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RESEARCH ARTICLE

Serum markers of apoptosis in the early period of heart transplantation

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Abstract

Context and objective: To assess the relationship between levels of serum markers of apoptosis and rejection grades in heart transplant (HTx).

Materials and methods: A prospective study was conducted in 91 HTx. We correlated apoptosis markers and biopsy samples. The apoptosis markers were: TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, sFas, sTNF-R1 and sTNF-R2.

Results: The only significant correlation with rejection grade was sFas ($r = 0.329$; $p = 0.005$). Cyclosporine showed a proapoptotic effect (sTNF-R1 0.02 and sTNF-R2 0.02) and everolimus an antiapoptotic effect (sTNF-R1 $r = -0.523$; $p = 0.0001$ and sTNF-R2 $r = -0.405$; $p = 0.0001$).

Conclusions: The utility of specific apoptosis markers in peripheral blood for diagnosis of acute cellular rejection is low. Everolimus may have an anti-apoptotic effect.

Keywords: Apoptosis, heart transplantation, rejection, biopsy, tumoral necrosis factor

Introduction

Advances in induction therapy and maintenance immunosuppression have led to improved survival in patients receiving a heart transplant (HTx). Despite this, acute cellular rejection (ACR) remains one of the leading causes of mortality in the first year after HTx (Stehlik et al. 2010). To date, no noninvasive method has been shown to have the necessary reliability to monitor ACR, so the performance of endomyocardial biopsies continues to be necessary during at least the first year after HTx (Sun et al. 2005; Arnau-Vives et al. 2004).

Histologically, ACR is characterized by a simple nonspecific inflammatory infiltration in mild cases to myocardial necrosis with extensive lymphocytic infiltration in more severe cases. In this inflammatory process are involved direct mechanisms of cellular death mediated primarily by lymphocytes, and other indirect mechanisms attributed to apoptosis. This inflammation usually remits to a large extent with resolution of rejection, though there seems to be a cumulative effect over the myocardium and on the development of cardiac allograft vasculopathy (Stoica et al. 2006).

Some studies have attempted to demonstrate the relationship of apoptosis with ACR in HTx and its possible utility in monitoring of ACR, but the results have been disparate (Szabolcs et al. 1996; Masri et al. 2003). Thus, studies such as those conducted by Koch et al. (2008) and Laguens et al. (1996) found markers and apoptotic cells in myocardiums with acute rejection, but did not find any relationship with rejection grade. On the other hand, Cristóbal et al. (2010) and Masri et al. (2001) did find a relationship between the

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presence of apoptosis markers and ACR, though it was not strong enough to substitute the performance of serial biopsies.

It is has been suggested that apoptotic phenomena occur during rejection that are manifested by the elevation of the levels of certain protein participating in this process. Therefore, there should be a relationship between the levels of these markers and rejection grades. On the other hand, apoptotic phenomena may be more related with other variables than with rejection, such as time since transplantation or even the immunomodulatory drugs.

The aim of this study was to assess the grade of acute cellular rejection in cardiac tissue samples and its correlation with specific serum markers of apoptosis, as well as the influence of immunosuppressive drugs and time since HTx on this whole process.

Materials and methods

Patients

A prospective, single-center study was conducted in 91 HTx recipients from January 2006 to September 2010. Patients who had undergone a protocol endomyocardial biopsy were recruited (first 14 months post-HTx). At the same time as biopsy, a blood sample was drawn from all patients. Samples from patients with any intercurrent disease (hemodynamic instability, ventricular function depression not due to rejection, infection, renal or hepatic dysfunction, presence of noncutaneous tumor-except for Kaposi sarcoma or melanoma, rejection with hemodynamic compromise and/or mediated by antibodies, and diagnosis of cardiac allograft vasculopathy) were excluded. Serum samples from patients with biopsies with insufficient material to classify correctly the grade of ACR were also not considered for analysis. In order to extract more reliable conclusions, samples with different rejection grades were chosen even if they did not imply treatment. Due to the exclusion criteria, the final number of samples included in the study was 72.

All patients gave their written consent for analysis of their samples and to participate in the study. The research project was approved by the clinical research ethics committee of our hospital.

Clinical variables analyzed

Clinical variables were age, sex, transplant etiology, time since HTx, arterial hypertension (HT), diabetes mellitus (DM), dyslipidemia (DL), treatment for these comorbidities and immunosuppressive medication. Patients were considered to have HT, DM and DL if they received treatment for these conditions. The drug families used in these cases were angiotensin-converting enzyme inhibitors for HT, insulin and metformin for DM and atorvastatin for DL. For the purpose of statistical analysis, the drugs that were being received at biopsy and serum sample collection were considered.

