Preliminary Communication

Epithelial cell polarity and improved early outcomes in delayed graft function: a pilot study of polyclonal vs monoclonal antibodies

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Abstract

**Background.** Polyclonal antibody preparations contain antibodies that bind not only to molecules on circulating lymphocytes but also to other sites that bear similar antigens. We hypothesized that this extra-antibody effect would increase the number of intact tubular epithelial cells in organs at high risk for delayed graft function (DGF).

**Methods.** We used immunohistochemistry to examine serial biopsy samples (time 0 and 7–10 days after transplantation) in 18 kidney transplant recipients with DGF. These individuals received either polyclonal rabbit antithymocyte sera or a monoclonal humanized anti-CD25 antibody as induction immunosuppression. We also examined their early clinical course over 6 months.

**Results.** Individuals treated with the polyclonal preparation demonstrated greater preservation of kidney epithelial cell polarity manifested by more intense and more localized basolateral distribution of E-cadherin ($P = 0.016$), $\beta$-catenin ($P = 0.008$) and Na-K ATPase ($P = 0.02$). These individuals were also more likely to maintain greater estimated glomerular filtration rates (eGFRs) at follow-up than patients treated with an anti-CD25 monoclonal antibody (6 month eGFR polyclonal: 55.5 ± 7.12 ml/min vs monoclonal: 43.33 ± 6.48 ml/min; $P = 0.002$).

**Conclusion.** Though a pilot study, these data suggest that a purified polyclonal antibody preparation may help conserve functional kidney mass during DGF with potential benefits on transplant function overall.

**Keywords:** antibody therapy; delayed graft function; epithelial polarity; kidney transplantation; transplant function

Introduction

Delayed graft function (DGF) is a compendium of injury to the allograft that ultimately results in an inability of the allograft to provide adequate kidney function to the recipient. The sources of injury are many. They include inflammation following procurement, operative technique causing vasospasm, intrarenal changes as a consequence of the perfusion solution and, if pulsatile perfusion is used, shear-stress-mediated events. Other factors contributing to DGF include cold ischaemia itself and warm ischaemia-induced changes in cellular integrity, apoptosis and necrosis. Ischaemia–reperfusion injury and even early immune-related injury also contribute to DGF. The end result of these events is a reduction in tubular epithelial cell number as damaged cells lose their cell–cell connections and slough off into the tubule.

DGF is associated with an increased incidence of biopsy-proven acute rejection and may play a role as a significant risk factor in the genesis of chronic allograft dysfunction [2]. The structural integrity of the tubular epithelial cells is probably a significant factor influencing these events and their sequelae. These cells are defined throughout the nephron by a polarized phenotype with vectorial transport processes and apical vs basolateral distribution of a number of different proteins integral to their function. They maintain their polarized structure surrounding the open space of the tubule through cell-based architecture that relies on gap junction complexes. E-cadherin and $\beta$-catenin are two proteins important for maintenance of these gap junctions in kidney epithelial cells.

Several recent and novel findings have refocused interest on the kidney epithelia as a key player in the rejection response. Tubulitis results, in part, from the binding of CD103-bearing lymphocytes binding to epithelial cells through E-cadherin [3]. Moreover, the maintenance of the cell architecture appears to be important in shielding or masking chemokines
and chemokine receptors associated with rejection in allografts. These observations suggest that an intact epithelial cell may be less likely to be either (i) a nidus for inflammation; or (ii) an immunogenic stimulus in the transplant setting.

Polyclonal antibody preparations often contain antibodies that bind to molecules on tubular epithelial cells as well as circulating lymphocytes. We hypothesized that polyclonal antibodies with this extra-antibody effect would, in effect, increase the number of intact-appearing tubular epithelial cells in organs at high risk for DGF. To address this hypothesis, we used immunohistochemistry to examine serial biopsy samples (time 0 and 7–10 days after transplantation) in a group of kidney transplant recipients with DGF who received either a polyclonal antibody preparation or a monoclonal humanized anti-CD25 antibody. Then, we examined their early clinical course and attempted to determine if there was a correlation between the immunohistochemical findings and clinical outcomes.

