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Biomarkers of tolerance and immunological monitoring in liver transplantation

V.E. Syutkin*, N.V. Borovkova, M.S. Novruzbekov

N.V. Sklifosovsky Research Institute for Emergency Medicine,

3 Bolshaya Sukharevskaya Sq., Moscow 129090 Russia

Correspondence to: Vladimir E. Syutkin, Dr. Med. Sci., Leading Research Associate, N.V. Sklifosovsky Research Institute for Emergency Medicine, e-mail: vladsyutkin@gmail.com

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Introduction*. We reviewed the literature data on clinical and laboratory parameters that allow predicting the development of operational tolerance in liver transplant recipients after their complete weaning from immunosuppressive therapy.*

The aim *was to identify possible biomarkers of tolerance in liver transplant recipients with the successful complete weaning from immunosuppression for subsequent implementation in routine clinical practice.*

The cellular, humoral, and molecular markers of the liver transplant recipients who were completely withdrawn from immunosuppressive therapy without the development of graft dysfunction were estimated. The

authors underlined the necessity of clinical trials for identifying biomarkers of the operational tolerance development.
Keywords: operational tolerance, liver transplantation, biomarkers

ACR, acute cellular rejection
CMV, cytomegalovirus
EBV, Epstein-Barr virus
HCV, hepatitis C virus
HLA, human leukocyte antigen
IS, immunosuppression
NK cells, natural killer cells
PBMC, peripheral blood mononuclear cells
PCR, polymerase chain reaction
Th17, T-helper cells
T-reg, regulatory T cells

Introduction

Successful organ transplantation became possible after the implementation in clinical practice of immunosuppressive agents, which impede the development of a rejection reaction and a subsequent graft loss. At the same time, a long-term continuous use of immunosuppressive drugs leads to the development of adverse effects associated with a decreased supervision of the immune system, the most threatening of them being malignant neoplasms. In this regard, an important task of transplantologists is the selection of optimal and adequate immunosuppressive therapy that reduces the risk of adverse reactions, on the one hand, and provides immunological tolerance to the transplanted organ, on the other one.

In 1993, T.Starzl et al. published an observational series of 6 liver recipients who were insufficiently compliant to immunosuppressive therapy and independently stopped taking the drugs. Those recipients maintained a normal liver transplant function for 5–13 years of follow-up [1]. This condition, in the absence of histological signs of progressive graft damage and/or rejection, was termed operational tolerance. Tolerance is a specific reaction of the body when the immune system does not respond to alien antigens by the immune response development. With an absent response to this antigen, the body's ability to respond to any other antigen is preserved. Observation of patients who had spontaneous operational tolerance did not reveal an increased risk of infections or malignant neoplasms, and their response to vaccination was similar to that in a healthy population.

The observed cases described by T.Starzl et al. convincingly showed the potential to the development of persistent tolerance in liver recipients, but at the same time there was a need for reliable diagnostic markers, which could serve as a sufficient ground to refrain from further immunosuppressive therapy.

The purpose of this review was, based on literature data, to identify possible biomarkers of tolerance in liver transplant recipients with successful complete withdrawal of immunosuppression (IS).

Clinical markers of tolerance

Following the first report by T.Starzl et al. (1993), several retrospective studies were published confirming the assumption that a complete discontinuation of IS in liver transplant recipients in some cases did not result in a rejection development [2–6]. The incidence rate of acute cell rejection (ACR) in those studies was 12–76%. But the ACR episodes per se were of a mild course in most cases and often resolved

after the return to the baseline IS without administering steroid boluses. Cases of chronic rejection were extremely rare (0–6%), and the graft loss was casual by nature [2, 4]. These studies demonstrated the possibility of IS discontinuation in stable liver transplant recipients, but the small sample size and/or lack of uniform, well-standardized protocols of patient selection, drug withdrawals and patient follow-up do not allow a qualitative generalization of the information. It is believed that the possibility of achieving operational tolerance in liver transplantation is about 20% [4, 7, 8].