Histologic samples of cardiac biopsies: rejection grades

Samples were fixed in paraffin. They were then cut and stained with hematoxylin-eosin and the grade of ACR analyzed according to the guidelines of the International Society of Heart and Lung Transplantation (ISHLT).

Biopsy samples were classified into 5 groups depending on the degree of lymphocytic infiltration and the presence of cellular necrosis. The 1990 ISHLT classification of ACR (Billingham et al. 1990) was taken as a reference as it provides a more detailed description of rejection grades. The adapted classification was as follows:

- Grade 0 (ISHLT 0): Absence of necrosis and infiltration (15 samples).
- Grade 1 (ISHLT 1A): Mild infiltration without necrosis (15 samples).
- Grade 2 (ISHLT 1B): Moderate-severe infiltration without necrosis (12 samples).
- Grade 3 (ISHLT 2): A single necrotic focus with or without infiltration (15 samples).
- Grade 4 (ISHLT ≥ 3): Two or more necrotic foci with or without infiltration (15 samples).

Only grade 4 was considered significant rejection and treated as such.

Sample processing: apoptosis markers

The molecules involved in regulation of the process of apoptosis that were determined in serum were TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, sFas, sTNF-R1 and sTNF-R2.

Venous blood was taken by venipuncture, centrifuged immediately and frozen at –80°C and only thawed once. Serum concentrations of TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, sFas, sTNF-R1 and sTNF-R2 were determined at a central laboratory by specific commercial sandwich enzyme-linked immunosorbent assay. TRAIL/Apo2L, TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 ELISA kits were from Gene-Probe® (France). Enzyme-linked immunosorbent assay Kit for Human Factor Related Apoptosis (FAS) was from Uscn Life Science Inc®, P.R. China. Human sTNF-R1 60kDa Platinum ELISA was from eBioscience®, Austria, and human sTNF-R2Inmunoassay was from R&D Systems®, USA.

The TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, sFas, sTNF-R1 and sTNF-R2 tests have a limit of detection of 64, 8, 6, 147, 64, 29, 50 and 0.6 pg/mL, respectively. Our intra-assay and inter-assay coefficients were 6.5 and 5.9% for TRAIL, 5.2 and 11% for TRAIL-R1, 2.3 and 7.0% for TRAIL-R2, 4.8 and 8.4% for TRAIL-R3, 5.4 and 11% for TRAIL-R4, 3.1 and 6.8% for sFas, 6.5 and 8.6% for sTNF-R1, and 4.7 and 4.3% for sTNF-R2.

Statistical analysis

According to the result of the Kolmogorov-Smirnov normality test, quantitative variables were expressed as mean and standard deviation or as median and interquartile

range. Comparisons of the parameters analyzed were made using a nonparametric analysis of variance (Kruskal-Wallis test). Since the variables of the samples did not follow a normal distribution, the nonparametric Spearman correlation coefficient was used to assess their degree of correlation. Assessment of the diagnostic capacity to detect rejection of the serological variables studied was done by obtaining the ROC curves, and is summarized in their area under the curve (95% confidence interval [CI]). An assessment was made of the change over time in the different study parameters. We performed a multivariate analysis to clarify the effect of each biomarker. In all cases, differences were considered statistically significant when $p < 0.05$. Statistical analysis was performed with the SPSS 10.0® program (SPSS Inc., Chicago, Ill, USA).

Results

Mean patient age was 41 ± 11 years, and 65.3% were men. The rest of the basal characteristics are shown in the Table 1.

In nearly all patients, the TRAIL-R2 marker was undetectable, so no results are presented for it. The median serum levels of all other markers are shown in Figure 1.

Of all the markers analyzed, the only marker significantly correlated with rejection grade was sFas (Table 2), which was correlated with grade 4 rejection ($r = 0.329$; $p = 0.005$). This association was also observed after performed a multivariate analysis HR 1.003 (1.001–1.005). Table 3. However, its discriminative capacity was low, with an area under the ROC curve of 0.646 (CI 0.475–0.816), Figure 2.

Table 1. Basal characteristics.

