodent chow (AO4, UAR Villemoisson-sur-Orge, France) and to tap water *ad libitum*.

Before bilateral renal ischemia, animals were anesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg b.w.; Nembutal, CEVA, Brussels, Belgium). After an ischemia of 35 minutes, the vascular clamps were removed and the kidneys observed for the normal return of blood flow. Preliminary experiments have shown that such an ischemic period was able to induce renal failure that was followed by complete kidney regeneration. The correlation between tubular regeneration and renal function recovery was already reported in a previous study from our group (Schaudies et al., 1993). The rats were sacrificed with an intra-peritoneal injection of pentobarbital every four hours during the first day of reperfusion and 2, 3, 7, 14 days post-ischemia. At time of sacrifice, blood samples were collected by aortic puncture and plasma creatinine concentration was determined using a colorimetric method. Four controls underwent similar treatment but without occlusion of the renal arteries.

Sample processing for histology

Immediately after sacrifice, the kidneys were fixed by immersion in Duboscq-Brazil fluid (alcoholic Bouin) for 48 hours at room temperature. Fixed tissue specimens were dehydrated and embedded in paraffin according to standard procedures. Paraffin sections of 4-5 μm thickness were cut on a Reichert Autocut 2040 microtome and placed on silane-coated glass slides. After rehydratation, sections were stained with PAS, hemalun and Luxol fast blue to allow the systemic examination of morphologic alterations induced by

ischemia.

Immunohistochemical detection of galectins

Interstitial cells in ischemic kidney were analyzed for the expression of galectins using a broad panel of primary antibodies (Table 1). As detailed elsewhere (André et al., 1999, 2008; Nagy et al., 2002, 2003; Dam et al., 2005; Lensch et al., 2006), the different galectins were purified by affinity chromatography and were subjected to rigorous purity and activity controls (André et al., 1999, 2001, 2006; Kopitz et al., 2003; Vrasidas et al., 2003; Morris et al., 2004). For each galectin, a polyclonal antibody was raised in rabbit and systematically tested by ELISA and Western blotting against galectins of the three families to eventually detect and remove cross-reactivity by chromatographic affinity depletion (detailed in Kaltner et al., 2002; Saal et al., 2005; Langbein et al., 2007).

Paraffin sections were immunostained following a slightly modified version of the streptavidine-biotin immunoperoxidase method (ABC method) detailed in previous publications (Schaudies et al., 1993; Toubeau et al., 1994; Saussez et al., 2005). Briefly, the sensitivity of the method was increased by microwave pretreatment of dewaxed sections in 0.01 M citrate buffer (pH 6.0) for 2 x 5 min. at a power of 900 W. Thereafter, the sections were incubated sequentially at room temperature in the following solutions: (1) primary antiserum at optimal dilutions (see Table 1) for 1 hour; (2) biotinylated swine anti-rabbit IgG (diluted 1:50) for 30 min. and (3) ABC complexes for 30 min. Biotinylated swine anti-rabbit antibody and ABC complexes came from Dakopatts (Glostrup, Denmark). Bound peroxidase activity was visualized by incubation with 0.02% 3,3'-diaminobenzidine - 0.01% H_2O_2 in PBS. The sections were finally counterstained with PAS, hemalun and Luxol fast blue and mounted in a permanent medium.

Controls for the specificity of immunolabeling included the omission of the primary antibody or the substitution of non-immune sera for the primary antibodies. In each case these controls were negative. In addition, we have checked that the antisera used in this study gave clear-cut immunostaining patterns on control tissue sections. In each case, the immunostaining patterns were in accordance with the observations reported in the literature for the normal histological distribution of each of these galectins (Wasano et al., 1990; Horie and Kadoya, 2004; Nio et al., 2006; Lohr et al., 2007, 2008).

Double-label immunostaining

The coexpression of galectins-1 and -3 with CD44, vimentin, nestin and CD68 by interstitial cells was analyzed by double-label immunofluorescence. Prior to immunostaining dewaxed rehydrated sections were pretreated for antigen unmasking (see above). Thereafter, the sections were incubated for 1h. at room temperature in a mixture containing a rabbit antibody raised against one of the galectins and either a mouse monoclonal antibody (anti-CD44, anti-CD68, anti-nestin) or a chicken polyclonal anti-vimentin antibody. The sections were then incubated for 30 min. in presence of a biotinylated swine anti-rabbit IgG antibody and FITC-conjugated goat anti-mouse IgG antibody (Dakopatts) both at a final dilution of 1:50. Finally, the immunostaining was completed by incubating for 30 min. with Texas red-conjugated streptavidine (Vector, Burlingame Laboratories, Inc., CA, USA) diluted 1:50. After final rinses in distilled water, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA).

As controls, serial sections were immunostained with one of the two antibodies, after or without microwave heating. The labeling patterns obtained in these conditions were compared to those produced by double-label immunostaining. The specificity of the immunoreactions was also tested by incubating the sections in the presence of non-immune rabbit or mouse sera or by omitting primary antibodies. These controls confirmed the specificity of the immunolabelings and the absence of cross-reactivity.