The results of the first two prospective, multicentre, and independently controlled clinical trials of IS discontinuation [9, 10] were free from the limitations inherent in the previous studies. In one of these studies, IS was discontinued in 20 carefully selected children, liver transplant recipients [10]. The normal function of the graft, for at least a year after a complete IS discontinuation, was preserved in 12 children. Histological examination of liver tissue 2 years after the complete IS withdrawal did not reveal any significant changes compared with the baseline data. The most significant clinical factor associated with a successful IS discontinuation was the time interval between transplantation and the start of IS withdrawal (101 months in the subgroup of children with operational tolerance compared to 73 months in the subgroup of children who did not succeed in achieving tolerance; p=0.03). None of the children developed irreversible damage of the graft.

Another study by A.Sanchez-Fueyo's group included 102 adult liver transplant recipients from Barcelona, Rome, and Brussels at least 3 years after transplantation [9]. In 41 cases, the IS was successfully discontinued; a stable transplant function retained for at least 12 months after the drug discontinuation. No signs of rejection were seen at histological examination of liver tissue samples obtained 12 and 36

months after the IS discontinuation. Successful cessation of IS was associated with the time elapsed since transplantation, the older age of the recipients during transplantation, and the male gender. In recipients who had had more than 10 years since transplantation, the successful IS discontinuation was possible in 79% of cases, while in a subgroup of patients who had had less than 6 years since transplantation, operational tolerance was achieved in less than 15% cases, which confirmed the results of the pediatric study. Recently, the final results of the A-WISH study were published, in which the withdrawal from IS was early and took place in the second year after transplantation [11]. Only 10 of 77 recipients (13%) succeeded in achieving operational tolerance (a complete IS withdrawal possible while maintaining normal transplant function for at least a year after the IS withdrawal). In a significant number of cases, serious adverse events were observed. The results of this study confirm the important role of the time elapsed after transplantation in the ability to achieve operational tolerance.

The results of these studies suggest that tolerance can be observed in 40-50% of recipients in case of a careful selection of liver transplant recipients considering the clinical and histological criteria (such as the time after transplantation for more than 3 years; the absence of recent rejection episodes, absent autoimmune diseases and inflammatory changes in the liver tissue), as well as provided carefully following the IS discontinuation protocol.

Due to the risks associated with a graft rejection, there is a need for an accurate prospective identification of individuals who have become operatively tolerant of their transplanted liver. This would ensure a personalized approach by a safe withdrawal of IS in individual recipients, and also study the mechanisms that determine the formation of tolerance,

thereby contributing to the deliberate induction of tolerance in those who do not develop it spontaneously.

Cellular markers of tolerance

The first attempts to identify biomarkers of tolerance in liver transplant recipients with a successful complete IS discontinuation were aimed at identifying specific circulating cells by immunophenotyping using a flow cytometer. So, in Japan, in living donor liver transplantation to children who developed operational tolerance, a higher concentration of regulatory T cells (T-reg) ($CD4^{+}CD25^{high+}FOXP3^{+}$) was detected in peripheral blood compared to the patients in whom immunosuppressive therapy could not be withdrawn. Also, recipients with tolerance showed an increased ratio Vdelta1/Vdelta2 of gamma-delta T-cells in blood compared to normal[12]. Another study demonstrated that the increase in the ratio of Vdelta1/Vdelta2 of gamma-delta T cells, previously detected in peripheral blood, was noted in the graft tissue [13]. A significant accumulation of T-regs was revealed in the liver graft tissue of tolerant recipients compared to intolerant ones [14].

Similar results were obtained when analyzing the adult tolerant recipients who were prospectively studied in the course of the gradual withdrawal of immunosuppression [15]. T-reg infiltration of the graft was found to be transient by nature, and the number of these cells returned to its baseline level by the third year after the IS withdrawal and did not correlate with parallel changes in peripheral blood.

