



Clinical Outcomes and Genetic Expression Profile in Human Liver Graft Dysfunction During Ischemia/Reperfusion Injury

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ABSTRACT

Introduction. This study aims to compare the molecular gene expression during ischemia reperfusion injury. Several surgical times were considered: in the beginning of the harvesting (T0), at the end of the cold ischemia period (T1), and after reperfusion (T2) and compared with graft dysfunction after liver transplant (OLT).

Methods. We studied 54 patients undergoing OLT. Clinical, laboratory data, and histologic data (Suzuki classification) as well as the Survival Outcomes Following Liver Transplantation (SOFT) score were used and compared with the molecular gene expression of the following genes: Interleukin (IL)-1b, IL-6, tumor necrosis factor- α , perforin, E-selectin (SELE), Fas-ligand, granzyme B, heme oxygenase-1, and nitric oxide synthetase.

Results. Fifteen patients presented with graft dysfunction according to SOFT criteria. No relevant data were obtained by comparing the variables graft dysfunction and histologic variables. We observed a statistically significant relation between SELE at T0 ($P = .013$) and IL-1 β at T0 ($P = .028$) and early graft dysfunction.

Conclusions. We conclude that several genetically determined proinflammatory expressions may play a critical role in the development of graft dysfunction after OLT.

THE ISCHEMIA/REPERFUSION INJURY (IRI) during liver transplantation (OLT) makes the graft more vulnerable by increasing immunogenicity, and rejection episodes, both earlier and later after OLT [1]. The clinical relevance of these phenomena is related to greater graft dysfunction and loss after OLT [2].

From a hemodynamic standpoint, IRI is well-characterized during OLT surgery, mainly by a decrease in systemic vascular resistance and need for continuous infusion of vasopressors [3–6]. However, the hallmark of IRI is the inflammatory response that lies beyond it.

A serial of molecular events occur after the depletion of cellular adenosine triphosphate during cellular injury and hypoxia. Under normal conditions, this dangerous molecular signaling is directed toward organ preservation and repair; because it is genetically determined, it can vary from patient to patient. During IRI, however, organ damage may be the result of an exacerbated inflammatory reaction. Overproduction of reactive oxygen species

occurs, acting during the reperfusion phase [7]. An inflammatory condition is then initiated, by activating Kupffer cells and T lymphocytes, along with increased production of proinflammatory cytokines (tumor necrosis factor- α , interleukin [IL]-1 β , interferon- γ , and others), chemokines (macrophage inflammatory protein-2, monocyte chemoattractant protein-1), adhesion molecules, and inflammatory cells recruitment, leading to liver infiltration by neutrophils, and, ultimately, cell death [8].

The authors studied different proinflammatory mediators to characterize the inflammation associated with liver IRI during OLT, as well as their genetic expression, which may result in different inflammatory profiles and different clinical

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outcomes, mainly regarding histologic characteristics and post-OLT graft dysfunction.

MATERIALS AND METHODS

Patients

From 2004 to 2011, 856 patients underwent OLT. Data collection was highly dependent on the availability of the surgical team, as well as on the transport of collected samples and laboratory convenience. For this reason, 54 patients were included, not selected by any method, and they expressed the average selection of recipients during the considered period of time. The ages of both recipients and donors were representative of the whole period considered.

We collected patient age, gender, main pathology, blood group, preoperative Model for End-Stage Liver Disease (MELD) score, Child–Pugh score, and Survival Outcomes Following Liver Transplantation (SOFT) score [9]. The highest aminotransferase value after OLT, cold ischemia time, warm ischemia time, and donor age were also collected, as well as the number of red cell packed units and fresh frozen plasma units used during the operation. Most donors were on pressors at the time of harvesting, but there were no detailed data on this issue. All donors had a body mass index of <30 kg/m².

Tissue Sample Collection

Tissue harvesting was performed by experienced surgical teams in the 4 OLT centers in Portugal, and in all the cases the preservation solution was Celsior (SangStat, Lyon, France) and the rapid Starzl technique was used [10]. Needle biopsies were collected from 54 liver grafts at 3 different times: at the beginning of the harvesting (T0), at the end of the cold ischemia period (T1), and post-reperfusion, at the end of the operation (T2).

