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Gene expression signature of tolerance and lymphocyte subsets in stable renal transplants: Results of a cross-sectional study

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ABSTRACT

Background: In kidney transplants operational tolerance has been associated with up-regulation of B cell differentiation genes and an increased number of total, naive and transitional peripheral B cells. The aim is to evaluate tolerance biomarkers in different cohorts of stable renal transplants under immunosuppression.
Methods: This is a cross-sectional study conducted in renal transplants. We evaluate genetic tolerance signature and lymphocyte subsets in stable transplants treated with calcineurin inhibitors (CNI) at 1 ($n = 15$), 5 ($n = 14$) and 10 ($n = 16$) years, and azathioprine-treated transplants followed 30 years ($n = 8$). Healthy volunteers ($n = 10$) and patients with chronic rejection ($n = 15$) served as controls.
Results: We confirm that peripheral expression of IGKV1D-13 and IGKV4-1 genes by RT-PCR distinguish tolerant ($n = 10$) from stable transplants ($n = 10$) provided by the International Tolerance Network. Tolerance signature was defined as the lowest expression for both genes in tolerant patients. In CNI-treated patients, genetic signature of tolerance and B cells showed a time-dependent increase not observed in azathioprine-treated patients ($p < 0.01$). Genetic tolerance signature was observed in 0% at 1, 7% at 5 and 25% at 10-years while it was not observed in azathioprine-treated and chronic rejection patients. Fifteen out of 16 CNI-treated transplants at 10 years were revaluated 3 months apart. Nine did not show the tolerance signature in any determination, 4 in one and 2 in both determinations. Genetic signature of tolerance was associated with an increase of total, naive and transitional B cells ($p < 0.05$).
Conclusions: IGKV1D-13 and IGKV4-1 gene expression and its linked B cell populations increase during follow up in CNI-treated patients. At 10 years, 2 out of 15 CNI treated patients consistently express biomarkers associated with true tolerance. In azathioprine-treated patients these biomarkers were down-regulated.

1. Introduction

Renal transplantation is the best available treatment for end-stage renal disease [1]. Short term graft survival has significantly improved thanks to the introduction of new immunosuppressive agents, but improvement of long term survival has been rather moderate [2,3]. Chronic humoral rejection, interstitial fibrosis/tubular atrophy, recurrence of the primary disease and death with a functioning graft constitute the major causes for late graft failure [4–6]. Long term immunosuppression, that is associated with complications such as cardiovascular disease, cancer, infections, diabetes, nephrotoxicity and inadequate control of the alloimmune response, may constitute a barrier to improve long term outcome [7–9]. Protocols using mild immunosuppressive regimens have been introduced in an attempt to reduce these side effects, but insufficient immunosuppression is associated with an increased risk for acute and chronic rejections [10,11]. The description of cases of operational tolerance, i.e., stable renal function lasting for at least one year after discontinuation of immunosuppression with preserved renal function, has arisen the interest of the transplant community.

Operational tolerance is a very rare condition in kidney recipients [12] but it is frequent in liver transplants [13]. Tolerance in kidney recipients is associated with up-regulation of multiple B cell differentiation genes and increased number of total, naive and transitional peripheral B cells, HLA-DR$^+$CD4$^+$ T cells and natural killer cells (NK) in comparison to stable grafts receiving immunosuppression or patients suffering from acute or chronic rejection [14–16]. Newell et al. reported that the overexpression of just three B cell-related genes (IGKV1D-13, IGKV4-1 and IGLL1) was sufficient to distinguish tolerant patients from stable renal allografts under immunosuppression. Additionally, the identification

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of these markers of operational tolerance also in some patients with stable grafts and still under immunosuppression suggested that it might be possible to identify those patients that could benefit from reduction or withdrawal of immunosuppression. Approximately, 3–4% of stable renal allografts under immunosuppression express this tolerance signature [12]. Living donation, older recipient age, good HLA matching and, especially, long-term follow up, were factors associated with this situation [17,18].

Clinical trials in liver transplantation have shown that immunosuppression can be withdrawn in approximately one third of patients [19,20]. A major difference between liver and renal transplantations is that acute rejection is not associated, if promptly treated, with a reduction in allograft long-term survival as it is the case in kidney transplantation. Thus, designing clinical trials to evaluate the utility of a tolerance signature to guide immunosuppression weaning protocols in kidney transplants constitutes a major goal in this field.

2. Objective

To evaluate the expression of the gene expression of tolerance signature previously described by Newell et al. [16] and B lymphocyte subsets in five groups of renal transplant patients receiving different immunosuppressive regimens at different times of follow up.

3. Patients and methods

3.1. Study design

This is a cross-sectional study to evaluate total blood candidate gene expression profiles and lymphocytes subsets previously associated with operational tolerance in stable renal transplants under immunosuppression. Healthy subjects and patients with chronic rejection served as controls. The study protocol was approved by the Ethics Committee of Hospital Universitari Vall d'Hebron. All patients gave written informed consent to participate in the study.

