Cold ischaemia, innate immunity and deterioration of the glomerular filtration barrier in antibody-mediated acute rejection

Èlia Ripoll1,*, Víctor Nacher2,*, August Vidal3, Esther Herrero1, Núria Bolaños1, Juan Torras1, Josep M. Grinyó1, Jesús Ruberte2 and Immaculada Herrero-Fresneda1

1Experimental Renal Transplant, Laboratory of Experimental Nephrology, Bellvitge Biomedical Research Institute, University Hospital of Bellvitge (IDIBELL-HUB), L’Hospitalet, Barcelona, Spain, 2Department of Animal Health and Anatomy, Center of Animal Biotechnology and Gene Therapy (CBATEG), Universitat Autònoma de Barcelona, Barcelona, Spain and 3Pathology Service, Hospital Universitari de Bellvitge, L’Hospitalet, Barcelona, Spain

Correspondence and offprint requests to: Immaculada Herrero-Fresneda; E-mail: iherrero@idibell.cat

*These authors contributed equally to this work.

Abstract

Background. In renal transplantation, cold ischaemia (CI) determines acute rejection through innate immunity among others. Acute rejection episodes are a risk factor for late allograft dysfunction and proteinuria. This implies some alteration of the glomerular filtration barrier (GFB). Besides its effects on acute rejection, we hypothesized that CI might somehow damage the GFB being directly responsible for late proteinuria.

Methods. On rat kidney allografts suffering from antibody-mediated acute rejection with or without CI and compared with syngeneic grafts, we quantified the gene expression of innate and adaptive immune mediators and assessed the capillary glomerular basement membranes (CapBM) by immunostaining collagen-IV (ColIV). ColIV was also assessed in equivalent groups from a previous chronic study followed up for 24 weeks.

Results. CI up-regulated enzymes critical in the stabilization of collagen chains, increasing ColIV deposition and thickening the CapBM. CI increased the C4d and IgG deposition of collagen chains, increasing ColIV deposition and chronic study followed up for 24 weeks.

Conclusions. Initial CI increased the ColIV deposition in CapBM, damaging the GFB and being responsible for part of the proteinuria associated with late allograft dysfunction. This deterioration of the GFB is related to the early innate immunity activation and subsequent up-regulation of CD40 in acute rejected grafts. In chronic rejected allografts, thickened CapBM may be a consequence of an unresolved immune-inflammatory response worsened by CI.

Keywords: AbAR CD40; cold ischaemia; collagen-IV; GFB; TLR4

Introduction

Cold ischaemia (CI) synergizes with allore cognition during the rejection process, providing an immunostimulatory signal that lowers the threshold for activation of the immune response and increases the immunogenicity of the graft [1, 2]. This synergism seems to be caused, at least in part, by the activation of innate immune mechanisms through Toll-like-receptor (TLR) signalling [3]. TLRs induce the transcription of inflammatory cytokines and stimulate the costimulatory signals [4, 5], which besides being activated by innate defence mechanisms, are central players in the adaptive immune response.

Initial CI also affects late allograft outcome [6]. In kidney, late allograft dysfunction is characterized by renal insufficiency and progressive proteinuria, with histological interstitial fibrosis and tubular atrophy (IF/TA) and transplant glomerulopathy (TGP). Proteinuria reflects some structural alteration of the glomerular filtration barrier (GFB) [7]. The GFB is made up of the fenestrated capillary endothelial cell, its basement membrane (CapBM) and the podocyte. The glomerular basement membrane (GBM) is a specialized part of the extracellular matrix containing type IV collagen (ColIV), fibronectin and heparansulphate among others. ColIV—the main constituent among the collagenous components of the GBM—has different alpha chains configuring its distinct triple helical isoforms [8, 9]. Two main isoforms can be found within the GBMs: the epithelial isoform (α3α4α5(IV)) located in the CapBM and the endothelial isoform (α1α2α3(IV)) found both in the CapBM and in the Bowman’s capsule (BowBM) [10]. Thus, the CapBM is composed of the α1α2α3(IV) and the α3α4α5(IV) isoforms. Alterations in any of these alpha chains are known to result in pathological syndromes characterized by haematuria and varying degrees of proteinuria [11, 12].
In a previous kidney transplant model with highly responding rat strains, we observed a humoural rejection pattern aggravated by CI [13, 14]. Additionally, CI also intensified late proteinuria in chronically damaged allografts [14]. It seems obvious that initial CI would consequently affect late allograft outcome because CI determines acute rejection, and acute rejection episodes are a risk factor for late renal allograft dysfunction [1, 6]. However, we wondered whether CI per se might be responsible for the late proteinuria observed in previous studies [14, 15].