In the case of familial amyloidotic polyneuropathy (FAP) grafts, informed consent of the donor patients and agreement of the ethical commission of the hospital was obtained.

All biopsy fragments were divided in half; 1 sample was processed for molecular marker expression and the other submitted to a comparative study by usual histologic procedure, following our hospital protocol.

Histologic Analysis

Tissue fragments were collected in vials containing formaldehyde, and processed in classic hematoxylin and eosin cuts. The histologic classification Suzuki classification [11] was used to quantify the lesions. In addition to the parameters integrating this classification (sinusoidal congestion, vacuolization/ballooning, and necrosis), 2 additional parameters were studied in this issue: the grade of steatosis and neutrophil infiltration. For steatosis, 4 grades were considered: none (0%, 0 points), slight (<30%, 1 point), moderate (30%–60%, 2 points), and intense (>60%, 3 points). Both macrovesicular and microvesicular steatosis were evaluated. Macrovesicular steatosis was considered a reversible condition, defined as a single vacuole pushing the nucleus aside; microvesicular steatosis was associated with deficiency of β -oxidation of the lipids, and defined as fine and multiple droplets dispersed in the cell cytoplasm. Both were considered able to amplify the IRI in a more relevant way, and enough to induce the phenomena of interference in the hepatic microcirculation (lipopeliosis, especially macrosteatosis).

Neutrophils infiltration was graded as scarce (≤ 5 cells per high-power field [HPF; 0 points]), 5–30 cells per HPF (1 point), or >30 to <60 cells per HPF (2 points). To the score provided by the

Table 1. General Characteristics of the Studied Population (n = 54)

Parameter	Result
Age (y), mean \pm SD	45 \pm 14
Male, n (%)	42 (78)
Blood group, n (%)	
A	28 (52)
B	3 (6)
AB	3 (6)
O	20 (37)
MELD (mean \pm SD)	16 \pm 8 (n = 36)
CTP score, n (%)	
A	20 (55)
B	10 (27)
C	6 (18)
Main disease, n (%)	
FAP	15 (27)
HCV	16 (29)
Alcoholic cirrhosis	14 (25)
Biliary diseases	4 (7)
Autoimmune diseases	2 (3)
Metabolic diseases	4 (7)
Acute liver failure	3 (5)
SOFT, n (%)	
Low risk	37 (69)
Low-moderate risk	12 (22)
High-moderate risk	5 (9)

Abbreviations: CPT, Child-Turcotte-Pugh; FAP, familial amyloid polyneuropathy; HCV, hepatitis C virus; MELD, Model for End-Stage Liver Disease; SOFT, Survival Outcomes Following Liver Transplantation.

Suzuki classification, we added 2 parameters (steatosis and neutrophil infiltration). According to this final score, the patients were divided in 2 groups: scores of 0–5 or ≥ 6 .

Molecular and Gene Expression

The tissue fragments were collected in vials containing RNA later solution (Ambion, Austin, TX). In all the samples, total RNA was extracted and converted in cDNA by reverse transcriptase, and gene expression analysis of a panel of proinflammatory genes was obtained: IL-1b, IL-6, tumor necrosis factor- α , perforin, E-selectin (SELE), Fas-ligand, granzyme B, heme oxygenase-1, and inducible nitric oxide synthetase (iNOS2A) by quantitative polymerase chain reaction (PCR) ct, using as reference the genetic expression of sample 1 (nonischemic).

Whenever possible, the service “Assays-on-Demand Gene Expression Products,” from Applied Biosystems (Foster City, CA), was used, to synthesize the group of “primers – Taqman probes” specific for each gene studied. A group of Taqman probes for the so-called housekeeping genes (β -actin) were used for normalization of the genetic quantification. The PCR kinetic analysis was performed in the sequence detection system of Applied Biosystems ABI Prism 7900. The relative analysis of genetic expression was performed as described by Livak et al [16]. Analysis of relative expression data using real time quantitative PCR and the 2^{-ct} method were performed.

