Expression of cytotoxic mediators (perforin, granzyme B, FAS, and FAS-I) in renal allograft biopsies

Expressão de mediadores citotóxicos (perforina, granzima B, FAS e FAS-I) em biópsias de aloenxerto renal

Therezinha Gauri Leitão¹, Luís Eduardo Becker², Ivone Braga de Oliveira³, Flávia Ramos de Siqueira⁴, Maria Regina Teixeira Araújo⁵, João Egidio Romão Junior⁶, Hugo Abensur⁷, Irene de Lourdes Noronha⁸

ABSTRACT

Objectives: To analyze the in situ expression of perforin, granzyme B, FAS-L and FAS in renal allograft biopsies by means of immunohistochemistry and correlate these findings with the degree of histologic rejection and allograft outcome. Methods: Ninety-six allograft biopsies were divided into three groups: acute rejection (n = 56), chronic rejection (n = 31), and cases with stable renal function (no rejection; n = 9). The expression of perforin, granzyme B, FAS-L, and FAS was evaluated by immunohistochemistry. Results: A significantly higher expression of perforin and granzyme B was observed in acute rejection biopsies (4.83 ± 0.65 and 30.05 ± 7.93 cells/mm²) compared to chronic rejection biopsies (0.71 ± 0.13 and 11.4 ± 3.84 cells/mm²; p < 0.001, and p <0.05, respectively), but this was not the case for FAS-L (24.44 ± 5.56 in acute rejection versus 18.87 ± 6.83 in chronic rejection). Perforin, granzyme B, and FAS-L expression was significantly higher in the acute rejection group compared to the no rejection and control groups. FAS expression was similar in all groups. A modest correlation between perforin expression and the severity of AR was observed (r = 0.28, p = 0.05). Perforin was the most reliable marker for acute rejection diagnosis, with 80% sensitivity and 84.3% specificity. Conclusion: The in situ expression of perforin, granzyme B, and FAS-L in AR reflects the presence of an active cytotoxic process. Additional allograft biopsies are necessary in order to evaluate the usefulness of these markers for allograft rejection monitoring.

Keywords: Kidney transplantation/immunology; Kidney/pathology; Immunohistochemistry; Antigens, CD95; Graft rejection

RESUMO

Objetivos: Analisar por imunoistoquímica a expressão in situ de perforina, granzima B, FAS-L e FAS em biópsias de enxerto renal, correlacionando esses achados com o grau histológico de rejeição e prognóstico do enxerto. Métodos: Biópsias renais (n = 96) foram divididas em três grupos: rejeição aguda (n = 56), rejeição crônica (n = 31), função renal estável (sem rejeição; n = 9). A expressão de perforina, granzima B, FAS-L e FAS foi avaliada por imunoistoquímica. Resultados: A expressão de perforina e granzima B foi significativamente maior na rejeição aguda (4,84 ± 0,65 e 30,05 ± 7,93) quando comparada à rejeição crônica (0,71 ± 0,13 cél./mm² e 11,40 ± 3,84; p < 0,001 e p< 0,05 respectivamente), mas não de FAS-L (24,44 ± 5,56 na rejeição aguda vs. 18,87 ± 6,83 na rejeição crônica). As marcas de perforina, granzima B e FAS-L na rejeição aguda foram significativamente maiores na rejeição aguda que nos casos sem rejeição e controle. A expressão de FAS foi semelhante entre os grupos. Houve modesta correlação entre a expressão de perforina e a gravidade da rejeição aguda (r = 0,28, p = 0,05). A perforina foi o marcador mais confiável para o diagnóstico de rejeição aguda, com 80% de sensibilidade e 84,4% de especificidade.
INTRODUCTION

Although the incidence of acute renal rejection has diminished with the advent of new immunosuppressive drugs, it is clear that these events, including the subclinical forms, have a negative impact on the long-term renal graft prognosis leading to chronic allograft nephropathy(1). Thus, survival of the kidney grafts depends, among other factors, on an adequate control of rejection. In this context, efforts aiming the understanding of the immune mechanisms involved in this complex process as well as the possibility of early diagnosis of rejection are of crucial relevance.

