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Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion

Daniela Cugini, Nadia Azzollini, Elena Gagliardini, Paola Cassis, Riccardo Bertini, Francesco Colotta, Marina Noris, Giuseppe Remuzzi, and Ariela Benigni

Transplant Research Center "Chiara Cucchi de Alessandri e Gilberto Crespi" Mario Negri Institute for Pharmacological Research, Villa Camozzi, Ranica, Bergamo, Italy; Azienda Ospedaliera Ospedali Riuniti di Bergamo, Bergamo, Italy; and Dompé SpA, L'Aquila, Italy

Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion.

Background. Ischemia/reperfusion (I/R) injury after organ transplantation is a major cause of delayed graft function. Following I/R, locally produced CXC chemokines attract and activate granulocytes, which in turn promote graft damage.

Methods. We examined the involvement of granulocyte recruitment via the CXCR2 pathway in a rat model of 4 hours cold ischemia followed by kidney transplantation. Serum creatinine and intragraft granulocyte infiltration were monitored in the early phase posttransplant. A CXCR2 inhibitor, repertaxin, was given to recipients before transplantation (at −24 hours or −8 hours or −2 hours), immediately before reperfusion and 2 hours later.

Results. An increase of granulocyte chemoattractant CINC-1/interleukin-8 (IL-8) mRNA expression after I/R both in syngeneic and allogeneic transplantation was associated with a marked infiltration of granulocytes in renal tissue. In syngeneic transplantation, Lewis rats given 15 mg/kg repertaxin 24 hours before surgery had granulocyte graft infiltration and serum creatinine levels significantly reduced in respect to vehicle-treated animals. Intermediate effects were observed with 5 mg/kg, whereas the dose of 30 mg/kg had toxic effects. We found that reducing the pretreatment time to 8 hours before surgery was still effective. Prevention of granulocyte infiltration and serum creatinine increase was also obtained in allogeneic transplantation, when Brown Norway recipients of Lewis kidneys were given 15 mg/kg repertaxin starting 8 hours before surgery.

Conclusion. Repertaxin treatment of the recipient animal was effective in preventing granulocyte infiltration and renal function impairment both in syngeneic and in allogeneic settings. The possibility to modulate I/R injury in this rat model opens new perspectives for preventing posttransplant delayed graft function in humans.

Key words: repertaxin, ischemia/reperfusion, CXCR2 inhibitor, transplantation, granulocytes.

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Ischemia/reperfusion (I/R) injury is an important cause of organ failure in transplant medicine due to the reestablishment of blood flow to ischemic tissues that causes greater injury than that induced by ischemia secondary to organ retrieval, storage, and surgery [1]. Impaired or delayed graft function occurs in as many as 50% cadaveric kidney recipients [2] and requires supportive measures, including prolongation of the length of the hospital stay as well as of the dialysis period with consequent amplification of the costs and difficulties in administration of immunosuppression. There is also evidence that the incidence of acute rejection affecting the long-term graft survival is higher in organs with delayed graft function than in those that function immediately [3, 4]. The lack of effective treatments for preventing or curing the occurrence of delayed graft function has prompted the transplant community to focus on identifying drugs that stabilize or reverse I/R injury. The mechanism(s) of injury in I/R involves activation of endothelial cells which are induced to express high levels of surface adhesion molecules, including P- and E-selectin and produce cytokines and chemokines capable to attract neutrophils and macrophages promoting a nonspecific host inflammatory response [5]. Activated granulocytes release vasoactive mediators in turn responsible for the decrease in renal blood flow. Subsequent major histocompatability complex (MHC) class II antigens up-regulation and enhanced T-cell recruitment instigate an antigen-dependent alloimmune response.

Among the early proinflammatory chemokines, interleukin-8 (IL-8), a member of CXC chemokines, in which two NH2-terminal cysteines are separated by a single nonconserved amino acid residue, is believed to play a key role in I/R injury [6]. IL-8 has a major effect in attracting neutrophils and T lymphocytes at the inflammation site [7, 8]. Rodents lack a homologue of IL-8 but possess other CXC chemokines acting through CXCR2, namely CINC-1 (also called KC or Gro-1), MIP-2, and

CINC-2, which share with IL-8 the properties of granulocyte chemoattractants.

Neutrophils rolling on P-selectin borne by endothelial cells can utilize IL-8 produced by endothelial cells to promote the conversion from rolling to stationary adhesion to endothelial layer [9]. Once adherent to the endothelium, granulocytes may in turn secrete reactive oxygen species as well as proteolytic enzymes [10]. Expression of IL-8 family members increases in ischemic tissues soon after reperfusion in experimental models of renal [11, 12], retinal [13], cardiac [14], and lung ischemia [6]. Finding that the administration of a neutralizing anti-IL-8 monoclonal antibody prevented neutrophil infiltration and tissue injury in a rabbit model of lung reperfusion injury would indicate the pathophysiologic role of locally produced IL-8 [6].

The present study was performed in a rat model of I/R renal injury after transplantation induced by preexposure of the graft to cold storage solution [15] to evaluate the involvement of granulocyte recruitment via the CXCR2 pathway in reperfusion injury of renal grafts. To address this issue we evaluated (*1*) the time course of renal infiltration of granulocytes together with CINC-1/IL-8 expression early after reperfusion in syngeneic or allogeneic strain combinations; and (*2*) the effect of the treatment with a specific inhibitor (repertaxin) of CXCR2 on intragraft granulocyte infiltration as well as on renal graft function.