We hypothesized that CI would somehow modify the CapBM during the acute rejection process by the intensification of the innate immunity, and this would probably be reflected in its late outcome. To assess this hypothesis, we considered the expression of some innate and adaptive immune mediators and evaluated the deposition of ColIV in the GBMs. All these parameters were determined on acute rejecting renal allografts from a preceding study [13] that underwent either CI or not, and they were compared with syngeneic grafts with or without previous CI. In addition, ColIV was also assessed in chronically rejected kidney allografts from another previous study followed up for 24 weeks [14].

We found that CI augments the complement and antibody deposits within grafts, up-regulates the gene expression of innate mediators and thickens the CapBM by the direct accumulation of ColIV. This deterioration of the GFB is related to the early innate immunity activation and subsequent up-regulation of CD40 in acute rejected grafts. In chronic rejected allografts, thickened CapBM may be a consequence of an unresolved immune-inflammatory response worsened by initial CI.

Materials and methods

Animals and surgical technique

Renal transplants were performed between inbred male rats (250 g body weight): Wistar-Agouti (WA) rats as recipients of Brown Norway (BN) or Wistar-Agouti kidneys for allogeneic or syngeneic transplants, respectively. For CI, kidneys were preserved in Euro-Collins (4°C, 2.5 h). Surgical technique was as previously described (www.renal-transplantation.com). Recipient rats were bi-nephrectomized at the moment of transplantation. Animals (Charles River by Harlan UK Limited) did not receive any immunosuppressant and were maintained in accordance with the Guidelines of the Committee on Care and Use of Laboratory Animals and Good Laboratory Practice.

Groups and follow-up

‘WA/WA’ (n = 10): immediate syngeneic transplant; ‘CI-WA/WA’ (n = 12): syngeneic transplant with CI; ‘BN/WA’ (n = 14): immediate allogeneic transplant and ‘CI-BN/WA’ (n = 10): allogeneic transplant with CI. Serum creatinine (sCr, μmol/L) was determined on blood samples collected from the tail vein every 2 days beginning the day after surgery. Allogeneic rats were followed up until their clinical appearance and sCr values recommended sacrifice. Syngeneic rats, with indefinite mean survival time, were sacrificed at the 7th day after transplantation to compare their grafts with those in the allogeneic groups. At sacrifice, plasma and serum samples were collected. Grafts were processed for histological and immunohistochemical studies and a part was immediately frozen in liquid nitrogen and stored at –80°C for western blot (WB) and gene expression assays.

Histological studies

Coronal graft slices (1–2 mm) were fixed in buffered formalin, dehydrated and embedded in paraffin. For light microscopy, tissue sections (3–4 μm) were stained with haematoxylin–eosin and periodic acid-Schiff. A pathologist blinded to the treatment groups assessed all sections following the Banff criteria [16].

Immunohistochemistry

Representative tissue sections were immunoperoxidase-stained for CD40 and C4d and immunofluorescence-stained for C3, IgG, TLR4, fibronectin and ColIV in paraffin-embedded sections as detailed in Supplementary material S1. C3 and IgG were directly observed under fluorescence light microscopy. Positive C4d, C3 and IgG samples were semiquantitatively scored from 0 to +4. CD40 immunostaining was only utilized to localize the CD40 protein expression.

ColIV fluorescence was examined with confocal microscopy (Leica TCS-SL spectral) and quantified with the AnalySIS® software (Soft Imaging System). At least 10 glomeruli were measured in each specimen (See details in figure legends).

Quantification of circulating donor-specific antibodies

The presence of circulating donor-specific antibodies (DSA) Class I and Class II were quantified as described in Supplementary material S2. A fluorescence increase of 15% with respect to the negative control was considered as positive. Results were expressed as percentage of positive cells with respect to the total CD3+ spleen cells.

