Original Article

Anti-glutathione S-transferase T1 antibody-mediated rejection in C4d-positive renal allograft recipients

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Abstract

Background. Chronic humoral rejection is a progressive form of graft injury, with defined diagnostic criteria, the crucial one being the evidence of circulating anti-donor antibodies. These antibodies are mainly directed against human leucocyte antigens (HLA), but other targets have also been described. We previously reported that antibodies against the Glutathione S-transferase T1 (GSTT1) enzyme appear in recipients without the GSTT1 gene who receive a graft from a GSTT1-positive donor. The primary aim of this study was to analyse the role of GSTT1 in cases of antibody-mediated rejection (AMR) in the absence of anti-HLA antibodies. A second objective was to describe the distribution of the GSTT1 enzyme in the human kidney.

Methods. Four renal biopsies from four renal transplanted patients with declined renal function and circulating anti-donor GSTT1 antibodies were studied for C4d deposits in sections of paraffin-embedded tissue samples. Anti-donor-specific HLA and MICA antibody detection was done with the Lumines platform and anti-GSTT1 antibodies were tested by indirect immunofluorescence on rat tissues and ELISA assay. DNA of the patients was extracted for GSTT1 genotyping.

Results. Four patients with the GSTT1 donor/recipient mismatch developed anti-GSTT1 antibodies 32, 42, 48 and 60 months after the transplant. One patient also had donor-specific anti-HLA antibodies. Their biopsies showed pathologic lesions compatible with chronic antibody-mediated rejection (CAMR), along with positive C4d deposition in peritubular capillaries in three of them, being no valuable in the other case.

Conclusion. This is the first study reporting an association between the appearance of chronic antibody-mediated renal allograft rejection and the occurrence of de novo production of anti-GSTT1 antibodies, in the absence of anti-HLA donor-specific antibodies. This fact suggests a potential role of the GSTT1 system in anti-graft immune response.

Keywords: antibody-mediated rejection; C4d; genetic mismatch; GSTT1

Short summary

Many studies have noted a strong association between circulating anti-donor HLA antibodies and chronic rejection. However, 10% of the C4d-positive cases had no detectable antidonor lymphocyte antibodies. We have tried to analyse the role of antibodies against Glutathione S-transferase T1 in renal transplant patients with chronic allograft rejection in some of the 10% unexplained cases without anti-HLA antibodies. As we reported in 2001, these antibodies are highly specific and appear in individuals lacking the GSTT1 gene when they are transplanted with a positive graft.

Introduction

Beginning in the 1990s, Halloran et al. brought renewed interest to the study of the clinical relevance of newly formed anti-donor-specific antibodies (DSA) in renal transplantation. They described a severe type of acute rejection (AR) mediated by anti-human leucocyte antigens (HLA) Class I antibodies [1]. Many studies have focused on the role played by HLA antibodies in humoral alloimmunity. More recently, research has focused on non-HLA systems in solid organ allograft rejection [2]. Feucht et al. reported in 1993 on the prognostic value of C4d deposits in peritubular capillaries (PTC) in renal biopsies of recipients with signs of graft rejection [3]. Since then, many studies have validated the usefulness of C4d deposits as a marker of humoral rejection [4].

According to Banff’05 the diagnostic criteria of late/chronic antibody-mediated rejection (AMR) include the following: morphological features including TG (duplication or ‘double contours’ in glomerular basement membranes and/or intimal fibrosis (IF)/tubular atrophy (TA) with or without duplication of the internal elastica); diffuse C4d deposition in PTC and the presence of DSA.
If only C4d deposits (with no DSA) or DSA (with no C4d) are present, a diagnosis ‘suggestive of chronic AMR’ can be made based on documented morphologic capillary changes [5].

In a cohort study carried out in our laboratory in 2005, we found that after kidney transplantation some patients with a null Glutathione S-transferase T1 (GSTT1) genotype who received a GSTT1-positive graft developed an immune response, with the production of donor-specific anti-GSTT1 antibodies [6]. After a follow-up of > 2 years, we detected no clinical symptoms related to the presence of the newly formed antibodies. This was a surprising result because in liver transplants the GSTT1 genetic mismatch and subsequent formation of anti-GSTT1 antibodies is highly associated with the risk of developing de novo immune hepatitis [7]. The GSTT1 enzyme is a phase II detoxifying enzyme encoded by a single gene that has two alleles, GSTT1*0 (null) and GSTT1*A (positive) [8]. Deletion of the GSTT1 gene, resulting in a complete lack of protein expression, occurs in 20% of the Caucasian population. This proportion varies among different ethnic populations (11–58%) [9].