The sections were observed on a Leitz Orthoplan fluorescence microscope equipped with a Ploem system for epi-illumination. Wavelengths of 490 nm and 596 nm for excitation and 525 nm and 615 nm for emission were used for the observation of FITC and Texas-Red, respectively. Microphotographs were recorded by a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland). The computer software (KS 400 imaging system, Carl Zeiss

Table 1. antibodies used for the immunohistochemical characterization of interstitial cells in rat kidney.

Primary Antibodies	Origin	Dilution
Anti-galectin antibodies	Rabbit, polyclonal, Pr Gabius	1:50
Anti-vimentin	Chicken, polyclonal - Chemicon, USA	1:1000
Anti-rat CD44	Mouse monoclonal (OX-49)* - BD Pharmingen	1:200
Anti-rat CD68	Mouse monoclonal (ED1)* - Serotec, UK	1:75
Anti-rodent nestin	Mouse monoclonal (rat-401)* - Chemicon -USA	1:100

*: Antibody producing clone

vision, Hallbergmoos, Germany) allowed the superimposition of images to detect colocalization and was also used to perform morphometric analysis (see below).

Histochemical localization of binding sites for tissue lectins

Preparation of biotinylated galectin derivatives

The different galectins were purified by affinity chromatography and subjected to rigorous purity and activity controls (see above). Biotinylation of galectins was carried out under activity-preserving conditions (Gabius et al., 1991), and the conjugates rigorously controlled for the maintenance of carbohydrate-binding activity in solid-phase and cell assays (André et al., 2004, 2006). The extent of biotinylation was determined by an established proteomics approach (Purkrábková et al., 2003).

Biotinylated galectin histochemistry

Tissue processing followed the protocol described above for the immunohistochemical detection of galectins. Renal tissue samples were subjected to an optimized protocol (Saussez et al., 2006) to determine the presence of accessible binding sites using the biotinylated galectins-1 and -3. Tissue sections were processed with biotinylated galectins following a slightly modified version of the standard streptavidine-biotin immunoperoxidase method (see above for ABC method). Briefly, the sections were exposed sequentially at room temperature to the following reagents: (1) biotinylated galectins (galectin-1 and -3 at 1/500) for 1 hour; (2) ABC complexes for 30 min. Bound peroxidase activity was visualized by incubation with a solution containing the chromogenic substrate (0.02% 3, 3'-diaminobenzidine-0.01% H_2O_2 in PBS). The sections were finally counterstained with PAS, hemalun and Luxol fast blue and mounted in a permanent medium. Controls for the specificity of galectin-dependent labeling included the omission of the incubation step with labeled lectin. Moreover, lactose/asialofetuin inhibition as routinely applied in histochemical glycan profiling by lectins was included to block ß-galactoside-specific binding and protein-epitope-dependent binding sensitive to lactose (Manning et al., 2004).

Morphological analysis

For each experimental group, we have analyzed the tissue distribution of both interstitial cells immunolocalized by the different primary antibodies tested in this study and tissue binding sites for lectins. For each histological section, 10 square fields (0.084 mm^2/field) selected at random were observed at x 400 magnification in each of the three regions of the kidney: cortex, outer stripe and inner stripe of the outer medulla (OSOM and ISOM). To analyze the immunolabelings obtained with the different galectins, we have scanned consecutive renal sections from each experimental animal. For galectins-1 and -3, the observations were expressed as the number of positive cells per mm^2 for each zone of the kidney. In every experimental group, individual values were pooled for each region of the kidney and presented in histograms as means ± SD. Due to problems of sensitivity, the mean staining intensity of positive cells (mean optical density) was not performed in this study.

In each field, we have also recorded the histological structures localized in the immediate vicinity of positive structures such as type of renal tubules, blood vessels or areas of interstitial fibrosis. For renal tubules, the degree of tubular injury was evaluated following an arbitrary scale described in a previous publication (Toubeau et al., 1994).

Statistical analysis

Results obtained from morphological analysis for galectins-1 and -3 were submitted to analysis of variance (ANOVA) and post hoc Dunnet's test by comparison to controls. Limit of significance was set at $p< 0.01$.

Results

Distribution of galectin-1 immunoreactivity after ischemia

In kidneys from control rats, galectin-1 immunoreactivity was detected in nerve bundles from the cortico-medullary junction, in media of arteries, in pericytes of descending vasa recta, in intraglomerular mesangial cells and in lipid-laden interstitial cells of the inner medulla. Within interstitium of cortex and outer medulla, galectin-1 presence is also observed in fusiform shaped cells (fibroblast-like) disposed in a peritubular network (Fig. 1a).

Two days after ischemia, the number of positive interstitial cells increased significantly within OSOM in the vicinity of altered tubules (Figs. 1c, 2). One week after reperfusion, the density of positive cells declined to reach control values (Figs. 1e, 2).

At long term (1-2 weeks), positive flat cells were restricted to the vicinity of a small number of chronically altered tubules (Fig. 1g).

Double-label immunofluorescence for galectin-1 and nestin showed that the two markers were coexpressed by fusiform cells localized at the periphery of necrotic tubules two days postischemia (Fig. 4a,b).

Distribution of galectin-3 immunoreactivity after ischemia

In kidneys from control animals, galectin-3 presence was seen in principal cells of collecting duct (Fig. 1b) and in urothelium of pelvis.

