NDT
Nephrology Dialysis Transplantation

Original Article

Label-retaining cells and tubular regeneration in postischaemic kidney

David Vansthertem[1], Nathalie Caron[2], Anne-Emilie Declèves[3], Stéphanie Cludts[1], Annabel Gossiaux[1], Denis Nonclercq[1], Bruno Flamion[4], Alexandre Legrand[2] and Gérard Toubeau[1]

[1]Laboratory of Histology, Faculty of Medicine and Pharmacy, University of Mons-Hainaut, B-7000 Mons, [2]Laboratory of Physiology, Faculty of Medicine, Facultés Universitaires Notre-Dame de la Paix, B-5000 Namur, [3]Laboratory of Physiology and Pharmacology, Faculty of Medicine and Pharmacy, University of Mons-Hainaut, B-7000 Mons and [4]Laboratory of Molecular Physiology, Faculty of Medicine, Facultés Universitaires Notre-Dame de la Paix, B-5000 Namur, Belgium

Abstract

Background. In this study, we have examined rat kidneys after ischaemic injury (35 min) with regard to the dynamics of S3 tubule regeneration.

Methods. One day before ischaemia, each rat received four successive i.p. injections of BrdU (5-bromo-2′-deoxyuridine: 80 mg/kg) at 2 h intervals. Groups of experimental animals ($n = 4$) were killed every 2 h during the first 24 h post-ischaemia as well as 2, 3, 7 and 14 days post-ischaemia. Renal sections were processed to characterize by immunohistochemistry the distribution and phenotype of BrdU-positive cells.

Results. Renal regeneration after ischaemia was associated with a typical sequence of transient events: (1) absence of immunostaining during the first 8 h after reperfusion; (2) between 8 and 16 h, detection of a small population of BrdU-positive cells (CD44$^+$, vimentin$^+$, CD45$^-$) restricted to the lumen of blood vessels characterized by the endothelial expression of selectin E; (3) between 16 and 24 h, progressive decrease of labelled cells in renal capillaries and a concomitant increase in the interstitial compartment; (4) after 1 day, labelled cells disappeared progressively from peritubular interstitium and were mainly observed in regenerating S3 tubules, and (5) after 3 days numerous positive cells were only present in regenerated tubules.

Conclusions. Our data suggest that positive cells (BrdU$^+$, CD44$^+$, vimentin$^+$ and CD45$^-$) observed in kidney tubules after ischaemia could originate from an extrarenal source and reach the renal parenchyma via blood vessels. We postulate that these immature cells migrate to injured tubules, proliferate and finally differentiate into mature epithelial cells leading to the replacement of a majority (>80%) of altered S3 cells.

Introduction

Renal ischaemia-reperfusion injury is one of the most frequent causes of acute renal failure both for native or transplanted organs. Renal pathology manifests itself as an acute tubular necrosis mainly affecting S3 segments localized in the outer stripe of outer medulla (OSOM) [1]. The recovery of a normal renal function after ischaemia requires the replacement of altered tubular cells by regenerating cells. However, the source of these regenerating cells remains controversial and still needs to be firmly established [2,3]. It is generally proposed that the origin of these tubular cells is surviving resident cells that dedifferentiate and proliferate to restore the normal architecture and functions of proximal tubules [4]. Recently, the discovery of bone marrow stem cells (BMSC) potentially able to differentiate into functional tubular cells [5–8] has led to re-evaluation of the processes involved in tubular regeneration. These observations suggest that BMSC could be rapidly mobilized from peripheral blood to reach the kidney where they would establish, differentiate and participate to tubular regeneration [9]. However, more recent publications have reported that the restoration of proximal tubules in post-ischaemic kidneys occurred independently of BMSC [10–12]. On the other hand, putative resident progenitor cells were previously identified in kidneys by their ability to retain BrdU in their DNA for long periods [13,14] or by expression of specific stem cells markers [15].

Whatever the source of kidney stem cells able to regenerate tubules might be, the various mechanisms described above suggest that the different cell types involved in tubular regeneration are able either to control their motility within the kidney or to modify their phenotype to interact with other cell types. In this regard, we have paid particular attention to events occurring in the renal interstitium after renal reperfusion. Indeed, interstitial cells seem to play

Correspondence and offprint requests to: Gérard Toubeau, Service d'Histologie, Faculté de Médecine et de Pharmacie, Université de Mons-Hainaut, Avenue du Champ de Mars, 6 (Pentagone) 1B, B-7000 Mons, Belgium. Tel: +32-65-37-3562; Fax: 32-65-37-3557; E-mail: gerard.toubeau@umh.ac.be

important roles not only in renal function but also in renal regeneration [16]. Interstitium is involved in renal pathologies and plays a pivotal role in determining the issue of renal failure [17]. Additionally, recent observations have shown that this interstitial compartment could be both a niche for a population of yet unidentified stem cells and/or harbour BMSC after migration through the blood [7,14].

In view of these points, the goal of the present study was to explore the potential involvement of extrarenal cells in postischaemic rat kidneys after *in vivo* BrdU labelling. To address this point we investigated by immunohistochemistry the dynamics of tubular regeneration in terms of time course and distribution of BrdU-labelled cells throughout the interstitium during a 2-week period following renal reperfusion. In parallel, we have investigated the immunohistochemical expression and distribution of selected markers such as structural proteins known to be critical in the development of normal kidney or potentially involved in kidney regeneration. The selected markers were: selectin E, a vascular adhesion molecule known to be involved in the capture of circulating cells [18,19], vimentin as a marker of differentiation [20,1]; CD44 [21,22] and its main ligand, hyaluronan [23–25], CD45, CD 90 [26,27] and CD 133 [28] as markers of stem cells.

The current study provided evidence suggesting that a population of extrarenal cells could be able to integrate and repair injured tubules by renewing a majority of altered epithelial cells.

Subjects and methods

Animals and treatment

All experiments were performed on 2-month-old male Wistar rats (200–250 g). Animals were maintained and treated in compliance with the guidelines specified by the Belgian Ministry of Trade and Agriculture (agreement LA1500021). Before bilateral renal ischaemia of 35 min, animals were anaesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg b.w.; Nembutal, CEVA, Brussels, Belgium). Preliminary experiments have shown that such an ischaemic period led to the development of an acute renal failure characterized by a massive tubular cell death (∼90%) mainly affecting S3 segments of proximal tubules. The renal alterations were followed by a process of regeneration leading to the restoration of tubular epithelium and of normal renal function [29].

In experiment 1, the rats of each experimental group ($n = 4$) received four successive i. p. injections of BrdU (80 mg/kg b.w.) at 2 h intervals and were submitted to renal ischaemia 24 h after the first BrdU injection. Animals were killed with a lethal injection of pentobarbital every 2 h during the first 24 h post-ischaemia as well as 2, 3, 7 and 14 days post-ischaemia. Control animals received BrdU following the same protocol and were killed 1 h after the last BrdU injection. In a second series of experiments, rats received one i.p. injection of cytarabine (100 mg/kg b.w.; Cytosar®, Pharmacia, Belgium) 2 h and 30 min or 20 h before a single BrdU administration, respectively (see the Discussion section). All experimental animals were submit-

ted to renal ischaemia 24 h after BrdU injection and were killed 2 days after reperfusion.

