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ORIGINAL ARTICLE

Prolonged cold ischemia accelerates cellular and humoral chronic rejection in a rat model of kidney allotransplantation

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Summary

One of the leading causes of long-term kidney graft loss is chronic allograft injury (CAI), a pathological process triggered by alloantigen-dependent and alloantigen-independent factors. Alloantigen-independent factors, such as cold ischemia (CI) may amplify the recipient immune response against the graft. We investigated the impact of prolonged cold ischemia and the subsequent delayed graft function on CAI in a fully MHC-mismatched rat model of kidney allotransplantation. Prolonged CI was associated with anticipation of proteinuria onset and graft function deterioration (ischemia: 90d; no ischemia: 150d), more severe tubular atrophy, interstitial fibrosis, and glomerulosclerosis, and increased mortality rate (180d survival, ischemia: 0%; no ischemia: 67%). In ischemic allografts, T and B cells were detected very early and were organized in inflammatory clusters. Higher expression of BAFF-R and TACI within the ischemic allografts indicates that B cells are mature and activated. As a consequence of B cell activity, anti-donor antibodies, glomerular C4d and IgG deposition, important features of chronic humoral rejection, appeared earlier in ischemic than in non-ischemic allograft recipients. Thus, prolonged CI time plays a main role in CAI development by triggering acceleration of cellular and humoral reactions of chronic rejection. Limiting CI time should be considered as a main target in kidney transplantation.

Introduction

Chronic allograft injury (CAI) remains the major obstacle to long-term allograft survival and, besides death of the recipient with graft function, it is the most common cause of renal transplant failure [1]. CAI is a multifactorial process and is caused by both alloantigen-dependent factors, such as the recognition of donor foreign antigens resulting in the activation of specific host immune response, and alloantigen-independent factors [1].

Research efforts have focused on improving HLA mismatches and reducing acute rejection to limit the development and progression of CAI. However, alloantigen-independent factors have recently received increasing attention as they may amplify the recipient immune response against the graft. One such factor is cold ischemia (CI). Indeed, solid organ transplantation is inevitably associated with a period of ischemia starting at the recovery of organs from the donor until their reperfusion in the recipient. The reintroduction of blood flow to the ischemic organ, although necessary to rescue the organ from necrosis and permanent loss of function may cause acute cellular injury [2]. Ischemia/reperfusion (I/R) injury is an important cause of renal graft dysfunction, leading

to a higher risk of early post-transplant complications including delayed graft function (DGF) and acute rejection [3,4]. In 1992 Land and coworkers indicated for the first time that an initial nonspecific injury to an allogeneic human renal allograft by I/R contributes both to acute rejection episodes and early irreversible graft loss and to chronic transplant failure [5].

The majority of the experimental studies on CAI has been conducted by using the model in the F344 ($RT.1^{lvl}$) to LW ($RT.1^{l}$) rat combination [6]. By using such a model we have previously documented that combining treatments that target immune and non-immune mechanisms of chronic graft loss (by MMF and losartan, respectively) preserve allograft structural integrity and function [7]. In the same model, Tullius *et al.* [8] studied the contribution of I/R injury on CAI and found that functional deterioration and structural changes worsened in ischemic allografts and progressed in parallel to increasing cold ischemia time. More recently, in the same model, Herrero-Fresneda *et al.* [9] showed that the initial parenchymal injury caused by cold ischemia is mainly responsible for chronic tubulointerstitial damage, whereas the vascular lesions are principally caused by alloantigen-dependent mechanisms. F344 and LW rats are two inbred strains which are considered haploidentical and differ only at minor histocompatibility (non-MHC) loci, an immunologic condition far from the clinical practice in human transplant patients. We and others recently developed a clinically relevant CAI model of kidney transplantation using a fully MHC-mismatched rat strain combination with Wistar-Furth rats ($RT.1^{u}$) as kidney donors and LW rats as recipients [10,11]. In the latter model, few studies have been carried out with the aim to verify whether or not CAI could be ameliorated by controlling alloantigen-dependent factors. Specifically, we documented that gene transfer of CTLA4Ig into the donor kidney prevents progressive proteinuria and protects transplanted kidneys from chronic renal structural injury [10]. A population of anergic T cells with regulatory activity, which eventually were responsible for long-term maintenance of graft survival, were found in recipient lymph nodes and in the graft as long as 120 days after transplantation [10]. Waaga-Gasser *et al.* [11] showed that transfer of donor-specific Th2 clones into recipient rats regulates alloimmune responses and protects allografts from progressive chronic rejection. However, no studies have assessed the impact of I/R injury on CAI in MHC-mismatched rat strain combinations.

We designed this study with the aim of evaluating the impact of prolonged cold ischemia and the subsequent delayed renal graft function on CAI in the context of fully MHC-mismatch.

Methods

Animals

Male inbred Wistar Furth ($RT1^{u}$, WF, Harlan, Bresso, Italy) and Lewis ($RT1^{l}$, LW, Charles River, Calco, Italy) rats weighing 210 to 250 g (8–10 weeks old) were used. These animals differ for class I, class II, and non-MHC genes. 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).