Repertaxin inhibits in a dose-dependent manner human neutrophil chemotaxis induced by IL-8 in vitro [16]. Repertaxin also inhibits the chemotaxis of rat neutrophils induced by CINC-1 [16, 17]. Mechanistic studies have shown that repertaxin acts as a noncompetitive allosteric inhibitor, blocking the intracellular signal transduction that follows engagement of IL-8 or CINC-1 with corresponding receptors present on neutrophil surface [16, 17]. Different doses of repertaxin and several times of drug administration were tested to find the most effective regimen in preventing granulocyte infiltration and renal impairment in our experimental model.

METHODS

Animals

Inbred adult male Lewis rats ($RT1^l$) (Charles River, Calco, Italy) were used as donor and recipients, respectively, in syngeneic kidney transplants. Brown Norway rats were used as recipient and Lewis as donor in allogeneic transplants.

Animal care and treatment were conducted in accordance with institutional guidelines in compliance with national (D.L. n.116, G.U. Suppl. 40, February 18, 1992, Circolare n.8, G.U. July 14 1994) and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 1987; "Guide for the Care and Use of Laboratory Animals," U.S. National Research Council, 1996). All animals were allowed free access to standard rat chow and tap water.

Experimental design

We first assessed the kinetics of CINC-1/IL-8 expression and granulocyte accumulation in the kidney after syngeneic or allogeneic transplantation (Fig. 1A). Either for syngeneic and allogeneic combinations, all the animals underwent a graft biopsy 1 hour after transplantation ($N = 6$); thereafter, rats were divided in two groups and sacrificed 3 hours after transplantation ($N = 3$) or 24 hours after transplantation ($N = 3$). Renal tissues were processed for CINC-1/IL-8 mRNA analysis and quantification of granulocyte infiltration. As control, Lewis kidneys exposed to cold ischemia but not grafted were used ($N = 3$).

To assess whether CXCR2 inhibition may prevent granulocyte infiltration and preserve renal function, different doses of a specific CXCR2 inhibitor, repertaxin, were tested in the syngeneic kidney transplantation model (Fig. 1B). Repertaxin (R(-)-2-(4-isobutylphenyl) propionyl methansulfonamide) salified with L-lysine was dissolved in saline. The recipient animals received a subcutaneous injection of repertaxin 24 hours before transplantation, an intravenous administration immediately before reperfusion of the transplanted kidney and a subcutaneous injection 2 hours after transplantation. Animals were divided in four groups: group 1 receiving 5 mg/kg repertaxin ($N = 5$), group 2 receiving 15 mg/kg repertaxin ($N = 6$), group 3 receiving 30 mg/kg repertaxin ($N = 3$) and group 4 receiving vehicle (saline) ($N = 8$) at the same time and route of administration of repertaxin. The doses of repertaxin were found effective in rat models of hepatic [16] and intestinal I/R injury [17]. Serum creatinine concentrations were evaluated at 16 and 24 hours. Animals were sacrificed at 24 hours after transplantation. At sacrifice kidneys were removed and processed for immunohistochemistry assessment of infiltrated granulocytes.

We further evaluated whether shortening the pretreatment time still protected the kidney after syngeneic transplantation (Fig. 1C). To this purpose, two groups of Lewis rats received a subcutaneous injection of 15 mg/kg of repertaxin 2 hours ($N = 8$) or 8 hours ($N = 4$) before transplantation, an intravenous administration immediately before reperfusion of the transplanted kidney and a subcutaneous injection 2 hours after transplantation. Serum creatinine was measured at 16 and 24 hours, time at which the animals were sacrificed. At sacrifice kidneys were removed and processed for immunohistochemistry assessment of infiltrated granulocytes.

Once established, the dose and the treatment schedule, the efficacy of repertaxin was tested after I/R injury