Quantification of plasmatic cytokines

For the quantitative measurement of interferon (INF-γ), interleukin (IL)-2, IL-4 and IL-10 in rat plasma, the SearchLight® Rat Cytokine/Chemokine Array and SuperSignal® ELISA Femto Chemiluminescent Substrate (Pierce Biotechnology Inc.) were used following the manufacturer’s instructions. The protein quantification is made by an enzyme-substrate reaction that produces a luminescent signal. This signal is proportional to the amount of each cytokine in the original sample and is detected with a cooled CCD camera. Results are expressed in pg/mL.

Western blot (WB)

To detect CD40 protein in kidney grafts, WB was performed incubating membranes with 1:50 anti-CD40 antibody (see details in Supplementary material S3). Control non-transplanted kidneys were used to normalize results. The membranes were exposed for 1 min to reveal the spots. The intensity of every spot was quantified on scanned films with the Quantity One quantification software (BioRad). Since WB was used as estimation, results were only normalized by the mean value of control non-transplanted kidney spots.

Quantification of gene expression in renal grafts

Total RNA was extracted and reverse transcribed to complementary DNA (cDNA). Tissue cDNA for CD40 (Table 1), HIF-1α, prolyl-4-hydroxylase (P4H)-α2, heat shock protein (HSP70), fibronectin, tumour growth factor-β (TGF-β), IL-12p40, tumour necrosis factor (TNF)-α, INF-γ, TLR4, MyD88 and 18S was amplified and quantified by real-time polymerase chain reaction as previously described [14] and detailed in Supplementary material S4. Pooled values of healthy non-transplanted kidneys were used as the reference value. Results were expressed as ‘many fold of the unknown sample’ with respect to the reference value (arbitrary units).

Statistical analysis

Overall survival was analysed using the Kaplan–Meier and log-rank methods. sCr differences at any time point, DSA, gene expression and plasma proteins were analysed by analysis of variance and subsequent Schefee’s test. For histological comparison of Banff classification, IgG, C3 and C4d deposits, chi-square P-value was calculated from the contingency table. Values of P <0.05 were considered as statistically significant. Data are presented as mean ± SEM.
A model of antibody-mediated humoural acute rejection (AbAR)

As we previously showed [13], survival and renal function differed between syngeneic non-rejecting (WA/WA and CI-WA/WA) and allogeneic rejecting groups (BN/WA and CI-BN/WA). While CI did not modify survival in syngeneic conditions, it increased mortality when added to a strong allogeneic background (Supplementary material S5). In this line, severe renal insufficiency developed from the 5th post-transplant day in rejecting groups, while serum creatinine in WA/WA and CI-WA/WA animals recovered pre-transplant baseline values (sCr = 53 ± 9 μmol/L) (Table 2).

Table 1. Rat CD40 oligonucleotides*

<table>
<thead>
<tr>
<th>Rat CD40 oligonucleotide</th>
<th>Fluorescent reporter and quencher dyes</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>FAM</td>
<td>5’-CCGGGAAACCAGACTTAGTTC-3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>FAM</td>
<td>5’-CGGTTGCCATGGGTCCTCTT-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>NFQ</td>
<td>5’-CAGTGGACACCTTGTTC-3’</td>
</tr>
</tbody>
</table>

*The primers and probe to detect the target sequence of rat CD40 (Gene Bank: AF241231.2) were designed with the File Builder software (Applied Biosystems).

Table 2. Determined parameters*

<table>
<thead>
<tr>
<th>Renal function and survival</th>
<th>WA/WA</th>
<th>CI-WA/WA</th>
<th>BN/WA</th>
<th>CI-BN/WA</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCr1</td>
<td>137 ± 33 (10)</td>
<td>233 ± 21 (12)</td>
<td>168 ± 33 (14)</td>
<td>223 ± 28 (10)</td>
</tr>
<tr>
<td>sCr3</td>
<td>127 ± 50 (10)</td>
<td>244 ± 52 (12)</td>
<td>142 ± 58 (12)</td>
<td>246 ± 100 (7)</td>
</tr>
<tr>
<td>sCr5</td>
<td>91 ± 28 (10)</td>
<td>119 ± 39 (12)</td>
<td>139 ± 13 (2)</td>
<td>121 ± 15 (5)</td>
</tr>
<tr>
<td>sCr7</td>
<td>62 ± 3 (10)</td>
<td>68 ± 4 (12)</td>
<td>440 ± 44 (12)</td>
<td>456 ± 79 (3)</td>
</tr>
<tr>
<td>sCr9</td>
<td>NA</td>
<td>NA</td>
<td>669 ± 68 (9)</td>
<td>697 ± 02 (2)</td>
</tr>
<tr>
<td>MST</td>
<td>NA</td>
<td>NA</td>
<td>8.6 ± 09 (5)</td>
<td>5.8 ± 1.3 (5)</td>
</tr>
<tr>
<td>Surv d7</td>
<td>100%</td>
<td>100%</td>
<td>85%*</td>
<td>50%*</td>
</tr>
</tbody>
</table>