Galectin-3 immunoreactivity was also localized in interstitial round cells interspersed among renal tubules

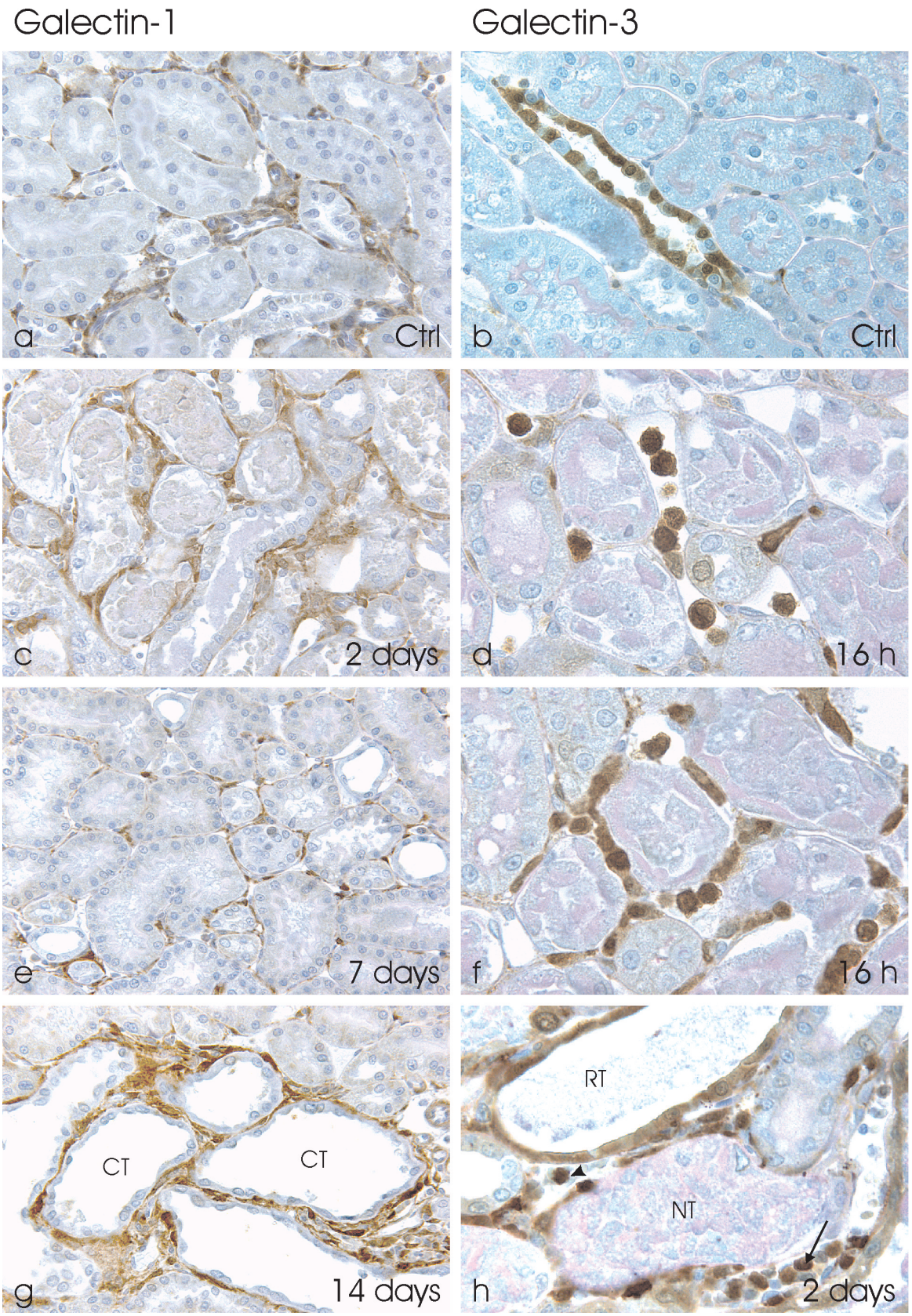

Fig. 1. Immunohistochemical localization of galectin-1 and galectin-3 in OSOM of controls **(a, b)** or of ischemic kidneys **(c-h)**. **a.** Distribution of galectin-1-positive cells in interstitium of OSOM in a control animal. Two days after ischemia **(c)** positive cells form a dense network around necrotic tubules. Later (**e**; one week), the distribution of galectin-1 appeared similar to controls. Long term (**g**; two weeks), galectin-1-positive interstitial cells are only present around residual enlarged S3 tubules (cystic tubules - CT). In controls **(b)**, galectin-3 presence is restricted to principal cells of collecting ducts. After 16 hours of reperfusion **(d, f)**, positive round cells were detected both in the lumen of peritubular capillaries **(d)** and in interstitium **(f)**. Longer term (**h**; 2 days), flattened epithelial cells of regenerating tubules (RT) appeared positive for galectin-3. Positive round cells were also detected in the lumen of capillaries (arrow) and in interstitium (arrow head). NT: necrotic tubules. a-c, e, g, x 320; d, x 550; f, x 600; h, x 450.

and in the lumen of peritubular capillaries. Twelve hours after ischemia, their number increased significantly at the junction OSOM-ISOM to reach a peak at 12-16 h (Fig. 3). These interstitial cells were in the vicinity of altered proximal tubules but also in the lumen of medullar blood vessels (Fig. 1d,f). Later, 20-24 h after ischemia, they infiltrated progressively OSOM to reach the basal region of cortex, displaying highest densities around S3 necrotic tubules. Later, its density declined slowly to reach control values after 7 days (Fig. 3).