We carried out immunohistochemical experiments to detect the distribution of the GSTT1 enzyme in human renal tissue and provide evidence for the importance of the GSTT1 genetic mismatch in renal transplantation. The introduction of this foreign antigen leads to a very specific GSTT1 genetic mismatch in renal transplantation. The introduction of this foreign antigen leads to a very specific immune response with the production of anti-GSTT1 antibodies. With the aim of evaluating the potential role of these new antibodies in the development of anti-GSTT1 chronic antibody-mediated rejection (CAMR), a re-evaluation of renal biopsies findings and the immunohistochemical staining of C4d on paraffin-embedded biopsies was carried out.

Patients and methods

Patients

The four patients included in this report are renal transplants with a long-term follow-up (3–5 years) who belong to a group of GSTT1 null recipients with a GSTT1 positive donor that produced anti-GSTT1 antibodies during their post-transplant evolution. They had a biopsy performed due to slowly rising creatinine of unclear origin. Concomitant serum samples were studied for HLA, MICA and GSTT1 DSA. Histology showed morphologic features compatible with AMR; they all had anti-GSTT1 antibodies at the time of biopsy and one patient also had anti-HLA DSA. During this time period ~46 additional patients were biopsied for similar reasons; 12 of them were C4d+, 7 had no detectable antibodies and 5 had anti-HLA Class I or II antibodies. Informed written consent was obtained from each patient.

Immunohistochemistry: detection of C4d

Three-micron-thick sections were deparaffinized and rehydrated. For antigen retrieval, slides were heated in a pressure cooker for 3 min in a citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), cooled and rinsed in distilled water. Sections were immersed in a 3% H2O2 aqueous solution for 30 min to quench endogenous peroxidase activity and then covered with 10% normal serum in Tris-buffered saline to block non-specific binding. Polyclonal anti-C4d (Biomedica, Vienna, Austria) antibody was added at a 1:10 dilution and incubated for 60 min at room temperature or at 4°C overnight. After several washes with Tris buffer, polymer conjugated to peroxidase (Masvision Universal Kit, Master Diagnostica SL, Granada, Spain) was added and incubated for 30 min. Visualization of the immunoreaction was completed using 3,3′-diaminobenzidine as the peroxidase chromogenic substrate. The slides were then counterstained with haematoxylin and mounted with DPX (BHD Laboratories, Poole, UK).

Immunohistochemistry: GSTT1 expression

We performed the same protocol as above with the following modifications: the primary antibody was a mouse monoclonal anti-human GSTT1 antibody used at a 1:500 or 1:3000 dilution (Abnova Corporation, Taipei, Taiwan). The secondary antibody was a biotinylated anti-mouse IgG (DakoCytomation, Glostrup, Denmark).

Detection of anti-donor HLA antibodies

Anti-donor-specific HLA antibody detection was done with the Luminex platform in conjunction with reagents from One Lambda Inc. (Canoga Park, CA, USA). For each patient sample, a total of 20 µl of serum was mixed between two wells, each containing either HLA Class I- or Class II-coated microspheres. Sera and microspheres were incubated for 30 min in a 96-well membrane filter plate. The specimens were then washed three times using a vacuum manifold system. Phycoerythrin (PE)-conjugated anti-human IgG was then added to each well and incubated for 30 min. All incubations were performed at room temperature, in the dark, on a rotating platform. The LUMINEX100 fluororanalyser was used for bead and data acquisition. Raw data were exported to the One Lambda LABScreen software for analysis and interpretation.

GSTT1 genotyping and anti-GSTT1 antibody detection

Genomic DNA was extracted from blood samples and PCR was performed in order to assign the null or positive genotype. Sera from patients in the risk group (null recipient/positive donor) were sequentially tested by indirect immunofluorescence on rat tissues and immunoblot as described [6] and by a new ELISA assay with the human recombinant protein GSTT1 (Biomedal, Sevilla, Spain).

Results

The patients reported in this study had received renal transplants and presented with histopathological features suggestive of CAMR, such as PTC multilayer basement membrane (two patients), chronic transplant glomerulopathy (two patients), chronic transplant arteriopathy (one patient) and interstitial fibrosis (IF)/TA (four patients). Other findings were capillaritis (three patients)
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Fig. 1. Immunohistochemical detection of C4d deposits in peritubular capillaries (PTC) and glomerular capillaries (GC) in paraffin sections of renal allograft biopsies. C4d+ staining in PTC was observed in Patients 1 and 3 (focal >25%), Patient 2 (indeterminate <10%) and Patient 4 (diffuse >50%). The section from Patient 4 also shows C4d+ staining in GC.