AbAR markers

| C4d+/n                      | 2/10    | 10/12b   | 14/14b     | 10/10b     |
| IgG+/n                      | 0/9     | 5/10b    | 10/12b     | 6/6b,c,d   |
| C3+/n                       | 0/9     | 2/10     | 11/14b     | 9/5b,c     |
| DSA+/n                      | 0/4     | 0/7      | 7/7b,c     | 6/6b,c,d   |
| %DSA-I                      | 0.05 ± 0.07 | 0.19 ± 0.11 | 98.5 ± 1.2b,c | 79.4 ± 2.3b,c |
| %DSA-II                     | 0.10 ± 0.17 | 0.03 ± 0.13 | 92.4 ± 9.3b,c | 89.7 ± 15.6b,c |
| hum/cell/n                  | 0/0/10  | 3/0/12   | 11/1/14b,c,d | 10/0/10b,c,d |

CI-induced ColIV synthesis

| HIF1α                       | 1.49 ± 0.40 | 1.68 ± 0.50 | 2.77 ± 0.36 | 3.96 ± 0.86b,c |
| P4Hoo2                      | 1.57 ± 0.34 | 1.40 ± 0.53 | 2.94 ± 0.74 | 6.52 ± 1.34b,c,d |

Innate response related

| Fibronectin                 | 1.75 ± 0.61 | 2.89 ± 0.53 | 3.01 ± 0.55 | 14.22 ± 7.06b,c,d |
| HSP70                       | 0.18 ± 0.05 | 0.27 ± 0.05 | 0.18 ± 0.04 | 0.54 ± 0.28b,c,d |
| TLR4                        | 0.32 ± 0.1  | 1.56 ± 0.1e | 32.2 ± 17.4 | 1846 ± 794b,c,d |
| MyD88                       | 0.15 ± 0.05 | 0.37 ± 0.18 | 2.67 ± 1.15 | 111 ± 47b,c,d   |

Cytokines

| IL12p40                     | 3.5 ± 1.1 | 3.3 ± 1.2 | 0.78 ± 0.4 | 0.82 ± 0.3     |
| TGFβ                        | 0.8 ± 0.3 | 0.5 ± 0.1 | 11.1 ± 2.6b,c,d | 14.4 ± 3.4b,c,d |
| IL10                        | 4.3 ± 0.5 | 3.8 ± 0.4 | 13.7 ± 1.5b,c | 11.8 ± 1.5b,c  |

*Survival and renal function: (n) indicates the number of surviving rats at each time point. Serum creatinine (sCr) is expressed in μmol/L. Syngeneic rats with and without CI were sacrificed at day 7 after transplantation in order to compare their grafts with those in allogeneic groups. For the statistical analysis, the mean survival time (MST, days) of these groups was estimated from our previous experience in 100 days. Antibody-mediated acute rejection: ’n’ indicates the number of assessed samples for each parameter in each group. DSA+ (both Class I and Class II) was determined on recipient (Wistar-Agouti) serum samples incubated with donor (Brown Norway) spleen cells and measured by flow cytometry. Donor splenocytes were isolated by Ficol® density gradient and used when fresh. Different controls were added: serum from non-transplanted WA rats as naive; serum from a transplanted WA rat with high anti-HLA antibody titre as positive and splenocytes from Lewis rats as negative. Serum from non-transplanted WA rats did not present DSA against BN splenocytes. From the histological examination and humoural markers determined, rejecting grafts were classified as humoural AbAR (hum) or cellular (cell), and they are shown with respect to the total number of samples in each group. Gene expression of CI- and innate immunity-related mediators: mRNA (fold/not transplanted kidneys) is expressed as mean ± SEM. Cytokines: the mRNA of IL12p40 and TGF-β was quantified by quantitative real-time polymerase chain reaction (fold/non-transplanted kidneys). Protein levels of IL-10 were quantified in plasma samples and expressed as pg/mL. NA, not applicable.

bPANOVA < 0.05 versus WA/WA.
cPANOVA < 0.05 versus CI-WA/WA.
dPANOVA < 0.05 versus BN/WA.
ePANOVA < 0.05 versus WA/WA.
fPANOVA < 0.05 versus WA/WA.