There were other retrospective studies that quantified the FOXP3 transcripts by the polymerase chain reaction (PCR) [16]. No differences were detected in the number of transcripts in peripheral blood mononuclear cells before the IS discontinuation between tolerant and intolerant patients. However, as IS was ceasing, tolerant patients showed

an increase in FOXP3 transcripts, while this did not occur in the patients with a lack of tolerance. Another study showed that FOXP3 levels in the graft were higher in the liver samples obtained from tolerant patients. This corresponded to an increase in the number of T-regs within the graft in the patients with tolerance compared to the patients with normal liver function [14].

A number of researchers consider natural killer cells (NK cells) to be important participants in the development of operational tolerance. The accumulation of NK cells in the liver in operational tolerance suggests their involvement in the development and maintenance of this clinical phenomenon. The hepatic compartment of NK cells is unique. If the content of NK cells in peripheral blood makes 5–15% of the entire population of lymphocytes, then they account for 30–50% of those in the liver tissue [17]. It is likely that the microenvironment in the liver affects the interaction of dendritic and NK cells, which is accompanied by the enrichment of the hepatic population of T-regulatory cells [18]. A detailed review of the cells involved in immune responses in the development of rejection and tolerance has recently been published [19].

Often, the development of operational tolerance is preceded by viral infections, the most common of which are EBV in children and HCV in adults. NK cells function at the junction of adaptive and innate immunity. In the process of escaping the innate immunity, viral infections such as EBV, CMV, and HCV negatively affect the development of an antigen-specific adaptive response, which leads to an increased population of T-regulatory cells in the graft tissue [20]. The relationship has been described between the development of post-transplant HCV infection over 5 years and T-reg markers and T-reg-associated cytokines, such as transforming growth factor beta and interleukin 10 [21]. Thus, it

is possible that the key role of NK cells in innate immunity may also be necessary to trigger and maintain allospecific tolerance.

A key role in predicting a possible IS discontinuation can be played by $CD4^+$ interleukin 17^+T helper cells (Th17). In a rat study, it was shown that Th17 cells cause a liver allograft rejection [22]. An increase in Th17 in liver transplant recipients during ACR compared to non-rejection recipients has also been reported [23]. Moreover, the Th17 differentiation in peripheral blood correlated with histological signs of rejection [24]. Prospective serial monitoring of immunological biomarkers undertaken by researchers from South Korea revealed that the T-reg/Th17, Th1/Th17, and CD8/Th17 ratios increased more considerably in patients with tolerance than in patients without tolerance amid lowering the dose of immunosuppressive drugs. Moreover, in patients with tolerance, the T-reg/Th17 ratio remained elevated during a 60-month follow-up period [25]. Another group of researchers emphasized the importance of plasmacytoid dendritic cell precursors as biomarkers of transplant tolerance [26].

The role of immunosuppressive drugs taken by the recipient in the development of operational tolerance is under discussion. Calcineurin inhibitors have remained the main drugs for maintenance IS after liver transplantation for several decades. Calcineurin inhibitors can inhibit tolerance by decreasing the number and function of regulatory T cells. Alternative immunosuppressants, such as proliferative signal inhibitors (mTORs), inhibit effector T cells, but retain the T-reg population and may contribute to a successful withdrawal from immunosuppressive therapy [27]. It was shown that the number of T-regs in peripheral blood was lower in liver transplant recipients receiving tacrolimus compared with the patients receiving monosuppression with sirolimus. The T-reg population increased as did the regulatory dendritic cell population after

replacing tacrolimus with sirolimus [28, 29]. French researchers compared the number and functional state of T-reg amid the IS conversion from tacrolimus to sirolimus (n = 5) or to everolimus (n = 10). Patients of both groups showed a steady increase in T-reg levels as early as after 3 months after the conversion to mTOR inhibitors, by average twice from the baseline. In contrast, in the control group of recipients who continued to receive tacrolimus, no significant changes in the T-reg population were observed. T-regs retained their functional ability to suppress activated T cells [30].

