

Immunohistochemical Profile of Acute Cellular Rejection in Renal Allograft

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Background : We wanted to find an adjunctive marker(s) in renal allograft biopsies for predicting acute cellular rejection (ACR), and so the expression patterns of immune-related molecules were compared between ACR, borderline ACR and non-ACR cases. **Methods :** The expression patterns of Fas ligand (FasL), HLA-DR, granzyme B, caspase-3, CD56, interferon stimulated growth factor-3 (ISGF-3), and CD53 were assessed via immunohistochemical study in 75 allograft biopsies from patients with ACR (n=19), borderline ACR (n=22), and non-ACR (n=34). **Results :** Compared to those of the non-ACR group, the ACR group revealed an elevated number of FasL positive interstitial inflammatory cells, HLA-DR positive tubular inflammatory cells, cytoplasmic caspase-3 positive tubular epithelial cells, granzyme B positive interstitial mononuclear inflammatory cells and CD53 positive interstitial inflammatory cells. The expression patterns of the borderline ACR group were similar to those of non-ACR group, except for the intensity of FasL in the tubular epithelial cells. **Conclusions :** Immunohistochemical investigations of the adjunctive markers FasL, HLA-DR, granzyme B, caspase-3 and CD56 can be useful for making the diagnosis of ACR.

Key Words : Renal transplantation; Graft rejection; Cellular immunity; Immunohistochemistry

Appropriate management of acute rejection is essential for preventing early graft loss and chronic rejection, which is one of the major causes of late graft loss. The diagnosis of acute rejection has traditionally relied heavily on histological assessment of the renal allograft biopsies. The diagnostic histological features of acute and chronic rejection are well defined, and classification schemes like the Banff schema are currently in wide use.^{1,2} Acute rejection is associated with cell-mediated and/or antibody-mediated immunity. The diagnosis of antibody-mediated rejection is aided by the immunohistochemical detection of the molecular marker C4d, a degradation product of an activated complement product. By comparison, acute cellular rejection (ACR) relies on the semiquantitative scoring of such histological features as tubulitis and arteritis. Most ACR cases with arteritis or with moderate to severe tubulitis are readily diagnosed. However, the borderline changes showing mild tubulitis are histological features that are suspicious for, but are not definitely diagnostic for ACR. Also confounding the diagnosis of ACR is the association of tubulitis with non-rejecting allograft nephropathies, such as IgA nephropathy (IgAN), which is one of the most com-

mon post-transplant glomerulopathies.³ Therefore, employing adjunctive markers would be helpful for making the diagnosis of ACR and administering the appropriate patient management.

ACR is induced by two distinct mechanisms. One mechanism involves CD8+ cytotoxic T cell-mediated graft destruction that kills targeted cells by activation of executioner caspases like caspase-3, and this is a consequence of granzyme-perforin- or Fas-Fas ligand (FasL)-dependent pathways.^{4,5} Another mechanism involves the activated CD4+ helper T cell-mediated delayed hypersensitivity reaction that upregulates the expression of class II MHC molecules, including HLA-DR, in allograft parenchymal cells like renal tubular epithelial cells.⁴ In addition to T lymphocytes, the infiltration of other inflammatory cells, including macrophages, plasma cells, neutrophils and natural killer cells, has also been reported.⁶

The expression of a variety of immune/inflammation-related molecules has previously been evaluated in the biopsy tissues of patients who are rejecting transplants. Immunohistochemical studies of allografts have revealed an increase of various molecules in acute rejection at the protein level. These molecules included

class II MHC antigens (HLA-DR, DP and DQ),⁷⁻¹³ the interleukin 2 receptor,^{7,12,13} adhesion molecules (I-CAM and V-CAM),^{7,8,10-12} apoptosis inducing molecules (FasL, perforin and granzyme A and B),^{14,15} and inflammatory cells (T lymphocytes, B lymphocytes, natural killer cells and macrophages).^{7,13,15} The molecules differentially expressed at the mRNA level have recently been evaluated by RT-PCR and cDNA microarray techniques.¹⁶⁻¹⁹ In addition to the previously described molecules HLA-DR, FasL and granzyme B, novel molecules have also been identified. They are interferon stimulated growth factor-3 (ISGF-3), a member of the interferon regulatory factor family, and CD53, a member of the tetraspan transmembrane family. Among them, HLA-DR has been proposed as an ACR marker, but its diagnostic potential needs further validation.²⁰