Kidney transplantation

Orthotopic kidney transplantation was performed according to our standard protocol [10]. The donor kidney and ureter were removed en bloc and flushed with Belzer solution (UW) containing 1000 U/ml heparin. Then the kidney was placed in iced Belzer solution with no direct contact with ice for the time of cold ischemia in a refrigerator at 4 °C to keep constant the temperature. Recipients were prepared by removal of the left kidney. Kidney grafts were washed with saline solution before transplant. An anastomosis was created between the recipient and donor renal artery as well as renal vein with end-to-end anastomosis. Vascular clamps were released after 30 min. Donor and recipient ureters were attached end-to-end. The native right kidney was then removed.

Experimental design

Figure 1 shows the experimental design of study 1 and study 2. Study 1 was designed as a survival study. LW rats, receiving a WF kidney allograft subjected ($n = 11$, CI-Allo group) or not ($n = 10$, no-CI-Allo group) to 16 h cold ischemia (CI), were followed for post-transplant survival. Renal graft function (as serum creatinine level, using an auto-analyzer) were measured monthly. At the same time points, animals were placed in individual metabolic cages for 24-h urine collection for determination of urine output and protein excretion. LW rats receiving a syngeneic non-ischemic kidney graft were used as isograft control group ($n = 6$, Isograft group). All allografted rats received cyclosporine A (CsA, 5 mg/kg per day intramuscularly; Novartis Farma, Milan, Italy) for the first 10 days after transplantation to prevent early acute rejection [10]. Four animals were sacrificed in each group 24 h after transplantation for histologic evaluation of ischemic injury.

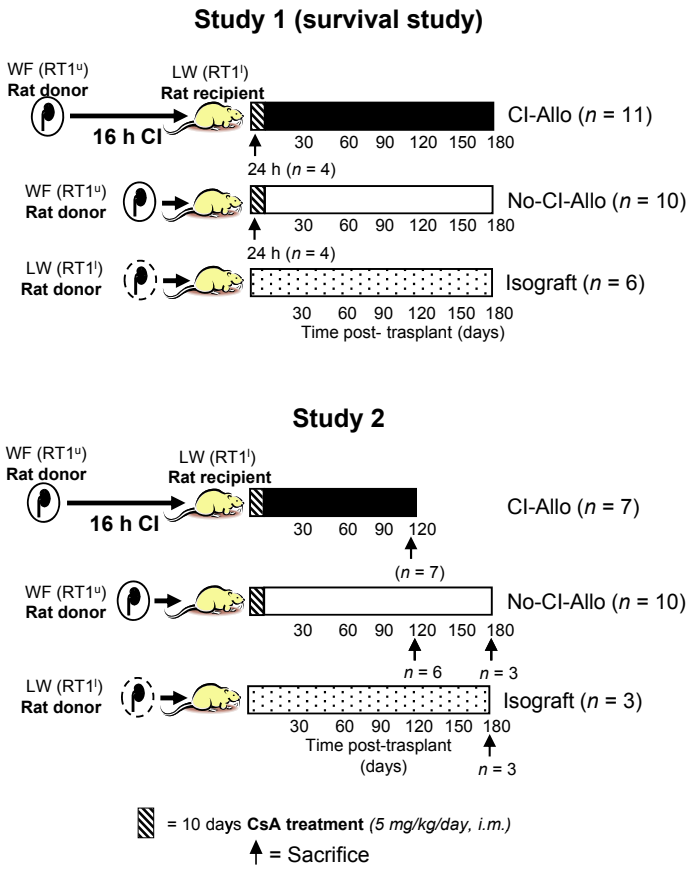

Figure 1 Schematic representation of the experimental design. CI-Allo: Wistar Furth (WF) kidneys subjected to 16 h cold ischemia (CI) and transplanted into allogeneic Lewis (LW) rat recipients. No-CI-Allo: WF kidneys transplanted into allogeneic LW recipients, without CI. Isograft: LW kidneys transplanted into syngeneic LW recipients, without CI. CsA, cyclosporine A. ↑ Sacrifice of surviving animals.

Since all the rats receiving an ischemic allograft died within 140 days post-transplant, the subsequent study (study 2) was carried out with a follow-up of 120 days. LW rats were given WF kidney allografts subjected ($n = 7$, CI-allo group) or not ($n = 10$, no-CI-Allo group) to 16 h cold ischemia. At the end of the follow-up, animals ($n = 7$ CI-allo group; $n = 6$ no-CI-Allo group) were sacrificed and the graft removed and processed for histology, immunohistology and molecular studies. Four animals in the no-CI-Allo group were followed until 180 days post-transplant, three of them survived until the end of the follow-up. LW rats receiving syngeneic non-ischemic kidney grafts were used as isograft control group ($n = 3$, Isograft group). Serum samples were collected at 30 and 90 days post-transplant for the detection of donor-specific antibodies.

The cold ischemia time of 16 h was chosen on the basis of preliminary experiments designed to find out a CI time able to induce delayed graft function. WF kidney allografts were subjected to 5, 8, 16, or 20 h CI time and then transplanted into LW recipient rats. As shown in Table 1, serum creatinine levels, monitored at 16 h, 24 h and 5 days post-transplant, showed that 16 h CI caused acute graft dysfunction followed by recovery within 5 days. Rats receiving an allograft subjected to 20 h CI did not recover from the acute graft dysfunction.

Renal histology

Duboscq-Brazil-fixed, paraffin-embedded kidney samples were cut (3-μm thick) and stained with hematoxylin/eosin and periodic acid-Schiff (PAS) reagent stain.