Thus, one of the candidates for the role of a biomarker for the development of tolerance after liver transplantation can be T-regs ($CD4^{+}CD25^{high+}FOXP3^{+}$), which concentration significantly increases both in the liver tissue and in the blood after the immunosuppressive therapy withdrawal in tolerant patients. At the same time, the increase in T-regs is not constant, which casts doubt on the reliability of this parameter.

Humoral markers of tolerance

It is known that the presence of antibodies against human leukocyte antigens of the organ donor (donor-specific antibodies) in a recipient is a risk factor for the development of the graft rejection. The issue of humoral rejection associated with the production of donor-specific antibodies deserves a special attention in kidney transplantation. The effect of anti-HLA (human leukocyte antigen) antibodies on the outcome of heart and lung transplantation has also been noted. In liver transplantation, donor-specific antibodies do not have such an importance, which is associated primarily with the peculiarities of the immunoglobulin metabolism. But meantime, when IS is discontinued,

monitoring of donor-specific antibodies in liver transplant recipients can be critical for the timely detection of a rejection risk [31].

In a recently published study, the investigators from the United States demonstrated that the appearance of de novo donor-specific antibodies in cadaveric liver transplant recipients undergoing the dose reduction of immunosuppressive therapy was associated with the rejection development. Moreover, regardless of the ongoing IS, the majority of *de novo*-formed donor-specific antibodies were directed against HLA class II, namely, against HLA–DQ (78.7%), including DQB1 (57.4%) and DQA1 (21.3%). At the same time, the appearance of *de novo*-formed donor-specific anti-HLA-A and anti-HLA-B was observed only with the complete withdrawal of the immunosuppressive therapy [32].

Molecular markers of tolerance

Studies by A. Sanchez-Fueyo from Barcelona, Spain, have shown that liver tolerance can be predicted by using molecular biomarkers. The profile of the expressed genes indicating the tolerance was first identified in the blood of functionally tolerant recipients and the corresponding controls. Mostly, those were the genes encoding gamma-delta cells and NK cells [33]. The results of that study were confirmed on the samples collected prior to IS discontinuation in the RISET Consortium trial [34]. Spanish scientists were the first (2008) to use microarray technology to determine the gene expression profile of peripheral blood mononuclear cells (PBMCs) in the recipients who achieved operational tolerance [34]. In a retrospective simultaneous study, the authors compared 16 tolerant recipients and 16 recipients in whom IS withdrawal failed due to the rejection development. They identified 462 positively and 166 negatively regulating genes. Real-time experiments, using microarray technology

and real-time PCR, confirmed the overrepresentation of transcripts, mainly expressed by gamma-delta cells and NK cells, in tolerant patients. Unfortunately, the determination of the PBMC antigen profile was not reproduced in any of three clinical centers participating in the study and could not reliably predict the outcome of IS withdrawal.

Interpreting the profile of genes derived from PBMCs was also difficult due to the retrospective study design and the lack of simultaneous molecular analysis of the graft tissue. Subsequently, both obstacles were overcome by the same research team in a prospective multicentre study of IS withdrawal in liver transplant recipients [35]. Of the 75 recipients who completed the study, the immunosuppressive therapy was successfully discontinued in 33. The study of PBMC genes once again confirmed transcriptional enrichment of natural killer cells and gamma-delta T cells; parallel comparisons were of particular interest, showing that the gene profile obtained from liver tissue was a more reliable, accurate, and reproducible biomarker of tolerance. Moreover, the expression profile of the genes obtained from the graft did not coincide with the genes identified in PBMCs.