In an attempt to identify a specific adjunctive molecular marker(s) of ACR that can be conveniently used for allograft biopsy specimens, we employed immunohistochemical methods to evaluate the expression patterns of various immune-related molecules (FasL, HLA-DR, granzyme B, caspase-3, CD56, ISGF-3 and CD53) in allografts of ACR, borderline ACR and non-ACR patients. To the best of our knowledge, this is the first study to explore the expression patterns and diagnostic usefulness of the newly described molecules CD53 and ISGF-3 in renal allografts at the protein level by immunohistochemical method.

MATERIALS AND METHODS

This study was approved by the Asan Medical Center Institutional Review Board. A total of 75 biopsy specimens from 68 patients were included in this retrospective study. Allograft biopsies were performed to identify the cause of acute renal dysfunction. Histological, immunofluorescence, immunohistochemical and ultrastructural studies using standard procedures, were em-

ployed to evaluate cell-mediated and antibody-mediated rejection and other pathologies.²¹ The specimens for histological examination were fixed in Bouin fixative, embedded in paraffin and then cut into 2 μ m sections. Subsequently, hematoxylin-eosin, periodic acid-Schiff, periodic acid-silver methenamine and Masson's trichrome staining was performed. Immunofluorescence study for IgG, IgM, IgA, C3, C4, C1q and fibrinogen was performed using snap-frozen acetone-fixed tissues. Immunohistochemical staining for C4d (Biomedica Gruppe, Vienna, Austria, 1:20 dilution) was performed to evaluate antibody-mediated rejection, which was regarded as positive when diffuse and strong staining was observed in more than 50% of the peritubular capillaries.²²

Immunohistochemical investigations of the molecular markers were performed on the residual tissues after performing the above conventional pathological studies. The expression of FasL was evaluated in 66 allograft biopsies and the expressions of HLA-DR, granzyme B, cleaved (Asp 175) caspase-3, which is an activated form of caspase-3, and CD56 were evaluated in 67 biopsies with using the Bouin-fixed paraffin-embedded tissues. The expression of ISGF-3 and CD53 was evaluated in 40 and 38 biopsies, respectively, with using snap-frozen acetone-fixed tissues. Immunohistochemical investigations were performed using standard procedures and a universal secondary antibody kit (iView™-DAB detection kit, Ventana Medical Systems, Inc., Tucson, AR, USA).²¹ All the sections were blocked for endogenous biotin to reduce the nonspecific immunopositivity (Endogenous Biotin Blocking Kit, Ventana Medical Systems, Inc., Tucson, AR, USA). Diaminobenzidine was used as a chromogen and the tissues were counterstained with hematoxylin.

For histological evaluation of FasL, HLA-DR, caspase-3, granzyme B and CD56, which were all expressed in a limited number of cells, the number of immunopositive cells was counted in the most active area at one high power field at a magnification

Table 1. Antibodies and studied expression patterns

Antibody	Dilution	Source	Expression patterns	
			Inflammatory cells	Tubular epithelial cells
FasL	1:100	LabVision Corp, Fremont, CA	Number	Number, intensity
HLA-DR	1:800	eBioscience, San Diego, CA	Number	Number
Caspase-3 ^a	1:200	Cell Signaling Technology, Inc., Beverly, MA	Number	Number, subcellular localization
Granzyme B	1:400	LabVision Corp, Fremont, CA	Number	NA ^b
CD56	1:400	Novocastra Laboratories Ltd, UK	Number	NA ^b
CD53	1:20	LabVision Corp., Fremont, CA	Extent, intensity	Extent
ISGF-3	1:50	Santa Cruz Biotechnology, Inc., California, CA	NA ^b	Extent

Number, the number of positive cells in one high power field magnification at $\times 400$ in the most active area.

