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ORIGINAL ARTICLE

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Selective intra-graft apoptosis and down-regulation of lymphocyte bcl-2, iNOs and CD95L expression in kidney–pancreas transplanted patients after anti-Thymoglobulin induction

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Abstract Intra-graft infiltrating cells apoptosis was evaluated in 20 consecutive kidney–pancreas transplanted (KP) patients without kidney rejection. Two fine-needle aspirated biopsy (FNAB) and two peripheral blood lymphocytes (PBL) samples were obtained 14 days after transplantation. Immunosuppression was based on anti-Thymoglobulins (ATG) induction for 7 days and cyclosporine/mofetil mycophenolate as maintenance therapy. Ten matched healthy subjects were chosen as controls for PBL. Lymphocyte phenotypes and activation markers, apoptotic rate and lymphocyte expression of pro/anti-apoptotic molecules were analysed by flow cytometry analysis (FACS). Lymphocyte phenotypes and activation markers: higher levels of CD8 and CD4DR were evident in the graft ($p < 0.05$) than in PBL, CD3CD25 in PBL were higher in transplanted patients than in controls. Apoptotic rate and lymphocyte expression of pro- and anti-apoptotic molecules: a higher expression of annexin V, together with reduced lymphocytes CD95L, iNOs and Bcl-2 expression (PBL = 97.7 ± 1.1% vs FNAB = 81.9 ± 15.1%; $p < 0.05$) were evident in the graft than in PBL. In KP patients intra-graft apoptosis and reduced anti-apoptotic molecules were evident after ATG induction.

Keywords Apoptosis · bcl-2 · Anti-Thymoglobulin induction · FNAB

Introduction

Lymphocytes are cells of the immune system that are highly sensitive to apoptotic triggers [1]. Interestingly, increased lymphocytes apoptosis follows the recovery of renal function after steroid therapy for a rejection episode and has been found in the presence of immune tolerance in mouse [1, 2]. On the contrary, lymphocyte apoptosis does not occur during acute myocardial rejection, which is conversely attributed to bcl-2 over-expression [3]. Among rejection-free patients, the intra-graft expression of some activation markers was increased as compared with the peripheral circulation [1]. This could be an indirect sign of an active process that leads to immune tolerance. During the immune response, lymphocytes produce cytokines, which can influence their own life span and immune response [4]. Between these cytokines, nitric oxide (NO) produced by

NO synthase (NOs) can influence apoptosis. In particular, activated T cells, which generate high levels of NO, are protected from CD95L-induced cell death [5, 6]. In vitro, chronically activated T cells progressively down-regulate NOs expression and become sensitive to apoptosis [5, 6]. Many pro and anti-apoptotic molecules could have a role in influencing lymphocyte life span. Particularly, CD95L and CD95 (pro-apoptotic molecules) could once activated induce an active lymphocytes death [7]. On the contrary, bcl-2, a mitochondrial anti-apoptotic molecule, could counteract the shortening of lymphocyte life, e.g. by blocking oxidative-induced cell death [8].

The aim of the study was to evaluate whether, in patients bearing a full-functioning kidney–pancreatic graft, previously undergoing immunosuppressive treatment based on a short-term anti-Thymoglobulin induction and with cyclosporine/mofetil mycophenolate as maintenance therapy, the cells infiltrating the graft would selectively express a high apoptotic rate.

Patients and methods

From June 1999 to June 2000 a series of twenty consecutive type-1 diabetic uremic patients who had undergone kidney–pancreas transplantation were studied. Only patients with no rejection episodes were chosen. Inclusion criteria were the absence of any infections in the post-operative periods, the recovery of a good renal function with no need of dialytic treatment, and the absence of re-laparotomy. Patients who experienced CMV infections in the early post-operative (i.e. 14 days) were not recruited. Patients with clear signs of systemic infection, lymphoproliferative disease, urinary infection, enhanced erythrocyte sedimentation velocity, or C-reactive protein were excluded from the study. All the patients gave their informed consent to be enrolled in the study. We did not require a specific Institutional Review Board approval, given that the work has a primarily clinical involvement and helps us in defining the right recovery of renal function and the absence of graft infiltrates. Control subjects were enrolled between our nurses, and those who gave the consent were chosen as controls. All kidney–pancreas transplanted patients considered for the study were insulin independent with a good renal function.

