Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury

Dirk K. Ysebaert¹, Kathleen E. De Greef¹, Sven R. Vercauteren¹, Manuela Ghielli², Gert A. Verpooten², Erik J. Eyskens¹ and Marc E. De Broe²

Departments of ¹Experimental Surgery, and ²Nephrology, University of Antwerp, Belgium

Abstract

Background. Leukocyte adhesion/infiltration in response to renal ischaemia/reperfusion (I/R) injury is a well-known but poorly understood phenomenon. The identification, kinetics, and exact role of these inflammatory cells in I/R injury and regeneration are still matters of debate.

Methods. Uninephrectomized rats were submitted to 60 min renal ischaemia by clamping of renal vessels.

Results. Severe acute renal failure was observed, with maximum functional impairment on day 2. By 12 h after the ischaemic event, up to 80% of proximal tubular cells in the outer stripe of outer medulla (OSOM) were already severely damaged. Proliferation (proliferating cell nuclear antigen (PCNA) staining) started after 24 h, reaching maximum activity on day 3. Regeneration of tubular morphology started on the 3rd day, and after 10 days 50% of tubules had regenerated completely. Interstitial leukocytes (OX-1 immuno-histochemical staining) were already prominent at day 1, thereafter gradually increasing with time. The so-called neutrophil-specific identification methods (myeloperoxidase (MPO), chloroacetate esterase, mAb HIS-48) proved to be non-specific, since they also stained for macrophages, as demonstrated by flow cytometry and the combination of these stainings with the macrophage-specific ED-1 staining. MPO activity was already significantly increased at 1 h post-I/R (439 ± 34%, P < 0.005), reaching its maximum activity after 12 h of I/R (1159 ± 138%, P < 0.0005), declining thereafter. On the other hand, neutrophil presence investigated by H&E staining revealed only a few neutrophils in glomeruli, medullary rays, and OSOM at 24 h after the ischaemic event (4.7 ± 4.2 cells/mm² vs controls = 2.3 ± 2.0 cells/mm² (n.s.)), and remained unchanged over the next 10 days. In contrast, significant monocyte/macrophage adhesion/infiltration (ED-1 staining) occurred at the OSOM at 24 h post-ischaemia (at 24 h, 120 ± 46 cells/mm² vs sham = 18 ± 4 cells/mm² (P < 0.05)), became prominent at day 5 (1034 ± 161 cells/mm² vs sham = 18 ± 18 cells/mm² (P < 0.05)), and almost disappeared after 10 days. CD4⁺ cells (W3/25) gradually increased from day 5, reaching a maximum at day 10. A few CD8⁺ cells (OX-8) were apparent from days 3 until 10, but no B-cells (OX-33) were observed.

Conclusions. After severe warm I/R renal injury, a pronounced acute tubular necrosis occurs during the first 12–24 h in the absence of a marked cellular infiltrate, but with an important renal MPO activity, reflecting the activation of the adhering inflammatory cells (polymorphonuclear cells (PMNs) and mainly monocytes/macrophages). Only later at the time and site (OSOM) of regeneration a sequential accumulation of monocytes/macrophages and T cells becomes prominent, in contrast with the low number of neutrophils found in the kidney during the 10-day post-ischaemic period. The non-specificity of the so-called neutrophil-specific identification methods (MPO activity, naph-thol AS-D chloroacetate esterase, or mAb HIS-48 staining), cross-reacting with monocytes/macrophages, explains the controversy in literature concerning the number of PMNs in post-ischaemic injury.

Keywords: damage; kidney; macrophages; myeloperoxidase; neutrophils; rat; regeneration

Introduction

Ischaemia/reperfusion (I/R) injury is a common clinical event, still associated with high mortality and morbidity [1], and lacking a specific therapy. Post-ischaemic acute tubular necrosis (ATN) is observed most frequently in patients after major surgery (cardiac and aortic operations), trauma, severe hypovolaemia, burns, and others [2]. In addition, delayed graft function of renal allografts is mainly caused by post-ischaemic ATN, with significant long-term graft survival [3]. Despite decades of laboratory and clinical investigation, the pathophysiology of I/R injury is still incompletely understood [1].
Leukocyte infiltrate after ischaemia/reperfusion injury