Materials and methods

Study population

The study population consisted of a subset of individuals who received cadaveric donor kidney transplants at our centre during 2001–2002. The donors were considered high risk for DGF based on increased donor body mass index (BMI) >30 kg/m² (n = 10), and/or non-heart-beating donor status (n = 6), and/or a prolonged cardiopulmonary resuscitation within 48 h of organ procurement, and/or stroke as cause of death. In addition, donors with a cold ischaemia time >24 h or older donors >55 years of age were also considered high risk for DGF. The transplant recipients were treated with either a monoclonal humanized anti-CD25 antibody (Simulect®, dosed on day 0 prior to reperfusion and on day 4) or polyclonal rabbit antithymocyte globulin (Thymoglobulin®, 1.5 mg/kg for 3–5 days, with the first dose being intra-operative as induction immunosuppression). Transplant recipients also received bolus corticosteroids for 3 days that subsequently were tapered to 30 mg of prednisone daily, mycophenolate mofetil 1000 mg twice daily (initially intravenous for 2–3 days then oral) and calcineurin inhibition with cyclosporine or tacrolimus, initiated as the serum creatinine neared or fell to <3.0 mg/dl (average day of introduction of calcineurin inhibition, post-operative day 12; range of post-operative days 7–30).

Data regarding kidney function (serum creatinine, β2-microglobulin levels, daily urine output) and rejection episodes were tabulated from the patient’s medical record. All events within 60 days were noted, as was the time to urine output >500 ml/day for two consecutive days. Follow-up serum creatinine values were assessed at 3 and 6 months, as were estimated glomerular filtration rates (eGFRs) [4] for each study subject. Dipstick proteinuria was assessed at 3 and 6 months post-transplant. The institutional review board at the University of Wisconsin Medical School approved this study.

Samples

Time 0 wedge biopsy samples, taken at the time of reperfusion, were obtained from transplanted allografts deemed by the transplant surgeon to be at high risk for DGF. Follow-up percutaneous needle biopsies were obtained at 7–10 days post-transplant as part of the standard clinical care of the transplanted patients if they had one or more of the following conditions: an increased serum creatinine, failure of the serum creatinine to fall, no significant urine output (<500 ml/day), a stalled serum creatinine at a level of >3.0 mg/dl, and a very slowly falling serum creatinine with a rise in β2-microglobulin levels. Study subjects provided consent for additional studies on the biopsy tissue obtained from the second biopsy. Two cores were obtained at the time of biopsy using an 18-gauge biopsy needle and a Biopry gun device. All biopsies were performed under ultrasonographic guidance. Halves of each core were cut and used for immunohistochemical studies to ensure that (i) cortical tissue was present and (ii) tissue similar to the pathological assessment was used for the immunohistochemical studies. The other halves of each core were used for standard pathological assessment of the tissue.

Each of the cores to be used for the study analyses was placed into paraffin. Immunohistochemistry was performed on paraffin sections from the biopsy samples. Samples were assessed for E-cadherin (1:300 dilution; Novocastra, Newcastle upon Tyne, UK), β-catenin (1:100 dilution; Novocastra) and sodium–potassium (Na–K) ATPase (1:50 dilution; Research Diagnostics, Inc., Flanders, NJ) protein localization.

Human tonsillar tissue and normal human kidney were used as control tissue samples for all studies. Pre-immune sera served as a negative control for immunohistochemical staining. Samples were incubated overnight at 4°C then washed four times for 5 min each with phosphate-buffered saline (PBS). Samples then were incubated with Mach II goat anti-mouse antibody (1:500 dilution; Biocare Medical, Walnut Creek, CA) for 1 h, washed again in PBS at 4°C and subsequently developed using the metal-enhanced diaminobenzidine (DAB) substrate kit (Pierce Biotechnology, Rockford, IL). Staining was graded from 0 to 4 based on localization [0 = absence of staining localized to the basolateral (BL) area, to 4 = the majority of staining localized to the BL domain] by a pathologist blinded to the clinical care circumstances.

Statistical analyses

Patient groups were categorized based on the type of induction antibody therapy. Differences between patient groups were assessed using Student’s t-test or the log-rank test when examining time to events. Scoring for protein intensity of staining and localization between groups was assessed by analysis of variance (ANOVA). Data are reported as mean ± SD. A P-value ≤ 0.05 was considered significant.