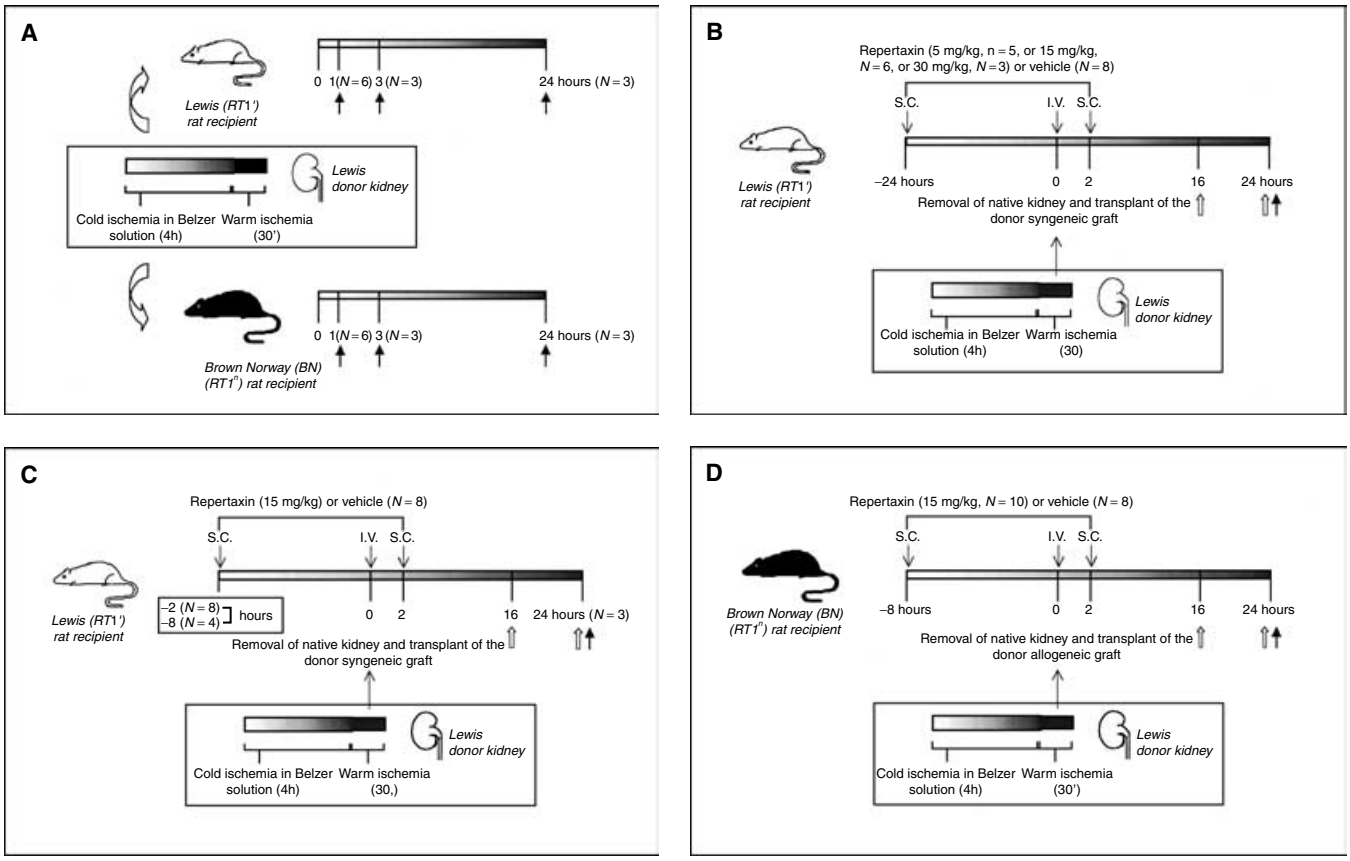

Fig. 1. Experimental design. (*A*) To assess the kinetics of CINC-1/interleukin-8 (IL-8) expression and intragraft granulocyte infiltration following ischemia/reperfusion (I/R) injury in syngeneic and allogeneic transplantation, rats recipients of syngeneic or allogeneic kidneys underwent a graft biopsy 1 hour after transplantation; theareafter, kidneys were taken at sacrifice 3 or 24 hours after transplantation. (*B*) Different doses of repertaxin were given to recipents rats to assess the effect of CXCR2 inhibition on granulocyte infiltration and renal function in syngeneic transplantation model. (*C*) Syngeneic recipent rats were given repertaxin at different pretreatment time before transplantation. (*D*) repertaxin was given to Brown Norway rats receiving a Lewis allogeneic kidney. Closed arrows are renal tissue taken for analysis; open arrows are measurement of serum creatinine levels.

in the allogeneic kidney transplantation model (Fig. 1D). We evaluated the effect of 15 mg/kg repertaxin given to Brown Norway rats as a subcutaneous injection 8 hours before transplantation, followed by an intravenous administration immediately before reperfusion and a subcutaneous injection 2 hours after transplantation ($N = 10$). As controls, Brown Norway rats receiving Lewis kidney were given vehicle ($N = 13$). Serum creatinine was measured at 16 and 24 hours, time at which animals were sacrificed. At sacrifice kidneys were removed and processed for immunohistochemistry assessment of infiltrated granulocytes.

Kidney transplantation

Kidney transplantation was performed as described previously [18]. Donor animals were anesthetized with Leptofen (Pharmacia, Uppsala, Sweden). The left kidney was prepared by freeing the ureter from the attachments. The renal artery was separated from the renal vein by blunt dissection. The donor kidney and ureter were removed en bloc and flushed with Belzer [University of Wisconsin (UW)] containing 1000 U/mL heparin. Then the kidney was placed in an iced Belzer (UW) solution for 4 hours (cold ischemia) until transplant. After ischemia time elapsed, recipient rats underwent removal of the native left kidney. Donor kidneys grafts were then washed with saline solution and transplanted. An anastomosis was created between the donor and recipient renal artery as well as renal vein with end-to-end anastomosis. Vascular clamps were released after 30 minutes (warm ischemia). Donor and recipient ureters were attached end-to-end. The native right kidney was then removed.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted from homogenated whole kidneys or biopsies in the presence of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). DNase-treated RNA was primed with random hexamers and reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen).

Quantitative real-time PCR was performed on a TaqMan ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Core reagents. To amplify rat CINC-1/IL-8 transcripts the following primers were used, based on Genbank sequence M86536.1, for (300 nmol/L) 5′-AAG ATT GTC CAA AAG ATG CTA AAG G-3′ and reverse (300 nmol/L) 5′-ATC GGT GCA ATC TAT CTT CTT TCT C-3′. Genomic DNA was not amplified since primers spanned an exon junction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene to assess the overall cDNA content. GAPDH primers were as follows: for (300 nmol/L) 5′-TCATCCCTGCATCCACTGGT-3′ and reverse (300 nmol/L) 5′-CTGGGATGACCTTGCCCAC-3′.

After an initial holding step of 2 minutes at 50°C and 10 minutes at 95°C, samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. All reactions were performed in triplicate. Similar amplification efficiencies were demonstrated for both the target and the housekeeping genes by analyzing serial cDNA dilutions, showing an absolute value of the slope of log input cDNA amount versus Δ threshold cycle (Ct) (Ct target Ct housekeeping gene) of <0.1. Thus, the ΔΔCt equation was used to compare the CINC-1/IL-8 expression in each sample with the expression in a control kidney taken as reference (calibrator).