Renal injury after ischaemia appears to be a consequence of tissue hypoxia from interrupted blood supply but also from the process of reperfusion, leading to an active inflammatory response [4]. Sublethal or even lethal injured proximal tubular and endothelial cells trigger this process through the release of cytokines and chemokines that will promote cellular infiltration. Leukocytes may play an important role in the mechanism of parenchymal injury after I/R as well as in the regeneration process, but their exact role is far from clear. Polymorphonuclear cells (PMNs) recruited during reperfusion have long been implicated as critical mediators of the early renal parenchymal injury in ischaemia ARF, as recently reviewed [5]. These assumptions were supported by morphological criteria (H&E stain [6]), enzymatic criteria (myeloperoxidase [7–9], chloroacetate esterase [10–13], mAb HIS-48 [14]) and labelling techniques (e.g. 111Indium labelling [15]), in most cases suggesting robust PMN recruitment in the post-ischaemic kidney [1]. These PMNs may contribute by potentiating an inflammatory response that leads to the generation of vasoconstrictor agents, cytokines, and toxic mediators such as reactive oxygen species and proteases [16,17]. I/R in rat myocardium [18], liver [19], and brain [20] have been correlated with the number of granulocytes adherent at the capillary walls. Also in the kidney, PMNs have been put forward as inducing or amplifying additional damage in post-ischaemic renal injury [7–9,12,13,15,21]. Several investigators, however, found that infiltration of the renal parenchyma by PMNs was not a prominent feature of experimental or human post-ischaemic ARF when PMNs in renal sections were counted using routine histology (H&E) [1].

The first step in unravelling this controversy consists in the careful analysis of the identification of these adhering/infiltrating cells and their kinetics and distribution, related to the course of tubular injury and repair after severe warm I/R.

Subjects and methods

Animals and experimental groups

After overnight fasting, the surgical procedures were carried out under anaesthesia with sodium pentobarbitone (60 mg/kg) in inbred male LEW rats (220–260 g). A midline abdominal incision was made and heparin (50 IU, i.p.) was administered. To help to maintain thermoregulation during ischaemia, the abdomen contents were replaced and covered with a wet dressing. No specific heating pad was used. Animals were randomly assigned to two groups: (i) left renal ischaemia, performed by cross-clamping the left renal pedicle for 60 min with a microvascular clamp, followed by right nephrectomy at the end of the ischaemia period (n = 40); (ii) right nephrectomy alone, without ischaemia (n = 40). In these control animals the left renal pedicle was dissected, but not clamped. Careful dissection was carried out to preserve the blood supply to the adrenal glands. The kidneys were inspected for ischaemia as well for good reperfusion for 2 min. This uninephrectomized model was chosen for analogy with the situation of renal transplantation; moreover, prior investigations have shown that contralateral nephrectomy enhances the functional and morphological recovery of the post-ischaemic kidney [22,23].

Post-operative animals were allowed to recover, each in a separate cage, at constant temperature (18°C) and humidity (45%) on a 12-h light/dark cycle. They received free tap water ad libitum, and standard laboratory rat pellets by the paired-feeding method. In this way non-ischaemic animals received the same protein and salt intake as post-ischaemic animals, which show greater post-operative anorexia. Animals were weighed and inspected daily. Sacrifice of 4 animals per experimental time point was done at hours 1, 2, 6 and 12 and on days 1, 2, 3, 5, 7, and 10. Blood samples were taken by heart puncture. All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals No. 85–23 (1985), and with approval of the Ethical Committee of the University of Antwerp.

Biochemical determinations:

Blood samples were allowed to clot and were centrifuged at high speed for 15 min. Serum was obtained and stored at −20°C until use. Serum creatinine values were determined in duplicate using a colorimetric method as modified by Jaffé (Creatinine Merckotest, Diagnostica Merck, Germany).

Tissue collection and fixation:

Immediately after sacrifice, tissue for analysis was collected from the left kidney. After dissection of the capsular fat, the kidney was weighed. Five sagittal tissue sections (1 mm thick) were made and fixed in formalin calcium, methacarn and Dubosq Brazil fixative. Two sections were stored in liquid nitrogen.

Morphological analysis of tubular injury, regeneration and cell proliferation:

The degree of injury in different tubular compartments was established on periodic acid-Schiff reagent (PAS)-haematoxylin-PCNA stained sections of methacarn-fixed and paraffin-embedded renal tissue. Proliferation was determined by immunohistochemical staining for the PCNA using the PC10 monoclonal antibody (Dako, Denmark). Sections were counterstained with PAS. Nuclei were stained with methyl green. Histological damage of the kidney was scored semi-quantitatively: 50 tubules in the outer stripe of the outer medulla (most sensitive zone for ischaemic injury) were assigned using a score-system ranging from 0 to 6 (score 0, normal tubule; score 1, (limited to) loss of brush border; score 2, <50% tubular damage, meaning less than 50% of the basal membrane; score 3, >50% tubular damage; score 4, total destruction of all epithelial cells, naked basement membrane; score 5, small rim of cytoplasm with large dark nuclei (PCNA+); score 6, increase in cytoplasm volume; evolution to score 1, uncompleted brush border and score 0, complete regenerated brush border). The proliferation was measured by counting the number of PCNA-immunoreactive nuclei in 25 circular-shaped proximal tubules.