Allorecognition, lymphocyte-T activation, and the subsequent inflammatory cell infiltration are key elements in the process of acute rejection of the allograft. In the effector phase of the immune response, the cytotoxic processes play an important role, in which activated CD8+ cells secrete granules containing perforin and granzyme B and simultaneously express FAS-L on their surfaces(2-3). These molecules are vital to the cytotoxic activity of CD8+, leading to tissue damage and apoptosis of the target-cell via different pathways.

Perforin, a lytic protein responsible for T CD8+ cell cytolytic activity, undergoes polymerization in the presence of calcium ions and promotes the formation of pores on the target-cell membrane. Besides allowing entry of the granzyme B into the cell, these pores induce changes in the cellular osmotic gradient, which leads to target-cell lysis(4).

Granzyme B is part of a family of serine proteases; when it is present in the target-cell cytoplasm, it promotes a cascade of nuclease-activating reactions that ultimately cause DNA fragmentation. Consequently, the target-cell undergoes apoptosis(5-6).

Human FAS is a transmembrane glycoprotein with similarities to human tumor necrosis factor receptors (TNF-R) and nerve growth factor receptors (NGF-R) and is expressed on the target-cell membrane. FAS-L is a type II transmembrane protein belonging to the superfamily that includes the tumor necrosis factor (TNF) and the nerve growth factor (NGF), and is expressed in activated T lymphocytes. This protein interacts with FAS, activating a cascade of biochemical reactions in the enzymatic machinery of the target-cell that culminates in DNA fragmentation and cell death by apoptosis(7).

Some studies have shown that the expression of messenger RNA for perforin and granzyme B in allograft biopsies is associated with acute rejection episodes(8-13). Detection of cytotoxic mediators in these studies was carried out using reverse transcriptase polymerase chain reaction (RT-PCR) techniques or real time PCR, which allows a semi-quantitative detection of transcriptional activity of these markers. In this study, the expression of these markers was measured in situ, by means of immunohistochemical techniques.

To date, the diagnosis of acute allograft rejection is still performed on basis of clinical and laboratory alterations, and confirmed by renal biopsy. There is no reliable test with high sensitivity and specificity. According to Banff’s classification, tubulitis and intimal arteritis are considered the most specific criteria for the histological diagnosis of acute rejection(14). The presence of interstitial inflammatory infiltrate is not considered a definitive criterion of acute rejection to the allograft. Therefore, monitoring of renal transplant patients has been focused on the identification of immune markers in the blood, urine, or even in biopsy fragments in order to establish a precise and early diagnosis of graft rejection, distinguishing it from other pathologies. The correlation between the degree of expression of these markers and the severity of the rejection by means of Banff’s classification histological criteria(14) is still not clear.

OBJECTIVE

The aim of this study was to identify and analyze the in situ expression of perforin, granzyme B, FAS, and FAS-L in renal allograft biopsies of transplant patients with the diagnosis of acute rejection, chronic rejection, and no rejection in order to analyze the association between the expression of these mediators and the clinical and histological diagnosis.

METHODS

Cases

In this study 96 biopsies of 76 patients submitted to renal transplantation at the Nephrology Clinic of the Hospital Beneficência Portuguesa de São Paulo were included.

Of the 76 transplanted patients, 44 received a kidney from a cadaver donor, 27 from a living related donor, biopsies obtained as “protocol biopsies“. As control group, normal renal tissue specimens were
obtained at the moment of donor nephrectomy (17 cases from living donors and 3 cases from cadaver donors).

All renal allograft biopsies had been performed for diagnostic purposes. For this study, all samples were recovered at the institution. There was no need of additional biopsies to provide specimens for this study.