Immunohistochemical analysis

The degree of granulocyte infiltration in glomerular and interstitial areas was determined by indirect immunofluorescence. A mouse antirat granulocyte monoclonal antibody (clone MOM/3F12/F2) (dilution 1:10) (Valter Occhiena, Torino, Italy) was used to stain infiltrating granulocytes. Tissue fragments were frozen in liquid nitrogen. Sections (3 μm thick) were cut using a Mikrom 500 O cryostat (Walldorf, Germany) and fixed in acetone. Tissue sections were blocked with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA), incubated overnight at 4°C with the primary antibody, washed with PBS, and then incubated with cyanate-3 (Cy3)-conjugated donkey antimouse IgG antibody (affinity-purified, absorbed with rat IgG) (5 μg/mL in PBS) (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at room temperature. The number of cells was counted in at least 20 glomeruli and 10 randomly selected high-power microscope field (×400) taking interstitial areas of each animals.

Statistical analysis

Differences between groups were analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis as appropriate. A P value of <0.05 was considered significant (two-tailed analysis). Results are reported as mean ± SE.

RESULTS

Kinetics of CINC-1/IL-8 expression and intragraft granulocyte infiltration following I/R injury of syngeneic and allogeneic transplantation

The kinetics of CINC-1/IL-8 expression was analyzed in kidneys pre-exposed to 4 hours of cold ischemia and subsequently grafted in a syngeneic or allogeneic rat strain combination. Real-time quantitative PCR on reverse transcribed RNA showed that CINC-1/IL-8 expression increased in both syngeneic and allogeneic groups as compared to the levels observed in control kidneys. The time course of CINC-1/IL-8 expression was similar in both groups reaching a peak at 3 hours after I/R (Fig. 2A). At this time point the CINC-1/IL-8 transcript levels in syngeneic and allogeneic groups were significantly higher than those in control kidneys ($P < 0.01$ and $P < 0.05$, respectively). CINC-1/IL-8 expression was higher in syngeneic than in allogeneic combination at each time point analyzed, a trend that has already been reported in a model of acute heart allograft rejection in mice [14].

Granulocyte infiltration after I/R injury was also evaluated in the same animals. Figure 2B summarizes the results of quantitative evaluation of granulocyte infiltration. A significant increase ($P < 0.05$) of the number of granulocytes was already observed 1 hour after I/R in intraglomerular and interstitial areas of the syngeneic group in respect to controls. The granulocyte infiltration remained stable at 3 hours and then slightly decreased at 24 hours, remaining significantly higher than that observed in the control group, however ($P < 0.05$). In the allogeneic group granulocyte infiltration both in intraglomerular and interstitial areas rose rapidly following I/R injury and remained significantly higher than that in control group at all time points analyzed ($P < 0.05$ at all time points in intraglomerular and interstitial areas). These results show that in the experimental model of I/R renal injury the significant increase of CINC-1/IL-8 levels is associated with a marked infiltration of granulocytes in renal tissue both in syngeneic and allogeneic transplantation.

Effect of different doses of repertaxin on intragraft granulocyte infiltration and renal function in syngeneic transplantation

Recipient animals received three administrations of the CXCR2 inhibitor repertaxin as follows: a subcutaneous injection 24 hours before transplantation, an intravenous administration immediately before reperfusion of the transplanted kidney, and a subcutaneous injection 2 hours after transplantation. The effect of different doses of repertaxin (5, 15, and 30 mg/kg, respectively) on granulocyte infiltration was determined 24 hours after syngeneic kidney transplantation (Fig. 3A). Treatment with