The assay of liver tissue using microarrays followed by the verification of gene expression by real-time PCR revealed a group of 10 genes (TFRC, PEBP1, MIF, CDHR2, SOCS1, IFNG, HAMP, SLC5A12, DAB2, HMOX1), which differential expression was significantly associated with tolerance. An unexpected observation was the overrepresentation of genes involved in iron metabolism (e.g., transferrin receptor 1 (TFRC), hepcidin (HAMP), macrophage inhibition factor (MIF). Moreover, this “iron signature” accurately predicted the outcome of the IS withdrawal regardless of any clinical parameters. Moreover, in contrast to recipients without tolerance, the recipients with operational tolerance had higher serum levels of hepcidin and ferritin and an

increased iron deposition in hepatocytes [35]. These results indicate the critical role of iron metabolism in the regulation of alloimmune reactions in the graft and provide a set of biomarkers for conducting clinical trials related to the attempt to cancel IS in liver transplantation.

The combination of 5 genes of the 10 above indicated, measured prior to the IS withdrawal, made it possible to accurately distinguish those liver transplant recipients in whom immunosuppressive therapy could be successfully discontinued from those in whom the IS withdrawal would be accompanied by a graft function impairment. This predictive set of genes included the following 5 genes: SOCS1, TFRC, PEBP1, MIF, CDHR2 and predicted the outcome of IS withdrawal with the sensitivity of 89%, specificity of 86%, the positive predictive value of 80% and negative predictive value of 92%. The gene profile differed from that obtained earlier in the study of blood mononuclear cells or the whole blood and was reproduced in all three clinical centers participating in the study. This set of genes was originally identified in 48 liver transplant recipients from Barcelona and confirmed in an independent cohort of 21 recipients in Brussels and Rome [35].

By combining data from many Centers, both pediatric and adult, from the deceased and living subjects included in the analysis, a Stanford group led by M. Sarwal developed a 13-gene set predictive of tolerance obtained by studying peripheral blood and showing very good prediction accuracy (sensitivity of 100%, specificity of 83 %). This predictive ability will apparently be sufficient to eliminate the need for obtaining the "gene signatures" from biopsy specimens, which were previously thought to have an advantage as tolerance biomarkers [36]. The combination of the three most significant genes in NK cells allowed us to build a logistic regression model with a combined AUC of 0.988. Individual AUCs for each gene were 0.70 for ERBB2, 0.83 for SENP6, and 0.87 for FEM1C.

Using this three-gene model by Q-PCR method, all 13 samples of tolerant patients and 12 of 13 samples of intolerant patients were correctly identified with an error rate of 3.84%.

Conclusion

So, a significant problem in finding biomarkers of tolerance in liver transplantation has been, first of all, the retrospective design of most studies that compare parameters in the patients who have empirically achieved operational tolerance with those whose immunosuppression cannot be withdrawn because of a relapse of graft rejection. There is very little information regarding biomarkers of operational tolerance before the start of a dose reduction or a complete withdrawal of immunosuppression.

In this area, we are still lacking enough prospective clinical trials where the immunosuppression withdrawal would be initiated basing on the results of biomarkers and better understanding of the immunological and local processes in the transplanted organ, the processes that are induced or act during immunosuppression withdrawal until a stable stage of tolerance has been achieved. In 2014, the LIFT study was launched, in which the selection of liver transplant recipients in an attempt to cancel immunosuppression was based on biomarkers. The publication of preliminary results is expected in 2020.

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Information about authors

Vladimir E. Syutkin, Dr. Med. Sci., Leading Research Associate, N.V. Sklifosovsky Research Institute for Emergency Medicine, https://orcid.org/0000-0001-8391-5211

Natalya V. Borovkova, Dr. Med. Sci., Head of the Scientific Department of Biotechnology and Transfusiology, N.V. Sklifosovsky Research Institute for Emergency Medicine, https://orcid.org/0000-0002-8897-7523

Murad S. Novruzbekov, Dr. Med. Sci., Head of the Scientific Department for Liver Transplantation, N.V. Sklifosovsky Research

Institute for Emergency Medicine, https://orcid.org/0000-0002-6362-7914

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