In a third experimentation, each rat received an i.p. injection of BrdU (80 mg/kg b.w.) 1 h prior to killing. The animals of each experimental group ($n = 4$) were killed at different time intervals (4 h to 2 weeks), time points chosen by reference to a previous publication [20].

Sample processing for histology

After killing, the kidneys were removed and fixed by immersion in Duboscq-Brazil fluid for 2 days. 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 rehydration, sections were stained with PAS, hemalun and Luxol fast blue to allow histologic examination.

Immunohistochemical procedures

The detection of BrdU incorporated into DNA was performed as previously detailed [20,30]. Briefly, dewaxed sections were treated successively with trypsin (50 min; 37°C) and HCl (30 min; 60°C). The sections were thereafter incubated with the mouse monoclonal anti-BrdU antibody (1/20 dilution; Dakopatts, Glostrup, Denmark) for 1 h. After incubation with the primary antibody, the sections were successively exposed for 30 min to a biotinylated goat anti-mouse IgG (1/50 dilution; Dakopatts) and to ABC complex (Dakopatts). Bound peroxidase activity was visualized by incubating the sections in a mixture of $3,3'$-diaminobenzidine and H_2O_2. After immunohistochemistry, tissue sections were counterstained as described above and mounted in a permanent medium. Controls for the specificity of immunolabelling included the omission of the primary antibody or the substitution of non-immune sera for the primary antibodies.

Interstitial cells in ischaemic kidney were analysed for the presence of various markers by using a broad panel of primary antibodies (Table 1). Tissue sections were immunostained following a slightly modified version of the streptavidin-biotin immunoperoxidase method (ABC method) detailed in previous publications [20,29] Briefly, the sections were incubated sequentially at room temperature in the following solutions: (1) primary antiserum at optimal dilutions (see Table 1) for 1 h; (2) biotinylated swine anti-rabbit IgG, biotinylated goat anti-mouse IgG (diluted 1:50) or biotinylated goat anti-chicken IgY (diluted 1:50) for 30 min and (3) ABC complexes for 30 min. A biotinylated goat anti-chicken antibody came from Vectors (Burlingame, USA) while other secondary antibodies and ABC complex 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.

The immunodetection of selectin E was obtained by exposure of the sections to the primary goat polyclonal anti-selectin E antibody (1/5 dilution; R&D Systems, USA) for 1 h followed by successive treatments with the rabbit

Table 1. Antibodies used for the immunohistochemical characterization of tubular and interstitial cells involved in kidney regeneration

Primary Antibodies	Origin	Dilution
Anti-BrdU (Bromodeoxyuridine)	Mouse monoclonal (Bu20a)[a]—Dako, DK	1:20
Anti-PCNA	Mouse monoclonal (PC 10)[a]—Dako, DK	1:40
Anti-vimentin	Chicken, polyclonal—Chemicon, USA	1:1000
Anti-selectin E	Goat polyclonal—R&D Systems, USA	1:5
Anti-rat CD44	Mouse monoclonal (OX-49)[a]—BD Pharmingen	1:200
Anti-rat CD45	Mouse monoclonal (MRC OX-1)[a]—Abcam, UK	1:50
Anti-rat CD68	Mouse monoclonal (ED1)[a]—Serotec, UK	1:75
Anti-rat CD90 (Thy1.1)	Mouse monoclonal (OX-7)[a]—Chemicon, USA	1:100
Anti-rat CD133	Rabbit, polyclonal—Abcam, UK	1:200
Anti-rat MHC classII RT Ia	Mouse monoclonal (OX-6)[a]—Abcam, UK	1:50
Anti-human α-smooth muscle actin	Rabbit polyclonal—Abcam, UK	1:200

[a]Antibody-producing clone.

anti-goat IgG antibody (1/50 dilution; Vectors laboratories, USA) and anti-rabbit Envision system (Dakopatts). Thereafter, the sections were treated as described above for interstitial cells immunohistochemistry.

The detection of hyaluronan (HA) was performed using a biotinylated HA-binding protein (HABP, Sigma-Aldrich, St. Louis, MO, USA) following a protocol previously described [25].

Double-label immunofluorescence was performed following protocols extensively described in a previous publication [31] and using antisera raised in different species to avoid cross-reactivity between secondary antibodies. Briefly, the sections were incubated for 1 h at room temperature with a mixture of two primary antibodies at the correct dilution. The sections were then incubated for 30 min at room temperature in the 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 at room temperature with Texas red-conjugated streptavidine (Vector Laboratories Inc., Burlingame CA, USA), diluted 1:50. After final rinses in distilled water, the sections were mounted in a Vectashield mounting medium (Vector Laboratories).

The coexpression of markers was also assessed by a method combining immunofluorescence and the ABC method followed by topographic staining. This technique was particularly effective to analyse the phenotype of BrdU positive cells and allowed a systemic examination of neighbouring histological structures. For the first step of the method, corresponding to immunofluorescence, dewaxed sections were rehydrated, pretreated at a high temperature for antigen unmasking and incubated in 0.5% casein-PBS for 20 min. The sections were then incubated sequentially at room temperature in the following solutions: (1) primary antiserum at optimal dilutions (see Table 1) for 1 h; (2) corresponding biotinylated secondary antibody; (3) horseradish-conjugated streptavidine (1/100) for 30 min and (4) FITC-conjugated tyramide (TSA Fluorescence Systems, Perkin Elmer, Boston, USA) for 10 min. After mounting in Vectashield, pictures of positive cells were obtained by a PC-driven digital camera (see below) while the coordinates of the corresponding fields on the histological section were simultaneously recorded. Before the second step, the sections were preliminary rinsed in ethanol, rehydrated and

incubated in a 0.1 M glycine–HCl buffer (pH 2.2) for 3 min to suppress non-specific binding by blocking proteins used in immunofluorescence [32]. The second marker was thereafter detected by applying the ABC method detailed above followed by a staining with PAS-Luxol fast blue. The superimposition of the immunofluorescence pattern and the positive structures stained by ABC (see below) revealed the respective distribution of both markers in renal tissue.

Morphological analysis

The sections were observed on a Leitz Orthoplan fluorescence microscope equipped with a Ploem system for epi-illumination. Pictures were obtained by a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland). The computer software (KS 400 imaging system, Carl Zeiss vision, Hallbergmoos, Germany) allowed the superimposition of images to detect co-localizations and was also used to perform morphometric analysis (see below).

For each experimental group, we analysed the tissue distribution of positive cells in each region of the kidney: cortex, outer stripe and inner stripe of the outer medulla (OSOM, ISOM) following methods previously described [20,29]. The observations were expressed as the number of positive-nuclei (BrdU) or positive-cells per mm^2 for each zone of the kidney and presented in diagrams as means ± SD. For each renal zone, the relative area occupied by HA was expressed as a percentage. The results obtained from morphological analysis were submitted to analysis of variance (ANOVA) and a *post hoc* Dunnet's test by comparison to controls with the limit of significance set at $P < 0.01$. We also evaluated the mean optical density corresponding to the mean immunostaining intensity obtained for BrdU positive nuclei using a method of computer-assisted quantification [33].