In 24-h kidneys, tubular damage (consisting of epithelial cell degeneration, brush border loss, cell detachment, luminal cell debris, luminal casts) was evaluated by a semi quantitative score accordingly to Dragun *et al.* [12]. Evaluation and scoring were done by two blinded investigators.

In 120 and 180-day kidneys the frequency of glomerular sclerosis was determined by examining all glomerular profiles contained within one coronal section (at least 80 glomeruli per kidney) and expressed as the percentage of the total number of glomeruli counted. Transplant glomerulopathy, tubular atrophy, and interstitial changes (inflammation and fibrosis, tubular cast and vascular intimal thickening) were graded on a scale of 0 to 4 + as reported [7,13].

Table 1. Serum creatinine levels (mg/dl) in allogeneic model of kidney transplant exposed to different cold ischemia (CI) time.

	Time post-transplant		
CI time	16 h	24 h	5 days
5 h	0.83	0.52	0.50
8 h	1.04	0.78	0.50
16 h	1.94	2.24	0.74
20 h	2.18	2.90	2.98

Mean values of two animals for each CI time tested are reported.

TUNEL staining

For analysis of apoptosis, terminal-deoxynucleotidyltransferase-mediated dUTP nick and labeling (TUNEL) was used (*in Situ* Cell Detection Kit, POD; Roche Applied-Science, Indianapolis, IN, USA) according to the manufacturer's protocol. The percentages of the numbers of TUNEL-positive nuclei to the numbers of total cell nuclei were counted in 20 non-overlapping random areas (400x) per section by two blinded investigators.

Nitrotyrosine staining

Oxidative damage was localized using a specific mouse monoclonal antibody against nitrotyrosine (Upstate Biotechnology Inc, Lake Placid, NY). Briefly, 3-μm paraffin sections were incubated with primary antibody (1:300), followed by biotinylated secondary antibody (horse anti-mouse IgG, 1:200, Vector Laboratories, Burlingame, CA, USA). The signal was developed with diaminobenzidine-Nickel (DAB, Vector Laboratories). The score (0 = absent; 1 = faint; 2 = moderate; 3 = intense) was calculated as a weighted mean. At least 20 non-overlapping fields for each section were examined by two blinded investigators.

Immunohistochemistry of intragraft infiltrating cells

A goat polyclonal antibody was used for the detection of the CD20 antigen present on the rat B cells (Santa Cruz, CA, USA); a monoclonal mouse antibody was used for the detection of CD3 cell surface glycoprotein expressed by T cells (eBioscience, San Diego, CA, USA, clone G4.18).

After microwave antigen retrieval, the sections were incubated overnight at 4 °C with the primary antibody (anti-CD20, 1:200; anti-CD3, 1:100), washed and then incubated with biotynilated horse anti-goat IgG antibody (1:200) (Vector Laboratories) or horse anti-mouse IgG antibody (1:200) (Vector Laboratories) for 1 h at room temperature. The signal was developed with DAB. For each marker, the number of cells was counted in at least 20 randomly selected high power microscope fields (×400) for each animal. We also performed double immunoperoxidase staining for CD20 and CD3 expression by combining the two methods and by revealing CD20 with DAB and CD3 with NovaRed (Vector Laboratories).

C4d and IgG staining

Frozen sections (3 μm thick) were post-fixed in acetone for 10 min at 4 °C. For C4d staining, a goat anti-human C4d pAb was incubated for 1 h at room temperature (1:25, Santa Cruz). Thereafter, sections were incubated for 1 h at room temperature with Cy3-conjugated $F(ab')_2$ fragment donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For IgG staining, sections were incubated with FITC-conjugated goat anti-rat IgG antibody (Sigma, Saint Louis, MO, USA). Negative controls were obtained by omitting the primary antibody on a second section present on all the slides. Evaluation and scoring was done by two blinded investigators. Around 30–40 glomeruli and 80 fields with tubuli for each sample were analyzed and the signal intensity was graded on a scale of 0–3 (0 = no staining; 1 = weak staining; 2 = staining of moderate intensity; 3 = strong staining).

Anti-donor antibodies detection by flow cytometry

Donor-specific antibodies detection was performed as previously described [14]. Briefly, donor WF or third party BN thymocytes were isolated, pelleted, and resuspended in 100 μl of diluted serum (serial dilutions from 1/10 to 1/500). Anti-donor IgG were then stained by incubating WF thymocytes (1×10^6 cells/sample) with FITC-conjugated goat anti-rat IgG (Sigma-Aldrich). The data (all with 1/100 serum dilution) are presented as the percentage of positively stained donor thymocytes. Unspecific staining of third party thymocytes (between 5 and 10% on average) was considered as background and subtracted.