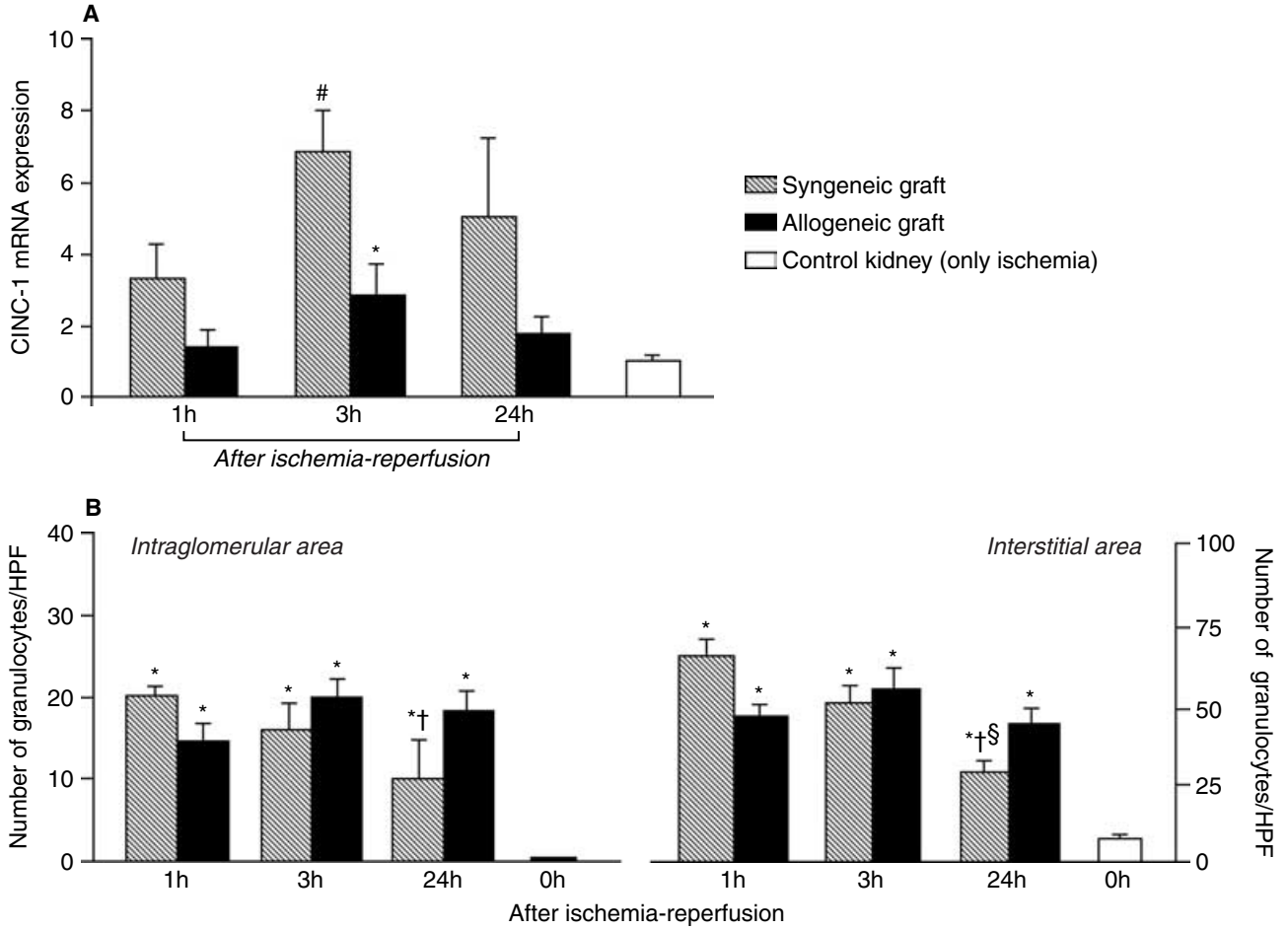

Fig. 2. Kinetics of CINC-1/interleukin-8 (IL-8) mRNA expression and granulocyte infiltration after renal ischemia/reperfusion (I/R). Syngeneic and allogeneic renal tissues were analyzed at different time points [1 hour ($N = 6$); 3 hours ($N = 3$); and 24 hours ($N = 3$)] after transplantation. (*A*) cDNA from kidneys were analyzed by real-time polymerase chain reaction (PCR) to quantify CINC-1/IL-8 mRNA expression. The expression level in the calibrator (control kidney, zero hour) is set to 1 (arbitrary unit). (*B*) Number of granulocytes counted in intraglomerular (left) and in interstitial (right) areas in at least 10 randomly selected high-power macroscopic fields (HPF). Data are mean ± SE. $^{\#}P < 0.01$ vs. control kidney; $^{*}P < 0.05$ vs. control kidney; †$P < 0.05$ vs. 1 hour; and §$P < 0.05$ vs. 3 hours.

repertaxin at the dose of 15 mg/kg significantly ($P < 0.05$) decreased granulocyte infiltration in intraglomerular and interstitial areas in respect to that observed in vehicle group. Granulocyte counts in animals receiving 5 and 30 mg/kg were fairly comparable to that of vehicle-treated animals.

Serum creatinine concentration was evaluated at 16 and 24 hours after syngeneic kidney transplantation (Fig. 3B). At 16 hours, serum creatinine concentration increase was significantly prevented by repertaxin at the dose of 15 mg/kg ($P < 0.001$). Animals given the dose of 30 mg/kg showed creatinine levels comparable to those measured in vehicle-treated animals. Of note, severe inflammation at the site of injection and hematuria was observed in animals given this dose, which might account for lack of effect of repertaxin on granulocyte infiltration and graft function. Twenty-four hours after transplantation, serum creatinine concentration increase was prevented by repertaxin at all the doses tested although to a different extent. The dose of 15 mg/kg gave the best protection on renal function impairment ($P < 0.001$ vs. vehicle); a lower but significant effect was also observed with 30 mg/kg ($P < 0.05$ vs. vehicle).

Overall, these data suggest that the most effective dose in preventing granulocyte infiltration and renal function impairment is 15 mg/kg.

Comparison of different pretreatment time of repertaxin on intragraft granulocyte infiltration and renal function in syngeneic transplantation

We then assessed whether shortening the pretreatment period with repertaxin could still result in prevention of granulocyte infiltration and renoprotection. Figure 4A