Kidney–pancreas transplanted patients were chosen to evaluate the tolerogenic effects of their immunosuppressive protocols which consisted of induction with Thymoglobulins and maintenance therapy with cyclosporine and mycophenolate mofetil. Two fine-needle aspirated biopsies of the kidney (FNAB) and peripheral blood lymphocytes (PBL) were obtained in all the transplanted patients 14 days after transplantation (Protocol FNAB). The PBL were obtained from 10 healthy subjects (Controls), matched for age (40.3 ± 1.9 years) and sex.

Immunosuppression

All the transplanted patients received the following immunosuppressive treatment: anti-Thymoglobulin (Thymoglobulin, IMTIX SANGSTAT: 7 days) according to lymphocyte blood count; cyclosporine (range 100–250 ng/ml); mycophenolate mofetil (dosage from 500 to 2000 mg/day); and prednisone (10 mg/day). In the first 4–6 days, cyclosporine was maintained intravenously to achieve therapeutic ranges (data not given). After 2 months, prednisone was reduced with complete withdrawn at six months. Furthermore, cyclosporine levels were minimized to reduce nephrotoxicity. In all the patients markers of immunocompetence were analysed [white blood cells count (WBC), CD3 levels, CD19 levels, immunoglobulins levels]. None of the patients developed or showed any sign of acute or chronic rejection. Rejection was excluded on the basis of clinical parameters, stable creatinine values, normal resistance index at echography, and absence of rejection at the FNAB analysis.

FACS analysis

The FNAB and PBL were immediately analysed at flow cytometry to avoid the senescence or death of infiltrating cells. Given that usually the number of cells recovered from tissues of fine-needle biopsy is very small, to obtain a sufficient number of cells we performed at least two FNAB in the same site, always with echography guidance.

The FACS analysis was performed as previously reported [9]. The following markers were evaluated: (a) lymphocyte phenotypes (CD3, CD19, CD4, CD8); (b) lymphocytes activation marker (CD4CD25, CD8CD25, CD4DR, CD8DR); (c) apoptotic rate of infiltrating cells (with annexin V expression and DNA ploidy assessment); and (d) lymphocyte expression of pro-apoptotic (CD95, CD95-L) and anti-apoptotic (bcl-2, iNOs) molecules. The following antibodies were used: IgG1 FITC; IgG2 PE; CD3 FITC; CD4 FITC; CD8 FITC; CD25 PE; HLA-DR PE (Becton Dickinson, San Jose, Calif.); Fas FITC and Fas-ligand FITC (Bender Med Systems Diagnostics, Vienna, Austria); annexin V FLUOS staining kit (Boehringer, Mannheim, Germany); and Bcl2 and iNOS FITC (Santa Cruz Inc., Santa Cruz, Calif.). A single and two-colour direct conjugate fluorescence staining procedure was used for the study of surface markers expression (CD3, CD4, CD8, CD25, HLA-DR, Fas), while a single and two-colour direct conjugate fluorescence staining procedure was used for the study of intracellular markers expression (IgG1, IgG2, Fas-ligand, Bcl2, iNOS). Permeabilization of cells

was performed using Fix and Perm (Caltag Laboratories, Burlingame, Calif.). Apoptotic cells detection was performed using annexin V FLUOS staining kit (Boehringer, Mannheim, Germany).

Statistical analysis

All the data were expressed as mean ± standard error. Data were tested for normal distribution with the Kolmogorov–Smirnov test and for homogeneity of variances with Levene's test. Two-sided unpaired Student's *t*-test (for parametric data) and Mann-Whitney U-test (for non-parametric data) were used to compare peripheral and intra-graft parameters. A p value of less than 0.05 (by two-tailed testing) was considered an indicator of statistical significance. Analysis of data was done using SPSS statistical package for windows (version 10.1; SPSS, Chicago, Ill.).

Results

General characteristics

All the patients had good renal function, none of them experienced rejection. The patients showed the following characteristics: age (42.3 ± 2.9 years); diabetes duration (24.0 ± 5.3 years); dialysis duration (22.7 ± 6.4 months); cold ischaemic time of kidney and pancreas (708.1 ± 64.6 and 735.0 ± 42.2 min, respectively); donor age (32.1 ± 3.4 years); hospitalisation time (28.8 ± 5.1 days); time on waiting list (33.0 ± 15.2 months); HLA match (2.6 ± 0.4); and PRA levels (3.2 ± 2.2%). An amelioration of creatinine levels was evident during the follow-up (Fig. 1A) together with normalization of blood glucose levels (data not shown). A statistical reduction of white blood cell count (WBC) was evident during the follow-up (Fig. 1B). This was associated with an increase in the CD3 levels (even not statistical) and a reduction of CD19 levels (Fig. 1C, D), while immunoglobulin levels remained stable during the follow-up (Fig. 1E, F).