Statistical Analysis and Groups for Comparison

An exploratory analysis was carried out for all variables. Categorical data are presented as frequencies and percentages, and continuous

Table 2. Results of Genetic Expression for the Different Studied Genes Related to the Variable Organ Dysfunction

Parameters	Dysfunction	Med (P25–P75)	P
iNOS at T0	No	0.14 (0.03–0.30)	.381
	Yes	0.21 (0.03–0.68)	
iNOS at T1	No	0.10 (0.03–0.19)	.512
	Yes	0.06 (0.01–0.57)	
iNOS at T2	No	0.06 (0.04–0.10)	.779
	Yes	0.05 (0.03–0.38)	
SELE at T0	No	0.84 (0.44–2.98)	.013
	Yes	2.69 (1.35–4.18)	
SELE at T1	No	1.45 (0.54–5.10)	.310
	Yes	2.05 (0.99–6.02)	
SELE at T2	No	2.34 (1.57–4.69)	.724
	Yes	2.35 (1.20–4.25)	
FASL at T0	No	0.97 (0.72–1.86)	.745
	Yes	1.00 (0.70–2.81)	
FASL at T1	No	0.70 (0.43–1.68)	.209
	Yes	0.45 (0.27–1.33)	
FASL at T2	No	0.22 (0.10–0.47)	.573
	Yes	0.29 (0.13–0.64)	
GRZB at T0	No	1.42 (0.86–2.70)	.566
	Yes	2.96 (0.58–4.91)	
GRZB at T1	No	1.36 (0.65–3.78)	.900
	Yes	0.53 (0.44–9.76)	
GRZB at T2	No	1.68 (0.96–4.41)	.742
	Yes	2.65 (0.78–5.33)	
HO-1 at T0	No	266.29 (146.74–385.47)	.323
	Yes	289.80 (179.39–652.55)	
HO-1 at T1	No	193.79 (147.75–275.31)	.798
	Yes	202.33 (135.82–460.55)	
HO-1 at T2	No	164.32 (125.05–353.36)	.742
	Yes	160.16 (99.72–270.90)	
IL-6 at T0	No	0.37 (0.10–2.50)	.354
	Yes	0.60 (0.20–3.54)	
IL-6 at T1	No	2.26 (0.74–4.85)	.423
	Yes	1.62 (0.23–4.97)	
IL-6 at T2	No	6.40 (2.38–14.36)	.634
	Yes	7.82 (3.31–12.10)	
IL-1 Beta at T0	No	9.84 (2.54–19.39)	.028
	Yes	19.53 (12.10–40.88)	
IL-1 Beta at T1	No	22.67 (8.47–46.82)	.682
	Yes	25.39 (7.87–217.55)	
IL-1 Beta at T2	No	36.35 (17.96–89.83)	.171
	Yes	61.38 (53.19–86.65)	

variables as mean or median, standard deviation (SD) or interquartile range (25th–75th percentile). Univariable analysis was done using nonparametric tests (χ^2 or Fisher exact test, Mann–Whitney and Kruskal–Wallis) because of the existence of outliers, high variability, and skewed distributions.

The significance level $\alpha = 0.05$ was considered and 95% CI were calculated as appropriate. All data were analyzed using the Statistical Package for the Social Sciences for Windows version 19.0 (SPSS Inc, Chicago IL). The main outcome considered was de graft dysfunction according to SOFT criteria, compared to histologic data and data from studied gene's expression. The preservation injury and early graft dysfunction according to Howard and Ploeg–Maring criteria were also evaluated, although not subjected to specific analysis.

RESULTS

The main characteristics of enrolled patients are presented in Table 1. In the studied cohort, we observed graft preservation injury in 5 cases, early graft injury (according to Ploeg–Maring criteria) in 9 patients, and 15 patients presented graft dysfunction according to SOFT criteria. The comparison between graft dysfunction and clinical variables are presented in Table 2. Although not attaining significance, we observed male patients were 3 times less likely to develop graft dysfunction. For cold ischemia time, graft dysfunction occurred 1.9 more often in patients with a cold ischemia time of >500 minutes, and 5 times more often in patients with a warm ischemia time of >90 minutes. In 2 of the 16 cases that experienced graft dysfunction, the liver graft came from a living donor with FAP. In only 8 cases of these 16 the donor was <50 years old. Among grafts that developed dysfunction, there was a donor–recipient gender mismatch, which affected female recipients (5/6) much more often than male recipients (4/10).