Renal adhesion/infiltration — leukocyte cell markers:

Sections (4 µm) were mounted on poly-l-lysine-coated microscope slides and treated for 5 min with 0.003% trypsin III (Sigma Chemical Co., St Louis, USA) in 10 mmol/L Tris–HCl
buffer pH 7.3. After washing in Tris saline buffer (TSB, 0.01 mol/l Tris–HCl pH 7.6 in 0.9% NaCl) and treatment with normal horse serum (1/5), the sections were incubated overnight with the primary antibody OX-1 (1/6000). This OX-1 mAb recognizes the rat leukocyte common antigen CD45, which is present on all marrow-derived leukocytes [24]. Positive cells were counted in 20 fields of 0.16 mm² and data were expressed as leukocytes/mm². Immuno-histochemical detection of monocytes/macrophages was performed on methacarn-fixed, paraffin-embedded renal-tissue sections using the ED1 monoclonal antibody (Serotec, UK). This antibody is directed to a cytoplasmic antigen of tissue macrophages and monocytes [25]. As control, a peripheral blood smear and a spleen section of the rat were stained. T-lymphocytes (Helper T-cells: W3/25, suppressor T-cells: OX-8), and B-lymphocytes (OX-33) were demonstrated on inactivated (step 3) and the ED-1 staining itself was developed. More in detail, 5 μm renal tissue cryosections were used, prefixed in formalin calcium (4% formaldehyde (BDH Chemical Ltd., Poole, UK)) buffered with 0.1 mol/l Na-cacodylate pH 7.4 containing 1% CaCl₂. Sections were incubated overnight with W3/25 (1/800), OX-8 (1/400) or OX-33 (1/100) (Serotec, Oxford, UK). Appropriate antibody dilutions were determined in preliminary experiments. The W3/25 monoclonal antibody recognizes the rat CD4-equivalent present on T-helper cells [26], and, in lower density, on some macrophages [27]. The OX-8 monoclonal antibody reacts with T-suppressor/cytotoxic and natural killer cells, and is directed to the rat CD8-equivalent [28]. The OX-33 monoclonal antibody reacts with only peripheral B-cells [29]. Positively stained cells were quantified in 10 randomly chosen OSOM microscope fields (magnification 125) in each animal and expressed as positive cells per square millimetre. Endogenous tissue peroxidase activity was inhibited by immersion in methanol for 15 min, followed by 30 min incubation with 0.03% H₂O₂ in TSB. After washing, subsequent incubations were performed with the avidin-biotin peroxidase complex (Vectorstain, Vector Laboratories Inc., Burlingame, USA) and 9-amino-ethylcarbazole as the chromogen supplemented with the H₂O₂ as substrate. As controls, a spleen section of the rat was also stained. Nuclei were counterstained with methyl green.

Myeloperoxidase (MPO) assessment of rat post-ischaemic kidney

This colorimetric method measures the activity of MPO found in the azurophilic granules present in PMNs [30]. Normal and post-ischaemic kidney at 1, 2, 6, 12, and 24 h post-reperfusion was homogenized in 5 mmol/l potassium phosphate buffer first and then centrifuged at 30 000 g for 30 min at 4°C prior to extraction. To avoid interference with supernatants from control and post-ischaemic kidney the pellets were washed twice [31]. The resulting pellets expressed MPO activity after suspending them in 50 mmol/l potassium phosphate buffer containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB). The pellets were centrifuged at 40 000 g for 15 min; 0.2 ml specimen was added to 0.8 ml 50 mmol/l potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% H₂O₂. Absorbency was measured at 460 nm for 2 min, and assay linearity was confirmed. MPO activity, normalized to protein content of the supernatant, was expressed as the percentage of levels in kidneys subjected to sham surgery.

Identification/quantification methods of PMNs

H&E staining

Histological detection of polymorphonuclear cells was performed on methacarn-fixed, paraffin-embedded renal-tissue sections. The H&E staining was used to identify and to quantify the infiltration of polymorphonuclear cells, based upon the localization of the cell and morphology of the nucleus of the cell. Positively stained cells were quantified in 75 randomly chosen microscope fields (magnification 1000) in cortex, outer stripe of outer medulla (+ medullary rays), and inner medulla, expressed as positive cells per square millimetre.