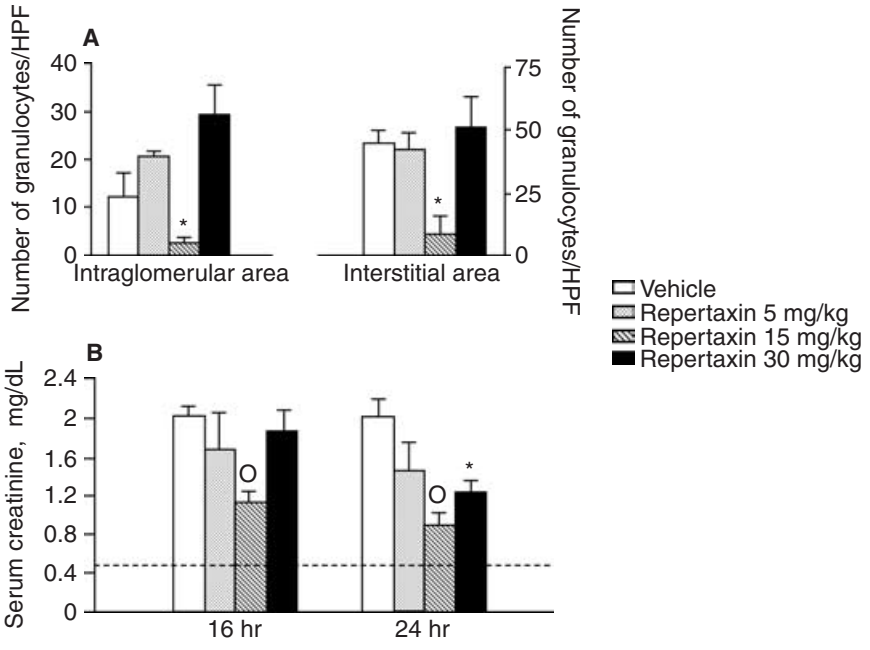

Fig. 3. Effect of different doses of repertaxin [5 mg/kg ($N = 5$); 15 mg/kg ($N = 6$); and 30 mg/kg ($N = 3$)] or vehicle ($N = 8$) on intragraft granulocyte infiltration and serum creatinine concentrations in syngeneic transplantation. (*A*) Number of granulocytes counted in intraglomerular (left) and in interstitial (right) areas 24 hours after reperfusion, in at least 10 randomly selected high power fields (HPF). (*B*) Dashed line represents normal serum creatinine values. After transplantation the serum creatinine concentrations were evaluated at 16 and 24 hours. Data are mean ± SE. $^{*}P < 0.05$; $^{\circ}P < 0.001$ vs. vehicle.

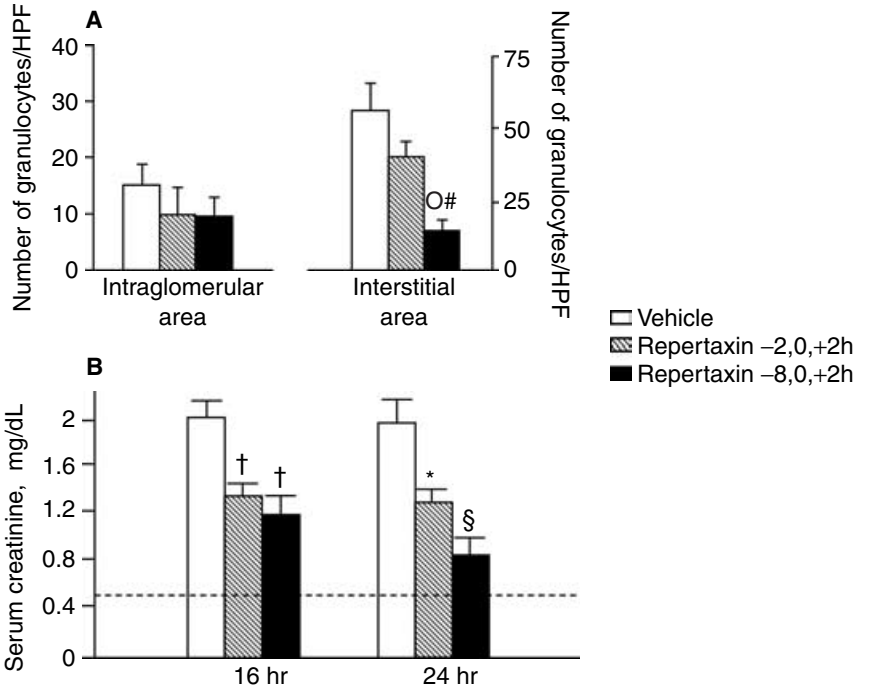

Fig. 4. Effect of different administration times of repertaxin 15mg/kg (−2, 0, +2 hours) ($N = 8$); (−8, 0, +2 hours) ($N = 4$); or vehicle ($N = 13$) on intragraft granulocyte infiltration and serum creatinine concentrations in syngeneic transplantation. (*A*) Number of granulocytes counted in intraglomerular (left) and in interstitial (right) areas 24 hours after reperfusion, in at least 10 randomly selected high power fields (HPF). (*B*) Dashed line represents normal serum creatinine values. After transplantation the serum creatinine concentrations were evaluated at 16 and 24 hours. Data are mean ± SE. $^{\dagger}P < 0.001$; $^{*}P < 0.01$; $^{\#}P < 0.05$; §$P < 0.005$ vs. vehicle; $^{\circ}P < 0.05$ vs. repertaxin at −2, 0, +2 hours.

summarizes the results of quantitative evaluation of granulocyte infiltration in the graft at sacrifice. Values of intraglomerular granulocyte accumulation were slightly affected by repertaxin given either at −2 hours or at −8 hours pretransplant. Eight hour pretreatment of the recipient induced a significant reduction of the number of granulocytes in interstitial area in respect to animals receiving vehicle ($P < 0.05$). A similar trend was observed in interstitial area of grafts from animals treated 2 hours before transplantation, although the difference in respect to rats receiving vehicle did not reach statistical significance.

