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TOLERANCE WITHOUT MICROCHIMERISM: IS THAT POSSIBLE IN LIVER TRANSPLANTATION?

KARACİĞER TRANSPLANTASYONUNDA MİKROKİMERİZM OLMADAN TOLERANS MÜMKÜN MÜDÜR?

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Öz

Amaç

Karaciğer transplantasyonu sonrası tolerans kavramı son yıllarda giderek popülarite kazanmaktadır. Bu çalışmada amacımız çok özenli parametrelerle seçilmiş yaklaşık on yıllık bir seride düşük doz immünosupressif kullanan bir grupta toleransın varlığını CD3+ hücrelerde mikrokimerizmin yokluğu ile kanıtlamaya çalışmaktır.

Gereç ve Yöntem

Mikrokimerizm yokluğunu araştırma yöntemi; retrospektif olarak donör kanlarından cross match yapılarak DNA izolasyonunun ardından, mikrosatellit markerlarının PCR ile amplifikasyonu ve dönör ve alıcı allelerinin karşılaştırılması ilkesine dayanıyordu.

Bulgular

Postoperatif takiplerinde organ reddi ya da buna bağlı komplikasyonları olmayan alkole bağlı sirozu olan 12 hastadan bir izogrup oluşturuldu. Bu hastalar immunsüpresif olarak takrolimus ve mikofenolat mofetil kullanıyordu ve ilaç kan düzeyleri 5 ng/l yani kabul edilebilir sınırın altında idi. Çalışmamızda hiçbir hastamızda kimerizm gözlemlemedik.

Sonuç

Biyokimyasal stabiliteyi koruyan mikrokimerizmin yokluğu yani transplantasyona tolerans olması durumunda immunsupressif tedavilerin kesilebilir olmasının düşünülebilirliği gündeme gelmektedir.Böylelikle immünsüpressif tedavilerin yan etkileri azalacak ve hastalar böbrek yetmezliği, metabolik bozukluklar, diyabet ve kanserlerin artmış riskinden korunacak, dolayısı ile yaşam kalitesi artmış ve transplantasyon sonrası kullanılan ilaçların ülke ekonomisine yükü azalmış olacaktır.

Anahtar Kelimeler: Mikrokimerizm, Karaciğer Nakli, Tolerans

Abstract

Objective

Itisuncoveredtherelationshipbetweenmicrochimerism and liver graft tolerance. Many studies inspired the concept of microchimerism and tolerance in solid organ transplantation (SOT). Our aim is to explore this aspect in a strictly selected patients' cohort on CD3+ cells to show the microhimerism disappearance prove the tolerance instead of presence of it.

Materials and Methods

We strictly selected liver transplantation patients treated with calcineurins inhibitors (cnis), with very low dose of drugs who has never developed documented rejection. The DNA extraction and

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microchimerism research method used was based on the discrimination of donor and recipient alleles by PCR amplification of microsatellite markers and capillary electrophoresis with fluorescence detection.

Results
Twelve patients with alcohol-induced cirrhosis without rejection or rejection-related complications during the post-operative course were selected. The immunosuppressive regimen included tacrolimus and mycophenolate mofetil. The blood residual tacrolimus concentration was under or equal 5 ng/L below normal. We described the count of white blood cells and selected CD3+ after the MACS separation procedure. In our study, we did not observe any microchimerism in none of the patients.

Discussion
We achieved that in case of absence of microchimerism with maintained biochemical stability we could choose to switch off the immunosuppressive therapy even the microchimerism accepted like a condition to the tolerance till our study. Consequently, it will be reduce the side effects of immunosuppressive treatment such as renal failure, metabolic disorders, diabetes mellitus and cancers and the patient's quality of life will be improved be decreased financial aspect of immunesuppressive treathment

Keywords: Microchimerism, Liver Transplantation, Tolerence

Introduction

Understanding the immunological phenomenons underlying the graft's tolerance could be considered as the 'Everest' of transplantation.

From the aspect of liver transplantation, some patients are described as tolerant in the non-observance of immunosuppressive drugs context or in the immunosuppressive drugs withdrawal controlled protocol.