The comparison between graft dysfunction and histologic variables is presented in Table 3. No relevant associations were found. Nonetheless, we observed that, in patients with ballooning at T0 and T1, the possibility to develop graft dysfunction was ≤ 7.5 times higher, and the presence of necrosis at T0 and T1 conferred a 2.3 times greater probability to develop graft dysfunction.

Laboratory and gene expression data are presented in Table 4. We observed a significant relationship between SELE at T0 ($P = .013$) and IL-1 β at T0 ($P = .028$) and early graft dysfunction. Significant associations were confirmed on multivariate analyses, but only regarding SELE expression at T0 (odds ratio [OR], 1.93; 95% CI, 1.06–3.50; $P = .030$).

Concerning the expression of IL-1 β at T0, the comparison between cases of patients with and without dysfunction revealed a P value of .028 (Med [P25–P75], no 9.84 [2.54–19.39]; yes, 19.53 [12.10–40.88], Mann–Whitney).

DISCUSSION

We have found that patients expressing higher levels of SELE and IL-1 β at T0 presented more severe early graft function after OLT, before flushing with the recipient's blood, at T0. This suggests that inflammatory phenomena may influence critically the severity of IRI during OLT. Interestingly, no histologic data correlated with IRI, although ballooning and necrosis were more frequent at T0, as well as with a cold ischemia time of >500 minutes and

Table 3. Multivariate Analysis for Clinical Variables and Demographic Data for Graft Dysfunction

Variables	OR	95% CI	P
Male gender	0.13	0.01–0.25	.077
Cold ischemia (min)	1.74	0.95–3.18	.074
Warm ischemia (min)	1.64	0.91–2.94	.099
SELE expression in T0	1.93	1.06–3.50	.030

Abbreviations: OR, odds ratio; SELE, E-selectin.

Table 4. Univariate Analysis of Gene Expression in All Time Points Based on Graft Dysfunction

Gene	All Patients (n = 54)	Patients Without Graft Dysfunction	Patients With Graft Dysfunction	P*
FASL median (P ₂₅ -P ₇₅)				
T0	0.98 (0.72-2.16)	0.97 (0.72-1.86)	1.00 (0.70-2.81)	.873
T1	0.68 (0.34-1.50)	0.70 (0.43-1.68)	0.45 (0.27-1.33)	.776
T2	0.25 (0.11-0.52)	0.22 (0.10-0.47)	0.29 (0.13-0.64)	.366
GRB median (P ₂₅ -P ₇₅)				
T0	1.51 (0.61-3.70)	1.42 (0.86-2.70)	2.96 (0.58-4.91)	.677
T1	1.33 (0.47-5.72)	1.36 (0.65-3.78)	0.53 (0.44-9.76)	.857
T2	2.08 (0.94-4.52)	1.68 (0.96-4.41)	2.65 (0.78-5.33)	.282
HO1 median (P ₂₅ -P ₇₅)				
T0	276.60 (153.41-419.45)	266.29 (146.74-385.47)	289.80 (179.39-652.55)	.167
T1	198.06 (139.91-291.16)	193.79 (147.75-275.31)	202.33 (135.82-460.55)	.801
T2	162.24 (120.97-315.76)	164.32 (125.05-353.36)	160.16 (99.72-270.90)	.900
IL-6 median (P ₂₅ -P ₇₅)				
T0	0.48 (0.15-2.64)	0.37 (0.10-2.50)	0.60 (0.20-3.54)	.282
T1	1.62 (0.50-4.77)	2.26 (0.74-4.85)	1.62 (0.23-4.97)	.571
T2	7.19 (2.76-13.42)	6.40 (2.38-14.36)	7.82 (3.31-12.10)	.662
IL-1b median (P ₂₅ -P ₇₅)				
T0	12.17 (2.83-24.01)	9.84 (2.54-19.39)	19.53 (12.10-40.88)	.143
T1	22.67 (8.31-49.51)	22.67 (8.47-46.82)	25.39 (7.87-217.55)	.342
T2	49.91 (22.40-85.84)	36.35 (17.96-89.83)	61.38 (53.19-86.65)	.788
iNOS2A median (P ₂₅ -P ₇₅)				
T0	0.15 (0.03-0.32)	0.14 (0.03-0.30)	0.21 (0.03-0.68)	.578
T1	0.09 (0.02-0.19)	0.10 (0.03-0.19)	0.06 (0.01-0.57)	.791
T2	0.06 (0.03-0.13)	0.06 (0.04-0.10)	0.05 (0.03-0.38)	.895
SEAL median (P ₂₅ -P ₇₅)				
T0	1.17 (0.49-3.26)	0.84 (0.44-2.98)	2.69 (1.35-4.18)	.214
T1	1.53 (0.61-5.45)	1.45 (0.54-5.10)	2.05 (0.99-6.02)	.517
T2	2.30 (1.40-4.56)	2.34 (1.57-4.69)	2.35 (1.20-4.25)	.319

