Perforin, Granzyme B, and Fas Ligand for Molecular Diagnosis of Acute Renal-Allograft Rejection: Analyses on Serial Biopsies Suggest Methodological Issues

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Background. The Perforin-Granzyme B and Fas/Fas Ligand apoptotic mechanisms are involved in the development of acute renal rejection (AR). We describe our experience of analyzing the expression of cytotoxic T-lymphotoxins (CTL) in biopsies and peripheral blood leukocytes (PBL) for the diagnosis of AR.
Methods. We analyzed Perforin (P), Granzyme B (GB) and Fas Ligand (FL) expression in 68 renal biopsies and 64 PBL using comparative kinetic RT-PCR and, for GAPDH and FL, we also replicated with real-time RT-PCR. The levels of expression were measured in different groups, such as T0 (biopsies before reperfusion and PBL in recipient before the transplant [Tx]), Td (biopsies and PBL collected for clinical purposes) and Tp (biopsies and PBL two months after Tx).
Results. A higher CTL expression was seen in nonrejecting (NR) biopsies in the first 2 months after Tx. P and FL were significantly more expressed during AR in all biopsies and in Td, while P remained upregulated in Tp. In PBL, there was no significant increase in CTL transcription during AR. A variable expression of CTL emerged in all T0 biopsies.
Conclusions. Two lytic pathways are activated in biopsies when AR occurs shortly after Tx, whereas the P/GB mechanism prevails if it occurs later on. Only P and FL in biopsies might be able to predict AR diagnosis, but with a considerable variability in each sample, possibly due to the small portion of tissue core, which may be inadequate for molecular diagnosis. CTL expression in PBL does not correlate with histological AR.

Keywords: Perforin, Granzyme B, Fas ligand, Acute rejection, Biopsies.

(*Transplantation* 2006;81: 1125–1132)

Acute rejection (AR) remains the major cause of renal allograft dysfunction in the first year after transplantation. Its occurrence is the strongest predictor of chronic allograft nephropathy (*1*, *2*). AR involves humoral immune (*3*) and host-mediated cellular responses. In particular, cytotoxic T-cell activation plays an important part in the response to major histocompatibility alloantigens during AR (*4*).

The diagnosis is currently based on clinical findings and biopsy histopathology. The distinctive histological feature of rejection is a T-lymphocyte-dominated leukocyte infiltrate in the cortical parenchyma (*5*). Hence the suggestion that analyzing the expression of a specific genes involved in T-cell activation may be a tool for diagnosing rejection.

Studies have investigated the expression of the cytotoxic T-lymphocyte activation markers Fas Ligand (FL), Perforin (P), and Granzyme B (GB) in renal biopsies, peripheral blood leukocytes (PBL) and urine of renal transplant (Tx) patients (*6–12*). The relevance of performing these analyses in blood or urine lymphocytes is self-evident, since this might spare the need for renal biopsies.

The present study describes our experience of analyzing the expression of these cytotoxic T-lymphocytoxins (CTL) in biopsies and PBL from renal Tx patients with AR. Biopsies were also collected from cadaveric donor kidneys before reperfusion to obtain the basal expression of these molecules for comparison with protocol (Tp) and diagnostic (Td) biopsies taken two months or a few days after Tx, respectively. Blood samples were drawn from recipients before Tx and at the time of Tp and Td biopsies to investigate the correlations with intragraft expression. We performed a comparative kinetic RT-PCR and also, for a few genes, a quantitative real-time RT-PCR to establish whether the two methods assure the same degree of accuracy.

MATERIALS AND METHODS

Patients gave their informed, written consent. The study was approved by our Ethical Committee. All patients received cadaveric kidneys. Only one core of tissue was taken during renal biopsy. Three different types of biopsies and PBL samples were available: those collected at time 0 before reperfusion (T0), at the time of a renal dysfunction suggesting AR (diagnostic biopsy, Td), and "per protocol" 2 months postTx (Tp) in patients with stable renal function, without any suspicion of AR. Due to the exploratory objective of our experience, only cases with clear-cut AR were considered in the analyses of data. Numbers of samples were:

→T0: 31 donor biopsies, 14–18 hr ischemia, and 20 PBL samples drawn from recipients before Tx;

This study was supported by the Italian Ministry for the University, Cofin 2000, protocol MM06198897-001.
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Received 16 December 2005. Revision requested 5 January 2006.
Accepted 13 January 2006.