The liver graft appears to be more prerogative in terms of immune tolerance than kidney, lung or heart with a low incidence of antibody-mediated rejection. Although tolerogenic mecanisms are not fully understood and strategies for immunesuppressive drug withdrawal should be selected carefully to avoid graft rejection (1)

In liver transplantation, the graft's rejection does not impair the organ's function definitely.

The significance of understanding or mastering the mechanisms of immunological tolerance or rejection is twofold. Firstly, it could propose an immunosuppressive program 'A la carte', adapted to each patient's profile and may reduce the side effects of immunosuppressive treatment such as renal failure, metabolic disorders, diabetes mellitus and cancers.

The second advantage would be financial since the costs related to the treatment and the management of complications could be reduced (2,3)

Many studies have dealt with tolerance in bone marrow transplantation and inspired the concept of microchimerism and tolerance in solid organ transplantation (SOT)(4). Studies revealing the relationship between microchimerism and liver graft tolerance are increasing.

In this study, which was carried out on a carefully selected small number of patients, we have attempted to shed light on microchimerism in homogeneous liver transplanted patients, who have not presented any graft rejection in their post-transplant course with minimal immunosuppressive treatment.

Our aim is to explore this aspect in a strictly selected group of non-immunological disease patients' cohort on CD3+ cells to show the microchimerism disappearance is real and prove the tolerance instead of its presence.

Material and Methods

This study was carried out in accordance with the approval of "Strasbourg University Clinical Research Ethics Committee" with date 16.10.2018

Selection of patients and exclusion criteria.
Our choice focused on a group of liver transplanted patients, grafted between 2005 and 2015, the period when the researchers worked at this hospital. Among 835 liver transplantations performed during that period, we made a selection of those only with alcoholic indications.

The patients grafted due to viral cirrhosis, auto-immune or metabolic diseases, fulminant hepatitis, and liver tumour were excluded from the group. We

have also excluded pediatric patients, split liver grafts and combined transplantations.

A second selection was operated regarding the immunosuppressive treatment. We have restricted our selection only to those patients who were treated with tacrolimus as calcineurins inhibitors (CNIs), with very low dose of drugs (less than < or = 5 ng/L as blood concentration), who has never developed documented rejection.

Donor blood samples
Donor blood samples were necessary to study microchimerism.

In the French organisation system of regulation and graft allocation, donor patients are identified by the CRISTAL number.

We did not take into account HLA compatibility for liver transplantation as opposed to kidney transplantation, however the local French Blood Establishment (Etablissement Français du Sang) of Strasbourg kept the blood donor samples identified by the CRISTAL number that allowed the realisation of microchimerism research in our center the recipient blood. We transplantedafter cross-match even for liver transplantation.

Immunosuppressive strategy
The immunosuppressive protocol usually combines FK506-Mycophenolate Mofetil and steroids. In renal failure cases, as a result of using the basiliximab at the induction, the introduction of calicineurin inhibitor's (CNIs) is delayed.

For long term immunosuppressive regimen, the dose of CNIs is reduced regarding the renal function and hepatic enzymes tests. Steroids are discontinued at the end of three months, except in autoimmune disease context. Mycophenolate mofetil is reduced or discontinued in case of digestive or haematologic intolerance. mTOR inhibitors are chosen in case of CNIs neurological intolerance or renal toxicity.

Consent
Patients were informed orally for the protocol program. A written document was also edited, describing the aim of the study and the steps of the research. If the patients accepted being part of the study, they had to give their consent orally and also in written by signing the consent document. This consent was also signed by the physician explaining the way the research would be led.

Two copies of the original consent document were made: one for the patient, the second for the patient's application; and the original was kept by the co-ordinator physician.

The consent guarantees the anonymity of the results. We specifically stressed the point on the importance to continue the immunosuppressive treatment.

Strasbourg University Ethic Council decision was required and acquired in the autumn session at the date of 3rd November 2018

No financial support was requested.