3.2. Patients

Patients with stable renal function (estimated glomerular filtration rate by MDRD-4 formula >40 mL/min and proteinuria below 0.3 g/day) receiving as maintenance immunosuppression the combination of a calcineurin inhibitor (either tacrolimus or cyclosporine) and an antimetabolite agent (either mycophenolate mofetil or enteric-coated mycophenolic acid) seen at our outpatient clinic between 1 and 30 of November 2011 were invited to participate. The following groups of patients with stable renal function were selected: a) follow up between 12 and 30 months (ST-CNI-1y); b) follow up between 60 and 84 months (ST-CNI-5y) and c) follow up between 120 and 144 months (ST-CNI-10y). Additionally, we also recruited d) a group of stable patients treated with azathioprine and prednisone (ST-AZA) with a very long follow up (between 325 and 364 months), e.) a group of patients diagnosed with chronic humoral rejection (CR) during the last year and f) a group of healthy controls (HC). The diagnosis of chronic humoral rejection was based on the presence of donor specific HLA antibodies (DSA) detected by the Luminex assay and a renal allograft biopsy showing typical rejection lesions (transplant glomerulopathy and/or microcirculation inflammation regardless of the presence of C4d deposition in peritubular capillaries). Patients from ST-CNI-10y group were re-assessed again, three months later, in February 2012.

3.3. Gene expression analysis

Total RNA was obtained using the ABI Tempus whole blood collection system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Blood collection was performed following the manufacturer's instructions. cDNA was synthesized using M-MLV retrotranscriptase enzyme. Template cDNA was added to Taqman Universal Master Mix (Applied Biosystems, Foster City, CA, USA) in a 12.5 μl reaction with specific primers and probe for each gene. The primers and probe sets for IGKV1D-13 and IGKV4-1 were purchased as Custom TaqMan® Expression Assays (Applied Biosystems, Foster City, CA, USA) and IGLL1 as Assay-on-Demand™ (Hs00252263_m1) according to their Ref Seq in http://www.ncbi.nlm.nih.gov/LocusLink; for endogenous housekeeping genes for controls were purchased as in the catalog of Assays-on-Demand™ (Hs99999907_m1 for B2M, Hs00920495_m1 for TBP and Hs01122445_g1 for YWHA). The sequence of primers and probes of Custom TaqMan® Expression Assays are given in Supplementary Table S1.

Gene expression was measured by qPCR using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the comparative Cq method. Final results were determined as follows: $2^{-(\Delta Cq\ sample\ -\ \Delta Cq\ calibrator)}$, where ΔCq values of the calibrator and sample are determined by subtracting the Cq value of the target gene from the geometric mean of three constitutive genes (YWHAZ, BM2 and TBP). Commercial RNA control was used as calibrator (Fist Choice Human Brain Reference RNA; Ambion). The calculations were done following the manufacturer's recommendations contained in Technical Bulletin #2 (Applied Biosystems, Foster City, CA, USA).

3.4. Validation of gene analysis determined by RT-PCR in a sample of patients from the International Tolerance Network

In order to validate gene expression measures for these genes from Sequenom MassARRAY QGE platform, 30 blood samples provided by the Tolerance Network (ITN) to Pangaea Biotech from 10 tolerant patients (TOL-INT), 10 stable patients (ST-INT) under immunosuppression and 10 healthy controls (HC-INT) were analyzed. IGLL1 gene was not amplified with employed primers. Accordingly, in this study the characterization of tolerance signature relied on IGKV1D-13 and IGKV4-1 gene expression. Correlation plots of log2-converted data confirmed that IGKV1D-13 and IGKV4-1 genes by RT-PCR accurately distinguish tolerant transplants (TOL-ITN) from stable transplants under immunosuppression (ST-ITN). As it is shown in Fig. 1, the cut-off value to allocate patients in TOL-ITN was established as $\log_2(2^{(-\Delta\Delta Cq)})$ IGKV1D-13 and IGKV4-1 > 7.75.

3.5. Peripheral blood lymphocyte subset immunophenotyping

For lymphocyte phenotyping on whole blood the following monoclonal antibodies multitest panels CD3-FITC/CD8-PE/CD45-PerCP/CD4APC and CD3-FITC/CD16-PE + 56/CD45-PerCP/CD19-APC (Becton Dickinson) were used. Regulatory T cells (Treg) were identified by staining with FoxP3-PE/CD4-FITC/CD25-APC/CD3-PercP. For B cell subsets, PBMCs were isolated by density gradient centrifugation in Ficoll-Paque Plus (Amersham Biosciences) and stained for CD19-PECy7/IgD-PE/IgM-APC and CD27-FITC, CD24-FITC, CD38-APC and CD86-PerCp-Cy5 in different panels. The following cell subsets were characterized: B naïve (CD19 + IGD + CD27-), B memory (CD19 + IGD + CD27+), B class switch (CD19 + IGD-CD27-), B transitional (CD19 + CD38 + CD24 + High), Bregs (CD19 + CD38 + CD24 + High) and Tregs (FOXP3+ GATED IN CD3 + CD4 + CD25 high).

Flow cytometric analysis was carried out on a BD FACSCANTOII and data were analyzed with FlowJo software (TriStar, Inc., Ashland, OR, USA).

3.6. HLA antibodies by Luminex assay

Anti-HLA antibodies were measured by the Luminex® technique. The assay applied was LIFECODES LifeScreen Deluxe (Gen-Probe, CA). Patients' Class I and II HLA antibodies were detected using beads coated with purified class I and II antigens. In patients with positive Class I and/or II HLA antibodies, donor specificity was evaluated by LIFECODES LSA

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