Figure 4B shows the effect of repertaxin given 2 or 8 hours before transplantation on renal function. At 16 hours posttransplant, the serum creatinine levels were reduced at a comparable extent in rats receiving the treatment 2 and 8 hours before transplantation, in respect to vehicle-treated animals ($P < 0.001$). However, 24 hours after transplantation, animals with −8 hours pretreatment had better preservation of renal function than rats

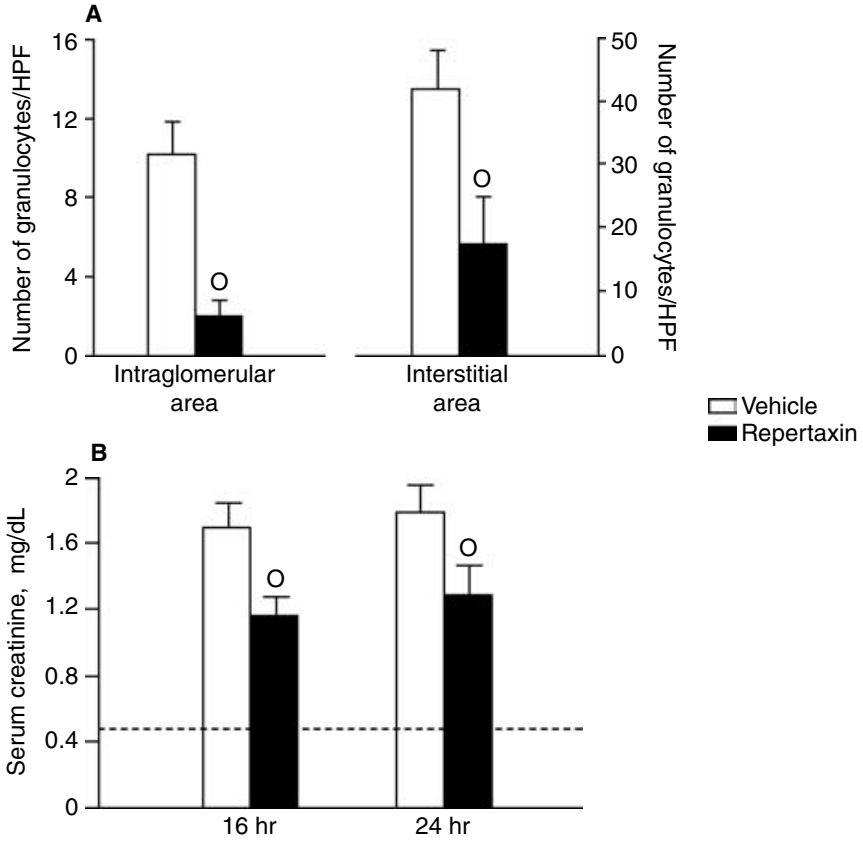

Fig. 5. Effect of repertaxin (15mg/kg) (−8, 0, +2 hours ($N = 10$) or vehicle ($N = 13$) on intragraft granulocyte infiltration and serum creatinine concentrations in allogeneic transplantation. (*A*) Number of granulocytes counted in intraglomerular (left) and in interstitial (right) areas 24 hours after reperfusion, in at least 10 randomly selected high power fields (HPF). (*B*) Dashed line represents normal serum creatinine values. After transplantation the serum creatinine concentrations were evaluated at 16 and 24 hours. Data are mean ± SE. $^{\circ}P < 0.05$ vs. vehicle.

receiving the compound 2 hours before transplantation ($P < 0.005$ vs. $P < 0.01$).

Effect of repertaxin on intragraft granulocyte infiltration and renal function in allogeneic transplantation

Once established that repertaxin effectively modulated I/R injury in syngeneic kidney transplantation, we assessed whether the treatment were of benefit in an experimental model of allogeneic rat kidney transplant. Repertaxin at the dose of 15 mg/kg was administered to animals with the same treatment regimen adopted for animals undergone syngeneic transplantation. Figure 5A shows the effect of repertaxin on the number of infiltrating granulocytes as detected by immunohistochemical staining. Repertaxin administered to a recipient of an allogeneic kidney significantly ($P < 0.05$) reduced the number of granulocytes infiltrating the intraglomerular and the interstitial areas of the kidney, as compared to that observed in vehicle-treated rats.

Figure 5B shows the effect of repertaxin on serum creatinine concentrations in rats 16 and 24 hours after receiving an allogeneic kidney transplant. A significant prevention of the serum creatinine level increase was observed both at 16 and 24 hours posttransplant in the groups receiving the CXCR2 inhibitor starting 8 hours before transplantation ($P < 0.05$ vs. vehicle group).

DISCUSSION

The main goal of this work was to gain insight into the impact of blocking granulocyte recruitment via the CXCR2 signaling in a model of renal posttransplant I/R injury in the rat by using a novel inhibitor of CXCR2 intracellular signal transduction, repertaxin L-lysine salt. After 4 hours of cold ischemia followed by organ transplantation, repertaxin administered to the recipient animals was effective in preventing renal function impairment both in syngeneic and in allogeneic settings. Concomitantly, the number of graft infiltrating granulocytes was lowered by repertaxin in respect to vehicle-treated rats, suggesting that the major therapeutic effect of the inhibitor was to limit granulocyte infiltration, whose massive presence in the graft contributes to renal dysfunction [19]. I/R injury is a pivotal pathogenetic mechanism in the development of acute renal failure associated with renal transplantation and appears to involve a complex interplay between renal hemodynamcs, tubular injury, and inflammatory processes. Activated neutrophils were implicated as participating in the pathogenesis of I/R injury through the generation of superoxide radicals and inflammatory cytokines resulting in endothelial cell damage and thereby renal blood flow decrease [20, 21]. As in acute inflammation, neutrophils infiltrate down a concentration gradient of chemotactic factor(s) after adherence to endothelial cells through adhesion molecules. The sig-

nals mediating transendothelial leukocyte migration [22], their recruitment and activation at the site of inflammation include.