Results

Distribution of BrdU-positive nuclei

In experiment 1 (BrdU administered 24 h before ischaemia), BrdU-positive nuclei in control rats were detected in the three regions of the kidney with a low density

both in tubular structures (30 $\pm$ 10 cells/mm^2) and in interstitium (15 $\pm$ 3 cells/mm^2) (Figures 1a and 3). After ischaemia, the distribution of BrdU-labelled nuclei appeared similar to the control group during the 8-h period following reperfusion. Nevertheless, a careful examination of kidney sections revealed that, 8 h after reperfusion, labelled nuclei were already present focally in altered S3 tubules and in the lumen of blood vessels (Figure 1b). Between 14 and 16 h after reperfusion, the density of BrdU-positive nuclei increased significantly in the basal region of OSOM (Figure 1c). A large majority of these positive nuclei were localized within interstitium (Figures 1d and 3b) and in the lumen of adjacent blood vessels (Figure 1e). In contrast, the number of positive tubular cells remained at a low level similar to the situation recorded at 8 h (Figure 3a). Later, i.e. 20 h after reperfusion, positive nuclei were distributed throughout OSOM but their localization remained consistently interstitial in close proximity of S3 altered tubules (165 $\pm$ 23 cells/mm^2). Frequently, altered tubular sections appeared surrounded by numerous BrdU-positive interstitial cells (Figure 1f). One day after ischaemia, a majority of positive nuclei were localized within altered tubules of OSOM (Figures 1j and 3a) while the density of positive interstitial cells fell to reach values slightly superior to controls (Figure 3b). Focally, positive oval-shaped nuclei were observed inside necrotic tubules (NT) in contact with the basement membrane (Figure 1g). From this period onwards, BrdU-positive cells were never detected in the lumen of blood vessels. Two days after reperfusion, the majority of positive nuclei (1765 $\pm$ 510 cells/mm^2) were restricted to regenerating S3 tubules of OSOM (Figures 1h, k and 3a). One or two weeks after ischaemia, this homogeneous distribution within OSOM remained identical in kidneys observed (Figures 2a–c and 3a) and was associated with a high density of BrdU-positive nuclei (2750 $\pm$ 400 cells/mm^2) within apparently normal tubular profiles (Figure 2a–c). Morphologically, each of these tubules was characterized by a high percentage of BrdU-positive nuclei (80–85% at 7 days) by comparison to unlabelled epithelial cells. The evaluation of the mean optical density of BrdU positive nuclei allowed the distinction of 1–2 classes of densities among positive nuclei at 48 h while the number of classes of densities climbed to 3–4 after 1 or 2 weeks. Numerous pale positive nuclei were consistently observed in the regenerated proximal tubules of the basal region of OSOM (Figures 1i, 1 and 2b). In contrast, after a time interval of 1–2 weeks, BrdU immunostaining in other renal areas (cortex, ISOM and inner medulla) was similar to the appearance of controls for both tubules and interstitium.

In the following experiment, in the kidneys from rats that received BrdU 20 h after cytarabine, the density of labelled nuclei in tubular structures of OSOM appeared very high (1195 $\pm$ 105 cells/mm^2), similar to the values recorded in animals not exposed to cytarabine and observed 2 days after reperfusion. In contrast, the administration of cytarabine 2 h and 30 min before BrdU induced a significant decrease of positive cells both in S3 tubules and in interstitium, to reach values similar to controls (29 $\pm$ 16 cells/mm^2).

The proliferative activity in the kidney after ischaemia was also evaluated by injecting BrdU 1 h before killing to detect S-phase cells during the successive steps of tubular regeneration. Within OSOM, the proliferative activity increased considerably in S3 regenerating tubules after 20 h and reached a peak at 48 h in regenerating tubules (Figure 3a). Thereafter, the rate of cell turnover progressively declined to reach after 7 days a rate similar to control values. When using such a protocol, a significant density of BrdU-positive cells was never detected during the first 20 h following reperfusion.

Distribution of selectin E immunoreactivity

As early as 8 h after renal reperfusion, the first positive capillary endothelia were detected around altered tubular profiles of OSOM (Figure 4a). Later, the density of positive vascular profiles rose progressively to reach maximum values 16–18 h after ischaemia (Figure 4d). Strikingly, the distribution of selectin E positive capillaries was limited to the periphery of altered S3 tubules within OSOM but also focally around altered cortical convoluted tubules. Histological examination also revealed characteristic images of labelled cells adhesion to the walls of capillaries (margination) during the 8- to 24-h period (Figure 1b, d, e). It is interesting to note that unaltered tubular profiles sometimes observed within OSOM after ischaemia were never surrounded by selectin E-positive capillaries. After 24 h, positive profiles became sparse to finally disappear at 48 h. After 1 week, selectin E remained expressed focally in capillaries surrounding chronically altered proximal tubules in the OSOM area.

Distribution of vimentin immunoreactivity after ischaemia

Twelve hours after ischaemia, vimentin-positive cells were already observed in altered S3 tubular sections of OSOM. Thereafter, the density of positive cells rose progressively and 48 h after ischaemia (Table 2) most regenerating tubules present in OSOM strongly expressed vimentin. Strikingly, vimentin immunoreactivity appeared to be restricted to immature epithelial cells lacking a brush border. However, at long term, vimentin was still expressed focally in dilated and ill-repaired renal tubules or in cystic tubules.