One-two days after ischemia, galectin-3-positive cells were observed in regenerating S3 tubules within OSOM. These cells, devoid of brush border, always showed the characteristics of immature epithelial cells (Fig.1h). Later, galectin-3 expression declined progressively in parallel to a return to a normal state of differentiation of tubular cells characterized by the reappearance of the brush border. Long term, from one week after reperfusion, S3 tubular cells appeared devoid of galectin-3 expression, except focally in chronically altered tubules.

At 16 hours after reperfusion, double immunostainings showed that galectin-3-positive cells also expressed vimentin (Fig. 4e,f) or CD44 (Fig. 4d). The immunolabeling corresponding to CD44 was restricted to the periphery of positive intravascular cells. Finally, double immunostaining for galectin-3 and CD68 (Fig. 4c) revealed two populations of intravascular and interstitial cells: a set positive for both markers specific of monocytes - macrophages while the other only gave a signal for galectin-3.

Immunoreactivities for other galectins after ischemia

The expression of galectins-2, -5, -6, -7 and -9 was never observed in normal rat or in the various cell types involved in tubular regeneration.

In control kidneys, epithelial cells of proximal convoluted tubules expressed both galectin-4 and -8, while distal tubules (cortex, OSOM and ISOM) expressed only galectin-8. After ischemia, the density and distribution of staining for these galectins were not modified and remained similar to controls.

Distribution of binding sites for galectins-1 and -3 after ischemia

In kidneys from control animals, the binding sites of biotinylated galectin-1 were mainly localized in the peritubular interstitium of OSOM. In contrast, the peritubular interstitium of both cortex and ISOM appeared negative, although the perivascular connective tissue around large blood vessels was positive. The distribution and density of sites reactive to galectin-1 were not modified by ischemia and remained similar to controls.

In control kidneys, the staining profiles of sites reactive to galectin-3 were observed in the interstitium of OSOM (Fig. 5a) and ISOM, while the cortex remained negative. As for biotinylated galectin-1, the connective tissue around arcuate blood vessels of the cortico-medullary junction appeared also positive (Fig. 5b). After ischemia, the distribution of galectin-3-binding sites was grossly similar to controls, although restricted areas of cortical interstitium appeared positive around S1-S2 altered tubules (12-48 h post ischemia –

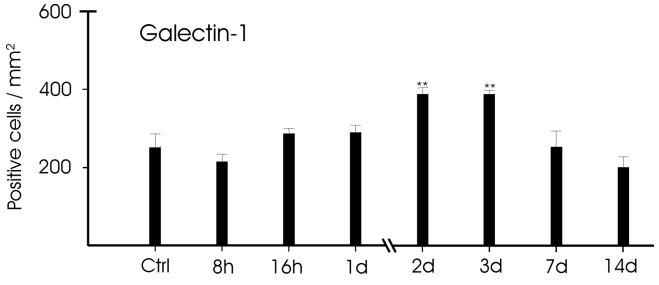

Fig. 2. Density of galectin-1-positive cells in interstitium of kidneys from control animals (Ctrl) or from animals sacrificed at increasing times (hours or days) after reperfusion. Each column corresponds to the mean number of labeled cells per mm^2 (± SD) determined after examination of OSOM in each experimental group (n=4) (**: p<0.01 *versus* control).

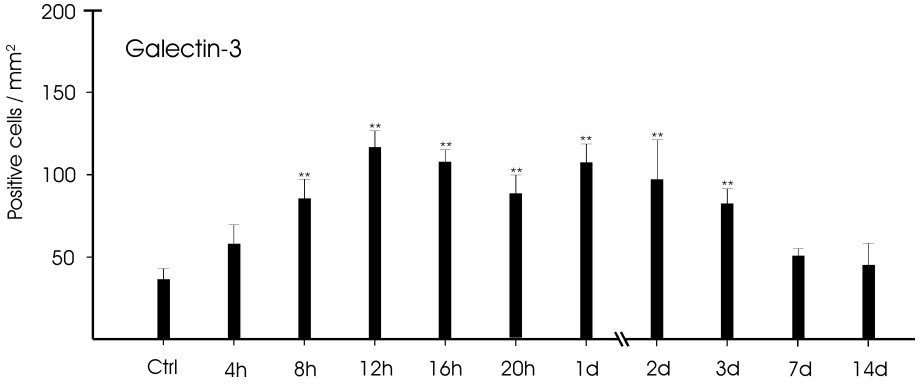

Fig. 3. Distribution of galectin-3-dependent immunoreactivity in the renal interstitium of OSOM from controls (Ctrl) or from animals sacrificed at increasing time intervals (hours or days) after ischemia/reperfusion. Each column corresponds to the mean number of galectin-3-immunoreactive round cells per mm^2 (± SD) after examination of each experimental group (n=4). (**: p<0.01 *versus* control).