*P value obtained by comparison of genes between groups of patients with and without graft dysfunction by logistic regression model.

a warm ischemia time of >90 minutes, a situation related to donor's condition and harvesting.

Unlike other selectins, SELE is expressed in an inducible manner, not under basal conditions, but in response to inflammatory stimulations, both chronic and acute, by endothelial cells, and not by hepatic sinusoids [12]. Among these stimuli is IL-1 β , a molecule involved in the precocious phases of the inflammation cascade [13].

Interestingly, the association between these 2 proinflammatory genes and the tendency toward graft dysfunction was only documented at T0, without any influence of recipient factors. It is recognized that any surgical manipulation triggers a molecular reaction through activation of Kupffer cells [14], explaining the enhanced expression of those 2 genes even before reperfusion, as an intrinsic quality associated with the graft itself [15]. The increase occurring at T0 in association with graft dysfunction represents a clinical implication with therapeutic potential.

In previous study [16] on this cohort, livers harvested from FAP patients presented less neutrophil infiltration at T0 ($P = .001$). We linked the neutrophil infiltration with gene expression of HO1 in biopsies taken at T2 ($P = .022$), as well as with the molecular expression of genes related to an attenuated proinflammatory reaction during IRI, namely, iNOS2A at T0 and HO1 at T2. We concluded that livers from FAP donors express differently the genes related

to attenuating proinflammatory reactions, and present less neutrophil infiltration during harvesting. These findings add to the previous description of a better short-term outcome in patients receiving FAP liver grafts. These differences prompted us to try to identify these inflammatory responses to organ dysfunction after OLT in the whole cohort.

The inflammatory process during IRI is well known [17,18]. Nonetheless, patients can react differently, according to a genetically mediated predisposition in the release of proinflammatory cytokines, which can explain, at least partially, the different and hardly preventable clinical features (mostly hemodynamic) and consequences (graft dysfunction and loss) of IRI. Nonetheless, obtained data point in 2 different, but proximal, directions. SELE is oriented to lymphocyte infiltration and IL-1 β to inflammation.

Selectins are members of cell adhesion molecules, whether those we studied are secreted by lymphocytes. They are linked to lymphocyte homing and promote cellular-mediated inflammation and infiltration, leading to acute and chronic inflammatory processes. Because we could not demonstrate these facts with histologic findings, it is hard to prove these basic aspects of SELE as a reference for IRI. Nonetheless, this description may reserve attention in further studies. The most studied process evolving SELE is metastatic tumor spread [19]. Diapedesis of tumor cells from the circulation into secondary sites is believed to occur through a mechanism

similar to that of leukocyte extraversion, in which there must be contact and then the cells roll along the endothelial cell layer. SELE is an adhesion molecule that is not expressed on normal endothelial cells; however, SELE is expressed transiently on the surface of vascular endothelium after stimulation with IL-1 and tumor necrosis factor- α . Their role in IRI has been described in both cellular infiltration and molecular signaling. Interfering with selectin produces a protective effect against liver IRI [20]. The improvement of hemodynamics and decreased leukocyte adherence after treatment with N-acetylcysteine might result from the shedding of selectins.