In kidneys, vimentin immunoreactivity was also localized in interstitial round cells interspersed among renal tubules. In controls, the density of these vimentin-positive round cells remained always very low (<20 cells/mm^2). Twelve hours after ischaemia, their number rose significantly in OSOM to reach a peak at 16 h (Figure 5). These interstitial cells were localized in the vicinity of altered proximal tubules (Figure 4e) but also in the lumen of peritubular capillaries (Figure 4b). After 16 h, the density of positives round cells declined to reach control values at 2 days (Figure 5). Double immunostaining for BrdU-positive nuclei (Figure 4c, f—BrdU 24 h before ischaemia) and vimentin showed that intravascular and interstitial vimentin-positive cells were also characterized by BrdU-positive nuclei. At 16 and 24 h, these round cells were PCNA negative within blood vessels and interstitium but became PCNA positive after incorporation into regenerating tubules (data not shown). The analysis of S-phase cells distribution within kidneys only revealed dividing S3 tubular cells
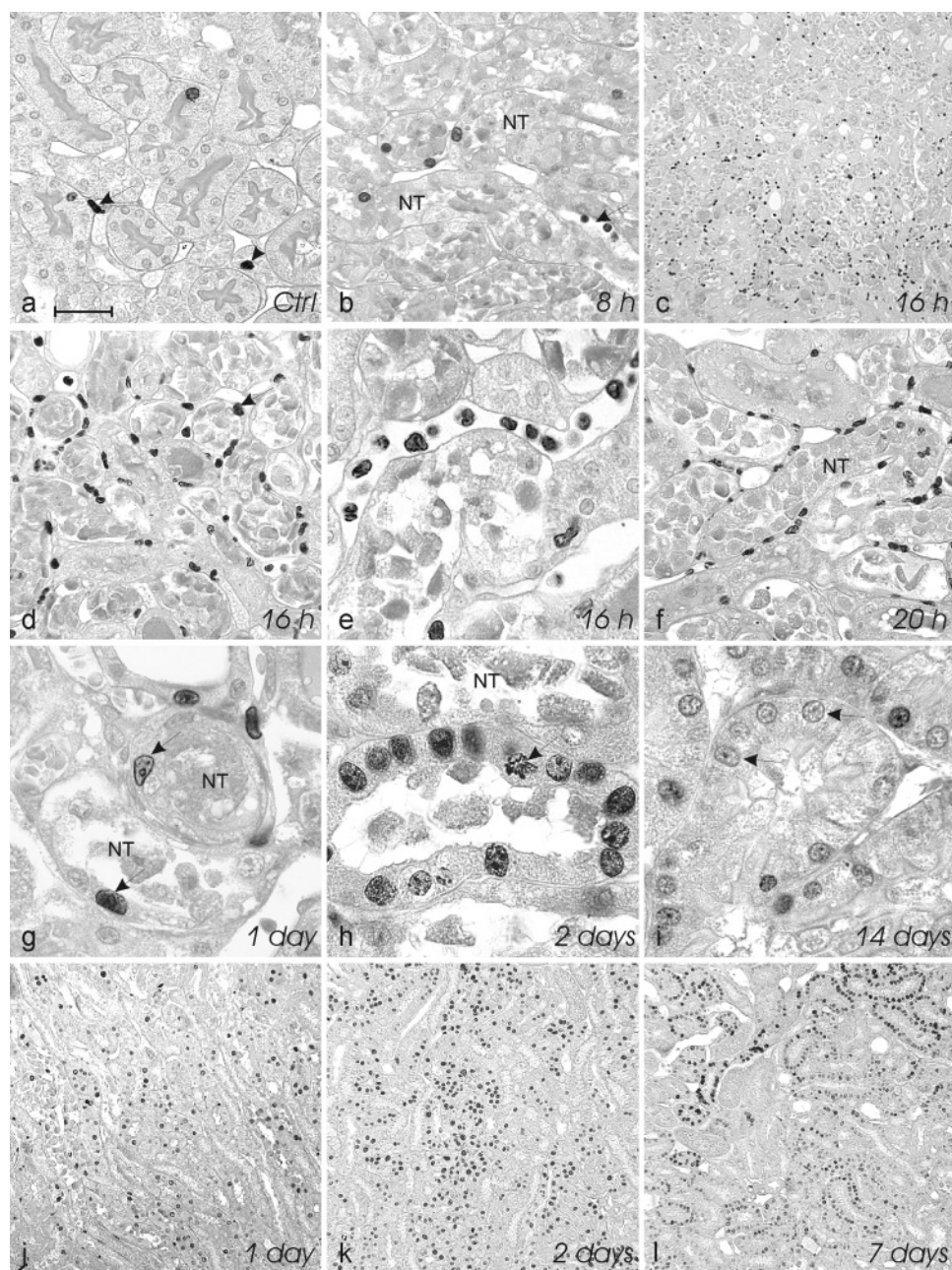
Fig. 1. Immunohistochemical demonstration of BrdU-containing nuclei in OSOM of controls (**a**) or of kidneys exposed to ischaemic insult (**b**–**l**). (**a**) In OSOM of control kidneys, sparse positive cells were localized in normal S3 and distal tubules (arrowhead) and focally in interstitium (arrow). Eight hours after ischaemia (**b**), the immunolabelling pattern is globally similar to controls but a small number of positive nuclei were detected in the wall of necrotic tubules (NT) and in the lumen of blood vessels (arrow). Sixteen hours after reperfusion (**c**–**e**), numerous BrdU positive nuclei were distributed within the basal region of OSOM (**c**). At higher magnification (**d**), these nuclei appeared scattered in interstitium on the periphery of necrotic tubules. Focally, positive nuclei were also observed inside the basal membrane of altered S3 tubules (arrow). At this point time, positive nuclei were frequently present in the lumen of vessels (**e**). Later, 20 h after ischaemia (**f**), the distribution of positive nuclei appeared unmodified but their density was higher, particularly in the vicinity of necrotic tubules (NT). One day after ischaemia (**g**), positive oval-shaped nuclei were observed focally inside necrotic tubules (**g**; NT) in contact with the basement membrane (arrows). Two days after reperfusion (**h**), the epithelial lining of regenerating S3 tubules contains a large number of BrdU-positive nuclei with focal images of mitosis (arrow). Two weeks after reperfusion (**i**), numerous pale positive nuclei were observed in normal epithelial cells of the proximal tubules located in the basal region of OSOM. The row of pictures (**j, k, l**) illustrates the distribution and the appearance of BrdU-positive nuclei in OSOM 1 day (**j**), 2 days (**k**) and 1 week (**l**) after reperfusion. A majority of positive nuclei are localized within S3 tubules. The density of positive nuclei increased from 1 day (**j**) to reach a maximum 1 week after reperfusion (**l**). Note that the staining intensity of nuclei (**k, l**) is different from the basal part of the picture (next to ISOM) to the top (upper part of OSOM) where nuclei exhibiting an intense black staining are more numerous. This is particularly obvious 1 week after reperfusion (**l**). Scale bar. **a**, **b**, **d**, **f**: 40 µm; **c**: 125 µm; **e**: 20 µm; **g**: 17 µm; **h**, **i**: 15 µm; **j**, **k**, **l**: 100 µm.
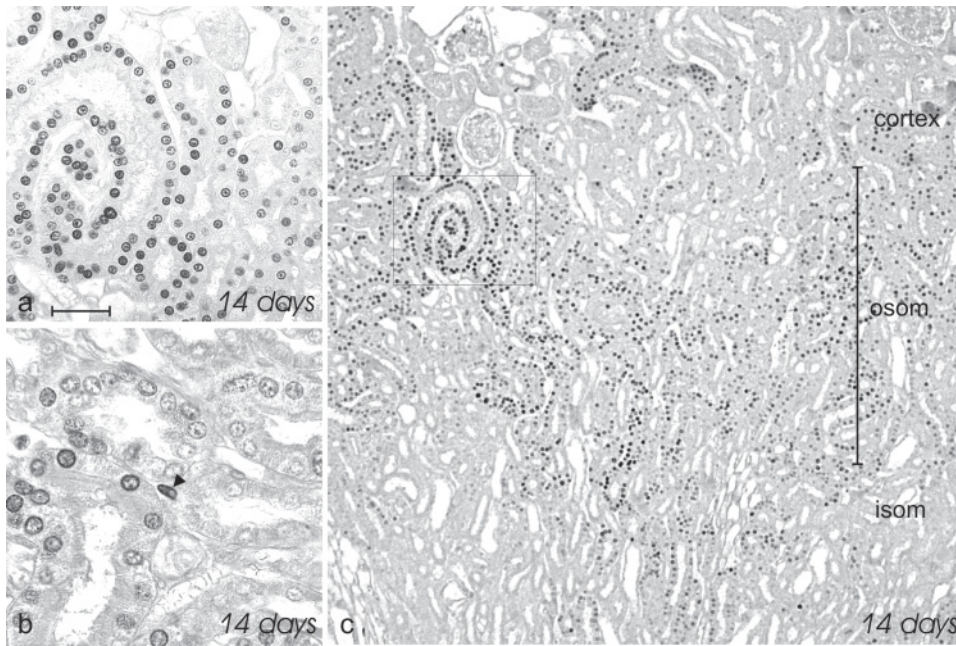
Fig. 2. Immunohistochemical demonstration of BrdU-containing nuclei in OSOM of kidneys 2 weeks after reperfusion (**a–c**). The density of BrdU-positive nuclei remained high within S3 tubules (**c**). At higher magnification, picture a (corresponding to the rectangular area of **c**) shows that in some tubular sections almost all nuclei are positive. Note that the intensity of nuclear labelling clearly varies among positive nuclei (**a, b**). In the same area, single positive nuclei were also observed in interstitium (b; arrow). Scale bar. **a**: 30 µm; **b**: 20 µm; **c**: 85 µm.
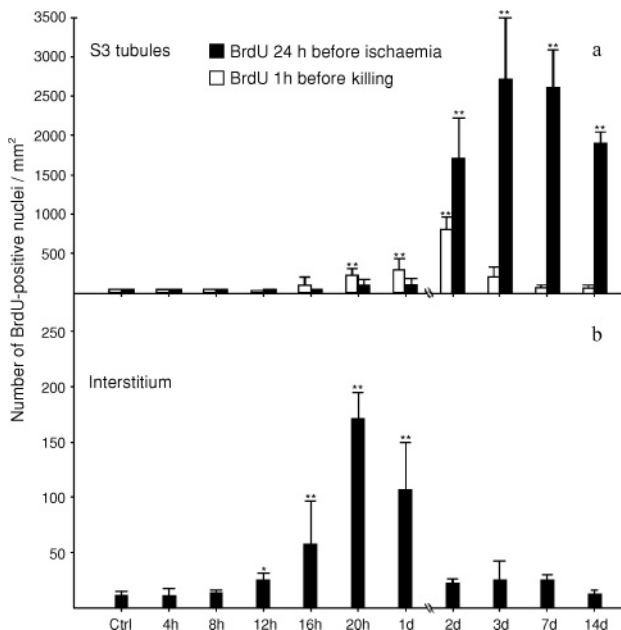