ED-1 staining on cryosections in combination with MPO staining

In brief, after incubation with the mAb ED-1 against macrophages (step 1), MPO was stained using the chromogen benzidine dihydrochloride (step 2). Thereafter, MPO was inactivated (step 3) and the ED-1 staining itself was developed (step 4) (Figure 1). More in detail, 5 μm renal tissue cryosections were used, prefixed in formalin calcium (4% formaldehyde (BDH Chemical Ltd., Poole, UK)) buffered with 0.1 M Na-cacodylate pH 7.4 containing 1% CaCl₂. In a first step, the sections were mounted on poly-l-lysine coated microscope slides and treated for 5 min with 0.003% trypsin III (Sigma Chemical Co., St Louis, USA) in 10 mmol/l Tris–HCl buffer pH 7.3. After washing in TSB with 1% bovine serum albumin and treatment with normal horse serum (1/5), the sections were incubated overnight with the primary mouse anti-rat antibody ED-1 (1/15000). The very specific antibody is directed to a cytoplasmic antigen of tissue macrophages, monocytes and dendritic cells [25]. Sections were then incubated for 30 min with biotinylated horse anti-mouse IgG. As second step, staining for the MPO was performed, using the chromogen benzidine dihydrochloride (30% ethanol, containing 1.5 g benzidine, pH 6.0) and hydrogen peroxide 0.03% as substrate. This shows the yellow-coloured intra-cytoplasmic granules. The sections were mounted with gelatine and photographed with a Leica microscope using the program KS 400 v. 2.0. In a third step, the sections were demounted to further develop the ED-1 mAb: after incubation of endogenous peroxidase using methanol for 15 min and H₂O₂ 0.03% in TSB for 30 min, sections were incubated with avidin–biotin labelled with peroxidase (Vectorstain, Vector Laboratories Inc., Burlingame, USA) and developed with 9-amino-ethylcarbazole as chromogen and H₂O₂ as substrate, which finally shows the ED-1 positive red-brownish-coloured cytoplasm of the macrophage. The sections were mounted with gelatine/glycerine and photographed again.

As part of control experiments the specific ED-1 staining was tested in peripheral blood. Rat blood samples were taken at moment of sacrifice. A peripheral blood smear was performed and fixed for 30 s in formalin calcium. Then the ED-1 staining (without MPO staining) was performed as described previously. A counterstaining with haematoxylin was performed.

ED-1 staining on cryosections in combination with naphthol AS-D chloroacetate esterase staining

In brief, after incubation with the mAb ED-1 against macrophages, the naphthol AS-D chloroacetate esterase reaction was performed. Thereafter, the ED-1 staining itself was developed. More in detail, ED-1 positive cells were demonstrated as described above; 4 μm renal tissue cryosections were used, prefixed in formalin calcium (4% formaldehyde (BDH Chemical Ltd., Poole, UK)) buffered with 0.1 M Na-cacodylate pH 7.4 containing 1% CaCl₂. In a first step, the sections were mounted on poly-l-lysine coated
microscope slides and treated for 5 min with 0.003% trypsin III (Sigma Chemical Co., St Louis, USA) in 10 mM Tris–HCl buffer pH 7.3. After washing in TSB with 1% bovine serum albumin and treatment with normal horse serum (1/5), the sections were incubated overnight with the primary mouse anti-rat antibody ED-1 (1/150 000). Sections were then incubated with biotinylated horse anti-rat ED-1 (1/15 000). Sections were then incubated with avidin–biotin horseradish peroxidase (Vectastain, Vector Laboratories Inc., Burlingame, USA) and developed with 9-amino-ethylcarbazole as chromogen and $H_2O_2$ as substrate.

In brief, first the primary antibody HIS-48 was demonstrated, photographs were taken, decoloured, and thereafter ED-1 staining was performed. More in detail, 5-µm renal tissue cryosections were prefixed and mounted as described above, and incubated overnight with HIS-48 (1/10) (monoclonal antibody against polymorphonuclear cells) [14]. As controls sections were also incubated with the primary mAb ED-1 (1/150 000) and mAb against $\alpha$-smooth muscle actin ($\alpha$-SMA) (1/30 000). After inhibition of endogenous peroxidase using methanol for 15 min and $H_2O_2$ 0.03% in TSB for 30 min, sections were incubated with biotinylated horse anti-mouse IgG for 30 min. Sections were then incubated with avidin–biotin horseradish peroxidase (Vectastain, Vector Laboratories Inc., Burlingame, USA) and developed with 9-amino-ethylcarbazole as chromogen and $H_2O_2$ as substrate.

HIS-48 (granulocytes) in combination with ED-1

In brief, first the primary antibody HIS-48 was demonstrated, photographs were taken, decoloured, and thereafter ED-1 staining was performed. More in detail, 5-µm renal tissue cryosections were prefixed and mounted as described above, and incubated overnight with HIS-48 (1/10) (monoclonal antibody against polymorphonuclear cells) [14]. As controls sections were also incubated with the primary mAb ED-1 (1/150 000) and mAb against $\alpha$-smooth muscle actin ($\alpha$-SMA) (1/30 000). After inhibition of endogenous peroxidase using methanol for 15 min and $H_2O_2$ 0.03% in TSB for 30 min, sections were incubated with biotinylated horse anti-mouse IgG for 30 min. Sections were then incubated with avidin–biotin horseradish peroxidase (Vectastain, Vector Laboratories Inc., Burlingame, USA) and developed with 9-amino-ethylcarbazole as chromogen and $H_2O_2$ as substrate.