Quantitative Real time RT-PCR

We obtained total RNA from frozen kidneys by homogenization followed by Trizol extraction (Life Technologies, Milan, Italy). RNA was treated with DNAse and reverse transcribed to cDNA by Superscript II (Life Technologies). We performed quantitative real time-PCR on the ABI PRISM 7300 Real Time-PCR System (PE Applied Biosystems, Foster City, CA, USA) with Power Syber Green Master Mix and primers specific for rat BAFF-R (primers: sense, 5′CCGAA GGAGTCCAGCAAGAGT-3′; antisense, 5′CAGTTAGGAG CTGAGGCATGAAG-3′) and TACI (primers: sense, 5′AGG CCGGATAACTTAGGAAGGT-3′; antisense, 5′GCTGGTC ACTGTTCATCCTCAA-3′). GAPDH served as housekeeping gene (primers: sense, 5′TCATCCCTGCATCCACTGGT-3′;

antisense, 5′CTGGGATGACCTTGCCCAC-3′;). We used the ΔΔ threshold cycle technique to calculate cDNA content in each sample using the cDNA expression in kidney from naïve WF rat as reference (calibrator).

Statistical analysis

Results were given as mean ± SE. For all parameters, the significance of differences between individual groups was analyzed by one-way ANOVA followed by the post hoc Student-Newman-Keuls test for pair-wise comparisons. Changes of the various parameters over time were evaluated by ANOVA for repeated measures followed by the post hoc Fisher PLSD test. All data were analyzed using MedCalc 10.0.1 statistical software. Survival data were analyzed by the log-rank test. Statistical significance was defined as $P < 0.05$.

Results

Prolonged cold ischemia time worsens graft function and survival in CAI

As shown in Fig. 2 panel A, 16 h cold ischemia time caused acute graft dysfunction as documented by the sudden increase in serum creatinine level at 24 h post-transplant. Rats receiving an ischemic allograft and sacrificed 24 h after transplant developed severe tissue injury. Indeed, histological analysis showed marked tubular alterations such as tubular necrosis, luminal congestion and loss of brush border in the ischemic grafts compared with the non-ischemic grafts (Fig. 2, panel B). At the same time, ischemic allografts were characterized by extensive nuclear changes consistent with apoptotic cell death involving around 25% of tubular cells whereas non-ischemic allografts showed few apoptotic tubular cells (Fig. 2, panel C). Moreover, as documented by the results of nitrotyrosine staining, oxidative stress was significantly stronger in tubuli of ischemic allografts as compared to control non-ischemic allografts (Fig 2, panel D).

Monitoring of graft function and survival documented that rats receiving an ischemic allograft, despite recovery of graft function within the first 7–15 days, displayed an increase in serum creatinine level from day 90, indicating that 16 h cold ischemia time accelerates the chronic loss of graft function (Fig. 2, panel A). All animals died within