ISSN 0041-1337/06/8108-1125
DOI: 10.1097/01.tp.0000208573.16839.67

→Td: 17 biopsies and 26 PBL samples performed 5–10 days postTx;

→Tp: 19 biopsies and 24 PBL samples.

In reference to Td and Tp biopsies, only patients whose graft started to function immediately were considered, provided they had an adequate renal biopsy (as specified below).

Patients characteristics are described in Table 1. All received similar triple immunosuppression (calcineurin inhibitors, mycophenolate mofetil, steroids).

Biopsies

Biopsies were divided into two parts, for histopathological analysis and for molecular analysis (approximately 1/3 of the tissue core). The procedure was performed under stereomicroscopic observation to assure the presence of glomeruli in both fragments.

The histopathological evaluation was performed by one of us (MLV) and confirmed by a second reviewer (DDP). That specimens devoted to histopathology were adequate according to Banff'97 criteria (*5*), was an inclusion criteria, otherwise the patient was not enrolled. Biopsies were processed according to Banff'97 guidelines.

Histology on the T0 biopsies revealed: 20% normal morphology, 13% mild fibrosis, 67% tubular cell detachment and intact basal membrane. According to Perico et al. (*13*) (Table 2), all T0 biopsies were graded 0 to 3.

Td and Tp biopsies were classified (Banff '97): 58% no rejection (NR), 39% AR (IA, IB, all with focal infiltrates), 3% cyclosporine A nephrotoxicity. In Td group, 11 cases were AR, 6 NR; in Tp group, 4 were AR (subclinical) and 15 NR. No evidence of type II or antibody-mediated rejection were present in biopsies (Table 1).

RNA Isolation

Total RNA was extracted from renal biopsies and PBL. Approximately 1/3 of the biopsy was set aside for molecular analysis and put into 200 μl of RNAzolB solution (BIOTEX, Houston, TX, USA) for RNA isolation. The RNA pellet was dissolved in 10 μl of diethyl pyrocarbonate water. One microliter of RNA was quantified using NanoDrop ND-100 Spectrophotometer. We evaluated the integrity of the RNA using the Agilent 2100 bioanalyzer (Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

For PBL, RNA was extracted from 1.5 ml of whole blood using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). DNase treatment was used to eliminate genomic contamination. RNA was eluted with 30 μl of RNAse-free water and one microliter was quantified as above.

Total RNA (100 ng) was retrotranscribed using random hexamers by Moloney murine leukemia virus transcriptase (PE Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μl.

Polymerase Reaction (PCR)

One microliter aliquots of RT reaction were used to amplify the following genes in different tubes: P, GB, FL and the housekeeping GAPDH. Oligonucleotide sequences were the following: GAPDH forward: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, reverse: 5′-CATGTGGGCCATGAGGTCCACCAC-3′; P forward: 5′-GCAATGTGCATGTGTCTGTG-3′, reverse: 5′-TCCGAGTGGCGCTCCCGGTA-3′; GB forward: 5′-GGGGAAGCTCCATAAATG TCACCT-3′, reverse: 5′-TACACACAAGAGGGCCTCCAGAGT-3′; FL forward: 5′-GGATTGGGCCTGGGGATGTTTCA-3′, reverse: 5′-TGTGGCTCAGGGGCAGGTTGTTG-3′.

Although primers were designed to span one or more introns within the genes, control negative reactions, without reverse transcriptase, were performed during the cDNA synthesis step to exclude genomic contamination. The amplification was carried out in a final volume of 50 μl containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U Jump Start Taq (Sigma Chemical Co.), and 0.4 μM primers. cDNAs were amplified in the following conditions:

-GAPDH 94°C for 45′′, 60°C for 45′′ and 72°C for 1′; -P 94°C for 45′′, 58°C for 1′ and 72°C for 1′; -GB and FL 94°C for 45′′, 68°C for 1′ and 72°C for 1′.

TABLE 1. Demographic and descriptive characteristics of study group

Donor age	*51 (21-70) yrs
Cold ischemia time	*16 (14-18) (hr)
Histological grading of T0 biopsies	0-3
Recipient age	*48 (30-63) yrs
Gender	male: 41 female: 10
Acute Rejection	11 Td – 4 Tp
No Rejection	6 Td – 15 Tp
Immunosuppression Cyclosporine A + mycophenolate mofetil + steroids Tacrolimus + mycophenolate mofetil + steroids	85% 15%

* Values expressed as mean and range.