^a: identifies cleaved (Asp175) caspase-3, an activated form of caspase-3; ^b: non-applicable due to immunonegativity.

of $\times 400$ (HPF). Because the CD53 and ISGF-3 were expressed in a large number of cells, the immunostaining results were described as an extent [negative, focal (<50%), or diffuse ($\geq 50\%$)] for the inflammatory cells or as a percentage for the tubular epithelial cells. The primary antibodies used for these investigations and the evaluated expression patterns are summarized in Table 1.

The data was analyzed using SPSS software (12.0 K). The Chi-square, Fischer's exact test, the Mann-Whitney U test, the Kruskal-Wallis test and Spearman's rank correlation coefficient were employed when appropriate. Differences were regarded as statistically significant at $p < 0.05$.

RESULTS

Clinical and pathologic data

The 75 biopsies were divided into three groups according to the Banff schema. They included 19 cases of ACR, 22 cases of borderline ACR and 34 cases of non-ACR.² The ACR cases consisted of 4 cases of IA, 11 of IB and 4 of IIA. The pathological features of the non-ACR cases included 13 cases of IgAN, 6 of chronic allograft nephropathy (CAN), 1 of BK nephropathy, 3 of acute pyelonephritis and 3 of acute tubular necrosis without C4d immunoreactivity. There were no pathological features in

8 of the non-ACR cases. The clinical and pathologic data from the 75 biopsies were summarized in Table 2. There were no differences among the three groups except for the expression of the humoral rejection marker C4d, which was observed more frequently in the rejecting allografts ($p = 0.008$, Fischer's exact test). While it was not clear whether the IgA deposits represented an

Table 2. Demographic, clinical and pathologic data

	ACR	Borderline ACR	Non-ACR
Biopsy	19	22	34
Patient (M/F)	14 (9/5)	21 (15/6)	33 (24/9)
Age (yr) ^a	37.8 \pm 8.0	41.8 \pm 10.9	41.6 \pm 11.7
Donor (L/C)	11/8	19/3	24/10
Donor age (yr) ^a	42.2 \pm 4.6	39.2 \pm 10.5	35.3 \pm 15.3
Duration (mo) ^b	33.2	75.2	38.6
	(0.3-110.9)	(1.0-156.4)	(0.5-163.4)
Matched HLA ^a	2.3 \pm 1.3	2.2 \pm 1.5	2.8 \pm 1.6
Baseline drug regimen			
Cyclosporine/FK506	10/9	16/6	23/12
MMF/AZT/CTX	7/9/1	7/8/1	19/7/3
Steroid (yes/no)	19/0	20/2	31/5
Cr level (mg/dl) at biopsy ^a	4.0 \pm 5.1	2.0 \pm 0.5	2.3 \pm 2.1
Cr elevation (%) ^a	213 \pm 109	147 \pm 56	171 \pm 191
C4d (%) ^c	6 (31.6%)	2 (9.5%)	1 (2.9%)
IgAN (%)	5 (26.3%)	10 (45.5%)	13 (38.2%)

M, male; F, female; L, living-related donor; C, unrelated cadaver donor; mo, months; MMF, mycophenolate mofetil; AZT, azathioprine; CTX, cyclosporin; Cr, creatinine; Cr elevation (%), percentage of peak Cr/baseline Cr; IgAN, IgA nephropathy.

^a: mean \pm standard deviation; ^b: median (range); ^c: $p = 0.008$.