Table 1. C4d deposits in biopsy and antibody detection in serum samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tx date</th>
<th>Biopsy date</th>
<th>C4d</th>
<th>HLA-DSA</th>
<th>GSTT1-DSA (titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22-7-00</td>
<td>26-4-06</td>
<td>Focal &gt;25%</td>
<td>4-5-06 negative</td>
<td>1-2-06/4-5-06 (1/160)</td>
</tr>
<tr>
<td>2</td>
<td>25-12-01</td>
<td>21-12-05</td>
<td>Indeterminate &gt;5%</td>
<td>16-11-05 negative</td>
<td>16-12-05 (&gt;1/320)</td>
</tr>
<tr>
<td>3</td>
<td>14-7-02</td>
<td>11-3-05</td>
<td>Focal &gt;25%</td>
<td>10-3-05/22-8-06 negative</td>
<td>10-3-05/28-8-05 (&gt;1/320)</td>
</tr>
<tr>
<td>4</td>
<td>31-10-01</td>
<td>14-11-06</td>
<td>Diffuse</td>
<td>7-11-06 Class II positive</td>
<td>7-11-06 (1/80)</td>
</tr>
</tbody>
</table>


with lymphocyte/monocyte accumulation (one patient) and lymphoplasmocytic inflammation (two patients). In renal biopsies C4d staining in PTC was observed in three patients, one of them with a diffuse pattern and the other two with a focal pattern (>25%) of PTC valvea in the renal tissue (Figure 1). All patients had a GSTT1 genetic mismatch (null recipient/positive donor) and as a result, at some point in their post-transplant period (42, 48, 32 and 60 months) they produced donor-specific anti-GSTT1 antibodies. These antibodies were absent prior to transplantation. All patient samples were negative for anti-HLA DSA, except Patient 4, who had both kinds of antibodies (anti-HLA Class II and anti-GSTT1) in the same serum sample (Table 1).

Patient 1 received a graft at the age of 60. Initial immunosuppression (IS) was a combination of prednisone, cyclosporine and mycophenolate mofetil (MMF). No AR episodes were registered. A renal biopsy performed 42 months after the transplant (Cr value of 2.7 mg/dl) was suspicious for chronic humoral rejection based on focal positive deposition of C4d in PTC and IF/TA Grade II. Lymphoplasmacytic interstitial inflammation and capillaritis Grade 2 with predominant monocytes accumulation were also present. Anti-rejection therapy was started with a steroid bolus and treated with tacrolimus. The patient exhibited some clinical progress, and there was a small reduction of Cr to 2.2 mg/dl (Table 2).

Patient 2 was transplanted when he was 20 years old. Baseline IS comprised daclizumab, prednisone, tacrolimus and MMF. There were no AR episodes, and a biopsy performed 48 months after the transplant (Cr value of 2.5 mg/dl) was suspicious for chronic humoral rejection based on splitting and multilayering structure of the peritubular capillary basement membranes in electron microscopy examination, with chronic transplant glomerulopathy, with no immune complexes in direct immunofluorescence evaluation and IF/TA Grade II. Lymphoplasmacytic interstitial inflammation, capillaritis and glomerulitis Grade 1 were additional findings. C4d staining was indeterminate. The IS dosages were increased and a slight
improvement was observed with a subsequent Cr of 1.7 mg/dl (Table 2).

Patient 3 was a paediatric transplant at the age of 13. Initial IS therapy was daclizumab, prednisone, tacrolimus and MMF. A biopsy performed 36 months after the transplant with a Cr value of 1.65 mg/dl showed IF/TA Grade III associated with chronic allograft arteriopathy and segmental and focal glomerulosclerosis changes. No electron microscopy examination was performed. No previous episodes of AR were recorded. There was a shift in the IS regimen from tacrolimus to sirolimus but with no subsequent clinical improvement, and Cr values progressively increased until terminal renal insufficiency occurred (Table 2).

Patient 4 was transplanted when he was 20 years old. Initial IS therapy consisted of prednisone, cyclosporine and MMF. The first biopsy was performed 60 months after the transplant with a Cr value of 2.5 mg/dl. Renal biopsy showed features suspicious of chronic humoral rejection based on the diffuse positive C4d staining in PTC, multilayering transformation of peritubular capillary basement membranes in the electron microscopy examination, chronic rejection glomerulopathy with no immune deposits in direct immunofluorescence examination and IF/TA Grade III. Glomerulitis Grade 2 and capillaritis Grade 3 with lymphocytic and monocytic accumulation were additional findings. Anti-rejection therapy was started with a steroid bolus, rituximab and subsequent conversion to tacrolimus. No previous AR episodes were recorded. Although there was an initial improvement, the clinical situation persists with the same altered Cr values (Table 2).

The patients have a four to five HLA mismatches, with the exception of the paediatric transplant patient, who had only two. Although the existence of previous AR episodes has been postulated as a risk factor for the development of chronic allograft nephropathy [10], none of the four patients described above experienced episodes of AR.