TABLE 2. Scoring system proposed by Perico et al. (*13*)

Glomerular global sclerosis •0 = none •1+ = <20% •2+ =20 to 50% •3+ = >50%	Tubular atrophy •0 = absent •1+ = <20% of tubuli affected •2+ = 20 to 50% •3+ = >50%
Interstitial fibrosis •0 = absent •1+ = <20% replacement by fibrous tissue •2+ = 20 to 50% •3+ = >50%	Arterial and arteriolar narrowing •0 = absent •1+ = increased wall thickness less than diameter of the lumen •2+ = wall thickness equal or slightly greater than diameter of the lumen •3+ = wall thickness far exceeds the diameter of the lumen
Final Grading : 0 to 3 mild lesions 4 to 6 moderate lesions 7 to 12 severe lesions	

Comparative RT/PCR

A kinetic analysis of the amplified products was applied to all samples for each gene to ensure that the signals derived only from the exponential amplification phase. For GAPDH, cDNA was submitted to the first 28 amplification cycles and an aliquot of 5 μl was drawn from each sample for electrophoretic analysis. Then tubes were submitted to two more amplification cycles and one more 5 μl aliquot was drawn. This procedure was repeated five times until a total of 36 cycles had been performed. PCR products obtained after 28, 30, 32, 34, and 36 cycles were analyzed by electrophoresis in polyacrylamide gel, 3% C with 5% glycerol and silver stained according to the standard protocol. The same procedure was applied to P, GB and FL for biopsies, starting from 36 to 44 cycles, and for blood samples, starting from 28 to 36 cycles. Densitometric analysis of the silver-stained bands was performed using Gel-Pro Analyzer software (Media Cybernetics, Silver Springs, MD, USA) and the quantity of the different mRNAs was expressed as the ratio between the optical density (OD) generated by the target gene and GAPDH. After determining the exponential reaction phase for each gene by kinetic PCR, we selected the appropriate cycle in which the PCR products had to be quantified. In biopsies, the analysis was performed at 32 cycles for GAPDH, 40 cycles for P, 38 cycles for GB and FL. In the PBL, it was performed at 34 cycles for GAPDH, 32 cycles for P and GB, 34 cycles for FL. We performed a duplicate for each sample and, in each experimental PCR assay, we used the same control cDNA in the biopsy and in the PBL to monitor and correct the variability.

Real-time PCR

Comparative kinetic PCR for GAPDH in biopsies and FL in PBL was replicated with real-time PCR. The same cDNA reaction and quantity were used for the two assays. GAPDH was assayed in 67 samples, FL in 70. A standard curve for the corresponding gene, constructed using dilutions of the specific purified amplification products (from 10^7 copies to 10^1), was loaded to monitor the variability in each experimental session. Briefly, PCR reactions for GAPDH and FL were verified by gel electrophoresis in the presence of only one product, purified with the MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and quantified by spectrophotometry. The number of copies/ml of standard were calculated according to the formula:

$$\text{copies/ml} = 6.023 \times 10^{23} \times C \times OD^{260}/Mwt$$

where $C = 5 \times 10^{-5}$ g/ml for DNA and MWt=molecular weight of PCR product (base pairs$\times 6.58 \times 10^2$ g).

Standard curves for GAPDH and FL were linear over the entire quantification range with the same correlation coefficient (r=0.98). The GAPDH and FL slopes were 3.87 and 3.6, respectively, in all assays. The excellent reproducibility of the standard curve demonstrated that these purified amplification products were as stable as those in the plasmids and enabled 10^1 copies of specific transcripts to be recognized from 5 ng of total RNA (data not shown).

Primer pairing was designed with the Beacon Designer Probe/Primer Design software (Bio-Rad Laboratories, Hercules, California).

For human GAPDH, the sequences were: forward 5′-GAAGGTGAAGGTCGGAGT-3′;reverse,5′-TGGCAACAATATCCACTTTACCA-3′. The size for the GAPDH PCR product was 92 bp. For FL, we used the same primer sequences as for comparative kinetic RT/PCR with a amplification product of 390 bp.