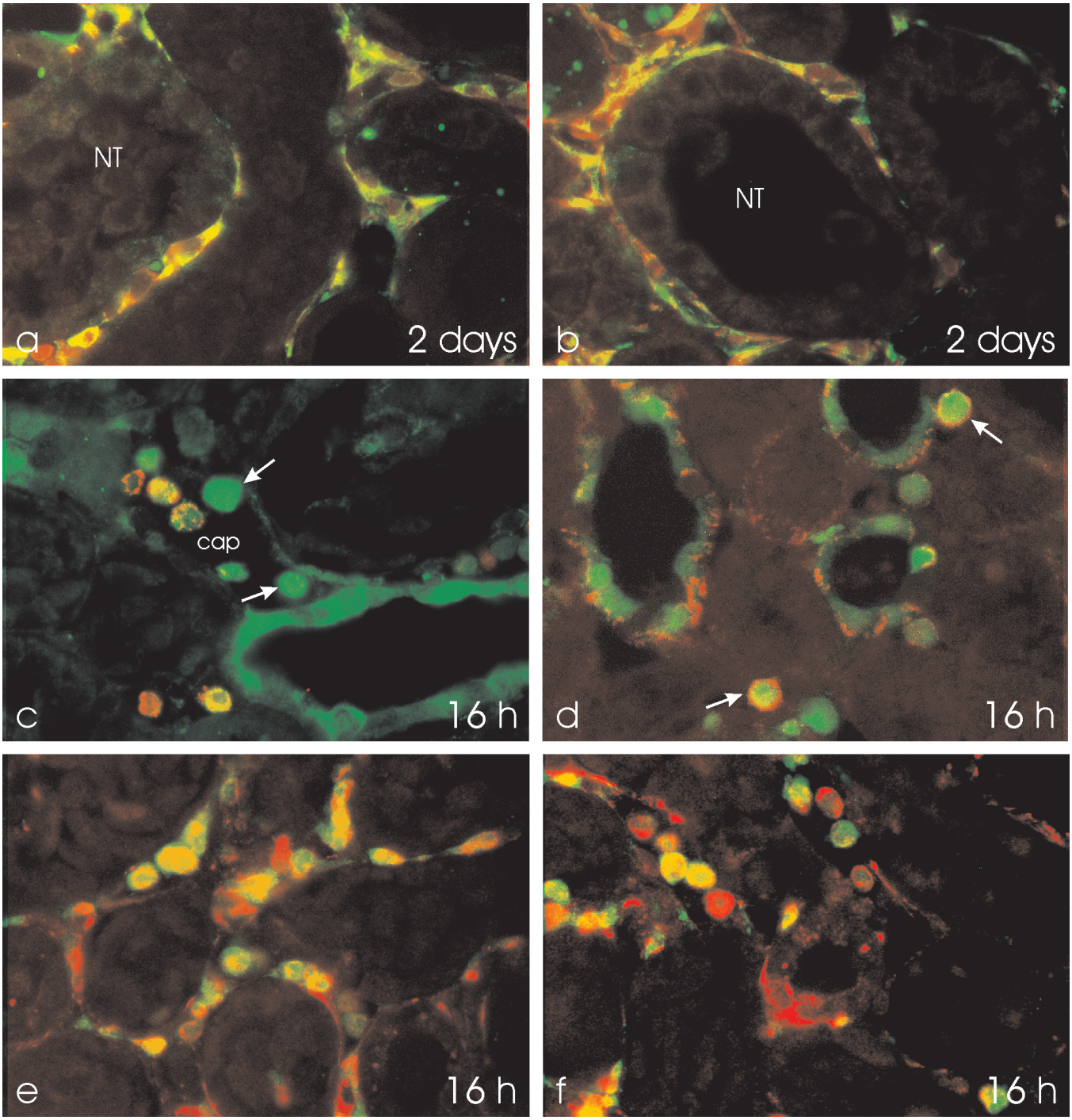

Fig. 4. a, b. Double immunostaining for nestin (green) and galectin-1 (red) in ischemic kidneys two days after reperfusion. Double-label positive fusiform cells (yellow) are localized at the periphery of necrotic tubules (NT). **c.** Double immunostaining for CD68 (red) and galectin-3 (green) 16 hours after reperfusion. Round cells in peritubular capillaries positive for both CD68 and galectin-3 appear yellow, while cells exclusively expressing galectin-3 appear green (arrows). cap: capillary. **d.** Double immunostaining for CD44 (red) and galectin-3 (green) 16 hours after reperfusion. The immunolabeling corresponding to CD44 receptors is restricted to the periphery of positive intravascular cells (arrows). **e, f.** Double immunostaining for vimentin (red) and galectin-3 (green) 16 hours after reperfusion. Positive cells co-expressing the two markers are localized in interstitium around altered tubules **(e)** or in the lumen of capillaries **(f)**. a, b, x 610; c-f, x 640