Results
Conventional histology showed well-preserved renal architecture in all WA/WA kidneys and only moderate tubulointerstitial damage in CI-WA/WA animals. The BN/WA kidneys showed characteristic features of cellular vascular rejection. CI-BN/WA kidneys displayed clear ischaemic tubulointerstitial damage and early vascular rejection with swollen endothelial cells and/or extensive areas of total endothelial denudation, suggesting humoral acute rejection (Figure 1).

The C4d and IgG deposits within peritubular capillaries significantly increased in response to CI in syngeneic rats. In the allogeneic combination, both the complement and especially the IgG deposits were augmented by CI. Ischaemia influenced the intragraft deposition of C4d and IgG but did not affect the incidence of DSA, which seems to depend on the allogeneic milieu. DSA were present in all the allografted animals but absent in the isografted rats. Some DSA+ BN/WA animals did not show histological features of humoral rejection. However, all CI-BN/WA grafts were DSA+ and displayed histological humoral rejection. Thus, the Banff classification resulted in significant differences between allogeneic and syngeneic groups, corroborating the AbAR pattern of the model (Table 2).

**Activation of innate and adaptive immunity**

To consider the innate immune response, we quantified the gene expression of TLR4 and MyD88 as well as the endogenous triggering factors HSP70 and fibronectin. Both HSP70 and fibronectin were up-regulated in the CI-BN/WA grafts with a trend towards an increase in HSP70 under the sole influence of CI. Accordingly, the maximum values of TLR4 and MyD88 were found in those kidneys, in which both immune and non-immune factors showed a synergistic effect on the innate response activation (Table 2).

---

**Fig. 1.** Haematoxylin and eosin representative photomicrographs. (A) WA/WA (×200), (B) CI-WA/WA (×200), (C) BN/WA (×200), (D) CI-BN/WA (×200). Details (×400) of fibrinoid necrosis (asterisk) and apoptotic nuclei remain (arrowhead) in the vessel wall of BN/WA (E) and denuded endothelium (arrow) in the vessel wall of CI-BN/WA (F).
The confocal evaluation of TLR4 and fibronectin immunostaining showed a clear colocalization of both proteins within the glomeruli of cold ischaemic grafts either at 1 or 24 weeks. In addition, there is a clear mark of TLR4 on the surface of infiltrated inflammatory cells (Figure 2 and Supplementary material S6). With respect to the cytokines involved in the immune-inflammatory reaction, both TNF-α and especially INF-γ messenger RNA (mRNA) were significantly elevated in the BN/W A and CI-BN/W A groups (results not shown). Interestingly, these rejecting groups also displayed high levels of TGF-β and low expression of IL12p40. The plasmatic protein levels of IL-10 showed a significant increase in both rejecting groups (Table 2), but there were no significant differences between plasmatic levels of IFN-γ, IL-2 and IL-4 (results not shown).

To assess the activation of the CD40 costimulatory pathway, we evaluated its mRNA and protein expression in tissue grafts. Immunohistochemistry located CD40 in tubular epithelial cells in all samples. In rejecting BN/W A and CI-BN/W A kidneys, it was also found in glomerular and wall vascular cells. The WB analysis showed larger spots in both BN/W A and CI-BN/W A samples while WA/W A and CI-W A/W A marks were similar to control non-transplanted kidneys. As corroborated by CD40 gene expression, allorecognition as the main factor over-expressing CD40 and CI did not further enhance it. However, CI increased CD40 expression under syngeneic conditions (Figure 3). Finally, there was a positive correlation between CD40 mRNA and both DSA-I and -II (Supplementary material S7).

Alteration of the GFB

We assessed the possible alteration of the GFB, focussing on the changes in CollIV deposition in the CapBM. Since CollIV isoforms are different in the BowBM, we also assessed deposition there. To consider the role of CI on CollIV changes, we studied the HIF–P4H axis as well.