Real-time PCR was performed using the iCycler iQ Detection System (Bio-Rad Laboratories, Hercules, California) according to the manufacturer's instructions.

Reaction conditions were the same for each gene. Reactions were obtained in a 25 μl volume with 300 nM of each primers, 200 nM dNTPs, 3 mM $MgCl_2$, 1 U Jumpstart Taq DNA Polymerase (Sigma- Aldrich, Saint Louis, Missouri USA), 0,2× SYBR Green I dye (Sigma S9430). The thermal cycling profile consisted of: stage 1, 95°C for 5′; stage 2, 94°C for 30′′ followed by 60°C for 30′′ for GAPDH, or 68°C for 30′′ for FL. Stage 2 was repeated for 40 cycles. A duplicate was performed for each sample.

A melting curve analysis was also performed to confirm the amplification specificity for each gene.

Statistical Analysis

The statistical analysis was performed using the Kruskal-Wallis or Mann-Whitney tests and considering a $P<0.05$ as statistically significant. The χ^2 test was used for categorical data.

A regression analysis was conducted to compare data obtained as optical densities (OD) with comparative kinetic PCR and numbers of copies with real-time PCR for GAPDH and FL, and to analyze the relationship between the levels of expression of the three genes in biopsies and PBL.

The cutoff was set as the mean value of each gene OD in NR patient samples plus the 95% confidence interval, and was used to assess the sensitivity and specificity of CTL for AR.

RESULTS

Expression of CTL in Biopsies

In NR kidneys, the three genes show a time-related trend in that their expression increases in the first two months postTx, though it is only for P ($P<0.05$) and GB ($P<0.01$) that this increased expression is statistically significant (Fig. 1). The expression of the three genes in the T0 biopsies correlated neither with the cold ischemia time nor with the morphological damage. P overexpression in T0 biopsies was associated with biopsy-proven rejection (clinically manifest or silent) occurring in the following 2 months ($\chi^2=3.93$; $P<0.05$). On the contrary, it was not associated with delayed graft function (DGF). By allocating biopsies according to presence/absence of rejection, in Td biopsies, taken a few days postTx (mean 7.5 days, range 5–10), P and FL were expressed significantly more in AR, though the sensitivity and specificity were only acceptable for P (90% and 71% respectively, cut-off=0.26) (Fig. 2A), but low for FL (70% and 86% respectively, cut-off=0.031). In Tp biopsies showing AR (all these cases were subclinical), only P expression was significantly raised, albeit with a poor diagnostic value (sensitivity=25% and specificity=66%, cut-off=1.41) (Fig. 2B). In all samples, divided according to the presence/absence of rejection, irrespective of the time of biopsy, P and FL were overexpressed in rejecting patients ($P<0.03$; $P<0.04$, respectively), but with poor diagnostic value (P, sensitivity 36%, specificity 78%, cut-off=1.07; FL, 43% and 74%, cut-off=0.41) (Fig. 3). Although P showed a better diagnostic performance, when its time-related trend was analyzed in single patients, incongruous results could emerge. In particular, few patients revealed a greater variability between samples unrelated to their renal pathology (Fig. 4, A and B). In Td