Recipient's blood samples
For the study, four EDTA tubes of 7 ml, each filled with peripheral human blood, were required.

The patients were convened to the outpatient clinic for the routine visit.

With the classic blood check-up (blood, renal and liver tests), four blood tubes were ponctionned and send to the Hematological Laboratory for the MACS* separation (Miltenyi Biotec*)

Protocol of CD3+ cells separation
▪ Principle of MACS separation
CD3+ is expressed on all T cells and is associated with the T cell receptor.

The CD3+ cells are magnetalically labeled with CD3 MicroBeads.

Then the cell suspension is loaded onto a MACS* Column which is placed in the magnetic field of a MACS Separator. These magnetically labelled CD3+ are retained on the column and the unlabelled run through.

The column is removed from the magnetic field and then the magnetically CD3+ can be eluated as the positively selected cell fraction.

▪ Protocol
Peripheral blood mononuclear cells are isolated by density gradient centrifugation Ficoll.

- Magnetic labelling
- Volumes for magnetic labeling given are for up to 10E7 total cells.

- Pass cells through 30µm nylon mesh to remove cell clumps.

- Determine cell number.

- Centrifuge cell suspension at 300Xg for ten minutes. Pipette off supernatant completely.

- Suspend cell pellet in 80 µL of buffer per 10E7 total cells.

- Add 20 µLof CD3MicroBeads per 10E7 total cells.

- Mix and incubate for fifteen minutes at 4°C.

- Wash cells by adding 1-2 mL of buffer per 10E7 cells and centrifugate at 300Xg for ten minutes and pipette off supernatant completely.

- And resuspend up to 10E8 in 500µLof buffer.

- **Magnetic separation**
- Place an appropriate MACS*Column and a MACS* separator according to the number of total cells.

- Prepare column by rinsing with appropriate amount of buffer LS: 3mL.

- Apply cell suspension onto the column.

- Collect unlabeled cells which pass through and wash column with appropriate amount a buffer. Perform washing steps by adding buffer three times, each time once the column reservoir is empty.

- Collect the total effluent, which is the unlabelled cell fraction.

- Remove the column from the separator and place it on a suitable collection tube.

- Pipette appropriate amount of buffer onto the column. Immediately flush out fraction with the magnetically labeled cells by firmly applying the plunger supplied with the column.

- Centrifugate and control the count and the label

DNA extraction and microchimerism research
The method used was based on the discrimination of donor and recipient alleles by PCR amplification of microsatellite markers and capillary electrophoresis with fluorescence detection.

Genomic DNA was extracted from donor's and recipient's total leucocytes or positively selected CD3+ cell fraction from recipient using Qiamp DNAminiKit (Qiagen) on a robotic workstation Qiacube (Qiagen) according to the manufacturer's recommendations. DNA was obtained from purified CD3+ fraction using QiampDNA microKit (Qiagen) on manual protocol as recommended by the manufacturer.

DNA quantification was performed using UV absorption at 260nm on a Nanodrop spectrophotometer (Thermoscientific)

- **PCR amplification**

In order to screen for informative markers that enables discrimination between recipient and donor alleles, 21 highly polymorphic microsatellite markers, included di-, tri-, tetra- and penta-nucleotide repeat were co-amplified in 3 multiplex PCR. The forward primers were labelled with 5-FAM, VIC, NED or PET fluorescent marker at the 5'end. Multiplex PCR amplification was performed in a 50µl final volume containing 2x GeneAmp PCR buffer (Applied Biosystem), 5U AmpliTaq Gold (Applied Biosystem, Life technologies), 2.5mM MgCl2, 1M Betaïne, 70µM dNTPs, 4 pmoles of each primer and 40ng of template DNA. PCR amplification was performed in a TProfessional Basic Thermocycler (Biometra, Germany). After an initial incubation step at 95°C for 10min to active the hot-start Taq polymerase, the PCR protocol consisted of 30 cycles with denaturation at 95°C for 45s, annealing at 59°C for 90s, elongation at 65°C for 25s followed by a final elongation step at 65°C for 30min and finally 10°C for ever.