Table 3. Expression patterns of molecular markers

Markers	Expression	ACR	Borderline ACR	Non-ACR	p-value ACR vs non-ACR
FasL	Inflammatory cells ^a	1.1 \pm 1.0	0.2 \pm 0.5	0.3 \pm 0.8	0.001
	Tubular epithelial cells				
	Number ^a	6.2 \pm 9.6	6.5 \pm 10.1	15.5 \pm 28.0	NS
	Intensity (-/+ /++)	10/4/2	11/8/2	9/4/16	0.02
HLA-DR	Inflammatory cells				
	Interstitial ^a	123.7 \pm 121.1	65.5 \pm 75.1	65.6 \pm 78.3	NS
	Tubules ^a	5.1 \pm 4.4	1.7 \pm 2.2	1.7 \pm 2.6	0.001
	Tubular epithelial cells ^a	1.00 \pm 3.0	0.32 \pm 0.8	0.2 \pm 0.9	NS
Caspase-3	Inflammatory cells ^a	0.7 \pm 1.1	0.3 \pm 0.5	0.3 \pm 0.8	NS
	Tubular epithelial cells				
	Nucleus ^a	1.8 \pm 7.5	9.8 \pm 28.8	4.3 \pm 13.8	NS
	Cytoplasm ^a	0.5 \pm 0.6	0.1 \pm 0.2	0.1 \pm 0.4	0.026
Granzyme B	Neutrophils ^a	6.0 \pm 10.6	11.4 \pm 41.6	9.2 \pm 29.5	0.038
	Non-neutrophilic inflammatory cells ^a	4.6 \pm 3.8	2.1 \pm 2.4	1.8 \pm 2.1	0.002
CD56	Inflammatory cells ^a	1.1 \pm 2.1	1.7 \pm 1.9	1.1 \pm 1.6	NS
CD53	Inflammatory cells (-/F/D)	0/1/8	1/7/5	4/10/2	0.014
	Tubular epithelial cells (%)	11.3 \pm 18.9	20.0 \pm 26.8	27.2 \pm 37.1	NS
ISGF-3	Tubular epithelial cells (%)	21.9 \pm 32.0	31.2 \pm 34.2	35.6 \pm 39.9	NS

Numerical data represent mean \pm standard deviation. Cases with no available tissues for each immunostaining were excluded.

NS, statistically not significant; F, focal; D, diffuse.

^a: the number of cells in one high power field magnification at $\times 400$ in the most active area.