**Characteristics of the groups**

Renal allograft biopsies were divided into three groups according to the clinical-histological diagnosis: acute rejection (AR) group; chronic rejection group; no rejection group; An additional control group with normal renal tissue was also assessed.

**Processing of renal graft biopsy specimens**

Allograft biopsies were performed by percutaneous technique with VIM-Silverman type needles under ultrasound guidance. Two fragments were removed from each patient for routine histological and immunofluorescence evaluations. One fragment was fixed in formalin and the other was immediately frozen in liquid nitrogen. The formalin-fixed fragment was embedded in paraffin in an automatic tissue processor (Jung Histokinette 2000, Leica Products, Germany), passing through successive solutions of alcohol (50%, 70%, 96%, and 100%), xylol, and paraffin. The embedded specimen was submitted to 4 mm thick cuts in previously gelatinized slides and kept under refrigeration. The biopsy materials were stained with hematoxylin-eosin, PAS, Masson trichromium, and silver impregnation techniques and then sent for histopathological analysis and classification by Banff criteria\(^{(14)}\). After histopathology analysis, the paraffin block was sent for the immunohistochemical study.

For freezing, the fragments were covered with Tissue-Tek II (Sakura Co, Tokyo, Japan), frozen in liquid nitrogen, and stored at \(-70^\circ\)C until processing. Slides were made with a 5 mm thickness, fixed in acetone, and stored at \(-70^\circ\)C. These fragments were utilized for immunofluorescence and the remaining material was sent for immunohistochemical testing.

**Immunohistochemical technique**

For the immunohistochemical technique, the slides preserved in paraffin went through a deparaffinization process that involved heating in a 60\(^\circ\)C oven and immersion in xylol and in alcohol. Next, the slides were washed in distilled water and TBS and submitted to the immunohistochemical technique. The frozen tissue slides were removed from storage, placed in a humid chamber, and submitted to the technique after having been washed with tri-buffered saline solution (TBS).

The slides were submitted to immunohistochemical analysis using the biotin streptavidin/alkaline phosphatase process\(^{(15)}\). This method consists of an initial block of endogenous avidin and biotin for 15 minutes and a non-specific block with nonimmune horse serum (VECTOR, Burlingame, USA), for 30 minutes. Tissue embedded in paraffin were incubated with anti-granzyme B (CHEMICON, Temecula, USA), anti-FAS (SANTA CRUZ, Santa Cruz, USA), and anti-FAS-L (SANTA CRUZ, Santa Cruz, USA), and frozen slides were incubated with anti-perforin antibodies (Pharmacell, Paris, France). The antibodies were incubated for 90 minutes or overnight at 4\(^\circ\)C. Next, they were incubated with the biotinylated anti-mouse antibody (VECTOR, Burlingame, USA) for 45 minutes, followed by the biotin-streptavidin-alkaline phosphatase complex (DAKO, Copenhagen, Denmark) for 30 minutes. All washes were carried out using TBS.

For staining, the slides were incubated in a freshly prepared solution composed of naphthol AS-MX phosphate (SIGMA Chemical Co, St Louis, USA) dissolved in dimethylformamide (MERCK, Darmstadt, Germany) plus Fast Red dye (DAKO, Copenhagen, Denmark). In order to block endogenous alkaline phosphatase, levamisole (SIGMA Chemical Co, St Louis, USA) was added. This procedure was carried out under microscopy, and was interrupted with TBS. The slides were then counterstained with Mayers haemalum (MERCK, Darmstadt, Germany) for two minutes. Cover glasses were placed in previously heated Glycergel (DAKO, Copenhagen, Denmark).

**Analysis of results**

The slides were analyzed by optic microscopy with a 400x magnification over the entire biopsy specimen in order to count the number of positive cells (stained in red). The total number of positive cells was expressed relative to the total area of the biopsy. A previously established 5.096 correction factor was used to calculate the number of positive cells per square millimeter (mm\(^3\)).