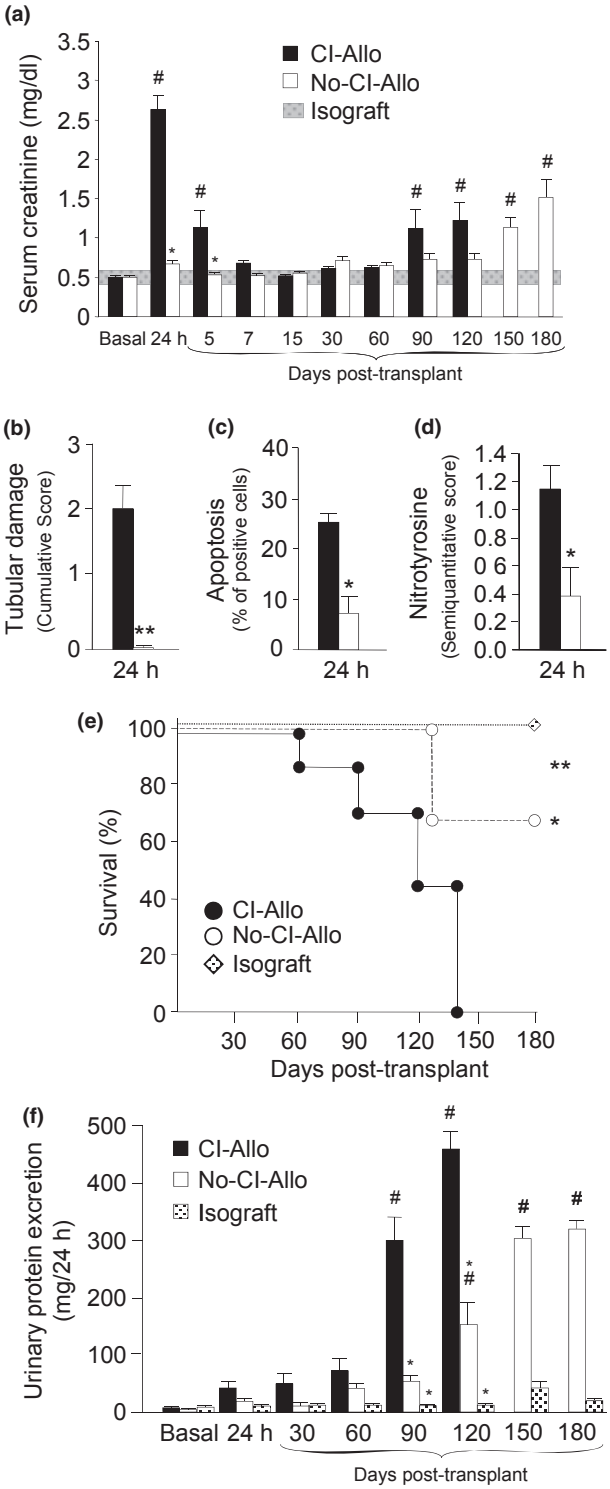

Figure 2 Effect of cold ischemia on graft function, survival and 24 h-tissue damage (Study 1). (a) Time course of serum creatinine levels (mg/dl). (b) Cumulative score of tubular damage on renal graft sections taken 24h post-transplant. (c) Percentages of apoptotic tubular cells on renal tissue taken 24 h post-transplant. (d) Semiquantitative score (0–3) of nitrotyrosine staining on renal tissue taken 24h post-transplant. (e) Percentages of animal survival. (f) 24-h urinary protein excretion. CI-Allo: WF kidneys subjected to 16 h cold ischemia (CI) and transplanted into allogeneic LW recipients. No-CI-Allo: WF kidneys transplanted into allogeneic LW recipients, without CI. Isograft: LW kidneys transplanted into syngeneic LW recipients, without CI. Values are mean ± SE. #$P < 0.05$ vs. basal and isograft; *$P < 0.05$ vs. CI-Allo; **$P < 0.01$ vs. CI-Allo.

day 140 post-transplant (Fig. 2, panel E). Control non-ischemic allografted rats had a later increase in serum creatinine level over baseline starting from day 150 post-transplant and four of six animals completed the 6-month follow-up (Fig. 2, panels A and E).

Sixteen hours cold ischemia time resulted in earlier onset of proteinuria (Fig. 2, panel F). Indeed, in rats receiving an ischemic allograft proteinuria significantly increased over the baseline already at 90 days post-transplant and was significantly higher than values measured at 90 and 120 days post-transplant in rats receiving a non-ischemic allograft (Fig. 2, panel F). The evolution of proteinuria in the control non-ischemic allograft group was characteristic of the pattern previously reported for this model with values significantly higher than baseline after 120-day follow-up [10].

Ischemic injury accelerates CAI development

The subsequent study was carried out for histology examination with a follow-up of 120 days (see experimental design, study 2, Fig. 1) ascribable to the high mortality recorded thereafter in the ischemic group. A sub-group of non-ischemic allografted rats was followed till 180 days.

At 120 days post-transplant, allografts subjected to 16 h cold ischemia displayed more severe histologic features of CAI than those found in non-ischemic allografts (Fig. 3 panels A, B, C and D). In detail, ischemic allografts displayed greater degrees of interstitial fibrosis and tubular atrophy than non-ischemic allografts. Glomerulopathy and vascular intimal thickening were found in ischemic allografts and almost all the glomeruli were affected by massive sclerosis. In non-ischemic allografts such severe histologic abnormalities became evident only 2 months later, at 180 days post-transplant (Fig. 3, panels A, B, and E).

By TUNEL staining we found that at 120 days post-transplant allografts subjected to 16 h cold ischemia showed marked tubular apoptosis whereas only a modest percentage of apoptotic tubuli was observed in non-ischemic allografts (Fig. 3, panel G). Non-ischemic allografts displayed a marked tubular apoptosis when rats reached 180 days of follow-up (Fig. 3, panel G).

Prolonged cold ischemia time increases T and B cell infiltration and activity in CAI

In ischemic allografts after 120-day follow-up, significantly higher numbers of T cells were found than in control non-ischemic allografts (Fig. 4, panel A). At the same time point, the number of infiltrating B cells was significantly higher in ischemic than in non-ischemic allografts (Fig. 4, panel A). Interestingly, in the ischemic allografts at 120 days post-transplant, B cells were organized to form aggregates whereas the few B cells which infiltrate the non-ischemic allografts were scattered and not well organized (Fig. 4, panels B, C, E, F). As shown by double staining for CD20 and CD3, such aggregates made of densely packed B