Fig. 3. Density of BrdU-positive nuclei in S3 tubules of OSOM (**A**) and in the corresponding interstitium (**B**) of kidneys from control animals (Ctrl) or from animals killed at increasing times (hours or days) after reperfusion. BrdU was administered to experimental animals 24 h before ischaemia (A, B: black columns) or 1 h before killing (A: open columns). Each column corresponds to the mean number of labelled nuclei per mm^2 (± SD) determined after the examination of OSOM in each experimental group ($n = 4$) (*$P < 0.05$; **$P < 0.01$).

(see above) suggesting that round cells remain quiescent within interstitium.

Distribution of CD44 immunoreactivity after ischaemia

Twelve hours after ischaemia the number of positive tubular cells rose significantly in S3 segments of OSOM to reach a peak at 48–72 h (Table 2). At this moment, the incidence of immunolabelled regenerating tubular profiles was very high (Figure 4k). These cells, always devoid of brush border, present the characteristics of immature epithelial cells. One week after reperfusion, CD44 expression declined abruptly in parallel with the return to a normal state of differentiation characterized by the reappearance of a brush border on proximal cells. At longer term, positive flat cells were restricted to a small number of chronically altered cystic tubules (Table 2).

In controls, scarce CD44-positive round-shaped cells appeared within interstitium in the three zones on the kidney. Eight hours after ischaemia, the number of positive circulating cells rose within small vessels at the junction OSOM–ISOM (Figure 4g) to reach a peak later (12–16 h) in the capillary network between NT (Figure 4h). Later, the density of intravascular round cells declined rapidly to reach control values after 1 day.

From 12 h after ischaemia, the density of positive cells rose within interstitium of the junction OSOM–ISOM to progressively infiltrate OSOM and the basal region of cortex (Figures 5 and 4j). After 2 days, the density of CD44-positive interstitial cells declined rapidly to resume the appearance of controls. Double-label immunofluorescence for vimentin and CD44 showed that the two markers are coexpressed both in intravascular cells (Figure 4i) and in interstitial peritubular cells (Figure 4l). As observed by double-label immunofluorescence, CD44-positive cells were negative for PCNA in both capillaries and interstitium (data not shown).
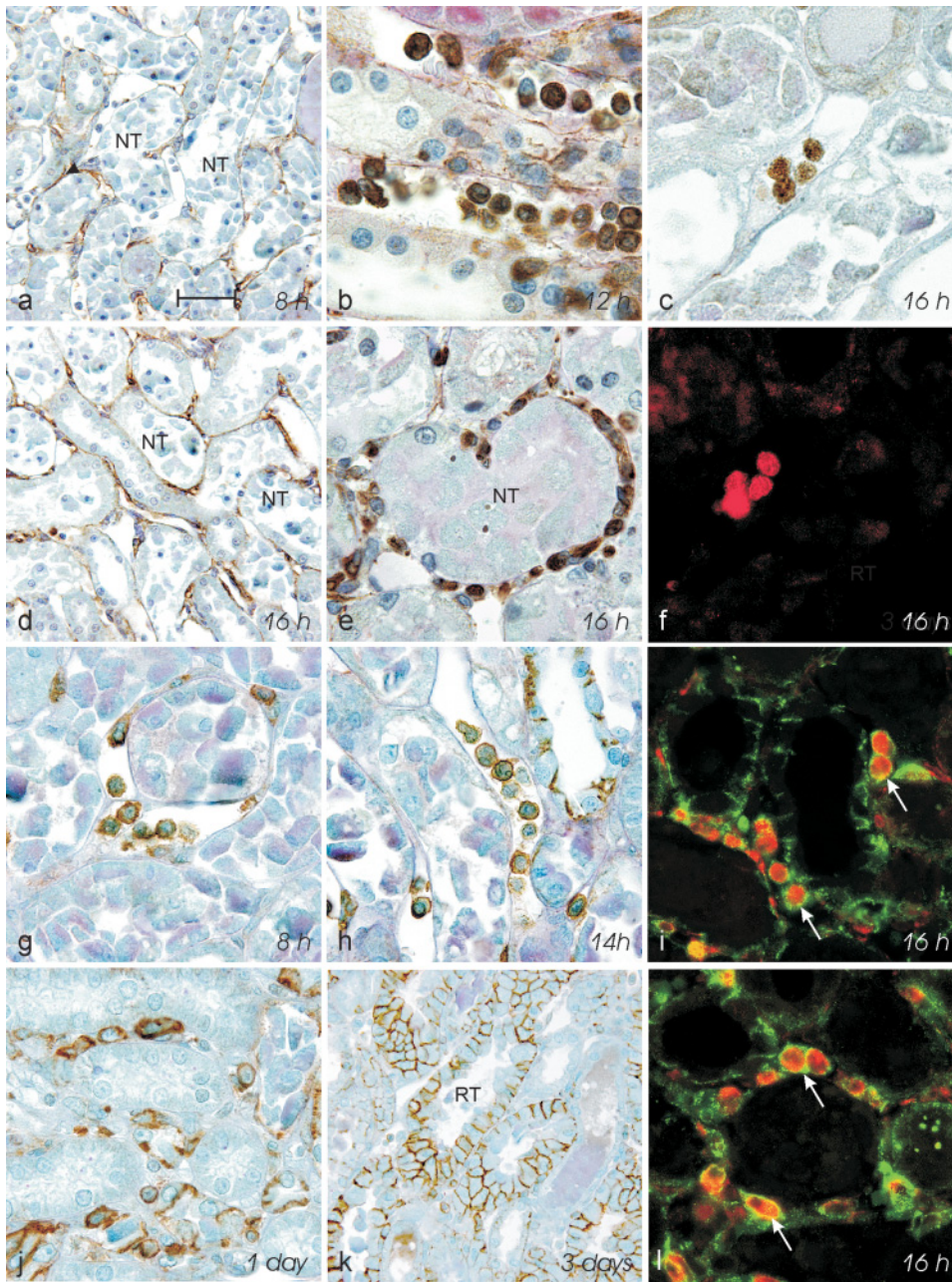
Fig. 4. (**a,d**) Distribution of selectin E immunoreactivity in vascular endothelial cells of kidneys from animals killed 8 h (**a**) or 16 h (**d**) post-ischaemia. Positive capillary profiles were observed within OSOM (a; arrow) adjacent to necrotic S3 tubules (NT). (**b,e**) Immunohistochemical demonstration of vimentin in ischaemic kidneys. Twelve hours after ischaemia (**b**) note the presence of positive round cells in the lumen of peritubular capillaries at the junction OSOM-ISOM. Later (e; 16 h after reperfusion) numerous positive cells were localized around necrotic tubules (NT) in OSOM. (**c,f**) Double immunostaining combining ABC method for BrdU-positive nuclei (**c**) and immunofluorescence for vimentin (f; red). The comparison of the two pictures shows that intravascular vimentin-positive cells are also characterized by BrdU-positive nuclei. (**g, h, j, k**) Distribution of CD44 positive cells within OSOM. Eight hours after reperfusion (**g**), the first positive round cells were detected in the lumen of peritubular capillaries to reach higher intravascular densities at 14 h (**h**). One day after ischaemia (**j**), numerous round cells had infiltrated the interstitium of OSOM. Three days after ischaemia (**k**), a majority of regenerating tubules (RT) within OSOM were characterized by a positive immature epithelial lining. (**i, l**) Double immunostaining for vimentin (red) and CD44 (green) in ischaemic kidneys 16 h after reperfusion. The two markers are coexpressed in intravascular cells (**i**) and in peritubular cells (**l**). The immunolabelling corresponding to CD44 receptors is restricted to the periphery of positive cells (arrows). Scale bar: **a**, **d**: 55 μm; **b**: 17 μm; **c**, **f**: 14 μm; **e**, **g**, **h**, **j**: 22 μm; **k**: 40 μm; **i**, **l**: 18 μm.