To investigate a possible association between the expression of cytotoxic mediators and the intensity of the rejection, a correlation was established between the number of positive cells for perforin, granzyme B, FAS-L, and the \(t\) score as per Banff’s classification\(^{(14)}\). Each slide from the AR group was scored from 0 to 3, according to the degree of tubulitis.each one of the markers in the different groups studied. From the
average number of positive cells/mm\(^2\) of the normal group, expression limit values were calculated for each mediator for the diagnosis of AR (mean plus twice the standard deviation).

**Statistical analysis**

The statistical analysis for quantification of the number of cells positive for perforin, granzyme B, FAS, and FAS-L in the four groups studied, was performed with the one-way ANOVA test after data normalization through logarithmic transformation, with p < 0.05 considered significant. All the quantitative data were expressed as mean ± standard error of mean (mean ± SEM).

Additionally, the correlation between the expression of these mediators in cases of AR and the Banff\(^{(14)}\) classification \(t\) score was analyzed using Sperman’s test, with \(a < 0.05\) considered significant.

The correlation between the time of chronic rejection and the expression of these mediators was made using a non-paired Student’s \(t\) test after data had been normalized with logarithmic transformation, with \(p < 0.05\) considered significant.

**RESULTS**

**Demographic data**

In the 96 biopsies analyzed, 56 had been diagnosed as “AR”; 31 as “chronic rejection”; and 9 as “no rejection,” and had been allocated to the respective groups. Additionally, 20 biopsies from normal renal tissue were studied, composing the control group. Mean age of patients was similar among the groups studied (AR; chronic rejection; no rejection; and control), as was the gender distribution. Prevalence of living donors was significantly higher in the control group when compared to the acute rejection and chronic rejection groups. Average postoperative time to biopsy was significantly higher in cases of chronic rejection relative to the other groups. These findings are summarized in table 1.

**Table 1. Demographic data of patients in different groups**

<table>
<thead>
<tr>
<th>Characteristics of the population</th>
<th>Acute rejection</th>
<th>Chronic rejection</th>
<th>No rejection</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>38</td>
<td>29</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>Total number of biopsies</td>
<td>56</td>
<td>31</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40 ± 2</td>
<td>33 ± 2</td>
<td>42 ± 3</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>Male</td>
<td>34 (89.5%)</td>
<td>19 (66%)</td>
<td>8 (89%)</td>
<td>11 (55%)</td>
</tr>
<tr>
<td>Living donor</td>
<td>15 (39%)(^*)</td>
<td>11 (38%)(^*)</td>
<td>6 (67%)</td>
<td>17 (85%)</td>
</tr>
<tr>
<td>PO of biopsy (days)</td>
<td>55 ± 8</td>
<td>2551 ± 544(^*)</td>
<td>57 ± 9</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^{*}\)\(p < 0.001\) vs. other groups.

\(^{\#}\)\(p < 0.05\) vs. control.

**Expression of cytotoxic mediators evaluated by immunohistochemistry**

Positivity for perforin was observed in mononuclear cells, especially in areas of interstitial infiltrates. Immunoreactivity for perforin, besides being present in the cytoplasm, was also observed as an intense reddish halo around positive cells (figure 1A). The expression of granzyme B was also detected in mononuclear cells, primarily in renal interstitium (figure 1B) and, in some cases, positive cells were found infiltrating glomeruli and invading tubules (tubulitis) (figure 1C). Positivity for FAS-L was intensely evident in cytoplasm of mononuclear cells (figure 1D) in the renal interstitium, similar to perforin.

Unlike the expression pattern of other abovementioned mediators, FAS expression was detected in tubular epithelium cells, with a greater tendency of staining distal tubules.