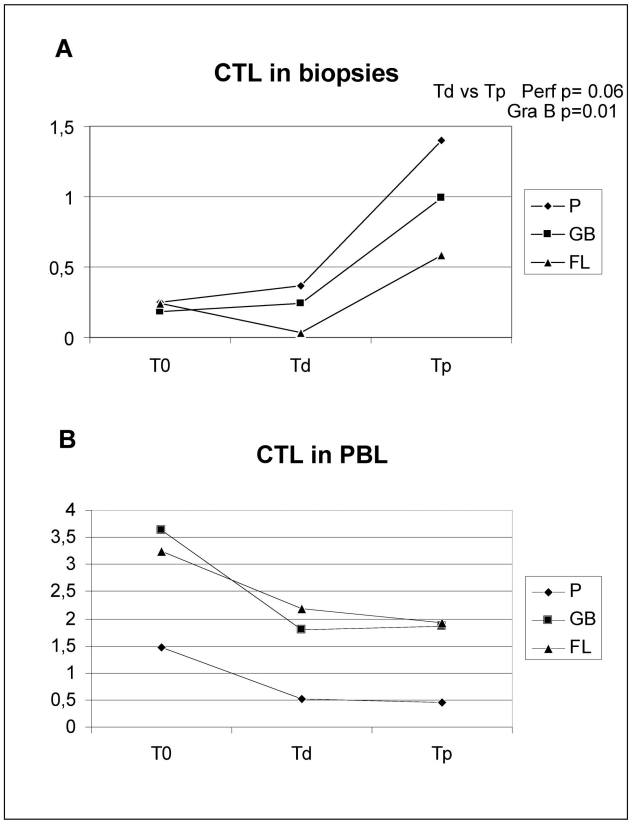

FIGURE 1. (A) Intragraft expression of P, GB and FL in T0 biopsies, Td biopsies (5–10 days) and Tp (60 days after Tx) in patients without AR. (B) Quantitative expression of P, GB and FL in PBL samples taken in concomitance with T0, Td and Tp biopsies in patients without AR.Values are given as mean OD of target gene cDNA per OD of GAPDH cDNA. CTL cytotoxic T-lymphocytoxins; PBL, peripheral blood lymphocytes.

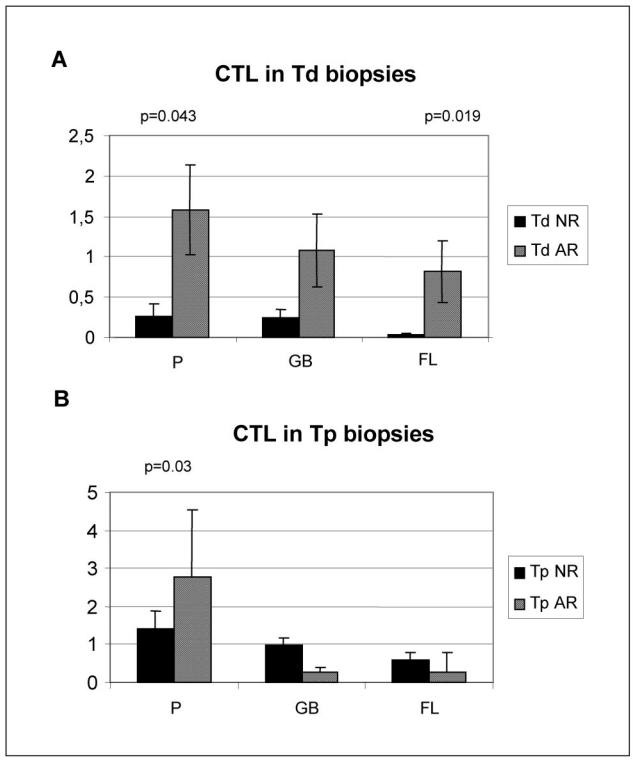

FIGURE 2. Quantitative analysis of P, GB and FL in biopsies performed at Td (A) and at Tp (B). Biopsies were classified according to the histological diagnosis of acute rejection AR and non rejection NR. Values are given as mean OD of the target gene cDNA per OD of GAPDH cDNA ± SE. CTL, cytotoxic T-lymphocytoxins; NR, no rejection; AR, acute rejection.

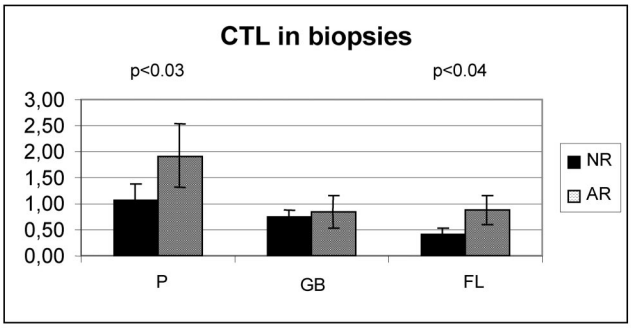

FIGURE 3. Quantitative analysis of P, GB and FL in all Tp and Td biopsies with histological diagnosis of acute rejection and nonrejection. Values are given as mean OD of the target gene cDNA per OD of GAPDH cDNA. Results are expressed as mean ± SE. CTL, cytotoxic T-lymphocytoxins; NR, no rejection; AR, acute rejection.