Age (years)	41 ± 11
Sex (male, %)	65.3
BMI	26.05 ± 4.35
HTx etiology (%)	
DCM	–34.7
Ischaemic	–44.4
Others	–20.9
Time since HTx (months)	7 ± 4
AHT (%)	37.7
Dyslipidemia (%)	43
Diabetes (%)	20.2
Smoking before HTx (%)	
No	–27.8
Yes	–30.6
Former	–41.7
Induction theraphy (%)	
Basiliximab	–48.6
Daclizumab	–51.4
Inmunosupressants (%)	
CsA	–45
Tac	–35
Eve	–22

BMI: body mass index; HTx: heart transplantation; AHT: arterial hypertension.

With regard to the influence of time since HTx, we found a significant correlation with the following molecules (Table 4): TRAIL ($r = 0.436$, $p = 0.0001$), TRAIL-R3 ($r = 0.311$; $p = 0.008$) and sTNF-R1 ($r = 0.240$; $p = 0.04$).

Finally, in the analysis of the influence of the drugs used in HTx, cyclosporine showed an apoptotic effect manifested in the following proteins (Table 5): sTNF-R1 ($r = 0.274$; $p = 0.02$) and sTNF-R2 ($r = 0.270$; $p = 0.02$). Conversely, everolimus showed an antiapoptotic effect according to the same markers, sTNF-R1 ($r = -0.523$; $p = 0.001$) and sTNF-R2 ($r = -0.405$; $p = 0.001$). The rest of the immunosuppressive drugs or those for treatment of HT, DM or DL did not show significant correlations.

Discussion

As we have demonstrated in this study, the analysis of apoptosis in serum shows a very weak correlation with the histopathological analysis of the cardiac biopsy, and therefore is not useful for monitoring acute cellular rejection (ACR). The most novel finding of this study was the presence of an antiapoptotic effect of everolimus.

To date, many attempts and methods have been tried to diagnose rejection without biopsies, but none has shown a sufficiently good correlation. This is the reason why guidelines still recommend performing biopsies in the first year after HTx with a class IB recommendation (Costanzo et al. 2010).

Apoptosis is a complex phenomenon and is implicated in numerous processes in the body. A large number of mediators are involved in apoptosis, and its activation may be effected by two pathways, the intrinsic pathway via so-called death receptors and the extrinsic or mitochondrial pathway. This has propitiated the use of different techniques in the different studies, and no technique can be considered the gold standard. The most widely used technique for quantification of apoptosis in tissue samples is the terminal deoxynucleotide nick end labeling (TUNEL staining) assay, based on the detection of DNA-3′ terminal fragments. Despite this, the TUNEL technique is not specific for detection of apoptosis. In fact, it has been shown that the TUNEL is positive not only in apoptotic cardiomyocytes but also in those with oncotic necrosis or even in health cardiomyocytes undergoing a process of active DNA repair (Kanoh et al. 1999; Ohno et al. 1998). Because of this, detection of DNA-3′ terminal fragments should also be accompanied by other techniques that confirm apoptosis.

To date, published studies have focused on demonstrating the presence of apoptotic mechanisms in cardiac tissue samples. In general, most studies show a higher number of apoptosis parameters as the rejection grade increases. However, few studies have analyzed the serum of patients with rejection searching for apoptosis markers and it seems that the correlation between apoptosis and rejection is weak. We chose determination in serum for several reasons. First, serum samples are easier to obtain than cardiac biopsies, avoiding the "patchy" effect