Lymphocyte phenotypes

Higher levels of CD3CD8 suppressor cells were evident in the graft than in periphery of the transplanted patients (PBL = 20.4 ± 3.3 vs FNAB = 27.7 ± 6.7%; $p < 0.01$).

Lymphocyte activation markers

A higher expression in lymphocytes of CD4DR, but not of CD3CD25, was evident in the graft as compared with periphery of transplanted patients (PBL = 3.3 ± 1.7 vs FNAB = 6.4 ± 5.6%, $p < 0.05$). CD3CD25 lymphocyte expression was higher in periphery of transplanted patients than in the controls (patients = 16.5 ± 2.5 vs controls = 0.1 ± 0.3%; $p < 0.01$). Also CD95 lymphocyte expression was higher in periphery of transplanted patients than in controls (patients = 56.7 ± 6.5 vs controls = 12.6 ± 12.5%, $p = 0.01$).

Apoptotic rate

An increased expression of annexin V was evident in the graft as compared with periphery (PBL = 0.7 ± 0.2 vs FNAB = 4.8 ± 1.2%; $p < 0.05$; Figs. 2A, 3A, B). The presence of lymphocyte apoptosis was confirmed with analysis of DNA, which revealed in lymphocytes a DNA peak different from diploid peak (data not shown).

Lymphocyte pro- and anti-apoptotic molecules

CD95 and CD95-L

No difference in CD95 expression in FNAB or PBL was evident. Furthermore, CD95-L lymphocyte expression was reduced in the graft (PBL = 85.8 ± 8.8 vs FNAB = 63.4 ± 20.1; $p < 0.05$; Fig. 2B).

iNOs

A reduced lymphocyte iNOs expression was more evident in the graft than in the periphery of the transplanted patients (PBL = 80.4 ± 7.5 vs FNAB = 63.0 ± 16.1%; $p < 0.05$; Fig. 2C).

Bcl-2

A reduced lymphocyte Bcl-2 expression was evident in the graft rather than in the periphery of the transplanted patients (PBL = 97.7 ± 1.1 vs FNAB = 81.9 ± 15.1%; $p < 0.05$; Figs. 2D, 3C, D).

Discussion

Our preliminary study shows the following:

1. The number of CD8 suppressor T lymphocytes is higher within the graft than in peripheral circulation of transplanted patients bearing a functioning kidney–pancreatic graft.
2. The CD4DR expression is higher in lymphocytes inside the graft than in periphery.