The pattern of staining of the GSTT1 protein in an autopsy sample from a normal GSTT1 positive kidney was a strong cytoplasmic expression in distal tubules and a less intense cytoplasmic expression in proximal tubules (Figure 2). In the renal biopsies from the four patients included in this study taken at the onset of the symptoms we observed a diffuse cytoplasmic expression pattern in distal tubules (Figure 3).

Discussion

The description of anti-GSTT1 in Patients 1, 2 and 3 further substantiates the idea that non-HLA antibodies are involved in antibody-mediated late renal allograft dysfunction. Patient 4 had both anti-GSTT1 and anti-HLA DSA; we therefore cannot say which of these antibodies has a decisive role in allograft rejection, or if perhaps both are involved in the process.

We propose that the GSTT1 enzyme expressed in the kidney of null transplanted patients is probably a target antigen in an active humoral rejection process mediated by anti-GSTT1 antibodies. Our initial hypothesis was that if GSTT1 is the target of a specific alloimmune response, this enzyme should be present in injured zones close to the lymphoplasmacytic infiltrates and also in nearby C4d deposits, where activation of the complement pathway occurs. If this is the case, we should find C4d deposits in PTC surrounding the positive tubules as a sign of an active humoral response. To look at this, we needed to be familiar with the location of GSTT1 in renal tissue, particularly in biopsies from the patients under study.

A series of papers from the early 1990s studied GST enzymes and their location in human renal tissue because they had been implicated in tumorigenesis and renal cell carcinomas [11–13]. At that time, only cytoplasmic classes alpha, mu, pi and microsomal GST had been identified. In those reports, the distribution of these enzymes was heterogeneous and sometimes very focal. In cases of glomerulonephritis, alpha-class GST was detected in proximal tubules, and pi-class GST was detected in distal tubules, podocytes and Bowman’s capsule.
Fig. 2. Immunohistochemical detection of the GSTT1 enzyme in paraffin sections of normal kidney tissue. A strong cytoplasmic expression in distal tubules and a less intense cytoplasmic expression in proximal tubules were observed. (A) 1:3000 primary antibody dilution. (B) 1:500 primary antibody dilution.

Fig. 3. Immunohistochemical detection of the GSTT1 enzyme in renal biopsies from the patients with CAMR. A cytoplasmic pattern is observed in the distal tubules while the proximal tubules are negative. The primary antibody dilution was 1:500.

In a very interesting report [14], Harrison et al. described the distribution of GST isoenzymes in human kidney as a possible marker of renal injury. They measured the release of cytoplasmic GST enzymes during injury and separated them into three classes: basic, acidic and neutral. They also identified a separate form, the microsomal GST. The authors reported that the presence of both acidic and basic GST in urine could be of diagnostic value in distinguishing between cyclosporine toxicity and transplant rejection, as cyclosporine is thought to be primarily a proximal tubule toxin. Later, new classes of this family of enzymes were described, as well as the existence of null alleles of the GSTT1 and GSTM1 genes [8].

In our patients, we observed expression of the GSTT1 enzyme principally in distal tubules. In accordance with the description of acidic and neutral GST, we did not observe GSTT1 expression in glomeruli. The finding that GSTT1 is not expressed in glomeruli is of particular interest, as this parallels the absence of C4d deposits in GC of patients with anti-GSTT1 antibodies alone, although the clinical relevance of this evidence is not yet clear to us.

We propose a simple model that allows us to predict which patients are at risk of developing anti-GSTT1 antibodies and subsequent risk of allograft rejection. This model is illustrated in a diagram adapted from Colvin and Smith [15], in which we have added a basic element crucial for this specific type of rejection, the GSTT1 donor/recipient genetic mismatch. The subsequent cascade of events in the original figure remains the same (Figure 4). Smith et al. [16] proposed a similar schema in a prospective
Study of non-human primates. In this study, alloantibodies were always detected before the C4d deposits, and both preceded chronic rejection. Although the prevalence of this kind of rejection appears low, we believe that this is only the tip of the iceberg. If we take the known frequencies of the null and positive GSTT1 alleles in our population we can estimate that about 16% of the total number of renal transplant patients will fall within the risk group. Based on our current data, 50% of them produce anti-GSTT1 antibodies de novo. Since this constitutes a long-term response and the policy in our hospital does not favour protocol biopsies, it will be only a matter of time to confirm the clinical consequences of a donor-specific response in which the target (non-HLA) antigen is well defined. In this era of ‘antibody mediated rejection’, anti-GSTT1 antibodies should definitely be considered as an important component of CAMR.

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Conflict of interest statement. None declared.

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