This rabbit anti-ED-1 FITC-labelled antibody was used in order to exclude aspecific staining with the previous secondary polyclonal horse anti-mouse antibody and avidin–biotin complexes. In another control experiment aspecific binding of ED-1 with the horse anti-mouse antibody, used during first staining step, was excluded using $\alpha$-SMA in the first staining.

Labelling of rat peripheral blood cells with mAb HIS48

Erythrocytes from 20 µl of heparinized blood were lysed (hypotonic buffer, 10 min, 20°C) and leukocytes were then
incubated (30 min, 4°C) with mAb HIS-48. A second incubation step (30 min, 4°C) with FITC-conjugated rabbit-anti-mouse IgG (RAM-FITC) followed washing of the cells (PBS + 0.1% BSA + 0.05% NaN₃). Cells were resuspended in washing buffer prior to analysis on a FACStar plus (Becton & Dickinson, Immunocytometry Systems). To identify positive cells, these were sorted onto poly-L-Lysine-coated glass slides and stained by May–Grünewald–Giemsa standard procedure.

Statistics

Data are presented as means ± standard deviation. They were compared with an one-way ANOVA analysis, and a Student–Newman–Keuls test was used to prove qualitative differences by using the software package SPSS. Significant differences were anticipated when \( P < 0.05 \).

Results

Animals (Figure 2 A-B)

During surgery, animals cooled from 38 to 28°C, as no specific heating pad was used. Post-ischaemic animals sustained a severe acute renal failure with a mortality of 15%, observed at days 3–4, while non-ischaemic animals all survived the right nephrectomy. Due to the paired feeding, all animals had comparative post-operative weight loss, which had not recovered during the 10-day study period. At sacrifice, the post-ischaemic kidneys showed a weight gain of 45% at day 5, which persisted, reflecting oedema and infiltration.

Kidney function (Figure 2C)

Unilateral nephrectomy, without any ischaemia, caused a slight increase in the serum creatinine (S-crea) levels on day 2 (S-crea_{nephrectomy day 0} = 0.75 mg/dl ± 0.05 vs S-crea_{nephrectomy day 2} = 1.22 mg/dl ± 0.13; \( P < 0.05 \)), and returned to normal on day 3. A period of 60 min of warm ischaemia caused a severe acute renal failure on the second day (S-crea_{ischaemia day 0} = 0.75 mg/dl ± 0.05 vs S-crea_{ischaemia day 2} = 4.0 mg/dl ± 1.9; \( P < 0.05 \)). Functional recovery of the kidney started after day 2 and was almost complete at day 7 (S-crea_{ischaemia day 7} = 1.06 mg/dl ± 0.11; not significant).

Damage and regeneration/PCNA positivity (Figures 3, 4)

The zones of most severe injury (PAS staining) and proliferation (PCNA staining), which are the OSOM and medullary rays, are exactly the same zones of most important leukocyte infiltration (OX-1 pan leukocyte staining) (Figure 3). The evolution of damage and regeneration of the kidney tubules was scored semiquantitatively (Figure 4). In cases of a unilateral nephrectomy without ischaemia of the unique kidney, no signs of damage or regeneration were seen in the remaining kidney during the study period of 10 days. No PCNA positivity or infiltrating leukocytes was noticed. In contrast to the non-ischaemic groups, 60 min of warm ischaemia resulted in remarkable damage of the proximal S3 segment and the thick ascending limb of the nephron in the OSOM and medullary rays. The first day after the ischaemia, almost half of the tubules showed a complete detachment (score 4) of their epithelial cells from their basement membrane. On day 2, the first signs of
regeneration appeared and they reached a maximum at day 3, together with a maximum of PCNA positivity. The characteristics of this early regeneration consisted of small non-differentiated cells with strong PCNA-positive nuclei, localized on the basement membrane of the proximal tubule and the thick ascending limbs (score 5). The following days, the volume of the cytoplasm of these cells increased (score 6), until finally the brush border recovered (back to score 0). The amount of PCNA-positive cells decreased in time and returned to baseline from the 5th day onwards. At the end of the investigated period (day 10), half of the proximal tubules were regenerated completely.

**Interstitial leukocytes (Figure 5)**

In the non-ischaemic group, no increase in number of interstitial leukocytes (OX-1 staining) could be seen within the study period of 10 days. Ischaemia of a unique kidney resulted in a gradually increasing interstitial leukocyte infiltrate in the OSOM during the 10 days investigation period. At day 10, the interstitial infiltrate was quite prominent (OX1\textsubscript{ischaemia} = 1733 ± 10 cells/mm\(^2\)).