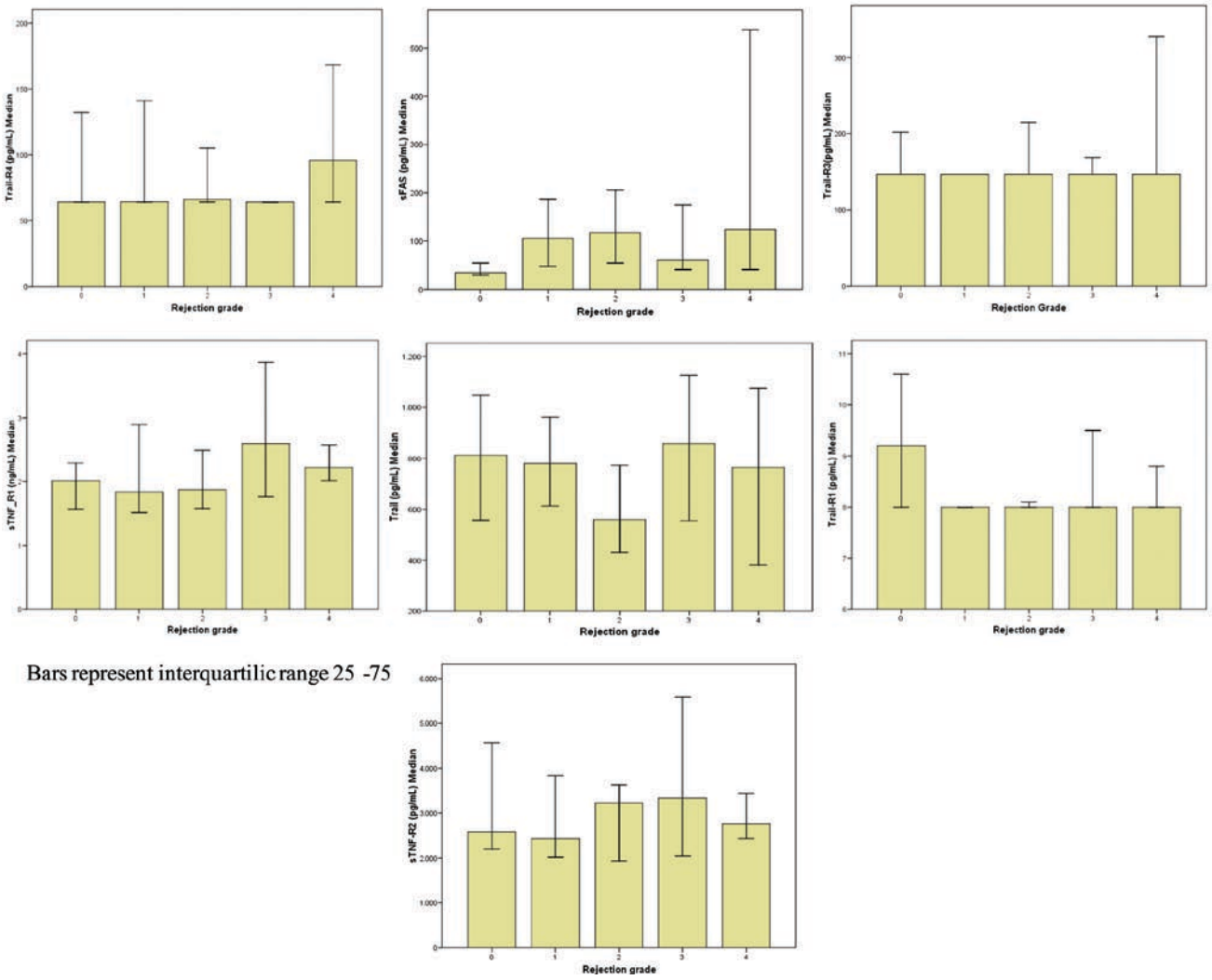

Figure 1. Correlations between the apoptosis markers and the rejection grade.

Table 2. Correlation of rejection grade and apoptosis markers.

Biomarker	TRAIL	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4	sFas	sTNF-R1	sTNF-R2
Spearman's r	0.029	0.215	NA	0.051	0.009	0.329	0.164	0.065
p	0.812	0.070	NA	0.672	0.941	0.005	0.168	0.589

NA: undetectable.

Table 3. Multivariate analysis: biomarkers and acute rejection.

Biomarker	HR	CI	p
Trail	1.001	0.999–1.002	0.457
Trail_R1	1.056	0.676–1.649	0.810
Trail_R3	1.000	0.998–1.002	0.985
Trail_R4	1.000	0.998–1.001	0.894
FAS	1.003	1.001–1.005	0.015
sTNF_R1	.658	0.207–2.095	0.479
sTNF_R2	1.000	0.999–1.001	0.912

of biopsies; serum processing is also simpler than histologic sample analysis, and finally, a potent and positive correlation of serum markers of apoptosis with rejection grade could initiate a method for monitoring rejection without invasive procedures.

The molecules analyzed by us all belong to the tumor necrosis factor-alpha gene superfamily, which is the most implicated family in apoptosis. TNF-R1 activates the apoptotic process by binding to TNF-α, Fas receptor by binding to Fas ligand (FasL), and the TRAIL-R1, TRAIL-R2 y TRAIL-R4 receptors by binding to TRAIL ligand. All these receptors have death domains and their activation triggers the recruitment of adaptor molecules (FADD and TRADD) that bind to these domains (Chinnaiyan et al. 1996; Kischkel et al. 2000), activating the caspase cascade and thus inducing apoptosis. The release of TNF-R1 (soluble form, sTNF-R1), Fas receptor (sFas) and TRAIL receptors from the cell membranes causes an antiapoptotic effect due to blockade of the binding of the apoptotic forms (TNF-α and FasL) to the receptors that are expressed on other cell membranes.