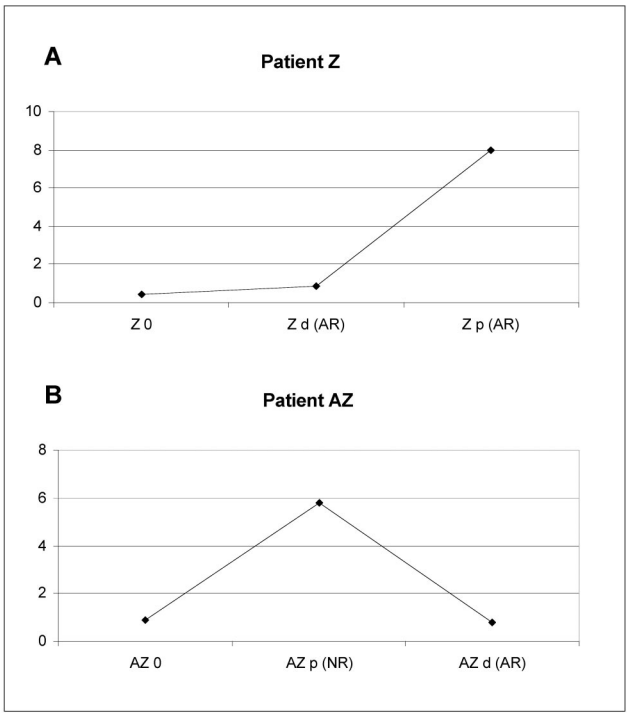

FIGURE 4. P quantitative expression in sequential biopsies performed in patient Z and AZ for diagnostic purpose (Z d and AZ d) or protocol (Z p and AZ p) with diagnosis of acute rejection AR or non rejection NR. It is evident the impossibility to establish a range of expression that recognizes single cases of acute rejection from nonrejection. Values are given as mean OD of the Perforin gene cDNA per OD of GAPDH cDNA. AZ d (AR) was a diagnostic biopsy performed >2 months from Tx. This time point value is shown only for making clear the concept. This value has not been included in the statistical analysis. NR, no rejection; AR, acute rejection.

biopsies, the combined analyses of two genes (either or both positive) identified AR with 100% sensitivity and 57% specificity for P+GB, 100% and 71% for P+FL, and 80% and 71% for GB+FL. By considering all three CTL (any two or all three positive), we raised a sensitivity of 80% and a specificity of 85%. The same analysis performed in all biopsies or in Tp raised lower sensitivities and specificities (data not shown).

Regression analysis between the three CTL in NR and AR biopsies demonstrated a significant, direct correlation only for GB/FL ($P=0.034$) and GB/FL ($P=0.004$) in AR and for P/GB ($P=0.004$) in NR.

Expression of CTL in PBL

The expression of the three genes decreased after Tx in the blood of nonrejecting patients (Fig. 4B). When samples were divided according to the presence/absence of rejection in the Td and Tp groups, there was a greater, although not significant transcription of the three genes in those with AR (data not shown). CTL expressions were correlated in both NR (P/GB r=0.58 $P<0.0004$, P/FL r=0.45 $P<0.01$, GB/FL r=0.86 $P=0.0000$) and AR (P/GB r=0.86 $P<0.0003$, P/FL r=0.87 $P=0.0000$, GB/FL r=0.83 $P<0.0001$).

Comparison of CTL Expression between Biopsies and PBL

Regression analysis of CTL transcript levels in biopsies and PBL samples showed no correlation in NR (P, $P=0.3$; GB, $P=0.34$; FL, $P=0.24$) or AR patients (P, $P=0.4$; GB, $P=0.72$; FL, $P=0.29$).

Comparison of Gene Expression by Comparative Kinetic RT-PCR and Real-time RT-PCR

The GAPDH data obtained as OD correlated significantly ($P<0.001$) with the number of gene copies obtained with the real-time procedure. The FL expression determined with the 2 quantitative techniques also revealed strict correlation ($P<0.0007$).