**ED-1 staining (monocytes/macrophages)**

As early as 24 h after I/R injury, an increased number of monocytes (ED-1 positive cells) was found in the renal interstitium (119 ± 46 cells/mm\(^2\) vs controls = 20.9 ± 7.3 cells/mm\(^2\) (P < 0.05)). Five days after the ischaemic event, a peak of ED-1 positive cells occurred (1034 ± 161 cells/mm\(^2\) vs controls = 18.4 ± 18.4 cells/mm\(^2\) (P < 0.05)).

**W3/25 staining (CD4\(^+\) molecule)**

A lymphocytic infiltration occurred from day 5 on in the ischaemic group, and persisted after 10 days.

**OX-8 staining (CD8\(^+\) molecule)**

The outer stripe of the outer medulla contained only a small but significant number of CD8\(^+\) cells, entering the ischaemic kidney at the 3rd day (35 ± 6.76 cells/mm\(^2\) vs controls = 6.8 ± 8.05 cells/mm\(^2\) (P < 0.05)) and persisting until day 10.
Fig. 4. (A) Renal damage and regeneration. Semiquantitative score of damage (score 1–3) and regeneration (score 4–6). (B) Number of PCNA-positive cells (per circular-shaped tubular cross-section) (*P<0.05).

**OX-33 staining (B-cells)**

The spleen section was clearly positive for B-cells. Neither the control kidney nor the ischaemic kidney contained B-cells.

**Early staining of PMNs vs monocytes/macrophages: analysis of 0–24 h (Figure 6)**

In all segments only scarce neutrophils (H&E staining) could be noticed at the early phase after the I/R injury, not significantly different from controls (OSOM, 8.37±7.68 cells/mm² vs controls = 2.41±2.84 cells/mm²; medullary rays, 2.50±2.02 cells/mm² vs controls = 2.47±2.16 cells/mm²; cortex, 4.73±1.90 cells/mm² vs controls = 2.91±2.34 cells/mm²). The few neutrophils that were noted were mainly located in the peritubular capillaries. As mentioned above, the number of monocytes/macrophages increased steadily with time, mainly in OSOM, and after 24 h was 12 times higher than the number of neutrophils present.

**MPO assessment of rat post-ischaemic kidney during the first 24 h post-I/R (Figure 6)**

MPO activity was expressed as the percentage of levels in kidneys subjected to sham surgery (=100%). MPO activity is already significantly increased after 1 h post-I/R (439±34%, P<0.005), reaching its maximum activity after 12 h of I/R (1159±138%, P<0.0005). However, kidney function, measured by S-creatinine, starts to be impaired after 6 h.

**Identification of infiltrating cells (Figure 7)**

**ED-1 staining on peripheral blood smear and kidney sections**

The ED-1 staining of peripheral blood monocytes showed ED-1-positive granules in the cytoplasm, whereas neutrophils and lymphocytes are negative for ED-1. Haematoxylin staining on a kidney section of a pyelonephritis kidney showed massive presence of intratubular PMNs, negative for ED-1 staining (data not shown).
ED-1 staining on cryosections in combination with MPO-staining

Figure 7A shows MPO staining of ED-1-positive cells, indicating that macrophages, like neutrophils, stain for MPO. By performing the staining procedure as described above but without incubation with the primary ED-1 mAb, no positive staining was observed, indicating no aspecific binding of the secondary mAbs and the avidin–biotin complexes, or residual MPO activity.

ED-1 staining on cryosections in combination with chloroacetate staining

Figure 7B shows naphthol AS-D chloroacetate esterase staining of ED-1-positive cells, which shows that macrophages, like neutrophils, stain with chloroacetate. By performing the staining procedure as described above but without incubation with the primary ED-1 mAb, no positive staining was observed, indicating no aspecific binding of the secondary mAbs and the avidin–biotin complexes, no residual activity of the endogenous peroxidase activity.

HIS-48 (granulocytes) in combination with ED-1

Figure 7C shows that ED-1-positive cells (macrophages) also stain for HIS-48. Different control experiments were performed (data not shown): first, by performing the double-staining procedure as described above but without incubation with the anti-ED-1 FITC mAb, no positivity was observed, indicating no
Fig. 6. Relationship of monocytes/macrophage and neutrophil adhesion/infiltration in cortex, OSOM and medulla, in relation to whole kidney MPO-activity and renal function (S-creatinine) during the first 24 h post-I/R. Left, Dark bars, number of monocytes/macrophages/mm². Light bars, number of PMNs/mm². Right, MPO activity in post-ischaemic kidney (dark bars), normalized to protein content of the supernatant, expressed as the percentage of levels in kidneys subjected to sham surgery (striped bar). Serum creatinine (mg/dl) of post-ischaemic animals (dark bars), vs controls without ischaemia (striped bar) (**P < 0.01; *P < 0.05).