Results

Population

Eighteen individuals were included in this pilot study. The characteristics of this study population are noted in Table 1. There were more men than women in each
The mean cold ischaemia time was not significantly different between groups. Panel-reactive antibody levels were also comparable between groups. There was a trend in donor age favouring individuals in the monoclonal group. The average number of haemodialysis sessions per patient was 4.5±2.4. It is of note that four individuals in the monoclonal group received at least one dose of polyclonal antibody therapy (one individual on day 3, one on day 4, one on day 6 and one on day 7) after the diagnosis of DGF. These individuals, however, were evaluated in the monoclonal cohort to maintain an intention-to-treat analysis.

Immunohistochemical studies

Time 0 biopsies from each cohort demonstrated a slight, but not statistically different intensity of BL staining for β-catenin and E-cadherin protein, with more of a BL distribution for both proteins in the anti-CD25-antibody-treated cohort. There was no significant difference in Na–K ATPase intensity of staining and distribution between the two cohorts in time 0 biopsies. Polyclonal antibody-treated individuals were more likely to have more intense staining and to maintain E-cadherin (Figure 1) \( (P=0.016; \text{polyclonal vs monoclonal}) \) and β-catenin staining (Figure 2) \( (P=0.0008; \text{polyclonal vs monoclonal}) \) in a BL distribution in follow-up biopsies at 7–10 days post-transplant. There was also a trend towards more intense staining and greater retention of Na–K ATPase in a BL distribution in polyclonal antibody-treated individuals (Figure 3) \( (P≤0.02) \) in biopsies 7–10 days post-transplant.

Including patients who received monoclonal then polyclonal therapy in the polyclonal cohort further skewed the results towards a beneficial effect of the polyclonal antibody (data not shown).

Clinical and functional outcomes

Individuals treated with polyclonal antisera tended to have a shorter time to urine output >500 ml/day for two consecutive days \( (8.7±8.8 \text{ days}) \) compared with those treated with an anti-CD25 antibody \( (12.4±6.3 \text{ days}) \) \( (P=\text{NS}) \). There was also a trend towards a faster rate of decline in β2-microglobulin levels between groups during the peri-operative course. Polyclonal antibody-treated individuals were more likely to reach a β2-microglobulin level ≤5.0 mg/l by day 10 post-transplant than anti-CD25-antibody-treated individuals \( (P=0.031) \).

<table>
<thead>
<tr>
<th>Category</th>
<th>Polyclonal induction</th>
<th>Monoclonal induction</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient number</td>
<td>6</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Recipient age</td>
<td>41±13 years</td>
<td>46±9 years</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient gender</td>
<td>4 M/2 F</td>
<td>9 M/3 F</td>
<td>NS</td>
</tr>
<tr>
<td>Panel-reactive</td>
<td>8±9%</td>
<td>12±7%</td>
<td>NS</td>
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<tr>
<td>antibody time</td>
<td>19±10.6 h</td>
<td>20±9.3 h</td>
<td>NS</td>
</tr>
<tr>
<td>Donor age</td>
<td>53±8.3 years</td>
<td>47±7.3 years</td>
<td>NS, ( P=0.07 )</td>
</tr>
</tbody>
</table>

Table 1. Demographics

Fig. 1. Representative E-cadherin follow-up biopsy. Top panel: tissue from an individual treated with a monoclonal anti-CD25 antibody at day 9 post-transplant \( (20×) \). Bottom panel: tissue from a biopsy from an individual treated with rabbit antithymocyte serum at day 9 post-transplant \( (20×) \). Staining and immunohistological scoring were determined as noted in Materials and methods.
There was a trend towards slightly lower discharge serum creatinine values in the polyclonal antibody treatment group (2.33 ± 0.69 mg/dl) vs the anti-CD25 antibody group (2.54 ± 0.59 mg/dl) (NS, \( P = 0.10 \)) (Table 2). The nadir serum creatinine values for the polyclonal cohort were less than the mean nadir serum creatinine in the anti-CD25 antibody cohort (polyclonal: 1.82 ± 0.37 g/dl; monoclonal: 2.41 ± 0.48 g/dl; \( P = 0.038 \)). Interestingly, despite similar values at discharge, eGFR in the polyclonal antibody cohort showed an increasing trend by 3 months post-transplant (Table 2) and, by 6 months post-transplant, it was
significantly greater than the eGFR in the anti-CD25 antibody-treated cohort \((P < 0.003)\) (Table 2). Moreover, six individuals had \(\geq 3+\) dipstick proteinuria in the anti-CD25 antibody-treated cohort during the first 6 months post-transplant vs only two individuals in the polyclonal antibody-treated group.