The CollIV deposition in the BowBM showed a non-significant trend towards thinning with ischaemia and allogenicity. In contrast, the CapBM became significantly thicker with ischaemia, especially in the CI-BN/W A grafts (Figure 4). In addition, the higher gene expression of

Fig. 2. Fibronectin and TLR4 colocalization. Confocal photomicrographs (×630) of fibronectin (green), TLR4 (red) and nuclei (blue) in samples of the acute [(A) WA/W A, (B) CI-W A/W A] and chronic models [(C) SynI, (D) AlloI]. Within the glomerulus of syngeneic animals, TLR4 was scarcely expressed in the epithelial cells of the Bowman’s capsule (A). Contrarily, in cold ischaemic kidneys, TLR4 was expressed in glomerular cells of the capillary tuft (podocytes, mesangial and even endothelial cells) and in the epithelial cells of the Bowman’s capsule where it clearly colocalizes with fibronectin (arrows in B to D). Some of these epithelial cells seem to express additional fibronectin as it is notably seen in D, where it forms an extra line surrounding the Bowman’s capsule by its urinary space. In addition, TLR4 is also specifically expressed by infiltrated inflammatory cells (arrow heads in B). Other representative images can be seen in Supplementary material S6.
Fig. 3. CD40 analysis. (A) Immunohistochemistry. Representative photomicrographs (×200) of each group; (a) WA/WA, (b) CI-WA/WA, (c) BN/WA, (d) CI-BN/WA. (B) WB. Representative spots of samples and control non-transplanted kidney. Asterisks correspond to post-mortem graft samples. CD40 spots appeared between Bands 5 and 6 of the protein ladder, corresponding to 43 kDa. The estimated quantification of spots was made by Quantity One quantification software (Biorad). The absolute intensity values for each spot were normalized with respect to the mean value of control non-transplanted spots. Results were expressed as the normalized mean intensity value ± SEM: 1.13 ± 0.12 WA/WA; 0.93 ± 0.02 CI-WA/WA; 1.77 ± 0.07ab BN/WA; 1.94 ± 0.10ab CI-BN/WA. *ANOVA < 0.05 versus WA/WA, bANOVA < 0.05 versus CI-WA/WA. (C) Gene expression. The graft CD40 mRNA was quantified by quantitative real-time polymerase chain reaction. Results (fold/non-transplanted kidneys) are expressed as mean ± SEM. aANOVA < 0.05 versus WA/WA, bANOVA < 0.05 versus CI-WA/WA. *ANOVA < 0.05 versus WA/WA, cANOVA < 0.05 versus CI-WA/WA.
HIF-1α and P4H-α2 in those grafts confirmed the relationship between ischaemia and ColIV synthesis (Table 2).

We also assessed the ColIV in equivalent groups from a previous chronic study made with the well-known Fischer-to-Lewis model followed up for 24 weeks in which, except for the syngeneic non-ischaemic group (Syn), all the animals (Synl, Allo, and AlloI) displayed progressive proteinuria and different degrees of TGP (14 and Supplementary material S8). In both chronically rejected groups Allo and AlloI, disruptions and double contours were observed at high resolution in the GBM. The examination of those samples from the chronic model revealed that both CI and alloreactivity induced a similar degree of ColIV accumulation within the CapBM, thickening the GFB (Figure 4).

**Discussion**

Little is known about the exact mechanisms by which ischaemia accelerates acute rejection and aggravates late graft outcome. The activation of innate immunity has been suggested as one of the possible mechanisms [17–20]. Here, we provide more evidence in that direction and point to the deterioration of the GFB as another possible mechanism.

Goldstein [21] postulated that the acute antigen-independent injury that occurs during organ transplantation releases innate immune ligands, the nature of which remains to be elucidated. Our results confirm prior data reporting HSP70 and fibronectin as being among these released TLR4 ligands. Following stress, the HSP70 could...
act as an endogenous ‘danger signal’ [22] and interact
with TLR4 to stimulate inflammatory cytokine production
[19, 23–25]. Cellular fibronectin, produced in response to
tissue injury and implicated in inflammation-associated
tissue remodelling, also binds to TLR4 [26] as showed
here for the first time within glomeruli of ischaemic
grafts. TLR4 and MyD88 are required for initiation of
ischaemia/reperfusion injury and both increase in
response to it [27, 28]. It has been said [29] that stimula-
tion of TLRs might not be essential when sufficient
antigen is present, as is the case in transplant. However,
our results show that the TLR4/MyD88 pathway is over-
activated only in ischaemic allografts, suggesting that
even in a strong allogeneic context, the effect of CI on
innate immunity may have more consequences than
was originally thought.