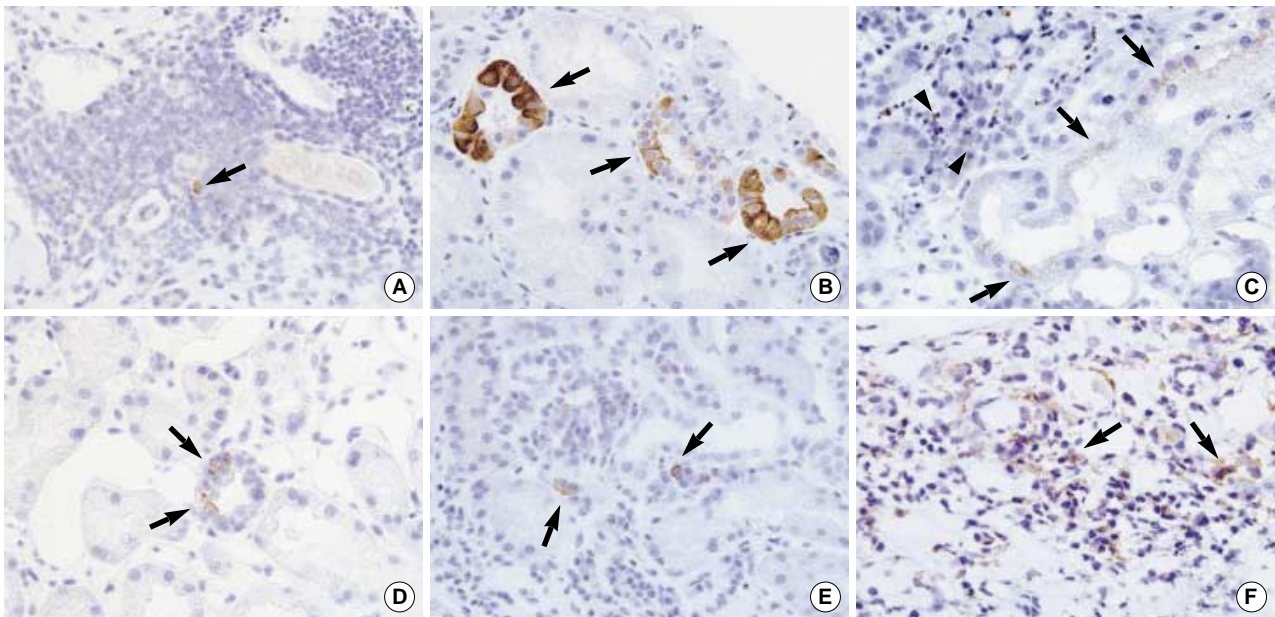


Fig. 1. Immunohistochemical studies reveal FasL positive interstitial mononuclear inflammatory cells (arrow) (A), FasL positive tubular epithelial cells (arrows) (B), HLA-DR positive tubular epithelial cells (arrows) and interstitial mononuclear inflammatory cells (arrowheads) (C), cytoplasmic caspase-3 positive tubular epithelial cells (arrows) (D), granzyme B positive interstitial mononuclear inflammatory cells (arrows) (E), and CD53 positive interstitial mononuclear inflammatory cells (arrows) (F) (A and C-F, ACR; B, non-ACR).

incidental finding or IgAN, a diffuse and predominantly mesangial IgA deposition was noted in 28 out of the total 75 cases (37%). It was more frequent among the borderline ACR cases (45.5%), although this difference was not statistically significant.

Expression patterns of the immune-related molecules in the ACR and non-ACR groups

Among the 7 molecules we tested for, FasL, HLA-DR, caspase-3, granzyme B and CD53 were differentially expressed between the ACR and non-ACR groups. The expression patterns of all the molecules we tested for the ACR, borderline ACR and non-ACR groups are summarized in Table 3.

FasL was expressed in the cytoplasm and cytoplasmic membrane of the inflammatory cells and tubular epithelial cells (Fig. 1A, B). The FasL positive interstitial mononuclear inflammatory cells were observed more frequently in the ACR group than the non-ACR group ($p=0.001$, Kruskal-Wallis test), although they were not abundant in either group. By comparison, the tubular epithelial cells expressed FasL at a stronger intensity in the non-ACR group than in the ACR group ($p=0.02$, Fischer's exact test). The greater number of FasL positive tubular epithelial cells in the non-ACR group was not statistically significant.

HLA-DR was expressed in the cytoplasm of the inflammatory cells and tubular epithelial cells (Fig. 1C). The number of HLA-

DR positive inflammatory cells that infiltrated the tubules was significantly higher in the ACR group than in the non-ACR group ($p=0.001$, Kruskal-Wallis test). The numbers of HLA-DR positive tubular epithelial cells and interstitial inflammatory cells tended to be higher in the ACR group, but this difference was not statistically significant.

Caspase-3 was expressed in the cytoplasm and/or nucleus of the tubular epithelial cells and inflammatory cells (Fig. 1D). Even in foci of severe tubulitis, only a limited number of tubular epithelial cells were positive for caspase-3. The number of tubular epithelial cells expressing caspase-3 in the cytoplasm was significantly larger in the ACR group than in the non-ACR group ($p=0.026$, Kruskal-Wallis test).

Granzyme B was expressed in the cytoplasm of the neutrophils and mononuclear inflammatory cells. The number of granzyme B positive neutrophils was larger in the non-ACR group than in the ACR group ($p=0.038$, Kruskal-Wallis test). After exclusion of the pyelonephritis cases in the non-ACR group, there was no statistically significant difference between the two groups. By comparison, granzyme B positive interstitial mononuclear inflammatory cells were more frequent in the ACR group than in the non-ACR group (Fig. 1E) ($p=0.002$, Kruskal-Wallis test).

CD53 was expressed in the cytoplasm of the interstitial mononuclear inflammatory cells and tubular epithelial cells (Fig. 1F). The ACR group revealed more diffuse CD53 positive mononu-

clear inflammatory cells than did the non-ACR group ($p=0.014$, Fischer's exact test). The number of tubular epithelial cells expressing CD53 was not significantly different between the ACR and non-ACR groups.

CD56, a natural killer cell marker, was expressed in a few mononuclear inflammatory cells in both groups. ISGF-3 was expressed only in the tubular epithelial cells. The expression patterns of CD56 and ISGF-3 did not show any statistically significant differences between the two groups.