Production of CXC chemokines such as IL-8, which is induced, in turn, by reactive oxygen metabolites generated upon reperfusion [23, 24]. Beyond their chemotactic activity on neutrophils, CXC chemokines may promote the release of lysosomal enzymes and superoxide anions by neutrophils, which concur to amplify the injury [10, 25]. In rodents the CXC chemokines CINC-1, MIP-2, and CINC-2 are thought to be the rat counterpart of human IL-8.

Here we first assessed the kinetics of CINC-1/IL-8 mRNA expression in the I/R transplanted kidney and found it markedly up-regulated by reperfusion, rising 1 hour after revascularization to reach a peak within 3 hours, in contrast to the very low levels of the chemokine in control kidneys only exposed to ischemia. Similar changes in renal tissue levels of IL-8 have been already reported in rats subjected to 60 minutes of renal ischemia. Renal levels of rat IL-8 evaluated by enzyme-linked immunosorbent assay (ELISA) reached the peak at 3 hours after reperfusion and decreased gradually thereafter [12]. In addition, in a model of retinal I/R injury produced by increasing intraocular pressure, the expression of IL-8 mRNA in the rat retinal vessels was induced with a comparable kinetics as for the kidney [13]. In other studies, reperfusion of the ischemic myocardium results in increased release of IL-8 in the rabbit [26] and elevated plasma levels of the proinflammatory chemokine were found in patients undergoing coronary artery bypass surgery [27]. In the present study, analysis of kidney RNA by real-time quantitative PCR indicated significantly higher CINC-1/IL-8 transcript levels both in isografts and allografts with respect to control kidneys. However, the increase was more evident in isografts than in allografts, in line with studies by Morita et al [14] in murine cardiac graft. Concomitant with CINC-1/IL-8 up-regulation, we observed as early as 1 hour after reperfusion an increase of granulocyte counts in interstitial and intraglomerular areas, which persisted along the experiment. It is plausible that additional chemokines acting through the CXCR2 receptor might contribute to granulocyte infiltration in kidney allografts, which might explain comparable numbers of granulocytes in isografts and in allografts, in the face of lower CINC-1/IL-8 expression levels in the latter.

As repertaxin blocks the activity of CXCR1 (in human) and CXCR2 receptors (in human and rat), it could potentially inhibit the biologic activity of all the chemokines acting through these molecules. Indeed previous in vitro studies showed that repertaxin inhibits the intracellular signal transduction induced by IL-8 in human polymorphonuclear cells and by CINC-1 and MIP-2 in rat polymorphonuclear cells [16, 17].

We found that repertaxin was capable to prevent graft granulocyte infiltration and to limit serum creatinine increase subsequent to I/R. Repertaxin allowed faster recovery of normal kidney function to the extent that 24 hours after transplantation serum creatinine levels in repertaxin-treated rats tended to normalize, whereas vehicle-treated animals still showed renal functional impairment. Within the range of the doses tested, we found that the effect of repertaxin was dose dependent: 5mg/kg of repertaxin only partially prevented serum creatinine increase, but had no effect on cellular infiltration. The most effective dose of repertaxin was 15mg/kg, while the local inflammation and hematuria induced with 30 mg/kg could have negatively affected the response to repertaxin.

We administered the compound to recipient rats three times starting at different time points before transplantation, to have an early block of CINC-1/IL-8 signaling in recipient granulocytes. Granulocyte graft infiltration was significantly reduced by repertaxin given 24 or 8 hours before transplantation, while reduction with −2 hours pretreatment was not significant, indicating that the duration of pretreatment time is crucial to allow drug to enter recipient polymorphonuclear cells and effectively inhibit CINC-1/IL-8 biologic activity. From these results, we suggest that the pretreatment at −8 hours followed by injection immediately before reperfusion and 2 hours after transplantation with the dose of 15 mg/kg was the best condition to prevent renal I/R injury. Furthermore, the effectiveness of repertaxin in I/R injury after allogeneic transplantation in preventing granulocyte graft infiltration and serum creatinine increase renders the present results more relevant since this setting more closely mimics the clinical condition.

Although leukocyte infiltration in response to I/R injury is a well-known phenomenon, the contribution of neutrophils to tissue damage is still controversial [28] to the extent that some studies reported a beneficial effect on graft function and tissue injury by preventing granulocyte accumulation [6, 29–34], whereas other studies showed no effect [35, 36]. Our finding that repertaxin blocks granulocyte infiltration and prevents serum creatinine level increase strongly suggests a causal role of granulocytes in renal function impairment by I/R in this experimental model.

Overall our results show that blocking the CXCR2 signaling by the use of repertaxin is effective in preventing renal dysfunction due to I/R in syngenic and allogenic transplantation.

Our finding is in line with very recent papers that reported the effectiveness of repertaxin in preventing organ damage in experimental models of rat hepatic [16] and intestinal [17] I/R injury.

The efficacy of repertaxin to modulate I/R injury opens new perspectives for preventing posttransplant

delayed graft function, that negatively affects both short- and long-term graft success of human transplantation.

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Reprint requests to Ariela Benigni, Ph.D., Department of Molecular Medicine, "Mario Negri" Institute for Pharmacological Research, Via Gavazzeni 11, 24125 Bergamo, Italy.
E-mail: abenigni@marionegri.it

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