**Quantification of cytotoxic mediator expression**

The quantitative evaluation of the number of positive cells for perforin showed that the expression of this marker was significantly higher in the AR group (4.84 ± 0.65 cells/mm\(^2\)) when compared to the chronic rejection (0.71 ± 0.13 cells/mm\(^2\); \(p < 0.01\)), no rejection (0.75 ± 0.27 cells/mm\(^2\); \(p < 0.01\)), and control (0.44 ± 0.14 cells/mm\(^2\); \(p < 0.001\)) groups (table 2). Additionally, for granzyme B, expression was notably higher in the AR group (30.05 ± 7.94 cells/mm\(^2\)) when compared to the chronic rejection (11.40 ± 3.84 cells/mm\(^2\); \(p < 0.01\)) and no rejection (5.41 ± 1.94 cells/mm\(^2\); \(p < 0.01\)) groups. Expression of granzyme B in the chronic rejection and no rejection groups was higher, particularly...
in comparison to the control group (0.47 ± 0.18; p < 0.05). There was no statistical difference in perforin and granzyme B expression between the chronic rejection and no rejection groups (figure 2).

Table 2. Analysis of expression of cytotoxic mediators according to severity of acute rejection by Banff classification "t" score

<table>
<thead>
<tr>
<th>Expression of cytotoxic mediators</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perforin (cells/mm²)*</td>
<td>1.91 ± 0.64</td>
<td>3.04 ± 0.87</td>
<td>5.08 ± 1.18</td>
<td>5.91 ± 2.36</td>
</tr>
<tr>
<td>Granzyme B (cells/mm²)</td>
<td>4.03 ± 3.60</td>
<td>14.73 ± 6.03</td>
<td>49.48 ± 20.82</td>
<td>7.40 ± 3.78</td>
</tr>
<tr>
<td>FAS-L (cells/mm²)</td>
<td>10.90 ± 4.56</td>
<td>25.74 ± 12.30</td>
<td>31.67 ± 16.08</td>
<td>23.99 ± 20.45</td>
</tr>
</tbody>
</table>

* r = 0.28 between "t" score and perforin expression p = 0.05.

In the quantitative evaluation for the expression of FAS-L, the number of positive cells was significantly higher in the AR group (24.44 ± 5.56 cells/mm²) compared to the no rejection group (5.64 ± 0.96 cells/mm²; p < 0.01), but did not reach statistical significance when compared to the chronic rejection group (18.87 ± 6.83 cells/mm²). The expression of FAS-L was significantly higher in groups AR, chronic rejection, and no rejection in comparison to the control group (1.13 ± 0.26 cells/mm²; p < 0.01) (figure 2).

No difference was noted in FAS expression among all the groups analyzed. Considering the homogenous expression pattern of this marker in renal tubules among the groups, quantification of this marker was not relevant for this study.

Correlation between expression of cytotoxic mediators and severity of the acute rejection

Considering the Banff(14) t score analysis in the acute rejection group, in the group of biopsies with a t score = 0, the mean was 1.91 ± 0.64 cells/mm² for perforin, 4.03 ± 3.60 cells/mm² for granzyme B, and 10.90 ± 4.56 cells/mm² for FAS-L. For a t score of 1, the mean was 3.04 ± 0.87 cells/mm² for perforin, 14.73 ± 6.03 cells/mm² for granzyme B, and 27.54 ± 12.30 cells/mm² for FAS-L.

Results for the group with a score of 2 were: 5.08 ± 1.18 cells/mm² for perforin, 49.48 ± 20.82 for granzyme B, and 31.67 ± 16.08 cells/mm² for FAS-L. For a score of 3, the mean was 5.91 ± 2.36 cells/mm² for perforin; 7.40 ± 3.78 cells/mm² for granzyme B, and 23.99 ± 20.45 cells/mm² for FAS-L. The data are displayed on Table 2.

With perforin, a modest correlation (r = 0.28) between the Banff(14) classification t score and the degree of marker expression was identified (p = 0.05). As for the other markers, this association was not evident.