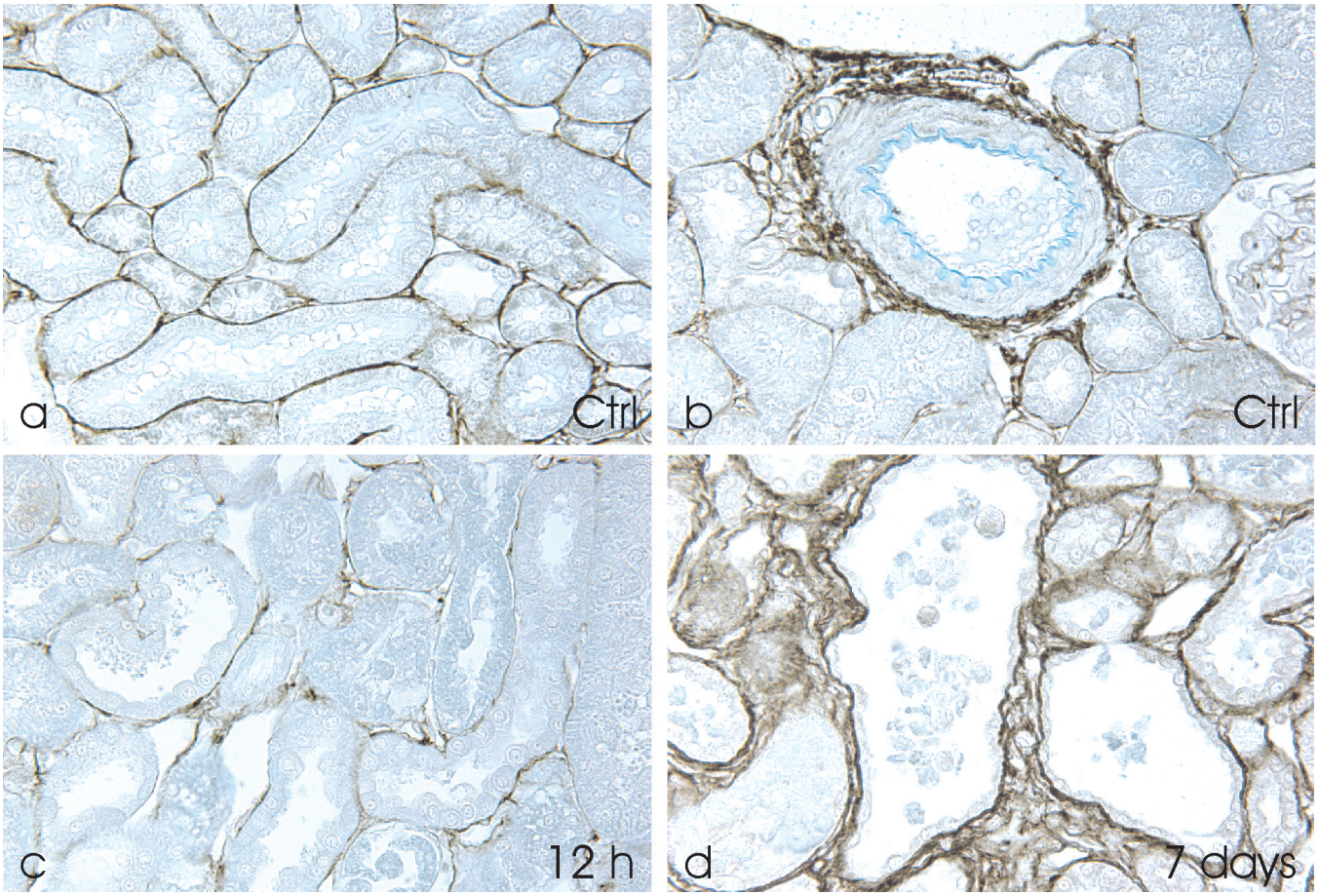

Fig. 5. Staining profiles for binding sites of galectin-3 in controls **(a, b)** or in ischemic kidneys **(c, d)**. In OSOM of controls, biotinylated-galectin-3 was detected in intertubular interstitium **(a)** and in perivascular tissue around blood vessels **(b)**. Twelve hours after ischemia **(c)** binding sites for galectin-3 were detected focally in cortical interstitium as a loose network around altered tubules. Long term (**d**; one week), reactivity for galectin-3 was mainly localized around residual cystic tubules within OSOM. x 275

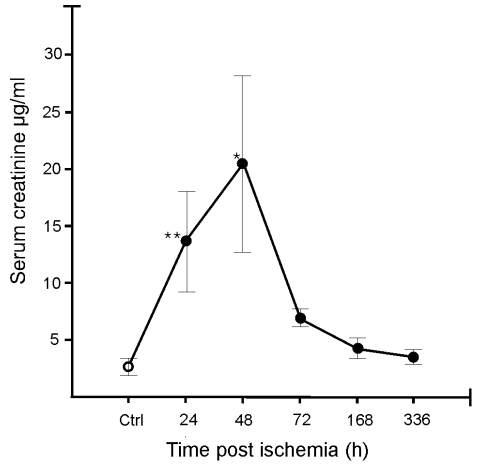

Fig. 6. Serum creatinine levels observed at different time intervals after reperfusion. Symbols indicate means ± SE (vertical bars) at different time points (n=4). Open circles (Ctrl) indicate mean baseline value (*: p<0.05; **: p<0.01).