One of the pathways activated by TLRs is the costimu-
latory signal. It is accepted that HSPs can interact with
antigen-presenting cells through different putative recep-
tors including CD40 [25]. Wang et al. [30] suggested that
HSP70 induces the proliferation of memory T cells through
the indirect regulation of CD40/CD40L expression in dendritic and T cells. Czapiga et al. [31] es-
established a link between endogenous danger signals and
acquired immune activation, pointing to CD40/CD40L as
a bridge between alloantigen-independent injury and cell-
mediated immune response. The nuclear staining of
CD40 found in our grafts would reflect the previous
binding of the CD40L to the CD40 receptor in the plasma
membrane, which is required for CD40 accumulation in
the nucleus [32, 33]. Other studies correlate TLRs with
costimulatory signals [34, 35] connecting ischaemia/reper-
fusion injury with either CD40 [36] or TLR4 [28]. Ours
is the first in which both CD40 and TLR4 are reported to
be up-regulated in response to CI. However, in contrast
to innate mediators, CI might not be decisive in CD40
expression when alloantigens are present.

Our results also demonstrate a positive correlation between
CD40 mRNA and circulating DSA. This does not
necessarily imply a cause–effect relationship. Rather,
it may suggest a simple association between CD40 and
the humoral AbAR pattern of the model.

The complement system, which plays a central role in
the humoral response, is another regulator of innate im-
munity. Cross-talk between complement and TLR con-
nects innate and adaptive responses [37]. The C3 fragment
is also known to promote TH2 responses [38]. Accord-
ingly, our rejected grafts displayed higher levels of IL-10
and TGF-β and a reduction in the pro-inflammatory TH1
subunit IL12p40, similar to what happens in liver dendritic
cells following ischaemia [39]. At early post-transplant,
TGF-β and IL-10 act as regulatory cytokines, reducing
graft inflammation and subsequent IF/TA [40, 41].

The ‘counter-regulation hypothesis’ [42] states that
continuous stimulation induces the production of ‘anti-
danger’ signals, such as IL-10 and TGF-β. Rieger and
Bar-Or [43] propose that memory B cells that integrate
sufficiently strong stimulatory signals, such as combined
engagement of TLR, B-cell receptor and CD40, produce
IL-10 to down-regulate the local immune response. Thus,
the gene expression profile of our ischaemic allografts,
added to their circulating DSA and IL-10, would reflect a
‘self-protecting response’ designed to limit excessive
tissue damage.