Analysis of sensitivity and specificity of the expression of cytotoxic mediators for the diagnosis of acute rejection

The results of this analysis showed that perforin is highly sensitive (80%) and specific (84.3%) for the diagnosis of AR. Although granzyme B has a good sensitivity (86.1%), it did not show a high specificity (35.5%). Similar to granzyme B, FAS-L was sensitive (72.9%), but not very specific (45.4%) for the diagnosis of AR. Combination analysis of the markers showed an increase in diagnostic sensitivity, but a loss in specificity (table 3).

Table 3. Analysis of sensitivity and specificity of cytotoxic mediators for diagnosis of acute rejection

<table>
<thead>
<tr>
<th>Mediators</th>
<th>Limit (cells/mm²)*</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perforin</td>
<td>1.53</td>
<td>80.0</td>
<td>84.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>1.93</td>
<td>86.1</td>
<td>35.5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>FAS-L</td>
<td>3.33</td>
<td>72.9</td>
<td>45.4</td>
<td>Ns</td>
</tr>
<tr>
<td>Perforin + Granzyme</td>
<td>-</td>
<td>83.9</td>
<td>46.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Perforin + FAS-L</td>
<td>-</td>
<td>82.4</td>
<td>56.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Granzyme + FAS-L</td>
<td>-</td>
<td>92.1</td>
<td>33.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Perforin Granzyme + FAS-L</td>
<td>-</td>
<td>87.7</td>
<td>36.2</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

* Limit value of expression obtained by the mean expression of mediators in the control group plus twice-standard deviation.
Expression of cytotoxic mediators in chronic rejection

The expression of cytotoxic mediators in the chronic rejection group, although significantly lower than in the AR group, was not related to post-transplantation time (table 4). The expression of perforin, granzyme B, and FAS-L was not significantly higher in patients who developed chronic rejection in less than three years post-transplant (0.85 ± 0.17, 12.91 ± 5.57 and 23.33 ± 9.66 cells/mm², respectively) than those diagnosed with chronic rejection more than three years post-transplant (0.41 ± 0.18, 8.40 ± 3.30 and 9.2 ± 4.34 cells/mm², respectively). The data are shown on table 4.

Table 4. Expression of perforin, granzyme B and FAS-L according to post-transplantation for diagnosis of chronic rejection

<table>
<thead>
<tr>
<th>Expression of mediators</th>
<th>Chronic rejection&lt;3 years</th>
<th>Chronic rejection≥3 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perforin (cells/mm²)</td>
<td>0.85 ± 0.17</td>
<td>0.41 ± 0.18</td>
</tr>
<tr>
<td>Granzyme B (cells/mm²)</td>
<td>12.91 ± 5.57</td>
<td>8.4 ± 3.30</td>
</tr>
<tr>
<td>FAS-L (cells/mm²)</td>
<td>23.3 ± 9.66</td>
<td>9.2 ± 4.34</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM.

DISCUSSION

Perforin and granzyme B are proteins produced by activated T CD8⁺ cells and that have a cytolytic affect on target-cells(4). This study characterized the expression of these mediators in renal allograft biopsies, demonstrating the presence of cytotoxic mechanisms in the rejection process. The expression of perforin and granzyme B was significantly increased in biopsies of the AR group. The results obtained in this study confirm the participation of the effector cytotoxic mechanisms (mediated by activated CD8⁺ lymphocytes) in the immune response to renal allograft. Additionally, the analysis of the expression of perforin and granzyme B by immunohistochemical technique proved capable of identifying cytotoxic mediator producing cells, and thus characterize in situ the presence of an active cytotoxic process of cell destruction.

As to the expression of FAS-L, findings showed that there was a non-significant increase of the expression of this marker in the AR group compared to the chronic rejection group, demonstrating that in spite of its high sensitivity, the specificity of this marker for acute rejection by immunohistochemistry is low. There are findings that underline the possibility that the presence of FAS-L is not absolutely necessary for mediation of rejection to the allograft, as was demonstrated by Larsen et al. in an experimental transplantation model using rats deficient for the production of FAS-L and that had acute rejection episodes.