Fig. 5c). Long term (1-2 weeks), a dense positive network was always observed in the vicinity of chronically altered cystic tubules concomitantly with an extension of the extracellular matrix in these areas (Fig. 5d).

Creatinine concentration and tubular regeneration

Serum creatinine levels (Fig. 6) rose from baseline values to 13.4±4.8 μg/ml at 24 h and 20.6±7.4 μg/ml at 48 h postischemia. These values returned to near normal levels after 72 h. In parallel, we also carefully examined kidney sections from all experimental animals to evaluate, for each time point, the state of tubular regeneration. Three days after ischemia, tubular regeneration led to the reepithelialization of most altered S3 tubules. Later, the kidneys that underwent ischemia appeared similar to those of control animals (compare figures 1a, b and 1e one week after ischemia). All these observations were in accordance with previously

published observations (Schaudies et al., 1993; Williams et al., 1997; Vansthertem et al., 2008) and indicate the recuperation of a normal renal physiology.

Discussion

Previous works of our group have demonstrated that renal regeneration after ischemia was associated with a typical sequence of transient events leading both to the restoration of normal tubular architecture and to the recovery of normal renal functions (Vansthertem et al., 2008). Altogether, our observations support the concept that immature cells originating from an extrarenal source could be able to migrate into renal parenchyma via blood vessels during the first 24 h post-ischemia. We suggested that these migrating cells reach injured tubules, proliferate and finally differentiate into mature epithelial cells leading to the replacement of a majority of altered S3 tubular cells. The main difference between this hypotheses and the current literature only concerns the origin of the cells that proliferate to regenerate altered tubules. Indeed, nowadays it is generally admitted that tubular repair mainly involves endogenous cells, such as surviving dedifferentiated tubular cells (Duffield and Bonventre, 2005), while we suggest an extrarenal origin. Beyond this controversy, it is interesting to remember that the intratubular mechanisms leading to complete tubular reparation are identical whatever the source of proliferating cells may be (Williams et al., 1997).

Vimentin and CD44 were expressed by these immature migrating cells, which appeared negative for other markers such as CD45, CD90 or CD133. In the current study we examined galectin presence and reactivity during the course of renal regeneration after an episode of moderate ischemia. Taken together, the presented data involving systematic monitoring of each step of tubular regeneration enabled us to answer the questions listed at the end of the introduction.

As already stated in the introduction, the functions ascribed to galectins are remarkably diverse and they have been implicated in the control of cellular proliferation - differentiation, e.g. in myogenic conversion (Lahm et al., 2004; Scott and Weinberg, 2004; Chan et al., 2006) and in the regulation of cell-matrix or cell-cell interactions (Kaltner and Stierstorfer, 1998; Ochieng et al., 2004; Gabius, 2008). Galectin-3 (formerly known as Mac-2) is expressed in a variety of epithelial cells (reviewed in Dumic et al., 2006) and is the major subtype of galectins expressed in the murine urinary system. In the kidney, its expression is mainly observed in the distal nephrons (Winyard et al., 1997; Kikuchi et al. 2004; Schwaderer et al., 2006) and the immunohistochemical analysis revealed that galectin-3 immunoreactivity was mainly localized in principal cells of collecting ducts and in the transitional epithelium of renal pelvis (Bichara et al., 2006; Nio et al., 2006; Schwaderer et al., 2006; Kim et al., 2007; Lohr et al., 2008). Our observations in control animals are consistent with the results reported in these studies and confirm the specificity of our antibodies.

Galectin-3 is involved in organogenesis and its expression has been demonstrated during nephrogenesis both in human and animal systems (Van den Brûle et al., 1997; Ochieng et al., 2004; Hughes, 2004). In this context, it appears to be implicated in the modulation of ureteric bud branching in organ culture of developing mouse kidney (Bullock et al., 2001). The role of galectin-3 in kidney development has also been studied by using a model of tubulogenesis *in vitro* (Bao and Hughes, 1995). From this model, it was assumed that galectin-3 could mediate adhesive forces limiting cell movement and reorganization during tubule formation. Galectin-3 is also presumed to play a key role in terminal differentiation of epithelial cells by binding and polymerizing a high molecular weight protein named hensin. This polymerized form of hensin is able to induce terminal differentiation but also to maintain polarity in the differentiated state (Hikita et al., 2000; Al-Awqati, 2008). As with galectin-1, the reactivity of galectin-3 with matrix glycoproteins enables these effectors to partake in cell-cell/matrix interactions (Gabius. 2006). Altogether, the diverse functions ascribed to galectin-3 are consistent with previously reported observations (see above) and suggest its involvement in tubular differentiation during ontogenesis and the remodeling process consecutive to ischemia. In the latter process, the immunocytochemical characteristics of interstitial round cells (gal-3 +, CD44 +, and vimentin +), along with their distribution within OSOM, suggest the involvement of a unique cell population during tubular regeneration. Interestingly, galectin-3 expression appeared to be upregulated during acute renal failure induced by folic acid or ischemia (Nishiyama et al., 2000), an observation consistent with the transient expression of galectin-3 in our model.