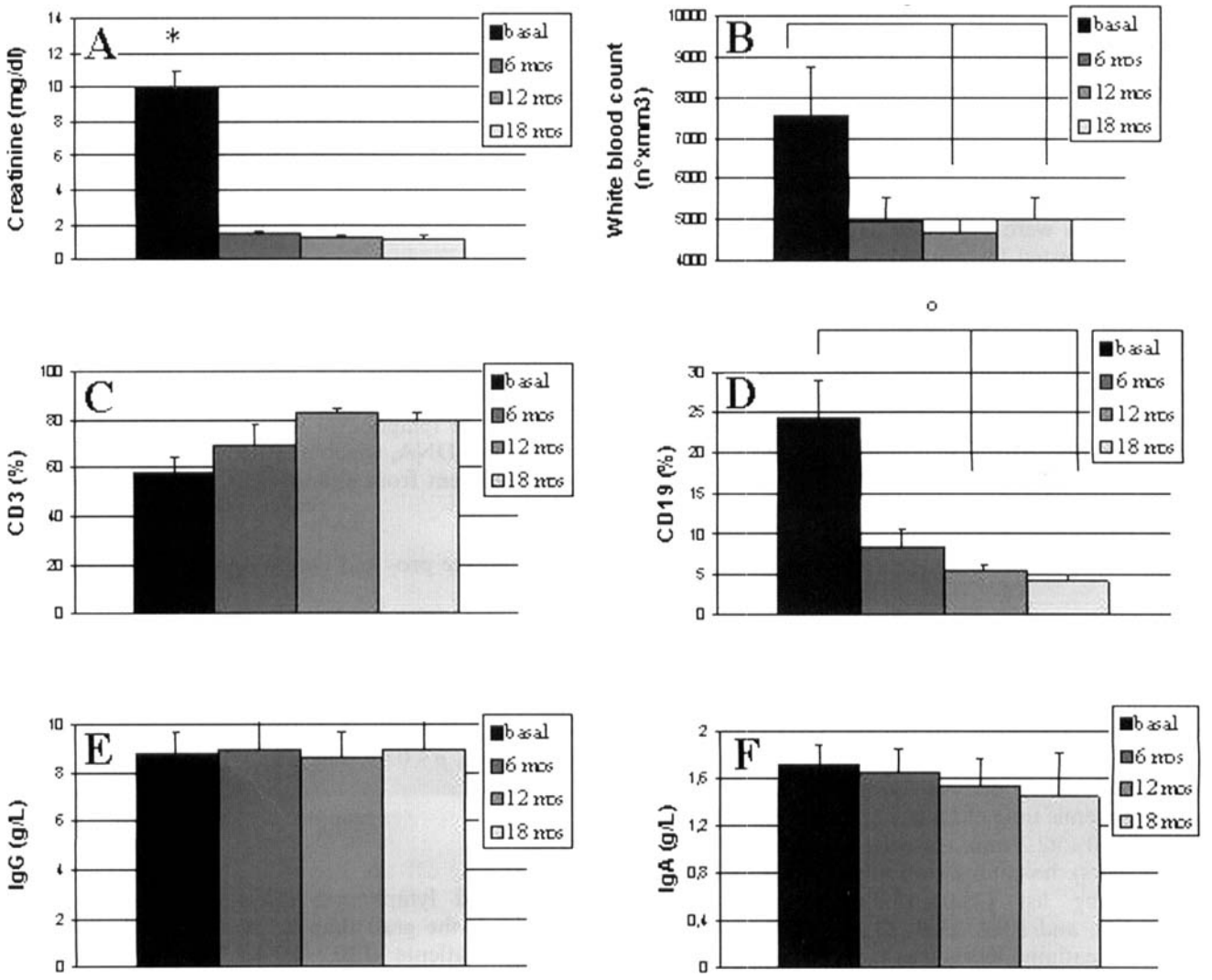


Fig. 1 **A** Creatinine levels (mg/dl). **B** White cell blood count (number×mm^3). **C** CD3 percentage. **D** CD19 percentage. **E** IgG levels and **F** IgA levels during the follow-up in the kidney–pancreas transplanted patients. * $p < 0.01$, ° $p < 0.05$

3. CD3CD25 activated lymphocytes are higher in peripheral circulation of transplanted patients than in normal controls.
4. In transplanted patients the expression of annexin V is higher in the cells infiltrating the graft than in the periphery.
5. A down-regulation of lymphocyte iNOs, Bcl-2, and CD95L expression is evident in the graft but not in the periphery of the transplanted patients. It is difficult to establish whether a correct immunocompetence was maintained during the entire follow-up, given that only few of the various immunological parameters were recorded.

These overall data suggest an ongoing process of late-lymphocyte activation. CD8-positive T suppressor cell recruitment within the graft appears to be evident, in association with a down-regulation of lymphocytes Bcl-2, iNOs, and CD95-L expression and a concomitant increase of apoptotic rate. Lymphocyte apoptosis, and modulation of surviving factors, might play a role in inducing graft acceptance in kidney–pancreas transplanted patients submitted to short-term anti-Thymoglobulin induction and undergoing cyclosporine/mofetil mycophenolate maintenance therapy.

Only T lymphocytes appear to be absolutely required for rejection. Lymphocytes are responsible for the production of cytokine which is necessary to stimulate the immune response and activate effectors cells [4]. The large amount of cytokine released during rejection can explain the increased lymphocyte activation and life span, and the resistance to steroid-induced cell death [10]. Even though proinflammatory cytokines are T-cell