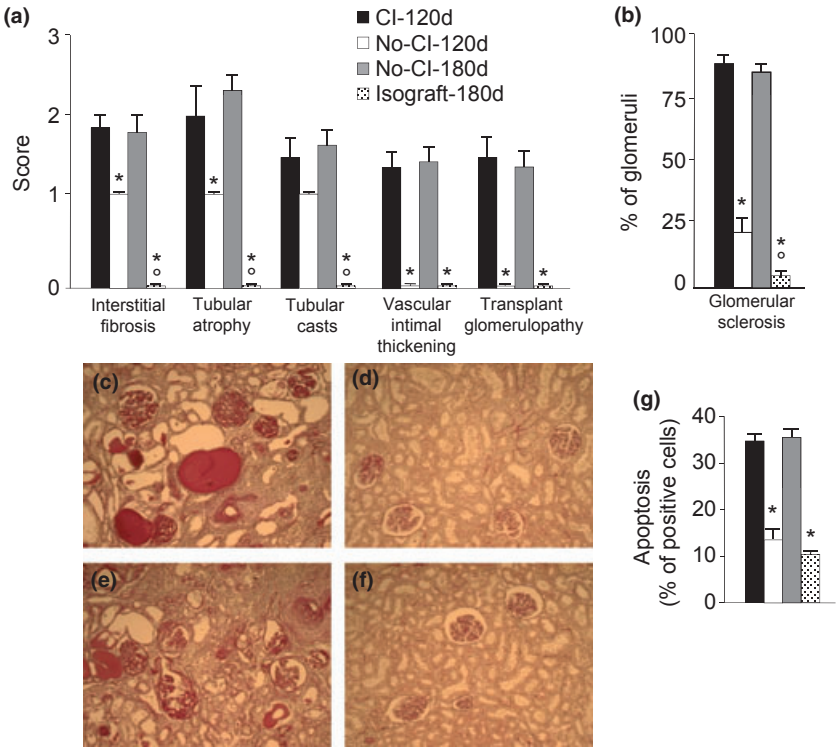

Figure 3 Effect of cold ischemia on histology and apoptosis. (a) Glomerular, vascular and tubulointerstitial injury. (b) Percentages of glomerular sclerosis. (c–f) Representative images of PAS staining on renal graft section (original magnification 200×). (c) CI-120d; (d) No-CI-120d; (e) No-CI-180d; (f) isograft-180d. (g) Percentages of apoptotic tubular cells on renal tissue taken at the end of follow-up. CI-120d: WF kidneys subjected to 16 h cold ischemia (CI) and transplanted into allogeneic LW recipients, taken 120 days post-transplant. No-CI-120d: WF kidneys transplanted into allogeneic LW recipients, without CI, taken 120 days post-transplant. No-CI-180d: WF kidneys transplanted into allogeneic LW recipients, without CI, taken 180 days post-transplant. Isograft-180d: LW kidneys transplanted into syngeneic LW recipients, without CI, taken 180 days post-transplant. Values are mean ± SE. *$P < 0.05$ vs. CI-120d and No-CI-180d; °$P < 0.05$ vs. No-CI-120d.

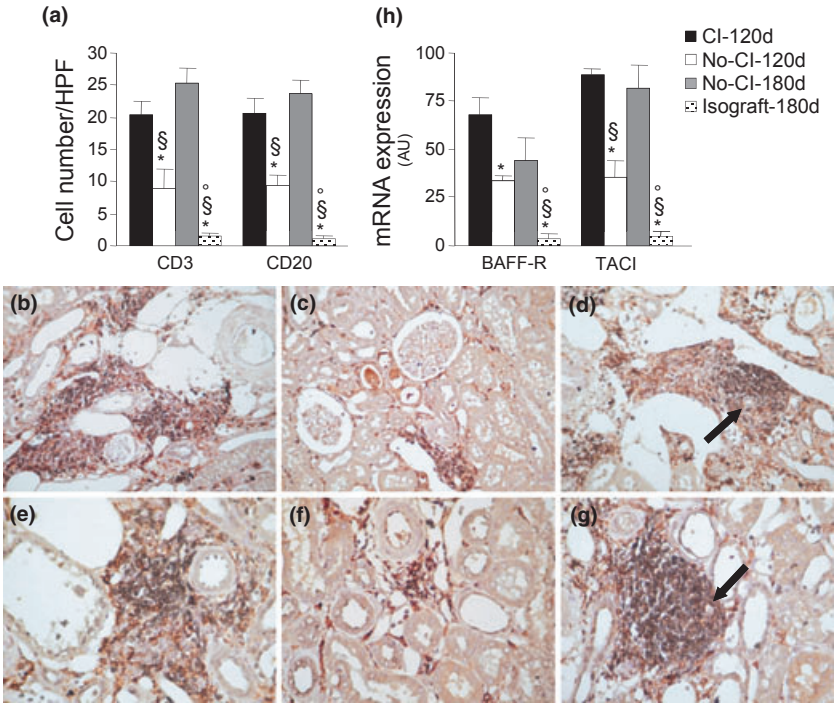

Figure 4 Effect of cold ischemia on intragraft T and B cell infiltration and activity. (a) $CD3^+$ T cells and $CD20^+$ B cells quantified as number of cells per high power field (HPF, magnification: 400×). (b–g) Representative images of double immunostaining for $CD3^+$ (NovaRed, dark red signal) and $CD20^+$ cells (DAB, brown-black signal). (b) and (e) CI-120d showing T/B aggregates defining both clusters and lymphoid follicles; (c) and (f) No-CI-120d showing scattered minimal T and B cell infiltration; (d) and (g) No-CI-180d showing T/B aggregates defining both clusters and lymphoid follicles; arrows: small vessels inside the cluster. (b–d) original magnification 200×, (e–g) original magnification 400×. (h) BAFF-R and TACI mRNA expression calculated with ΔΔCt method. Results are expressed as Arbitrary Unit (AU), taking the expression of BAFF-R and TACI of naïve untransplanted WF kidney as calibrator (AU = 1). CI-120d: WF kidneys subjected to 16 h cold ischemia (CI) and transplanted into allogeneic LW recipients, taken 120 days post-transplant. No-CI-120d: WF kidneys transplanted into allogeneic LW recipients, without CI, taken 120 days post-transplant. No-CI-180d: WF kidneys transplanted into allogeneic LW recipients, without CI, taken 180 days post-transplant. Isograft-180d: LW kidneys transplanted into syngeneic LW recipients, without CI, taken 180 days post-transplant. Values are mean ± SE. $^*P < 0.05$ vs. CI-120d; $^\circ P < 0.05$ vs. No-CI-120d; $\S P < 0.05$ vs. No-CI-180d.

cells were surrounded by margins made of T cells thus defining inflammatory clusters as well as structures similar to lymphoid follicles (Fig. 4, panels B and E). In non-ischemic allografts B/T aggregates were found at 180 but not at 120 days post-transplant (Fig. 4, panels D and G). Rare small vessels were detected both inside and nearby the B/T cell clusters both in ischemic (120 days) and non-ischemic (180 days) allografts (Fig. 4, panels D and G, arrows).