Results

Twelve patients (5 male and 7 female) were included in the protocol. All participians was adult (aged 44-63) and mean age was 53.5. Only patients with alcohol-induced cirrhosis without rejection or rejection-related complications during the post-operative course were selected.

The immunosuppressive regimen included tacrolimus and mycophenolate mofetil. Table 1 represents the donor/recipients data and the time of follow-up since the date of LTx. All liver transplantations were performed in isogroup.

The blood residual tacrolimus concentration was under or equal 5 ng/L below acceptable levels. Hepatic enzymes tests at the time of consultation were analyzed (Table 2). For patient n°7, we can observe anicteric cholestasis because of occasional alcohol abuse. A mild elevation of alkaline phosphatase is often observed in liver transplanted patients (Patients nos. 3, 8 and 12).

Table 1 Donor and recipients data

N° Patient	Year of LTx	D/R Age At time of LTx	Follow-up time (in days)	D/R Sex	D/R blood group	Cross match
1	2005	19/47	3885	M/M	B/B	N
2	2010	45/53	2178	M/F	A/A	N
3	2007	45/57	3300	F/M	B/B	N
4	2008	65/61	3008	M/M	O/O	N
5	2011	41/56	1675	M/F	A/A	N
6	2008	39/57	3041	F/F	O/O	N
7	2010	63/46	2283	F/F	A/A	N
8	2010	45/63	2299	F/F	O/O	N
9	2012	41/44	1478	F/F	O/O	P
10	2015	63/47	536	M/M	O/O	N
11	2014	28/54	886	M/M	O/O	N
12	2015	54/57	408	F/F	A/A	N

Abbreviations: LTx, Liver Transplantation – D, donor – R, recipients – N, negative – P, positive – M, male – F, female

Table 2 Liver enzymes test at the time of blood punction for the study of microchimerism. The values indicated in parenthesis are the normal values according the biochemical laboratory

N° Patient	FK506 blood residual concentration (5-15) ng/L	SGOT (15-40) UI/L	SGPT (10-49) UI/L	ALP (41-117) UI/L	GGT (11-69) UI/L	Total bilirubin (1.7-21) µmol/L
1	3.7	14	12	9	28	7.7
2	1.3	21	18	83	22	11.5
3	3.8	25	27	139	31	9.9
4	2.5	25	15	79	42	4.3
5	4.2	17	14	71	19	9.6
6	4.7	28	15	71	31	11.7
7	4.7	28	18	422	392	21.5
8	2.6	14	13	133	25	9.9
9	5	19	13	86	9	7.6
10	4.8	24	30	69	11	6.5
11	4.9	20	20	112	25	10.6
12	4.7	16	13	135	14	6.5

Table 3 describes the count of white blood cells and CD3+ after the MACS separation procedure. Patients n°7 and n°11 present leucopenia. For patient n°7, because of alcohol abuse, we can suppose a hyperpsplenism phenomenon and for n°11, a potential toxicity of mycophenolate mofetil is implied.

Results of DNA extraction and microchimerism research

In this retrospective study, we did not observe any microchimerism in none of the patients.

We have chosen patient n°1 to illustrate the results. Figure 1 represents the chimerism research after PCR amplification of the R9 discriminant microsatellite. The first graph above represents the donor peaks at 238.85 and 273.24 wave lengths. The graph below represents the recipient peaks at 259.79 and 277.05 wave lengths. The two last graphs confirm the absence of donor DNA in the recipient blood.

For a better accuracy of these results, a dilution was performed to a level of 0.5% of sensibility. Figure 2 illustrates the manipulation. The small peaks at 238 are considered as shadows and are not considered for the presence of donor in the recipient blood.