DISCUSSION

Renal rejection is diagnosed on the basis of clinical observation and histological examination of a biopsy. The diagnosis may be uncertain if histology shows intermediate picture, e.g. borderline changes. Studies have investigated whether diagnosis of AR (even subclinical forms) can be improved by analyzing the expression of various immune activation transcripts in biopsies (*8*, *12*, *15–17*). A study on 18 genes involved in host-mediated cellular response in renal biopsies from AR patients found a high expression of P, GB and FL by comparison with NR (*17*). Given their role, it makes sense for these genes to be over-expressed in the kidney of AR patients. P and GB are expressed and stored in the granules of T and NK cells. Alloantigens induce fusion of granules with cell membranes and activation of a apoptosis pathway. P perforates membranes, enabling GB to enter and catalyze target proteins that lead to DNA fragmentation and cell death (*18*). The interaction between FL and Fas-antigen triggers a distinct cytolytic pathway leading to caspase-activated apoptosis (*19*). Following the initial report (*8*), the involvement of the two cytolytic pathways in AR was demonstrated by quantitative PCR and immunohistochemistry in PBL (*10*, *15*, *20–22*), urine leukocytes (*11*, *23*), and fine needle

samples (*9*). In all these specimens, the expression of at least one of the three CTL correlated with the patient's renal pathology.

The relevance of these findings is obvious, since molecular analyses on blood or urine samples are a non-invasive alternative to renal biopsies for diagnosing AR.

Our goal was to replicate previous observations. We tested the expression of the three CTL in renal biopsies and PBL of Tx patients. P and FL were significantly more expressed in all AR biopsies than in NR patients (Fig. 3). This was particularly true of Td biopsies (taken 5–10 days postTx), where the two genes were maximally expressed (Fig. 2A), suggesting that both lytic pathways are activated when AR occurs shortly after Tx. Previous studies examining the P/GB pathway found that it was the main culprit responsible for CD4+ mediated cytotoxicity and led to renal tubular cell destruction (*24–27*). The Fas-FL system, on the other hand, might play a different part, involving peripheral tolerance, activation of the cell suicide responsible for the down-regulation of immune responses (*28*, *29*) and apoptosis of tubular cells (*9*, *10*). FL is expressed on T lymphocytes, macrophages, neutrophils, B and NK cells, but it has recently been found in renal tubular cells too (*30*, *31*), where it induces the elimination of antigen-activated CD4+ lymphocytes in a process known as activation-induced cell death (AICD) (*32*). Thus, the FL over-expression at Td in cases of AR (Fig. 2A) might indicate the initiation of renal damage, but it may also reveal the early activation of immune privileged conditions due to the apoptosis of infiltrating mononuclear cells.

Unlike Td biopsies, only P expression was significantly elevated after a longer period, i.e. at Tp, suggesting that the P/GB mechanism prevailed (Fig. 2B). While the P/GB loop has reportedly invariably been activated in AR (*24–27*, *33*), contrasting are the results on FL expression in renal Tx biopsies. Some found a low expression of Fas and FL in AR renal biopsies while other CTL had a more important role (*24*, *25*), as in our patients at Tp; others recorded an FL overexpression (*8*, *17*, *33*). Unfortunately, these studies differ in terms of enrolment criteria and timing of the biopsy. It may well be that FL expression varies over time, thus explaining these divergent results.

It is worth noting that our analysis on the expression of CTL in T0 biopsies established the basal levels in the cadaveric donor kidney. All three genes were variably expressed in T0 biopsies, suggesting that lytic mechanisms had already been activated. This phenomenon seems to be unrelated to either cold ischemia time or tubular morphological damage, and does not predict DGF.

That apoptosis is involved in renal damage caused by ischemia-reperfusion is known (*34*), but in this condition, apoptosis—which is an active process requiring ATP—occurs mainly during and after kidney reperfusion (*35*), so it could hardly be responsible for our findings because biopsy was performed before engrafting.

FL can be synthesized by resident renal cells (*30*, *31*), but this is not the case of P and GB, so the latter's expression indicates the presence of CD4+ lymphocytes in the kidney of cadaveric donors. Circulating cells expressing CTL may be disclosed in these donors (not shown), albeit at much lower levels than in the kidney. This suggests that their renal expression mirrors some specific renal phenomenon. We have no explanation for this, but we speculate that the brain death storm observed in cadaveric donors may explain our unexpected findings.

The expression of CTL in T0 biopsies means there are favorable conditions for the development of tissue lesions. We found that this basal P, GB and FL expression was destined to increase during the first two months after Tx in NR patients with stable clinical conditions (Fig. 1A), and that P over-expression in T0 was associated with a risk of AR in the 2 months postTx.