Expression patterns of immune-related molecules in the borderline ACR group

The 5 markers differentially expressed between the ACR and non-ACR groups also revealed different expression patterns in the borderline ACR group when compared to the ACR or non-ACR groups (Table 3). Among the 6 expression patterns of the 5 markers in the borderline ACR group, 5 patterns were similar to those of the non-ACR group, whereas only 1 was similar to those of the ACR group. Those 5 patterns similar to the non-ACR group were FasL in interstitial inflammatory cells, HLA-DR in tubular inflammatory cells, cytoplasmic caspase-3 in tubular epithelial cells, and granzyme B and CD53 in interstitial inflammatory cells. The intensity of FasL immunoreactivity in the tubular epithelial cells was similar to that of the ACR group.

DISCUSSION

Although the overexpression of CD53 and ISGF-3 at the mRNA level in ACR has been reported by cDNA microarray studies,^{17,23} our study is the first to explore their expression patterns at the protein level in ACR, borderline ACR and non-ACR cases. Only the CD53 expression was up-regulated in ACR and the ISGF-3 expression was not different among the three groups.

A unique immunologic event against a donor kidney initiates the rejection phenomenon, resulting in mixed inflammatory cell infiltration, tubulitis and occasionally vasculitis. The inflammatory cells in ACR mostly consist of T lymphocytes and macrophages, although plasma cells, neutrophils and natural killer cells may also be present.⁶ It has been known that granzyme B was expressed in cytotoxic T cells, natural killer cells and neutrophils. The granzyme B was involved in target cell apoptosis during cell-mediated cytotoxicity. The present study demonstrated an increase of the granzyme B expression in mononuclear inflammatory cells in the ACR group, as compared to the borderline

ACR and non-ACR groups, while the expression of the natural killer cell marker CD56 was not different among the three groups. This finding suggests the increased activity of cytotoxic T lymphocytes has more an important role for ACR than do the natural killer cells.

It's been reported that upregulation of the HLA-DR expression was present in the rejecting tubular epithelial cells and inflammatory cells, and this correlated with cellular infiltration and late graft dysfunction during acute rejection.^{8-11,16,24} Like the previous reports, the present study also demonstrated an increased HLA-DR expression in the ACR group.

In addition to HLA-DR, this study showed that expression of FasL in inflammatory cells and tubular epithelial cells can also be used as an immunohistochemical marker of ACR. A dual role for the Fas/FasL pathway has been suggested in transplantation-induced processes. Engagement of the FasL on the cytotoxic T lymphocytes with the Fas on the graft cells activates a cell death pathway and results in tissue damage.²⁵ FasL also plays a role in peripheral tolerance, and its expression was found in those tissues that were traditionally thought of as being immunologically privileged, such as sperm cells in the testes and the cells in the anterior chamber of the eye.^{26,27} As expected, two distinct FasL expression patterns were noted in the present study. FasL positive interstitial inflammatory cells were more frequently found in the ACR group than in the non-ACR group, whereas FasL positive tubular epithelial cells revealed stronger intensity in the non-ACR group than that in the ACR group. These findings are consistent with the proposed dual role of the Fas/FasL pathway in transplantation rejection.

Pathologically, borderline ACR is accompanied by mild interstitial and tubular inflammatory cell infiltration, and the extent of this lies between ACR and non-ACR. It is interesting that the expression patterns of the molecules in the inflammatory cells of the borderline ACR group resembled those of the non-ACR group. In contrast, the intensity of FasL in the tubular epithelial cells of the borderline ACR group is similar to that of the ACR group. This suggests rejection-related molecular alteration of the tubular epithelial cells may be predominant or it may precede that of the infiltrating inflammatory cells in borderline ACR.

In summary, the present study has shown that HLA-DR, FasL, caspase-3, granzyme B and CD53 were differentially expressed among the ACR, borderline ACR and non-ACR groups, suggesting their potential use as adjunctive immunohistochemical markers for ACR. A further prospective study with a larger number of cases would be beneficial for validating the results from this current, small retrospective study.

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