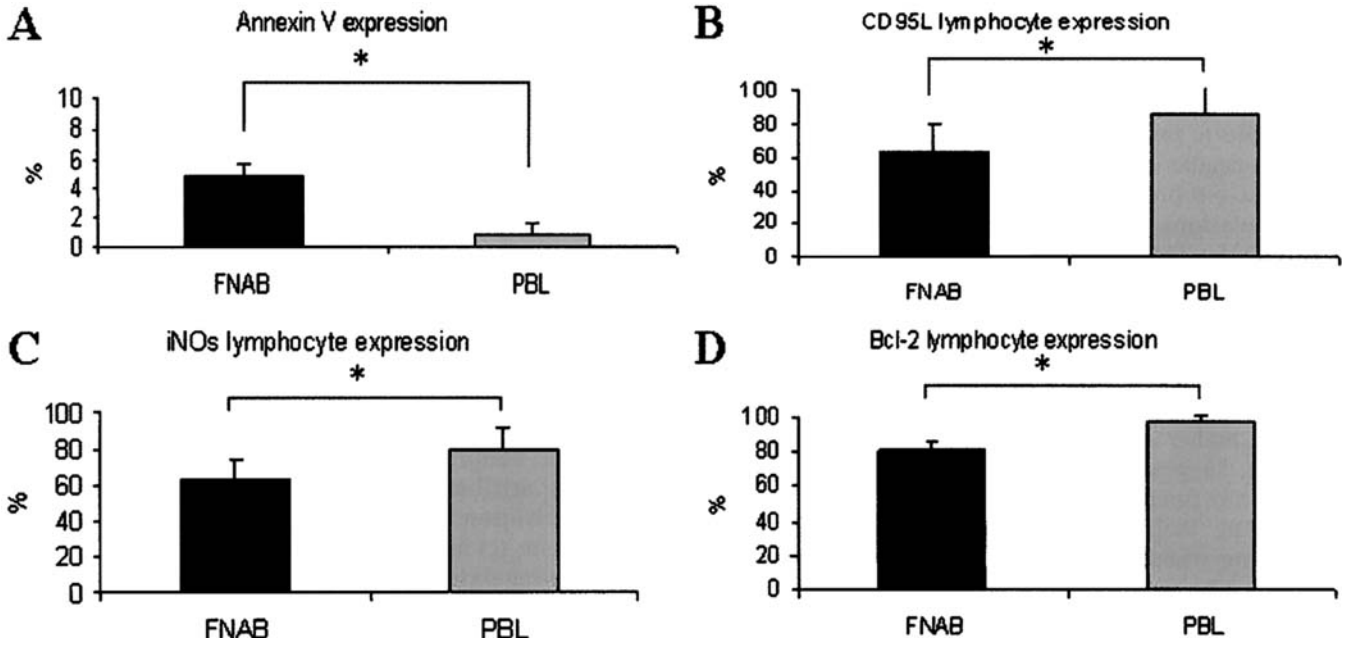
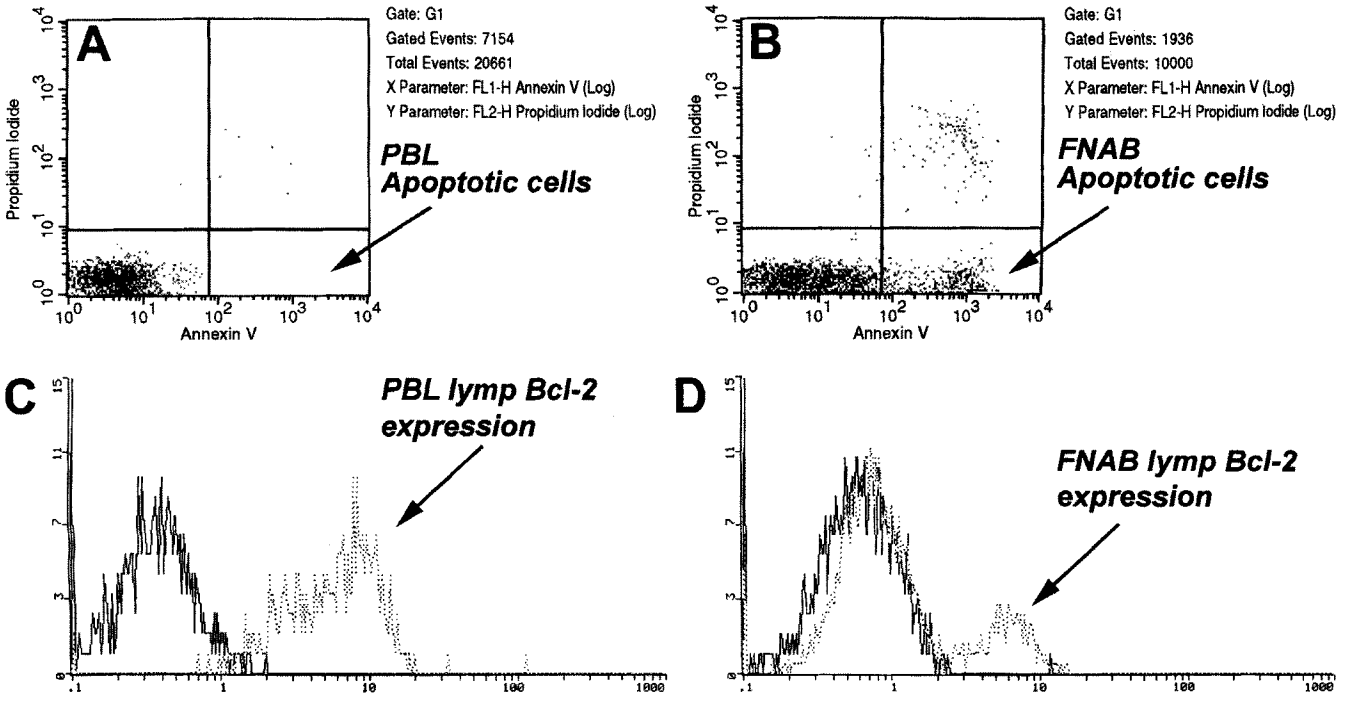