Endogenous concentrations of the soluble receptors of these three ligands, both those from death domain molecules (sTNF-R1, sFas, TRAIL-R1, TRAIL-R2 and TRAIL-R3) and those from molecules without these domains (sTNF-R2 and TRAIL-R3), play a modulatory role in biological function of TNF-α, FasL and TRAIL, since they reflect the degree of activation of these systems.

The choice of these mediators was based on the fact that they represent one of the two pathways of activation of apoptosis and the evidence accumulated in the scientific literature. Thus, in heart failure and after an acute myocardial infarction, it has been shown that markers such as sFas, sTNF-α and their receptors are elevated in serum and are correlated with symptom severity and disease prognosis (Nishigaki et al. 1997; Rauchhaus et al. 2000; Deswal et al. 2001; Ridker et al. 2001). In all these studies, an increase in apoptotic processes was put forward as one of the mechanisms implicated in increased mortality.

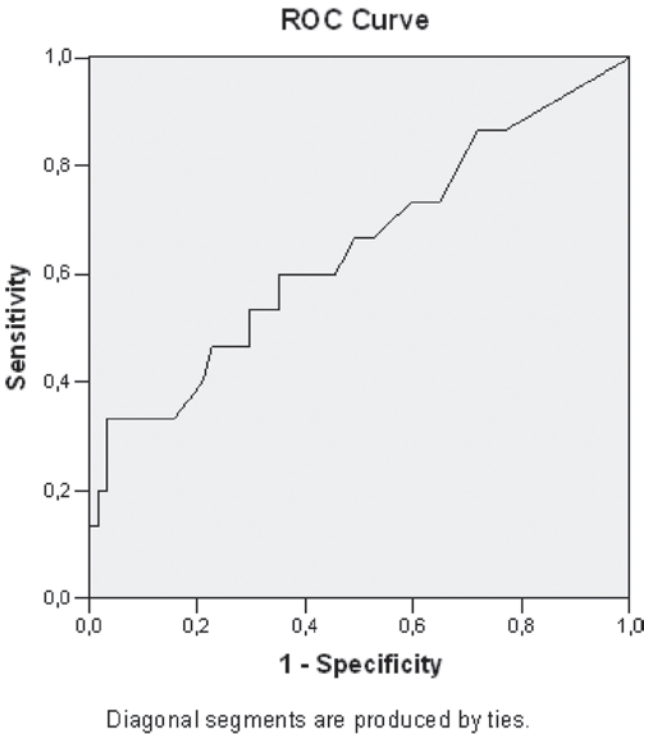

Figure 2. Discriminative capacity for sFas.

Although to a lesser extent, the presence of apoptosis has also been documented in the field of transplants with the markers used. Thus, elevated serum levels of sFas, TRAIL-R4 and TRAIL-R5 have been found in renal transplantation, but without a clear relationship to the presence of rejection (Song et al. 2004; Carpio et al. 2006). Other studies have investigated the presence of apoptosis in biopsy samples from HTx, and though the results are variables, they generally show greater apoptotic activity in those samples with histological signs of ACR[8]. It is for this reason that, in a second step, authors such as Cristóbal et al.(2010) or Cholllet-Martin et al. (1990) have attempted, although in few patients and unsuccessfully, to correlate rejection grade and apoptosis in biopsies with serum markers of apoptosis.

Immunosuppressants prevent ACR and so it can be assumed that they inhibit the process of apoptosis. Our results show that cyclosporine has an apoptotic effect, tacrolimus is neutral, and surprisingly, everolimus proved to be an antiapoptotic agent. It is known that calcineurin inhibitors are the most potent immunosuppressant agents, so if they promote apoptosis or have a neutral effect it means that they prevent ACR by pathways not mediated by apoptosis. Everolimus is an immunosuppressant agent belonging to the family of the proliferation signal inhibitors, whose main characteristics in the field of transplantation are the lack of renal toxicity, an antitumoral effect and delayed development of cardiac allograft vasculopathy in HTx. Additionally, everolimus is being used with some success in the treatment of cancers such as kidney cancer (Siebels et al. 2011). At the doses

Table 4. Correlation of time since heart transplantation and apoptosis markers.