Analysis of FAS expression showed that this molecule could be present in different situations, regardless of an association with the cytotoxic process. There seems to be a tendency towards the expression of this molecule in tissues diagnosed with rejection, since this expression is rare in normal biopsies(16).

The significantly higher expression of granzyme B and FAS-L in the no rejection group as compared to controls suggests an active cytotoxic process in the graft in this situation. This process was not detectable by the clinical features or even by histology with habitual staining methods.

Previous studies demonstrated that using PCR technique the expression of mRNA for perforin, granzyme B, and FAS-L in allograft biopsies is increased in acute rejection cases(9-13,17-18). Similarity, Vasconcellos et al. described an increased expression of mRNA for perforin and granzyme B in peripheral blood leukocytes in acute rejection cases(13). Some studies emphasize the possible utilization of the expression of these cytotoxic markers for diagnostic purposes, applied to non-invasive methods(13,19). On the other hand, the possibility of analyzing these markers in situ in allograft biopsies, with quantification and exact location of their production, highlights the importance of this study, especially regarding the use of biopsies as the main diagnostic method of graft rejection(20-21).

Concerning a correlation between the degree of cytotoxic mediator expression and the severity of the rejection, this study demonstrated a weak, albeit positive, association between the expression of perforin and the Banff t score(14). This association was not seen with the other mediators, primarily because of the great variability in expression of mediators in the acute rejection group. It is possible that in an analysis of a larger number of cases this correlation could be established. The study of cytotoxic mediator expression, as was proposed by Lipman et al. (22), seems to point more and more towards the role of these mediators as instruments for predicting cytotoxicity, especially under circumstances with a mononuclear infiltrate, but the diagnosis of rejection cannot be established by Banff criteria(14) as in cases of borderline alterations.

Conversely, in chronic rejection cases, there was no significant correlation between the expression of perforin, granzyme B, and FAS-L with post-transplant time. There is a tendency towards a higher expression of these mediators in cases with chronic rejection established in less than three years after the transplant, a fact that could be confirmed with a larger number of patients. These data indicate a possibility of an acute component, mediated by effector cellular mechanism dependent on CD8⁺ cells activated in cases of chronic rejection, particularly in early phases. In fact, several studies related initial episodes of acute or subclinical
Expression of cytotoxic mediators (perforin, granzyme B, FAS, and FAS-L) in renal allograft biopsies

rejection with the subsequent appearance of chronic rejection, suggesting that cytotoxic mediators might have a role in the development of chronic rejection. In addition, the presence of cytotoxic mechanisms in chronic rejection situations suggests the need for a reevaluation of the immunosuppressive regimen in these cases. Although increasing relevance has been given to the non-immune mechanisms involved in chronic rejection, the most important risk factors leading to allograft failure in this situation are the immunological risk factors.

The analysis of sensitivity and specificity of three of the mediators studied (perforin, granzyme, and FAS-L) regarding acute rejection showed that the immunohistochemical technique is sensitive enough to reveal the expression of these molecules when an active cytotoxicity process is present. The immunohistochemical technique for perforin, granzyme B, and FAS-L proved capable of characterizing the destruction pathway of the allograft and furthermore, identified the exact localization of this process on the biopsy tissue. Thus, this study demonstrated that the biopsy is still the most effective method for post-transplant monitoring, since it clearly recognizes the cells involved in the allograft rejection process.

CONCLUSION

This study demonstrated the expression of perforin, granzyme B, and FAS-L in allograft biopsies, particularly in cases with a clinical-histological diagnosis of acute rejection. The presence of these markers in chronic rejection cases, especially during early phases, reveals the presence of an active cytotoxic process, suggesting, in these cases, the need for a reevaluation of the immunosuppression regimen. The data point to the possibility of using these molecules as rejection markers that could become valuable instruments in post-transplant monitoring.

REFERENCES