Fig. 2 A–D Flow cytometry (FACS) analysis of peripheral and fine-needle (*FNAB*) samples shows a reduction of anti-apoptotic molecules in FNAB as compared with periphery in the kidney–pancreas transplanted patients * $p < 0.05$.

growth factors, they simultaneously inhibit T-cell expansion and promote the apoptosis of activated T cells, such that they limit the immune responses and maintain T cell homeostasis [10]. Interestingly, previous studies have shown an increased number of activated T lymphocytes in well-functioning rejection-free grafts. The presence of activated lymphocytes in the graft might play an important role in the induction of tolerance [11]. It is likely that a tight balance exists between T-lymphocyte activation, sustained cytokine release, Bcl-2 and iNOs over-expression from a side, and T-lymphocyte

Fig. 3 A,B Flow cytometry (FACS) analysis of peripheral and fine-needle (*FNAB*) samples shows an increase of apoptotic cells (PBL = 0.5% vs FNAB = 6.1%), together with **C,D** reduced lymphocytes Bcl-2 expression in FNAB (18.9%) as compared with periphery (97.0%) in a kidney–pancreas transplanted patient



apoptosis, Bcl-2, and iNOs down-regulation from the other side. Such balance could be claimed as responsible for regulation of T-cell fate. The drop of iNOs, Bcl-2, and CD95-L expression could commit T lymphocytes to an apoptotic program.

Fine-needle aspirated biopsy is a safe, easy, and reproducible procedure, which allows studying the cell subpopulations infiltrating the graft [12]. By using the CD3CD25/CD4DR ratio of graft-infiltrating cells and peripheral blood cells, a good level of accuracy for the diagnosis of rejection was achieved [12]. We did not find any difference in CD3CD25 expression between the graft and the periphery; conversely, for CD4DR a difference was detected. It is noteworthy that transplanted patients showed higher levels of CD3CD25 in periphery than controls, suggesting possibly an ongoing immune process. Unfortunately, these were not characterized as CD4/CD8; their characterization could be helpful in evaluating whether this is an expression of a tolerance process. The major cause of lymphocyte depletion was the apoptosis of T cells in the peripheral circulation and lymph nodes [13]. It is possible that the combined effect of anti-Thymoglobulin induction, and MMF maintenance therapy, might induce T-activated lymphocyte depletion perhaps by modulating pro- or anti-apoptotic molecules, given that mofetil mycophenolate inhibits DNA synthesis in T lymphocytes inducing apoptosis. It is of interest to observe that the use of cyclosporine did not inhibit lymphocyte intra-graft apoptosis in our model.

Conclusion

In conclusion, our study shows that the recipients of full-functioning kidney or kidney–pancreatic grafts, previously treated with short-term anti-Thymoglobulin induction, manifest a selective intra-graft lymphocyte apoptosis attributed to: (a) latent intra-graft lymphocytes activation; (b) CD8 T-suppressor intra-graft recruitment; (c) increased intra-graft apoptotic rate; and (d) down-regulation of lymphocyte anti-apoptotic molecules. Such events might lead to the deletion of alloreactive lymphocytes and participate in the acceptance of the graft.

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