The role of IL-1 β can be more interesting. IL-1 β is cleaved by caspase 1 (IL-1 β convertase) and is linked to inflammation, cell proliferation and differentiation, and apoptosis. This is an inflammatory process, now recognized as an autoinflammatory process, and observed as a mechanism involved in some autoinflammatory diseases [21,22]. Autoinflammatory diseases diverge from autoimmune diseases, mediated by abnormal cell-mediated injury, mainly lymphocytes and macrophages. They are the result of uncontrolled release of inflammatory mediators, as the result of intracellular inflammasomes, a complex of proinflammatory-producing protein complexes, produced when cells sign the “danger” of damage. These phenomena are mediated genetically and can vary from person to person. The caspase 1 complex is a part of this inflammatory pathway and it may be related to IRI phenomena, regardless of the organ involved, including OLT. It originates in large, multiprotein complexes that sense danger signals through specific receptors. These protein complexes are known as inflammasomes, and are determined genetically. Regarding liver inflammatory processes, a number of conditions have been linked to this inflammatory pathway. Inflammasomes regulate cell fate and pyroptosis, which differ from apoptosis by damaging plasma cell membrane and lack of chromatin condensation, leading to the secretion of various danger molecules. The clinical entities involved are drug-induced liver injury, endotoxin-induced liver injury, and alcoholic and nonalcoholic fatty liver diseases [23]. IRI processes, which are linked with liver resection, liver manipulation, hypovolemia, and OLT, have been linked to this proinflammatory pathway as well. The data presented herein support these findings, demonstrating the importance of inflammasomes and IL-1 β release in the process of IRI. Other known data, such as cold ischemia time, warm ischemia time, and other specific donor conditions, cannot be ignored and are described widely [24,25]. Nonetheless, the different clinical outcomes of IRI and the different clinical presentation, can be, at least partially, attributed to different gene expression of IL-1 β in donor livers, leading to a more significant inflammatory process during IRI in OLT [26,27].

In most cases of dysfunction in this cohort, there was a gender mismatch, which affected female recipients (5/6) much more than male recipients (4/10). There are in literature very

few reports on this issue, and most are related to lesser graft survival, specifically regarding male recipients and female donors [28,29]; however, the arguments presented are related with the hormonal milieu of the donor and its action on the colangiocytes [30].

The only recipient pathology directly implicated with graft survival concerning gender mismatch is chronic hepatic disease with hepatitis C virus infection [31], wherein a female donor is an independent predictor of fibrotic evolution and graft loss [32]. However, this issue does not apply to this study, because only 1 of the 12 female recipients in group of 54 patients presented with fibrosis. There are some reports on poor prognosis in female recipients with donor mismatch, which is considered an independent risk factor for primary graft nonfunction [33]. The causes are yet to be clarified, but the role of minor histocompatibility antigens such as H-Y antigen, codified by genes from Y chromosome, has been suggested in literature; further studies are awaited [34].

Study Limitations

The main limitation of this study is the small number of patients enrolled. Many variables can be accounted for, but no previous selection was performed, and patients were enrolled randomly. In this regard, more studies with greater numbers of participants are needed.

We decided not to exclude FAP grafts from deceased donor grafts; it is well-known that FAP grafts experience less damaging phenomena, all previous to harvesting. As we previously described, histologic data differ in these 2 types of graft. Accordingly, proinflammatory data can also differ. It should be also pointed out that gene expression related to histologic findings was different from that described herein. This matter should be elucidated further.

In conclusion, several genetically determined proinflammatory expressions may play a critical role in the development of graft dysfunction after OLT. We found that gene expression of IL-1 β and SELE in donor livers were linked with liver dysfunction after OLT.

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