Table 2. Distribution of markers used for the characterization of tubular and interstitial cells of OSOM involved in renal regeneration

Period post-ischaemia	Controls	8‑12 h	16 h	20 h	1 day	2 days	7 days	CT‑NT
BrdU[1]	★ •	★ •	★ •	★ ○	★ ○	★ ○	★ ○	– –
Vimentin	★ ○	★ ○	★ ○	★ ○	★ ○	★ ○	★ ○	– ○
CD44	★ ○	★ ○	★ ○	★ ○	★ ○	★ ○	★ ○	– ○
MHC II	– –	★ –	– –	– –	– –	– –	– –	★ –
CD68	– –	★ –	– –	– –	– –	– –	– –	★ –
Alpha-actin	– –	– –	– –	– –	– –	– –	– –	★ –
Hyaluronan	★	★	★	★	★	★	★	★

Long lasting Cystic (CT) or Necrotic (NT) tubules

Density of positive interstitial cells
- – : Absence of positive cells
- ★ : < 50 cells / mm²
- ★ : 50–100 cells / mm²
- ★ : > 100 cells / mm²

Density of positive tubular cells
- – : Absence of positive cells
- ○ : < 50 cells / mm²
- O : 50–100 cells / mm²
- O : > 100 cells / mm²

BrdU – Density of positive tubular cells
- • : < 100 cells / mm²
- ○ : 100–1000 cells / mm²
- O : 1000–2000 cells / mm²
- O : > 2000 cells / mm²

Hyaluronan – % of scanned area
- ★ : < 2%   ★ : 2–5%   ★ : > 5 %
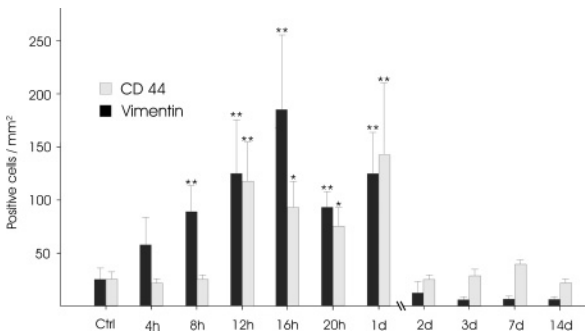
[1] BrdU administered 24 h before ischaemia

Fig. 5. Distribution of vimentin and CD44 immunoreactive-round cells in the vascular and interstitial compartments of OSOM from control animals (Ctrl) or from animals killed at increasing time intervals (hours or days) after reperfusion. Each column corresponds to the mean (± SD) determined after the examination of OSOM for each experimental group ($n = 4$) (*$P < 0.05$; **$P < 0.01$ versus control).

Fig. 6. Schematic representation of the distribution of BrdU positive nuclei and of positive cells (CD44, vimentin) in OSOM of kidney during a 2-week period consecutive to ischaemia (h: hours, d: days). Renal regeneration after ischaemia is associated with a typical sequence of successive events: (I) absence of positive cells; (II) positive cells restricted to the lumen of blood capillaries; (III) positive cells observed in the same time in peritubular interstitium and in vessels and (IV) positive cells in tubular epithelium. The horizontal pecked line corresponds to both the period of increased deposition of hyaluronan in interstitium and the period of selectin E expression by endothelium of capillaries.

Distribution of hyaluronan after ischaemia

In controls, hyaluronan is clearly visible within interstitium of ISOM and inner medulla but is absent from cortex and OSOM. From 8 h after ischaemia, the density of hyaluronan climbed progressively within interstitium of OSOM to remain at higher values between 12 and 48 h after reperfusion (Table 2). During this period, hyaluronan appears localized around NT. Later, its density declined slowly to reach control va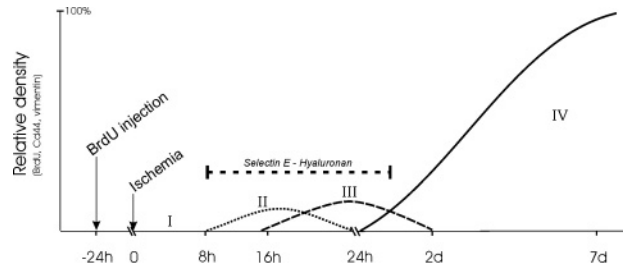lues after 3 days. Altogether, the higher density of hyaluronan within OSOM corresponds to the presence of CD44-positive cells in capillaries and interstitium.