While some of the differences in intrarenal CTL expression are significant, they are not seen in PBL. CTL transcripts are increased in circulating cells during AR, though not to a statistically significant degree, and they strictly correlate one to the other because, when one of these is activated, the other two are transcripted in the same way. At intrarenal level, the only positive correlation is between GB and FL in AR patients.

Activation of the three genes in PBL is probably due to conditions other than AR, such as uremia. While over-transcription of the three CTL in PBL is partly reversed by Tx, as long as renal function is restored (Fig. 1B), in the kidney their expression follows a rising trend, possibly because of the activation of lytic mechanisms or induction of tolerance.

Concerning the diagnostic prospects (*36*, *10*), i.e. the use of PBL markers to improve the effectiveness and reduce invasiveness of AR diagnosis, our results are discouraging since the gene expression profile in PBL fails to represent intrarenal conditions. Our results cast doubts on the feasibility of using these PBL markers for the AR diagnosis, contrarily to others (*10*, *22*). Instead, we agree with Simon et al. (*20*), who found that best diagnostic results were obtained from samples taken in the earliest stages of Tx, but we only confirmed this in biopsies.

Referring to renal tissues, we could not confirm that molecular methods increase AR diagnostic accuracy. Unlike Strehlau et al. (*8*), who observed a 100% sensitivity and specificity for the analysis of combinations of the three CTL for the AR, we attained a lower sensitivity (80%) and specificity (85%). This study, however, was not designed in a way to assess whether molecular methods increase AR diagnostic accuracy. In order to do this, all biopsies, including Banff borderline infiltrates, must be included and biopsy histology and CTL expression correlated with the gold standard of the rejection diagnosis- that is, the retrospective diagnosis when follow-up makes it clear which patients have behaved as rejection.

Indeed, we observed a consistent expression of these CTL in NR patients and in T0 biopsies (Fig. 1A), and each group (T0, Tp, Td) disclosed a marked variability in the expression of the three genes as demonstrated by the high SEM (Figs. 2 and 3).

Since P performed better in diagnostic terms than FL and GB, we reviewed our data focusing only on the time-related changes (T0, Td and Tp biopsies) in transcription of this gene. As shown in Figure 4, which regards two particular cases, the variability in P gene expression was so high that it was impossible to distinguish AR from NR cases.

Although we only enrolled AR cases with biopsies adequate for Banff '97 (*5*) and all with focal infiltrates our findings suggest that the portion of tissue (only 1/3 of the core)

used in the molecular diagnosis may be inadequate to test for the presence/absence of rejection. A reason for our discouraging results might lie in a different performance of our quantitative procedures. Most of previous studies in this field were carried out with competitive RT-PCR (*9*, *10*, *12*, *17*) and more recently, with real-time RT-PCR (*20–23*, *33*). Two considerations make us confident of the reliability of our conclusions based on the comparative RT/PCR, however. First, we used specific strategies in each experimental session to enable inter- and intra-assay comparisons. Second, gene expression data obtained with real-time RT-PCR and comparative kinetic RT-PCR had robust statistical correlation, a finding supported by a previous study of ours (*38*).

The present study has a number of limitations. First, the number of patients was small, but we tried to collect a homogeneous group. Actually the immunosuppressive regimen was very similar in all subjects. Furthermore we did not enrol patients with DGF, and borderline changes. Second, we did not comply with Banff's guidelines which suggest collecting two renal biopsy cores. However, the local Ethical Committee did not allowed us to do so for protocol renal biopsies. Thus, in reference to the Td biopsies (which actually gave two cores per patients), to avoid the introduction of a bias, we decided to blindly consider for the aim of the present analysis only one biopsy core randomly chosen among the two collected for diagnostic purposes. Third, since we did not examine cases with borderline changes, we cannot extend our conclusion on the clinical role of molecular studies to this very important group of cases.

In conclusion, we found that the two cytotoxic pathways are involved in AR, but their use as molecular markers may be hindered by a certain time-related variability in their expression. These methods may also demand a sizable quantity of renal tissue to ensure an adequate sensitivity.

Further studies are needed to investigate the time pattern of gene activation following Tx, the quantity of tissue required, to investigate clinical outcomes of NR patients with high renal CTL and to look for other more predictive AR molecular markers.

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