Table 3 The white blood cells count after MACS separation

N° patient	Count		Lymphocyt T	
	Leucocyt (10^6/L)	Lymphocyt (10^6/L)	CD3+ (10^6/L)	CD3+/CD4+ (10^6/L)
1	4500	977 (21.7%)	743 (76%)	498
2	5500	1854 (33.7%)	1372 (74%)	1168
3	5900	1475 (25%)	1018 (69%)	708
4	7100	987 (13.9%)	543 (55%)	345
5	5200	1430 (27.5%)	1201 (84%)	729
6	6400	2029 (31.7%)	1157 (57%)	832
7	2200	748 (34%)	658 (88%)	277
8	7400	1110 (15%)	888 (80%)	555
9	6100	2318 (38%)	1808 (78%)	974
10	5400	1836 (34%)	1248 (68%)	569
11	2500	650 (26%)	436 (67%)	195
12	9900	5148 (52%)	4736 (92%)	721

Discussion

Liver transplantation is the only therapy for end-stage liver diseases. The development of immunosuppression with the cyclosporine in the eighties improved the outcome of LTx in terms of rejection management, and overall graft and patient survival. However, the immunosuppressive treatment is associated with many side effects, such as renal insufficiency, metabolic and cardiovascular disorders. The transplanted patients are also exposed to a higher risk of developing infectious diseases and malignancies (1). The financial impact of treatment for thousands of patients and the treatment of side affects are important. If we can stop this treatment, the gain of country will be considerable

Thus the mechanisms of antigen/antibody immune response could help to the understanding of tolerance induction. It is also assumed that the immunosuppression protocol influence a tolerance state. One of the most promising methods is cell-based strategies for immune tolerance induction, as chimerism induced by hematopoietic stem cells and adoptive transfer of regulatory macrophages, regulatory T cells, regulatory dendritic cells, regulatory B cells, and mesenchymal stromal cells (2,3).

Tolerance in transplantation represents the Grail of the subject and constitutes a sheer challenging area

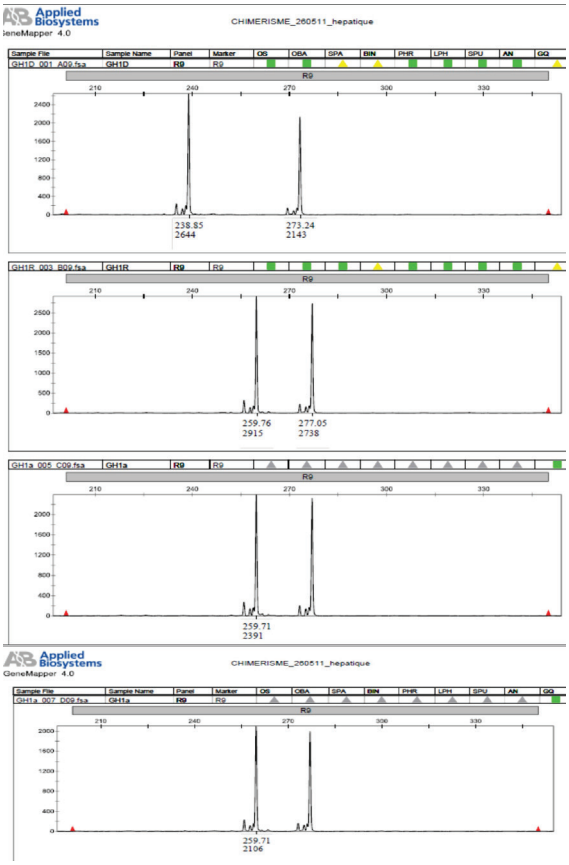

Figure 1
Chimerism research after PCR amplification for patient n°1.