Distribution of MHCII, α-smooth muscle actin and CD68 immunoreactivities after ischaemia

After ischaemia, MHCII-positive cells appeared temporarily in OSOM 8 h after reperfusion (Table 2) mainly around blood vessels of the cortico-medullary junction. At 12 h,

MHCII immunostaining had resumed the same appearance as controls. At long term, these cells were disposed in clusters associated with inflammatory infiltrates and restricted to chronically altered areas of OSOM (Table 2).

In control kidneys, the immunolabelling with an anti-α-smooth muscle actin antibody was limited to the media of arteriolar vessels and to pericytes of the ascending vasa recta. Two weeks after reperfusion, interstitial α-actin positive cells were exclusively found in the vicinity of necrotic or cystic tubules (Table 2).

From 12 h after reperfusion, clusters of CD68-positive cells were only observed focally on the periphery of NT in OSOM. After 2 weeks, infiltrates of CD68 positive cells persisted in chronically altered areas such as residual NT (Table 2).

Finally, three other markers known to be expressed by leukocytes (CD45—common leukocyte antigen) and by some categories of stem cells (CD90 and CD133) were also tested in our model. However, the expression of these markers was never observed in the round cells described above or in regenerating tubules.

Altogether, the examination of distribution and density of BrdU$^+$, CD44$^+$ and vimentin$^+$ cells during a 2-week period consecutive to ischaemia revealed a typical sequence of events (Figure 6): (1) absence of immunostaining during the first 8 h after reperfusion; (2) between 8 and 16 h, detection of a small population of positive cells in the lumen of blood vessels; (3) between 16 and 24 h, a progressive decrease of positive cells in blood vessels and a concomitant increase in interstitium; (4) after 1 day, labelled cells disappeared progressively from peritubular interstitium but were increasingly detected in regenerating tubules and (5) after 1 week, BrdU-positive nuclei were restricted to epithelial lining of mature proximal tubules. Selectin E and hyaluronan were expressed during a limited period (8–48 h post-ischaemia) corresponding to the presence of labelled cells in renal vessels and interstitium.

Discussion

The model of bilateral renal ischaemia in the rat leads to the development of an acute tubular necrosis mainly affecting proximal straight tubules (S3 segments) in OSOM. Two weeks after ischaemia, a majority of proximal tubules are completely repaired [29]. In the present study, we adopted a protocol originally designed to detect interstitial or tubular stem cells and/or BMSC harboured in the kidney, cell subpopulations characterized by very long cell-cycle times (label-retaining cells) [13,14,34]. In this context, BrdU was injected in untreated animals 24 h before ischaemia and the kidneys examined during a 2-week period after ischaemia. This protocol led to the detection of a significant number of BrdU-positive nuclei very early after reperfusion (8–18 h) in blood vessels and within interstitium of OSOM. This observation was unexpected since the time interval 8–18 h corresponds to a period characterized by a very low level of S-phase cells (BrdU 1 h before killing—Figure 3a). This time course of events suggests that BrdU was not incorporated immediately into renal cells but rather in an extra-renal rapidly cycling population of cells migrating later within the kidney after ischaemia. Another striking observation that emerges from our experimental results is that the density of labelled interstitial cells is low at 12 h (30 $\pm$ 8 cells/mm^2), but climbs abruptly to higher values (165 $\pm$ 23 cells/mm^2) 20 h after reperfusion. This rapid increase of density during a limited period of time (8 h) does not seem to be consistent with a succession of cellular divisions (2–3 cycles) but rather points to an involvement of extrarenal migrating cells. The typical sequence of transient events (Figure 6) also indicates that the transit of BrdU-positive cells from blood through interstitium is an inconspicuous event of short duration (maximum 16 h) in comparison to the extended period needed for complete tubular regeneration (1–2 weeks).

Our study also showed that selectin E was expressed in the endothelium of blood vessels during a short period of time (8–24 h) corresponding to the presence of labelled cells in interstitium and in capillaries localized at the periphery of altered S3 tubules. This limited expression of selectin E after tissue injury (8–24 h) was described previously [18,19] and characterized by a similar time course. The expression of selectin E, activated by various stimuli, is normally involved in interactions between circulating leucocytes and endothelia such as margination and diapedesis. Finally, it results from a comparative analysis of the data at the same time points (between animals receiving BrdU 24 h before ischaemia versus 1 h before killing) that migrating cells remain quiescent during their migration through interstitium. It is also interesting to note that the short duration of this step of migration ($\sim$12 h) does not appear to be consistent with a cycle of cell division. This suggests that these migrating cells start to divide actively only after implantation in altered tubules until complete epithelial restoration. This hypothesis seems to be corroborated by the evaluation of the mean optical density of positive tubular nuclei. Indeed, this increased number of positive nuclei was accompanied by a concomitant decrease of the immunolabelling density in BrdU-positive nuclei. We therefore suggest that the increase of positive nuclei during tubular regeneration results from the successive divisions of cells originally labelled by BrdU before ischaemia. During the first stages of tubular regeneration, these cycling cells, as early descendants of migrating cells, could be considered as transit-amplifying cells retaining growth capacity while acquiring a more differentiated phenotype. Another observation that emerges from our experimental results is that this increasing number of labelled nuclei during the process of regeneration is not only consistent with the massive tubular cell death ($\sim$90%) affecting S3 segments but also with both a small population of migrating extrarenal cells ($\sim$130–150 cells/mm^2) and a limited number of successive cellular divisions (3–4 cycles). Another consequence of this process is that a high number of BrdU-positive nuclei ($\sim$3000 cells/mm^2) remained detectable within fully differentiated tubules 1–2 weeks after ischaemia. If we assume that these fully differentiated tubular cells have achieved 3–4 cycles of division, what we observed at long term is a population of mature and fully differentiated epithelial cells retaining the label for long periods. In this context, the detection of S-phase cells (BrdU 1 h before killing—Figure 3a) only revealed dividing tubular cells but never disclosed

migrating extrarenal bone marrow-derived cells because they remained quiescent until their implantation within altered tubules. This observation led to the hypothesis, frequently proposed, that tubular repair is a mechanism that requires dedifferentiation of surviving intratubular cells [4,10,11].

One can speculate that our observations could simply result from the persistence of a pool of plasmatic BrdU remaining available for incorporation in S-phase cells after renal reperfusion. This appears unlikely since previously published observations [35–37] have demonstrated that parenterally administered BrdU undergoes rapid and extensive degradation in the liver (80% after 1 h) and that only a minor part of the administered BrdU remains transiently available (8–11 min) for incorporation into various tissues. Assuming this rapid clearance of BrdU, we concluded that the majority of BrdU-positive cells detected in the kidney after ischaemia could originate from a rapidly cycling extrarenal cell population.