Biomarker	TRAIL	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4	sFas	sTNF-R1	sTNF-R2
Spearman's r	0.436	0.043	NA	0.311	0.007	0.034	0.240	0.205
p	0.001	0.720	NA	0.008	0.956	0.777	0.042	0.084

NA: undetectable.

Table 5. Correlation between immunosupressants and apoptosis markers.

Biomarker	CsA	Tac	Eve
TRAIL	$r=0.160$; $p=0.178$	$r=-0.184$; $p=0.122$	$r=0.059$; $p=0.624$
TRAIL-R1	$r=-0.024$; $p=0.842$	$r=0.084$; $p=0.483$	$r=-0.171$; $p=0.151$
TRAIL-R2	NA	NA	NA
TRAIL-R3	$r=-0.165$; $p=0.167$	$r=0.143$; $p=0.230$	$r=0.070$; $p=0.561$
TRAIL-R4	$r=-0.131$; $p=0.274$	$r=0.120$; $p=0.314$	$r=0.036$; $p=0.761$
sFAS	$r=0.054$; $p=0.650$	$r=-0.076$; $p=0.524$	$r=0.060$; $p=0.615$
sTNF-R1	$r=0.274$; $p=0.020$	$r=-0.097$; $p=0.416$	$r=-0.523$; $p=0.001$
sTNF-R2	$r=0.270$; $p=0.022$	$r=-0.134$; $p=0.261$	$r=-0.405$; $p=0.001$

CsA: cyclosporine; Tac: tacrolimus; Eve: everolimus; NA: undetectable.

used in oncology (much higher than in transplants), everolimus has a clear apoptotic and hence antitumoral effect. However, there are no studies on apoptosis and everolimus at the doses used in HTx. We therefore think that while everolimus cannot be regarded as an anti-apoptotic drug, at the doses used in HTx its effect differs from that seen at the doses used in oncology (Thaunat et al 2005; Tanji et al. 2011).

There are few studies that have evaluated the influence of time since HTx and the degree of apoptosis. In general, the studies that have evaluated it have documented a trend to increased expression of apoptosis markers with the passage of time (Cristóbal et al. 2010). Similarly, we found that some molecules, such as TRAIL, TRAIL-R3 or sTNF-R1, were more elevated in the serum of patients with a longer time since HTx. In contrast, the incidence of ACR is lower the greater the time since HTx. These findings reveal the current lack of knowledge in this field and the need to further our knowledge of apoptosis in rejection of heart transplants.

None of the comorbidities studied, HT, DM or DL, were related with apoptosis. There are studies showing that patients with these comorbidities have raised levels of apoptosis markers. Close monitoring in HTx recipients tends to ensure that these comorbidities are well controlled, which is the reason we think they were not shown to be apoptotic.

The present study has various limitations. We should first mention that it involves HTx at different times in its course and that patients with intercurrent diseases at biopsy were excluded from the sample. Immunosuppressive therapy only varied by one drug (cyclosporine, tacrolimus or everolimus), so we could not assess the effect of the corticosteroids or mycophenolate mofetil. Regarding the determination of apoptosis in serum, we used more novel and hence less validated techniques, in addition to analyzing only the extrinsic pathway. Despite this, we think that none of the limitations invalidates the results obtained given the high number of patients and the significances obtained.

Conclusions

The results of this study are negative, because, obwohl there is a certain correlation between serum markers of apoptosis and acute cellular rejection grades in cardiac biopsies, this correlation is low and does not allow noninvasive monitoring of rejection. Similarly, some of these mediators are related with other clinical and pharmacological variables that need to be analyzed in depth, particularly the role of immunosuppressants in the regulation of apoptosis.

The authors have contributed as follows: Ignacio J. Sánchez-Lázaro: drafting article; concept/desing; statistics. Luis Almenar-Bonet: drafting article; concept/desing; statistics. Ana Romero-Pelechano: data collection; drafting article. Manuel Portoles-Sanz: data collection. Luis Martínez-Dolz: critical revision of article. Esther Roselló-Lleti: drafting article; data collection. José Ramón-González-Juanatey: critical revision of article. Miguel Rivera-Otero: critical revision of article. Antonio Salvador-Sanz: critical revision of article. There are no potential conflicts of interest related to the manuscript for any of the authors.

Declaration of interest

The authors report no conflicts of interest.

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