Aspecific binding of the second staining procedure. However, the ED-1 itself might bind with parts of the primary staining procedure. Therefore, a second control staining was performed by a double-immuno-histochemical staining combining mAb against α-SMA in the first staining with ED-1 FITC in the second staining. The typical pattern of peritubular and periglomerular α-SMA staining and also that in the walls of large blood vessels is different from the typical interstitial pattern of macrophage staining. If in our control staining ED-1 should bind non-specifically to the secondary biotinylated horse anti-mouse antibody of the first staining, the ED-1 staining would have had the staining pattern of α-SMA, which was not the case. Hence the secondary staining for ED-1 was specific for the monocyte/macrophage cell type. In order to exclude aspecificity or decreased activity of a FITC-labelled antibody, a control staining was performed by combining the ED-1 in the first staining with the ED-1 FITC-labelled antibody in the second staining. Not surprisingly, although less intensive, all cells positive for ED-1 were also positive for the FITC-labelled antibody.

Labelling of rat peripheral blood cells with mAb HIS48 (Figure 8)

The HIS-48-labelled subsets were sorted according to a selection procedure based on fluorescence intensity in a negative, a weakly positive, and a strongly positive subpopulation. In these weakly and strongly HIS-48-labelled subsets the nuclear morphology was analysed and the distribution of lymphocytes,
monocytes, and PMNs was counted. The most strongly HIS-48-labelled cells were identified as monocytes/macrophages, while the weakly labelled cells were identified as PMNs.

**Discussion**

Renal ischaemia/reperfusion injury is a major cause of ARF in the native as well as in the transplanted organ,
Fig. 8. Labelling of rat peripheral blood cells with mAb HIS-48. (A) Flow cytometric labelling of peripheral blood cells with mAb HIS-48 shows cell in a wide intensity range (M1) but especially a weakly and a strongly (M3) labelled cell subset. (B) These labelled subsets were sorted according to a selection procedure based on fluorescence intensity. (C) Analysis of nuclear morphology reveals a majority of PMNs in the weakly HIS-48 labelled subset and a majority of monocytes in the strongly HIS-48 labelled subset.

and is associated with a high mortality and morbidity. There is no specific treatment for this devastating clinical syndrome, reflecting, in part, the relatively poor understanding of the disease pathophysiology [1]. Leukocyte adhesion/infiltration and in situ proliferation in response to I/R injury is a well-known but ill-defined phenomenon. Sublethal damaged epithelial cells in proximal tubules and thick ascending limbs have the capacity of liberating chemotactic substances [33] which, through upregulation of adhesion molecules at the site of endothelial cells, may facilitate a leukocyte adhesion/infiltration, the role of which in injury or in the regeneration process after I/R is still not clarified.

The presence of these leukocytes, particularly of neutrophils, is generally considered as a damaging event, exacerbating I/R damage [17]. Recent experiments that interfere with leukocyte activation and adhesion/infiltration (mAbs to ICAM-1 and/or LFA-1, ICAM-1 antisense oligonucleotides, etc.) have shown that the kidney can be functionally protected against post-I/R injury [7,34]. On the other hand, phagocytes are also regarded as important scavengers of apoptotic cells or necrotic debris, and their presence in the kidney following I/R injury could alternatively represent a repair process [1]. Yet these infiltrating inflammatory cells may be a source of growth-stimulating substances [35], implying a role in the repair process after ARF [36–38].

Although many data are available concerning the overall cellular infiltrate after injury, only scarce information is available on the careful identification and time course of the different subsets of these adhering/infiltrating and proliferating leukocytes [5]. Recently, a mixed mononuclear cell (macrophage and CD4+ cell) infiltration of the kidney, within a few days after I/R, was described after 45 min warm ischaemia [39] and after experimental cold ischaemia [40], with limited information, however, as to its exact topographical localization and kinetics. In the latter and many other studies, no important neutrophilic infiltration was mentioned, while even very recently, others, using enzymatic tests and membrane markers described mainly neutrophilic adhesion/infiltration [12,13,21,34]. These findings underscore discussion in the literature concerning the number and role of neutrophils after renal I/R. Our results contribute to the unravelling of this controversy.