The source of these extrarenal cells and their phenotype remains however to be identified. In view of the ability of BMSC to differentiate into different cell lineages, we have hypothesized that some of these extrarenal bone marrow-derived cells could participate to the tubular regeneration. Previous studies have observed the distribution of radioactive ^{82}Br activity in the DNA of various rat tissues 1 h after the administration of labelled BrdU-^{82}Br [36]. As expected, the ^{82}Br activity was high in bone marrow as compared to the low levels detected in kidney. In this context, we have examined the effects of cytarabine on BrdU incorporation during tubular regeneration. Cytarabine, an antimetabolic drug blocking DNA synthesis, is commonly used as a chemotherapeutic agent for bone marrow suppression. It was previously demonstrated [38] that haematopoietic stem cells cease to incorporate BrdU 2–3 h after cytarabine administration. This is followed by a rapid increase of BrdU incorporation reaching normal values at 20 h. In this experiment, BrdU was administered 2 h and 30 min after cytarabine injection while kidney ischaemia was performed 24 h after BrdU administration. Under these conditions, the administration of cytarabine induced a dramatic decrease of positive cells density in S3 tubules, to reach values similar to controls, when observed 2 days after ischaemia. This experiment demonstrates that BrdU, in the presence of cytarabine, was not incorporated in a population of cells normally involved in tubular regeneration and strongly suggests that an unidentified population of BMSC could be involved in kidney regeneration. Cytarabine was injected in control animals 24 h before ischaemia. Consequently, due to the short half-life of this compound, cytarabine could not be incorporated in kidney since the number of S-phase cells is extremely low in control animals.

However, the source of proliferating cells that repopulate altered nephrons remains controversial. A survey of the literature reveals that kidney regeneration may result from three different mechanisms such as (i) proliferation and differentiation of surviving dedifferentiated tubular cells [4,11,12], (ii) activation of resident stem cells [13,14] or (iii) differentiation of cells of extrarenal origin such as circulating BMSC [6,7,9,39]. The present study provides the first detailed description of a time course of events that is consistent with an involvement of extrarenal bone marrow-derived cells during regenerative hyperplasia. However, in contrast with our study, a majority of previously published observations appear less supportive of a major role for extrarenal bone marrow-derived cells in this regenerative process. A common feature of these studies resides in experimental protocols that involve infusion of whole bone marrow [8] or transplantation of purified bone marrow subpopulations [10,39]. Using different approaches for tracking donor-derived cells, these authors found that implanted cells were not observed to incorporate significantly and differentiate into repairing tubular segments. These studies led to the conclusion that tubular repair mainly involves proliferation of endogenous cells (such as dedifferentiated tubular cells) and that incorporation of unidentified subpopulations of BMSC within the kidney is only followed by an improvement of renal function (renoprotection) [11,40,41]. On the other hand, the present work provides evidence that both BrdU-positive cells migration and E-selectin expression are transient events occurring immediately (8–20 h) after renal reperfusion. Consequently, we suggest that the difficulty to detect a significant number of bone marrow-derived cells in renal tubules of rodents could result, at least in part, from the choice of inappropriate time slots to perform transplantation.

In a second step of this study, we have characterized the BrdU-positive cells involved in tubular regeneration by the use of antisera raised against two markers: vimentin and CD44. Vimentin is an intermediate filament expressed in mesenchymal tissues and used as a marker of differentiation. In a normal adult kidney, vimentin is absent from mature epithelial cells but is expressed transiently by regenerating proximal tubular cells [1,20]. CD44 glycoprotein, a cell surface receptor for the glycosaminoglycan hyaluronan, is involved in cell–cell or cell–matrix interactions [24]. Beyond its function of adhesion molecule CD44 is implicated in various processes such as cell migration, tissue repair or cancer metastasis and appears upregulated in kidney during regeneration of proximal tubules [42]. On the other hand, CD44 is also a marker of stem cells such as undifferentiated mesenchymal stem cells (MSC) [43] in contrast to myeloblastic stem cells mainly expressing CD45 [44]. Other markers such as CD45 expressed by leukocytes and their precursors [44] or CD90 and CD133 known to be expressed by some stem cell categories [45,46] were also tested in our model but were never evidenced in cell categories involved in tubular regeneration.

As illustrated in Figure 4, we observed a massive accumulation of vimentin- and CD44-positive cells within blood vessels very soon (8–16 h) after reperfusion. These cells were, however, restricted to the vicinity of altered S3 tubules, generally bound to the endothelium of capillaries (margination) thus suggesting a process of diapedesis. Additionally, we showed that selectin E was expressed transiently by capillaries of OSOM during the same time interval. Altogether these observations suggest that the selective capture of vimentin/CD44-positive extrarenal bone marrow-derived cells could be mediated by selectin E, a process currently observed during inflammation [18,19,47]. Later, 16–24 h after reperfusion, these cells disappeared

progressively from capillaries and were only noticed in the interstitial compartment around necrotic tubules. Concomitantly, we observed a transitory increase of hyaluronan in the extracellular matrix of OSOM suggesting that hyaluronan could play a pivotal role during the transit of CD44-positive cells through the interstitial compartment. CD44 expression associated with the hyaluronan overproduction seems to provide a general mechanism for leukocyte recruitment both in bone marrow and in peripheral tissues [21,48]. It was also reported that interactions between CD44 and hyaluronan could lead to the incorporation of MSC in injured kidneys. Stem cells from CD44 knockout mice appear unable to localize into injured kidneys and this resulted in an impairment of both morphological and functional recovery of the organ [39]. Altogether, these data suggest a pivotal role for CD44 and its ligand hyaluronan in the process of renal regeneration [49,50].

As demonstrated by PCNA/CD44 double-label immunofluorescence, these extrarenal cells remained quiescent during their transit across the interstitial compartment. One day after reperfusion, vimentin/CD44-positive cells began to colonize necrotic tubules where they started to divide actively. Indeed, 48 h after reperfusion we observed a maximum of BrdU incorporation in undifferentiated epithelial cells within regenerating S3 tubules (Figure 3a). This phase of proliferation led to a rapid re-epithelialization of necrotic tubules. The final differentiation of mature epithelial cells is characterized by the restoration of a brush border but also by the loss of vimentin and CD44 expression [1,20,42]. Finally, as evidenced by double-label immunohistochemistry, these two markers were also expressed by cells involved in tubular regeneration and characterized by BrdU positive nuclei.

In a last step of our study, we have investigated the distribution of various cell types by performing immunohistochemistry for markers of monocytes/macrophages (CD68), dendritic cells (MHCII) and myofibroblasts (α-SMA). Monocytes/macrophages were only present focally around large blood vessels very early after reperfusion (6–8 h). Later, they decreased rapidly except in chronically altered areas suggesting that they are not involved in tubular regeneration. Accordingly, infiltrates of dendritic cells, lymphocytes or of neutrophils were never noticed during the 2-week post-ischaemic period while myofibrobasts were restricted to chronically altered areas. In this respect, it is worth noting that our model of moderate warm ischaemia is characterized by a rapid tubular regeneration in the absence of fibrosis or of inflammatory infiltrates [51,52].

In conclusion, the present observations suggest a massive intervention of extrarenal cells in the process of renal reparation and provide new insight into the fundamental mechanisms of tubular regeneration consecutive to ischaemia. It remains to answer the question of the precise nature and origin of these potential bone marrow-derived stem cells, although the immunohistochemical characteristics of round cells (vimentin$^+$, CD44$^+$ and CD45$^-$) could point to an involvement of exogenous MSC in renal regeneration as recently proposed by Herrera and coll [53].

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