Messenger RNA expression for BAFF-R and TACI were significantly higher in ischemic than in non-ischemic allografts (Fig. 4, panel H). Consistent with a scenario of B cell activation, in the ischemic allografts about 30% of the glomeruli displayed strong staining for IgG (Fig. 5, panel A). C4d deposition was evident on peritubular and glomerular capillaries (Fig. 5, panels E, F and I). In non-ischemic allografts, IgG and C4d depositions were very faint at 120 days post-transplant (Fig. 5, panels B, E, G and J) but increased at 180 days post-transplant (Fig. 5, panels C, E, H and K).

Circulating donor-specific antibodies were detected in the serum of rats receiving an ischemic allograft earlier (30 days) than in rats receiving a non-ischemic allograft (90 days, Fig. 5, panel L).

Discussion

In the current study we investigated the impact of prolonged cold ischemia (CI) causing acute delayed graft function (DGF) on functional and morphologic signs of CAI in fully mismatched kidney allotransplantation. As compared to control non-ischemic allograft, we found that prolonged cold ischemia time is associated with acceleration of proteinuria onset and graft function deterioration, more severe tubular atrophy, interstitial fibrosis, and glomerulosclerosis, and increased mortality rate. Consistent with our findings, Herrero-Fresneda *et al.* [9] have shown that rats receiving an ischemic graft displayed at 6 months after transplantation a deterioration of graft

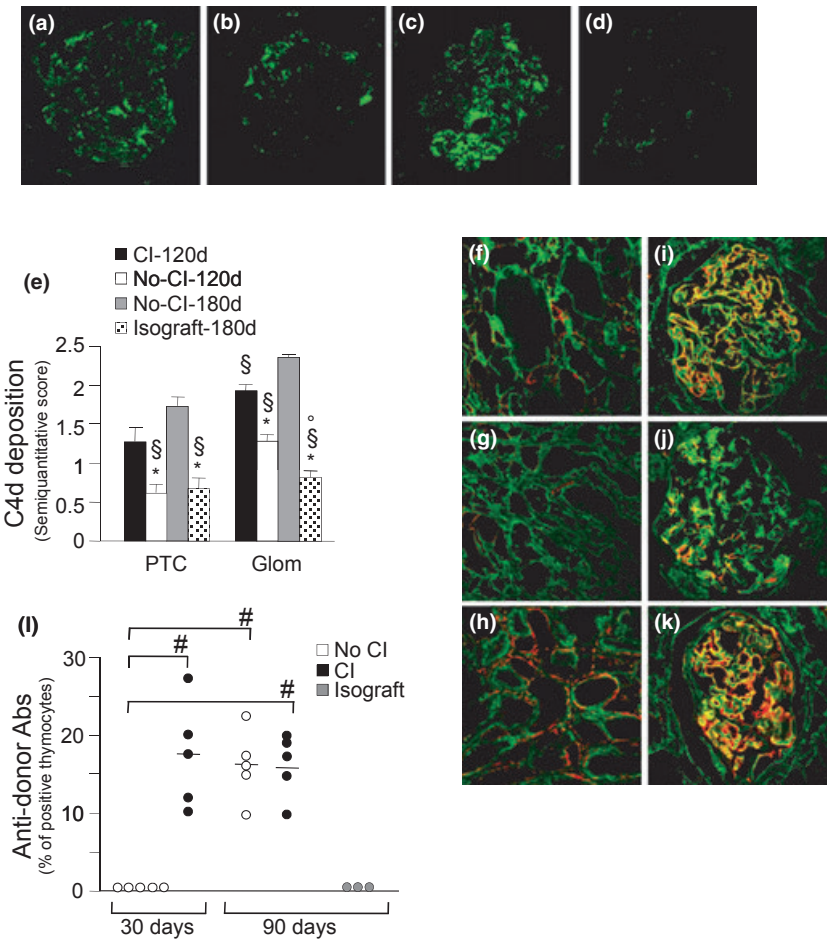

Figure 5 Effect of cold ischemia on IgG and C4d depositions and on circulating donor-specific antibodies. (a–d) Representative images for IgG glomerular deposition. (a) CI-120d showing moderate IgG staining; (b) No-CI-120d showing faint IgG staining; (c) No-CI-180d showing moderate/strong IgG staining and (d) isograft-180d showing no signal for IgG staining. (e) Semiquantitative score (0–3) of C4d staining in peritubular capillaries (PTC) and glomeruli (Glom). (f–k) Representative images of C4d immunofluorescence staining on peritubular capillaries (PTC, f–h) and on glomeruli (i-k). (f) and (i) CI-120d; (g) and (j) No-CI-120d; (h) and (k) No-CI-180d. C4d staining: red, wheat germ agglutinin: green, merge: yellow; original magnification 400×. (l) serum binding to donor target cells. CI-120d: WF kidney subjected to 16 h cold ischemia (CI) and transplanted into allogeneic LW recipients, taken 120 days post-transplant. No-CI-120d: WF kidney transplanted into allogeneic LW recipients, without CI, taken 120 days post-transplant. No-CI-180d: WF kidney transplanted into allogeneic LW recipients, without CI, taken 180 days post-transplant. Isograft-180d: LW kidneys transplanted into syngeneic LW recipients, without CI, taken 180 days post-transplant. Values are mean ± SE. $*P < 0.05$ vs. CI-120d; $°P < 0.05$ vs. No-CI-180d. $\#P < 0.05$ vs. No-CI-30d.

function and morphology more severe than that in rats receiving a non-ischemic graft.