First, during the first 24–48 h the detailed identification and quantification of the interstitial leukocytes in the rat kidney after severe warm I/R injury showed a pronounced acute tubular necrosis, with only a moderate monocyte/macrophage infiltration and proliferation. It is important to stress that the decrease in body temperature from 37 °C to 28 °C during anaesthesia and surgery might change kinetics and other properties of the cellular infiltrate, especially at early time points after ischaemia. Despite there being no significant increase of PMNs (identified by routine H&E staining) and the slightly increased monocytes/macrophages (ED-1 staining) during the first 12 h, the MPO activity, generally related to presence of PMNs [7,9,30,31], was significantly increased in the first hours post-ischaemia. This early (1 h) to maximal (12 h) MPO activity of the post-ischaemic kidney remains to be explained in the absence of significant increased number of inflammatory cells. It could be argued that PMNs and monocytes/macrophages do not infiltrate early after reperfusion but only adhere long enough to the activ-
ated endothelium to release their enzymes (like MPO) and to interfere with microcirculation with blockage of the vascular flow in the capillary network (‘no reflow’) [41,42], followed by re-entry to the circulation (‘hit-and-run’ phenomenon). In this respect, MPO activity reflects in fact more the activation of the adhering inflammatory cells and eventual of residential interstitial macrophages, resulting from post-ischaemic chemotactic and other cytokine activation, rather than infiltration itself. After this first critical period of 24 h, the number monocytes/macrophages was 12–25 times higher than the scarcely present PMNs, which remained comparable to control values. This indicates that firm adhesion and diapedesis has taken place, generating the classical infiltration and proliferation observed in many conditions (ischaemia, HgCl₂, obstruction, etc.). It could be suggested therefore that the immediate beneficial functional effect of anti-adhesion therapy with for example antibodies to ICAM-1 and LFA-1, has to be explained at the level of early intravascular (microcirculation) leukocyte activation, and adhesion, but not at the level of the interstitial infiltration, which appears slightly later, at a time when the functional recovery is already operational.

Secondly, we could demonstrate that MPO activity is not a specific measure of PMN presence, since MPO content of post-ischaemic tissue reflects not only presence of PMNs but at least also of monocytes/macrophages and/or of activated residential interstitial macrophages [30,43,44]. In addition, by combined histochemical and immunohistochemical methods, we could demonstrate naphthol AS-D chloroacetate esterase activity in macrophages, present in the renal interstitium after I/R. Again, the latter staining is extensively used as specific for PMNs [10,12,21]. Finally, staining of ED-1 (specific for macrophages) followed by HIS-48, a monoclonal antibody considered to be specific for polymorphonuclear cells, showed that macrophages also react with mAb HIS-48. Flow cytometric analysis of circulating white blood cells demonstrating the high affinity of macrophages for the anti-HIS-48 antibody, supported this result. Hence, none of the so-called cell-specific stainings for PMNs have proved to be specific for this particular cell type, and cross-react with monocytes/macrophages. The H&E staining (morphology of cell nucleus) remains the gold standard for identification of PMNs. MPO assays or chloroacetate esterase stainings should be regarded as tools to quantitate both PMNs and monocytes/macrophages. These observations explain the long lasting and frequently observed dissociation between the use of H&E staining vs enzymatic and/or membrane markers in the identification in the number of neutrophils [1,45].

Three days post-I/R, functional recovery starts, at the moment of maximum tubular cell proliferation. Only later, after 5–10 days, the mononuclear infiltrate becomes quite prominent, consisting of a sequential accumulation and proliferation of monocytes/macrophages and helper T-cells, a few suppressor/cytotoxic T-cells and no B-cells. These cells are most prominent at the site of maximal damage/regeneration, which is the OSOM and medullary rays. The total number of adhering/infiltrating PMNs in the different nephron segments remained unchanged up to 10 days post-I/R, their absolute number in the interstitium remaining far beyond that of other inflammatory cells.

In conclusion, the non-specificity of the so-called neutrophil-specific identification methods (MPO-activity, naphthol AS-D chloroacetate esterase or mAb HIS-48 staining), cross-reacting with monocytes/macrophages, explains the controversy in the literature concerning the number of PMNs in post-ischaemic injury. The only reliable method to quantitate PMNs remains H&E staining. Twelve to 24 h after severe warm I/R renal injury, a pronounced acute tubular necrosis occurs in the absence of a marked cellular infiltrate, along with a striking increase of MPO activity. This important MPO activity in this very early critical post-ischaemia period most probably reflects the activation of the adhering inflammatory cells (PMNs and monocytes/macrophages). Only at the later time of regeneration a sequential infiltration/proliferation of monocytes/macrophages and T-cells becomes prominent. The total number of PMNs remained low during the first 10 days post-I/R. These observations underscore an early effect of the few activated neutrophils, associated with the higher number of activated monocytes/macrophages.

Acknowledgements. Our appreciation goes to Simone Dauwe for excellent morphological slide preparation, Marleen Nysten for technical assistance with the animal experiments, Dirk De Weerdt for the excellent illustrations, and K. Achten for his excellent operative skills. Also the critical appraisal of the morphology by N. Buyssens is greatly appreciated. Part of this work was supported by a grant of the Fund for Scientific Research (FWO No 15.045.96N).

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