There is a proven relationship between DSA and TGP
[44]. Both in patients and in the F344 to Lew model,
Joosten et al. [45, 46] support the idea that it is mediated
by humoral immune responses against GBM’s com-
ponents. Other data related complement, antibody-
mediated cell injury and CD40 with regulation of matrix
metabolism and glomerular damage [38, 44, 47, 48].
Given the convergence of these factors in our allografts, it
seemed interesting to hypothesize that their GFB might
be damaged. We found that CI increased ColIV in the
CapBM. This is in agreement with other studies, in which
thickened BM was described following acute renal
damage. In human renal graft biopsies, Utsumi et al. [49]
found increased ColIV in BM, suggesting that changes in
ColIV during acute rejection were also due to ischaemic
damage and might be a defence mechanism to protect the
kidney from the immune attack. Moreover, they suggested
that such changes still remain after graft function recovery.
Wieczorek et al. [50] showed progressive synthesis of
collagens in the evolution from acute to chronic rejection.
In this line, ColIV was also increased here in chronically
rejected grafts, linking proteinuria [14] with the alteration
of the CapBM. De Heer et al. [51] have already shown
the presence of anti-GBM antibodies in the same F344 to
Lew model of chronic allograft nephropathy. Taking into
account that the F344-to-Lew model is highly reproduc-
ible, one can assume the presence of such circulating anti-
GBM antibodies in our chronically followed animals.
Ivaný et al. [52], in chronic renal allografts, reported
thickening of the glomerular capillaries related with
antigen-independent factors. Here, the influence of CI per-
se on the CapBM thickening was supported by the ColIV
increase in CI-WA/WA and SynI grafts. The up-regulation
of HIF-1α and P4H-α2 in CI-BN/WA kidneys con-
nects the previous injury of the graft with the changes in
the epithelial ColIV isoforms. The P4H is a critical enzyme for the stabilization
of collagen chains [53]. Exposure to hypoxia increases
the P4H gene through HIF, enhancing the formation of 4-
hydroxyproline, and thereby increasing the ColIV protein
levels [53]. In glomerular capillaries, the endothelial and
the epithelial ColIV isoforms are in close contact [12, 54].
Because the endothelial isoform is more prone to proteol-
ysis [12], the endothelial-rich BM (such as that of cerebral
microvessels [55] or BowBM) becomes softer under the
metalloproteases (MMP) attack [56]. In contrast, thicken-
ing of CapBM results from the accumulation of ColIV
promoted by CI directly through HIF and P4H. It is also
possible that ColIV is indirectly increased here by tissue
repair. Since ColIV accumulation in basal lamina is suffi-
cient to stimulate the restoration of injured cells [57], and,
together with fibronectin, it is up-regulated after injury
and localized to the regenerating cells [58], the elevated
fibronectin and ColIV found here may well be a marker of
damage/repair. Moreover, this tissue damage/repair cycle
would be consistent with the ‘self-protecting response’
exhibited by ischaemic allografts.

Our results point to CI as causative agent of ColIV
deposition in the CapBM it activates innate immunity and
up-regulates CD40. The inflammatory mediators thus induced are further amplified by the subsequent adaptive response. Increase in many of these mediators could be an attempt at self-protection as a reaction to cell damage, but it may also be its cause, perpetuating a cycle of self-damage/repair that would eventually lead to late allograft dysfunction. In chronically rejected allografts, thickened CapBM could be a consequence of an unresolved initial immune–inflammatory response.

Supplementary data

Supplementary data are available online at the NDT website. Colour versions of Figures 1–4 are available online as supplementary data.

Acknowledgements. We thank the Serveis Científico-Tècnicus (UB, Bellvitge) and David Ramos ( Morphological Analysis Unit, CBATEG, UAB) for technical support.

Conflict of interest statement. E. R. is the recipient of a fellowship from IDIBELL. I.-H.-F. is a researcher from ‘Programa Estabilización Investigadores’ financed by ISCIII and Departament de Salut Generalitat Catalunya. This work was supported by grants from Instituto de Salud Carlos III/FIS (PI03/0082, FIS06/0630 and PS09/00107).

References

Deceased-donor kidney perfusate and urine biomarkers for kidney allograft outcomes: a systematic review

Ronik S. Bhangoo1,2, Isaac E. Hall1,2, Peter P. Reese3 and Chirag R. Parikh1,2

1Section of Nephrology, Department of Medicine, Yale School of Medicine, New Haven, CT, USA, 2Clinical Epidemiology Research Center, VAMC, West Haven, CT, USA and 3Renal Division, Department of Internal Medicine, University of Pennsylvania, Philadelphia, PA, USA

Correspondence and offprint requests to: Chirag R. Parikh; E-mail: Chirag.Parikh@Yale.edu

Abstract
Background. Accurate and reliable assessment of kidney quality before transplantation is needed to predict recipient outcomes and to optimize management and allocation of the allograft. The aim of this study was to systematically review the published literature on biomarkers in two mediums (the perfusate from deceased-donor kidneys receiving machine perfusion and deceased-donor urine) that were evaluated for their possible association with outcomes after kidney transplantation.

Methods. We searched the Ovid Medline and Scopus databases using broad keywords related to deceased-donor biomarkers in kidney transplantation (limited to humans and the English language). Studies were included if they involved deceased-donor kidneys, measured perfusate or urine biomarkers and studied a possible relationship between biomarker concentrations and kidney allograft outcomes. Each included article was assessed for methodological quality.

Results. Of 1430 abstracts screened, 29 studies met the inclusion criteria. Of these, 23 were studies of perfusate (16

doi: 10.1093/ndt/gfr806
Advance Access publication 11 April 2012

Published by Oxford University Press on behalf of ERA-EDTA 2012.