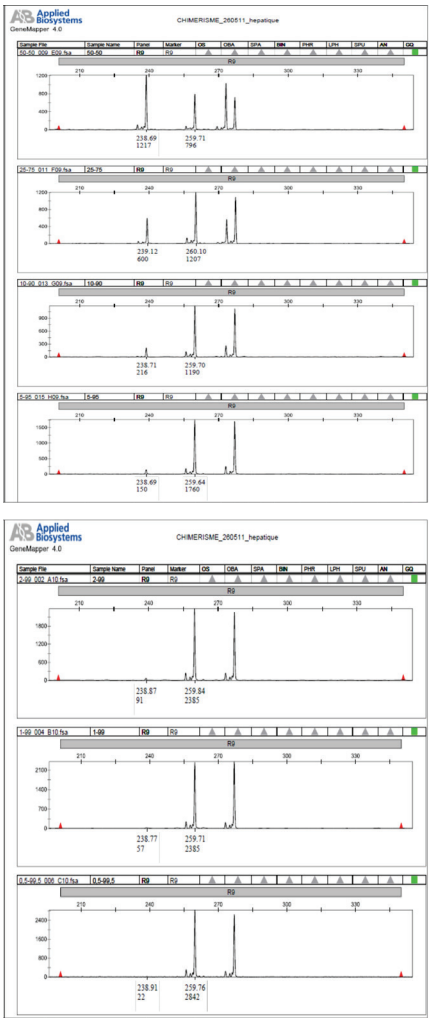

Figure 2
The dilution program for a sensibility of 0.5% (patient n°1)

(4, 5). Immunosuppressive withdrawal was observed in a few cases and graft tolerance was observed in non-observance situations. This possibility would open a new way in transplantation, proposing a tolerance therapy “A la carte” and alleviating morbidity and mortality, related to the use of such drugs (6-8).

It is described that microchimerism was a condition for liver graft tolerance and observed such phenomenon in “old” kidney transplanted patient (9-11). An interesting clinical case was reported with a complete hematopoietic chimerism and tolerance in a nine-year old child (12). Prospective clinical trial of weaning of immunosuppression in liver transplant recipients was described a cohort of 23 patients free from immunosuppressive drugs among 104 liver transplantations, however it requires a permanent management and follow-up of rejections events (13-14). Accordingly to a publication their findings suggest that chronic rejection does not occur in pediatric liver transplant recipients receiving tacrolimus-based immunosuppression, provided baseline immunosuppression is maintained (15,16). Protocols inducing mixed chimerism are described with infusion of donor bone marrow into recipients, however these protocols have revealed to have toxic side effects. (17-18). Among adult liver transplant patients, age at time of immunosuppression withdrawal and length of time since transplant are both significant predictors of successful immunosuppression withdrawal, which suggests that immunosenescence and immune exhaustion play a role in tolerance this development. Remarkably, in patients 20 years posttransplant, more than 90% will not have rejection after withdrawal of immunosuppression(19)

With its particular anatomical situation, the liver is an interesting and mysterious organ. The liver has

hematopoietic properties from the embryologic area and is perfused with a double circulation from the portal vein draining the intestine, which contacts with many digestive antigens and the arterial circulation (20). The other circuit is the bile drainage against the current, which could play a role of epuration. The sinusoids are borded with liver sinusoidal endothelial cells, which behave as antigen presenting cells. The Kuppfer cells play a role of clearance with the phagocytosis activity of activated T cells. The hepatic dendritic cells also are pointed to be involved in tolerance with a potential of extrahepatic migration and may influence systemic immune response (21). The liver is also composed of Natural Killer T cells that have been suggested to mediate pro-tolerant effects.

Our centre experiences a large series of liver and kidney transplanted patients. We aimed to study the presence of microchimerism in an extremely selected cohort of patients. We have based our study only on those patients with alcoholic indications to avoid other immunological interferences in the research due to autoimmune or viral diseases. The twelve patients included in the study were selected according the initial disease, the absence of proved rejection, with normal hepatic biology and with a reduced immunosuppressive therapy composed with tacrolimus and mycophenolate mofetil.

The microchimerism research was realized on blood samples. Considering our results, we do not observe microchimerism in these tolerant patients. Our study is realized in a one time-point and may not reflect a dynamic phenomenon from the time of transplantation. We aimed and achieved that in case of absence of microchimerism with maintained biochemical stability and independently of time after transplantation we could choose to switch off the immunosuppressive therapy even the microchimerism accepted like a condition to the tolerance till our study (22-23). Consequently, it will be reduce the side effects of immunosuppressive treatment such as renal failure, metabolic disorders, diabetes mellitus and cancers and the patient's quality of life will be improved.

Conflict of Interest

The authors have no conflicts of interest to declare.

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