### Acute rejection

During the course of the study, six of the anti-CD25 antibody-treated individuals had biopsy-proven acute cellular rejection (two each Banff grade 1A and Banff grade 1B, and two Banff grade 2A rejections). There was also one humoral rejection episode diagnosed in an individual who received anti-CD25 antibody induction. There were two episodes of biopsy-proven acute cellular rejection (one each Banff grade 1B and Banff grade 2A) in the polyclonal cohort. All of the acute rejection episodes were diagnosed within the first 60 days post-transplant.

### Discussion

DGF continues to be problematic in kidney transplantation. This fact is made even more prominent by the constant search for more donors, e.g. extended criteria donors. It is possible, therefore, that DGF will become even more common in the context of kidney transplantation. This is important as this condition is associated with acute rejection [2,5] and has an effect on chronic allograft dysfunction and long-term outcomes. Our findings in this pilot trial corroborate the benefit demonstrated in other studies that have examined polyclonal antibody induction treatment [6–9]. Moreover, we have identified a potentially important and novel determinant of efficacy, i.e. more rapid recovery of tubular cell polarity in the setting of polyclonal antibody therapy.

DGF results from a complex set of events. These include graft injury at procurement; changes due to cold ischaemia and ischaemia–reperfusion; inflammation propagated by the kidney in response to these events; hypoxic tubular epithelial cell damage; and immune-mediated effects related to the donor–recipient interaction. The initial thought is that antibody treatment would mitigate these events by reducing T-cell activation [10,11]. Rather than focus on the effects of antibody induction treatment on recipient mononuclear cells, we examined whether antibody treatment might have an effect in preserving donor kidney epithelial cell polarity.

Our choice to examine epithelial cells makes sense in the context of a study such as this. Recent studies have noted the importance of the tubular epithelial cells as prognosticators of outcome in the setting of DGF [12]. Myers and colleagues examined post-transplant biopsies to assess segmental distribution of Na–K ATPase [13]. Their premise was that loss of cell polarity could activate tubuloglomerular feedback and contribute to the depressed GFR in post-ischaemic acute renal failure. They noted aberrant Na–K ATPase distribution, especially in the proximal tubule. Our findings extend those observations in highlighting the abnormal distribution of other proximal tubule epithelial cell proteins that define polarity, i.e. \(\beta\)-catenin and E-cadherin.

E-cadherin is a major gap junction protein in epithelial cells, with a central role in cell–cell adhesion and cell polarity [14]. Intact E-cadherin and \(\beta\)-catenin complexes bind to \(\alpha\)-catenin, leading to protein complexes that constitute key and integral components of the zonula occludens in tubular epithelial cells. Disruption of these complexes through injury often leads to the translocation of these proteins towards the nucleus [15]. Furthermore, loss of E-cadherin is associated with epithelial–mesenchymal transdifferentiation [16], a process whereby tubular epithelial cells could transform into fibroblasts with consequences for the micro-architecture of the kidney parenchyma. This is presumably an early event in the genesis of atubular glomeruli, a finding that characterizes chronic allograft dysfunction, both experimentally and clinically, and contributes to decreased kidney function [17]. Thus, the presence of these proteins in the basolateral cell domain suggests that the tubular epithelial cell is intact and, hence, likely to perform its normal functions.

The differential effects between the monoclonal and polyclonal regimens are notable, even in the context of this pilot study. The rabbit antithymocyte globulin preparation used in this study has been examined \textit{in vitro}. It downregulated adhesion molecules and integrins involved in immune responses, e.g. LFA-1, ICAM-1, VLA-4 and \(\alpha_4\beta_7\) integrins, leading to their intracellular internalization, thereby limiting their potential for cell–cell interactions [18]. Some of these molecules are expressed in kidney epithelia or in interstitial fibroblasts adjacent to the epithelia [19]. This suggests that the rabbit antithymocyte globulin