Long time periods (from 6/7 to 24 h) of cold preservation tested in experimental models, showed that ischemia-induced cellular stress, probably caused by nutrient and energy deprivation, leads to tissue necrosis and apoptosis [15–17], which trigger recruitment and activation of inflammatory and T cells [16–18]. In kidneys from experimental animals, cold ischemia duration correlated with early dysfunction and injury (present data and 19). Machine perfusion has been shown to be a better method of preservation compared with static cold storage because of its ability to support higher levels of ATP and remove waste products [20,21]. In rat livers, perfusion at 21 °C enabled the liver to build up its energy stores more effectively than cold perfusion resulting in rapid initial function during reperfusion [22]. In addition, using a combination of hypothermic storage followed by normothermic perfusion before transplantation restored energy metabolism and reversed damage in kidneys from experimental animals and patients [20,23].

A prolonged cold ischemia time has been considered as a risk factor in kidney allo-transplantation, especially when grafts were from old donors which are more vulnerable to ischemic injury. Tullius *et al.* [8] showed that the impact of cold ischemia on chronic graft dysfunction and histologic

changes was especially detrimental in kidney grafts from old donor animals (12 months), whereas grafts from young donors (3 months) were partially protected showing only moderate changes. At variance, we observed detrimental impact of cold ischemia on CAI in kidney grafts from young donors. A possible explanation of discrepancy between our and previous findings is that the more prolonged ischemia time we used (16 vs. 2 h) overrode the capacity of young kidneys of protecting themselves from ischemia/reperfusion injury. Consistent with this interpretation, recent data [24] in a great cohort of kidney transplant patients documented that a prolonged cold ischemia time (>19 h) contributes to long-term graft loss even in patients receiving a graft from young donors (<50 years of age).

Chronic allograft injury depends on both alloantigen-independent factors, such as the ischemic injury, and alloantigen-dependent factors, such as the unavoidable recognition of donor foreign antigens. The present results show that cold ischemia strongly impacts cellular and humoral immune response. Indeed, T and B cells could be detected in ischemic allografts very early, when non-ischemic allografts were almost free of such infiltrates, and were organized in inflammatory clusters. That such organized inflammatory aggregates may have a role in chronic rejection in human patients has been recently suggested by Thaunat *et al.* [25] who performed a comprehensive analysis of human kidney grafts explanted because of terminal chronic rejection. In chronically rejected kidneys they found inflammatory infiltrate organized into ectopic lymphoid tissue, composed of a core of B cells surrounded by T cells, which harbors the maturation of a local humoral response. It was hypothesized that lymphocytic clusters, like the secondary lymphoid organs, serve to amplify the local immune response within the graft.

Mature B cells play a crucial role in mediating chronic rejection both by acting as antigen presenting cells and by producing donor-specific antibodies [26]. Our data of higher expression of BAFF-R and TACI within the ischemic allografts, indicate that infiltrating B cells were mature and activated, since these two receptors are expressed by mature B cells and are important for B cell activation and class-switching recombination [27,28]. This suggests that a prolonged cold ischemia time renders the graft more vulnerable to humoral rejection. Chronic humoral rejection evolves over the time and is characterized by a sequence of four stages: (i) production of donor-specific antibodies, (ii) deposition of C4d in peritubular capillaries, (iii) development of transplant glomerulopathy, and (iv) loss of graft function [26,29,30]. Of note, allografts previously subjected to prolonged cold ischemia displayed all the features of chronic humoral rejection at earlier time points than non-ischemic allografts, and donor-specific antibodies were detected as early as 30 days post-transplantation. It is tempting to speculate that ischemic injury may lead to unmasking or altering graft antigens, thus lowering the threshold for activation of immune response and accelerating the process of chronic humoral rejection.

Solid organ transplantation is inevitably associated with a period of ischemia. In Eurotransplant region the mean cold ischemia time is 20 h, when kidneys are from cadaveric and are not transplanted locally. Static cold preservation remains the current standard of care and a kidney subjected to prolonged cold ischemia time is more prone to early post-transplant complications [3,4]. Herein we document that prolonged cold ischemia time greatly impacts also on long-term allograft outcome, triggering acceleration of cellular and humoral reactions. Maintaining cold ischemia time as brief as possible should be a main target in kidney transplantation.

Authorship

MN, SA and PC: performed design of the study and interpretation of the data. SA, SS: wrote the paper. NA and MM: performed kidney transplantations, monitored serum creatinine and proteinuria levels. SS, PS and PC: performed immunohistochemical experiments for CD3, CD20, nitrotyrosine, TUNEL staining and worked on data analysis and presentation. RLP: performed real time-PCR analysis. FR: performed the analyses of anti-donor antibodies. MA: performed experimental work on histological analysis. MN: participated in discussion and critical revision of the manuscript.

All the authors contributed to the final version of the manuscript and have seen and approved the final version.

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