Galectin-3 is expressed in a variety of tissues, including epithelial cells and fibroblasts, but also in leukocytes such as neutrophils, monocytes/macrophages and eosinophils (Liu. 2000; Dumic et al., 2006; Lohr et al., 2008; Liu et al., 2009). In particular, galectin-3 seems to play a role in renal ischemia-reperfusion injury by being involved in the secretion of macrophage-related chemokines, pro-inflammatory cytokines (Fernandes Bertocchi et al., 2008) or in promotion of renal fibrosis (Henderson et al., 2008). In inflammatory sites, these leukocytes are able to transmigrate to the interstitial matrix following a multistep cascade, including initial rolling followed by firm adhesion and transendothelial migration into extravascular sites of inflammation (Sato et al., 2002; Rao et al., 2007). Rolling, which is the first step of vascular adhesion, is mediated by molecules of the selectin family (P-and E-selectins) expressed by endothelium of postcapillary venules. In the present study, we have disclosed an intravascular population of round cells galectin-3(+), CD44 (+) apparently able to transmigrate to interstitium in the vicinity of necrotic tubules. In this context, recent studies suggest a connection between regulation of cell adhesion and

surface lattice formation induced by galectin-3 which is able to oligomerize when in contact with suitable ligands (Ahmad et al., 2004b; Yu et al., 2007; Friedrichs et al., 2008). Indeed, these studies suggest a critical role for galectin-3 which can even enhance interactions with endothelial cells by uncovering adhesion molecules that are otherwise concealed. The expression of galectin-3 also appears consistent with both the transendothelial migration of Gal-3$^+$, CD44$^+$, vimentin positive cells and the recolonization of altered tubules (Ochieng et al., 1998, 2004). This recolonization of necrotic tubules is immediately followed by a phase of active proliferation (24-48 after reperfusion) leading to the reepithelialization of altered tubules (Vanstheretem et al., 2008). Later these cells differentiate to mature S3 epithelial cells characterized by both the reappearance of a brush border and the simultaneous loss of galectin-3, CD44 and vimentin expression. One week postischemia, the expression of the three markers totally disappeared from S3 tubules of OSOM, except focally in chronically altered tubules.

Galectin-1 is a pluripotent effector of diverse cell functions expressed by many cell types, e.g. mesenchymal cells, here an inductor of myogenic conversion (Scott and Weinberg, 2004; Chan et al., 2006). In the present study, galectin-1 was detected only in peritubular interstitial tissue with a transient overexpression during the first week postischemia. Although galectin-1 can be expressed on the level of various stem cells, including embryonic or mesenchymal stem cells (Silva et al., 2003; Lee and Han, 2008), this protein was never expressed by epithelial cells of regenerating tubules or by galectin-3-positive cells supposed to be involved in tubular regeneration. However, galectin-1 may contribute to cell-extracellular matrix adhesion and could thus modulate interstitial migration of galectin-3-positive cells from blood vessels to altered tubules. Additionally, this galectin could also promote the proliferation of these migrating cells (Sakaguchi et al., 2007; Lee at al., 2009) or be implicated in the initiation of differentiation (Goldring et al., 2002). An emerging function in human kidneys concerns intracellular signaling in podocytes after binding nephrin (Shimizu et al. 2009). Globally, our findings suggest that galectin-1 expression is predominantly located in the connective tissue of the kidney, an observation which is consistent with differential expression profile of galectins-1 and -3 during nephrogenesis (Van den Brûle et al., 1997; Hughes, 2004).

During a short period of time post-ischemia (48 h), a population of galectin-1-positive cells also expressed nestin. This protein was originally identified in neuroepithelial stem cells but also appeared expressed during kidney development. After injury, in a model of ureteric obstruction, the expression of nestin was correlated with the degree of interstitial fibrosis and located mainly in hypoxic regions of the kidney (Sakairi et al., 2007). In the present study, the limited expression of nestin seems thus consistent with our model of moderate ischemia, which is characterized by a complete tubular regeneration and the absence of interstitial fibrosis. Moreover, two to three days post-ischemia, the coexpression of galectin-1 and nestin is concomitant with a transient episode of proliferation occurring in interstitium at that time (Chou et al., 2003; Sakaguchi et al., 2007; Vanstheretem et al., 2008).

Testing the different biotinylated galectins enabled us to detect accessibility of binding sites and to monitor their spatial and temporal patterns of localization. The binding sites for galectin-1 were mainly localized in interstitium of OSOM while the sites reactive to galectin-3 were observed in the interstitium of OSOM and ISOM. The distribution and density of sites reactive to galectins-1 and -3 were not modified by ischemia and remained similar to controls.

In conclusion, we have extended our previous immunohistochemical study by analyzing the spatial and temporal localization of different galectins during kidney regeneration after moderate ischemia. We answer the issues raised in the introduction by showing that: (i) immunodetection of galectins-1 and -3 revealed distinct staining profiles which were different during the process of regeneration; (ii) binding sites for these galectins were primarily localized in the ECM; (iii) their distribution and density were not altered by ischemia; (iv) galectin-3 appeared expressed by circulating cells probably involved in tubular regeneration, whereas galectin-1 expression was restricted to peritubular interstitium.

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