### Table 2. Functional outcomes

<table>
<thead>
<tr>
<th>Category</th>
<th>Polyclonal induction</th>
<th>Monoclonal induction</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discharge serum creatinine</td>
<td>2.33 ± 0.69 mg/dl</td>
<td>2.54 ± 0.59 mg/dl</td>
<td>NS, (P = 0.10)</td>
</tr>
<tr>
<td>eGFR at discharge</td>
<td>50.1 ± 8.5 ml/min</td>
<td>43.2 ± 10.4 ml/min</td>
<td>NS, (P = 0.13)</td>
</tr>
<tr>
<td>eGFR at 3 months</td>
<td>52 ± 9.9 ml/min</td>
<td>43.3 ± 11.5 ml/min</td>
<td>NS, (P = 0.1064)</td>
</tr>
<tr>
<td>eGFR at 6 month</td>
<td>55.5 ± 7.1 ml/min</td>
<td>44.75 ± 6.48 ml/min</td>
<td>(P &lt; 0.003)</td>
</tr>
<tr>
<td>(\geq 3+) dipstick proteinuria at 3 or 6 months</td>
<td>(n = 2)</td>
<td>(n = 6)</td>
<td></td>
</tr>
</tbody>
</table>
preparation in this study could bind to the kidney parenchyma and thereby prevent cell damage directly, a hypothesis that has been tested successfully in part in vitro [20]. Maybe as important is the fact that this antibody preparation was given intra-operatively. This route of administration recently was shown to improve outcomes in contrast to post-operative administration [8].

The rejection rate in our monoclonal cohort was 50%, significantly greater than that reported in trials with this form of induction antibody therapy. There is no doubt that the increased frequency of biopsies might be considered as oversampling the population, and cellular infiltrates read out on biopsy as rejection might actually be simply consistent with a healing kidney [21]. However, it is notable that the rejection rate we report is consistent with the report from Qureshi et al. [1] who identified a 57% rejection rate in patients biopsied during DGF to identify silent rejection, as opposed to a 15.1% rejection rate in DGF patients who were not biopsied. Thus, silent rejection might actually be more prominent in DGF in general.

It is interesting that recent data implicate E-cadherin in acute rejection events; yet, we noted more intense staining in our samples treated with polyclonal antisera and better outcomes. This protein serves as the principal ligand for CD103, an integrin heterodimer [22]. We hypothesize that cell disruption through other events, e.g. cell necrosis, either exposes E-cadherin to the surrounding microenvironment or alters the intracellular and membrane milieu to provide access for this integrin to bind to allograft epithelial domains. Thus, a protective effect in limiting cell injury would limit such access. Alternatively, polyclonal antisera possibly mask these integrins through a binding effect, alter their cell surface expression or alter their ability to interact with E-cadherin. Further clinical and in vitro studies will be necessary to elucidate which of these mechanisms of action are actually at work.

Certain caveats have to be accepted in a study such as this. This is a small pilot, single-centre, non-randomized study. The patient selection criteria were clinically determined based on the surgeon’s decision at the time of the procedure. Moreover, the fact that several patients received sequential monoclonal then polyclonal therapy points to the clinical nature of this study rather than a true trial structure. Yet, to exclude those patients would have led to a skewing of the results further in favour of the polyclonal therapy. It also would have detracted from the ‘clinical’ nature of the study and certainly introduced an increased likelihood of type I error. For that reason, we chose to assess the population in an intention-to-treat manner. The fact that follow-up biopsies were taken at days 7–10 post-transplant further substantiates this clinical care approach. It is for that reason, however, that we chose to examine a novel histological outcome. If predictive, its validity would, in essence, be even greater as it would have been developed through clinical practice. Our analysis supports the idea that a clinically usable measure of kidney histology may help predict early outcomes in the setting of DGF and the beneficial impact of one therapy vs another. Yet, we have tried to be cautious in our conclusions. We have noted only an association between treatment, maintenance of epithelial cell polarity and clinical function. There is no means in this analysis to define causality. That will require a broader based prospective examination of the effects of antibody therapy on DGF. Finally, these data only examine two therapies. There are other accepted and novel antibody treatments now available for use in kidney transplantation and the effects of these other medications. This type of surrogate marker, though novel, would allow for new antibody preparations to be tested with a clinical and histological marker of efficacy.

In summary, these data, though preliminary, are provocative. They suggest that polyclonal antibody preparations may have kidney-specific effects that either as a discrete mechanism of action or as a result of altered lymphocyte activity lead to maintenance of tubular epithelial cell integrity. This, in fact, may be important for the beneficial effects of this polyclonal antibody preparation in leading to resolution of DGF with the least immunological toll possible on the allograft.

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