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Immunosuppressive activity of serum taken from a liver transplant recipient after withdrawal of immunosuppressants

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Abstract

In orthotopic liver transplantation (OLT), tolerance is induced in a certain combination of donors and a recipient in rats and, in some clinical cases, rejection has not occurred in OLT patients after weaning off immunosuppression. However, this mechanism has not yet been elucidated. Among our cases of liver transplantation (LTx), one OLT patient (Patient A) has not required immunosuppressive drugs for the last 5 years, following post-transplant lymphoproliferative disease (PTLD). This patient's serum interleukin-2 levels were undetectable following withdrawal of immunosuppressants. The same serum taken after discontinuing the immunosuppressants inhibited concanavalin A blast cultured cells and up-regulated the IL-4/IFN-γ gene expression ratio. These results suggested that other proteins were induced following withdrawal of immunosuppressants. Proteomic assay demonstrated 12 differentiated spots exclusive to this patient where immunosuppressants have been discontinued. Haptoglobin, found to have immunosuppressive activity in vitro, may play an important role in the maintenance of drug-free tolerance as a natural immunological suppressor after cessation of immunosuppression. Proteomic analysis will allow us to develop a novel weaning protocol for patients on long-term immunosuppression to avoid major immunosuppressant-related complications.
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Keywords: Drug-free tolerance; Liver transplantation; Post-transplant lymphoproliferative disease; Two-dimensional gel electrophoresis

1. Introduction

In solid organ transplantation, rejection is inevitable and must be treated with long-term immunosuppressive agents, such as tacrolimus and cyclosporin A, which cause deleterious side effects [1–3]. It is unethical to randomly terminate immunosuppressive therapy among transplant recipients simply to investigate its natural course. In our series of 245 living donor liver transplantations (LDLT), one recipient has not received any immunosuppressive agent in the last 5 years following a diagnosis of post-transplant lymphoproliferative disease (PTLD). This successful cessation of immunosuppressive drug therapy, although incidental, was found to be one of the most effective therapies for lymphoma after transplantation. If a reliable marker for allograft tolerance is available allowing safe termination of immunosuppressants at a stage following transplantation, similar recipients may avoid developing PTLD.

Our objective is to investigate cytokines and proteins in the serum of this immunosuppressant-free liver transplant recipient, which may act as tolerance markers. The development of effective monitoring markers which may indicate when

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immunosuppressants can be safely withdrawn will result in less recipient exposure to these immunosuppressants and avoid immunosuppressant-related complications.

2. Materials and methods

2.1. Serum collection

All serum samples were stored at −70 °C until analysis. Serum samples were taken after obtaining informed consent from the parents or guardians of the patients, as well as ethics approval from the Chang Gung Memorial Hospital.

2.2. Patient characteristics

Fifteen biliary atresia (BA) patients (10 males and 5 females; mean age=5.5 years; range=4–7 years) underwent LDLT and treated with immunosuppressant were enrolled in this study. One of these patients, who underwent Kasai's operation in 1990 at the age of 2 months, was able to be completely weaned from immunosuppressants. In 1994, the patient underwent living related liver transplantation (LRLT) and was treated with an immunosuppressive regimen consisting of a calcineurin inhibitor (cyclosporin A, CsA) and steroids. After 5 years, the patient suffered PTLD, which required the cessation of the immunosuppressive drugs as well as comprehensive chemotherapy. Nevertheless, this patient has not needed immunosuppressive drugs for 5 years since the remission of the PTLD. Sera of this patient were collected from 1994 (before liver transplantation) to 2004. Sera of all healthy volunteers (n=10; 5 males and 5 females, mean age=32 years; range=27–36 years) were obtained from donors for liver transplantation. All participants provided informed consent for the use of sera in these studies.

2.3. Time course of IL-2 level in the serum of a drug-free patient

The concentration of interleukin-2 (IL-2) in the serum of a drug-free patient was determined by standard enzyme-linked immunosorbent assay (ELISA) using an antibody preloaded kit (Pierce-Endogen, Rockford, IL, USA) at different numbers of days before and after weaning off immunosuppressants.

2.4. 5-(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling

Peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers were labelled by 5-(6)-[carboxyfluorescein diacetate succinimidyl ester] (CFSE, Sigma, St. Louis, MO, USA) as previously described [4]. Briefly, a 5-mM stock solution of CFSE in dimethyl sulfoxide (DMSO, Sigma) was thawed and diluted to 5 μM in a volume of phosphate-buffered saline (PBS) equal to that in which the responder cells (1×10^7 cells/ml in PBS) were suspended and incubated at 37 °C for 10 min. The labelling process was quenched by adding an equal volume of heat-inactivated fetal bovine serum (FBS, GIBCO™ Invitrogen Corporation, Carlsbad, CA, USA) to the sample. After 1 min, the CFSE-labeled cells were washed twice, recounted and adjusted to a concentration of 1×10^6 cells/ml in AIM-V serum-free culture medium (GIBCO™ Invitrogen), and then stimulated with 2.5 μg/ml of concanavalin A (ConA, Sigma) and 10 ng/ml of phorbol myristate acetate (PMA, Sigma). An equal number of cells (1×10^6 cells/ml) was prepared in a total volume of 200 μl per well in 96-well round-bottom microculture plates. Sera of different groups (normal controls, BA patients under the treatment of immunosuppression and patient A before and after weaning of immunosuppression) were supplemented into the ConA blast culture at different concentrations (2% and 4%) and were co-cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cultured cells from each well were harvested after 4 days. CsA (10 μg/ml) was added into ConA blast culture as a positive control. Fluorescence staining was performed on an Epics® ALTRA™ flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA) using EXPO32 software. The residual cells were stored at −70 °C until analysis.

2.5. RNA isolation and reverse transcription

RNA was extracted from PBMCs with a ConA blast culture using TRIzol® reagent (GIBCO™ Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from total RNA employing ImProm-II™ Reverse Transcriptase (Promega Biosciences, Inc., San Luis Obispo, CA, USA) following the manufacturer's instructions. The cDNA were then amplified by polymerase chain reaction (PCR) in a total volume of 50 μl containing 3 μl of cDNA, 1 μl of the 10-μM PCR primer, 5 μl of 25-mM dNTP, 5 μl of 10-fold PCR buffer, 35.5 μl of H_2O, and 0.5 U *Taq* enzyme (Roche Molecular Biochemicals, Mannheim, Germany). The human-specific PCR primers are shown in Table 1. The PCR reaction was hot-started at 94 °C for 2 min to denature all cDNA samples. After the initial denaturation step, the cDNA samples were subjected to rounds of denaturation (94 °C for 40 s), annealing at 55 °C to 60 °C for 30 s, and extension at 72 °C for 30 s. Each reaction was completed by heating at 72 °C for the final 10 min. The samples were amplified with different cycle numbers based on optimal amplification efficiencies: 30 (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), 22 (interferon gamma (IFN-γ) and IL-2) and 30 (IL-4). The amplification products were separated by electrophoresis on 2% TBE agarose gels and stained with ethidium bromide. The gel profiles were visualized (photographed) with ultraviolet illumination gel documentation (UVItec, Cambridge, UK) and analyzed with ultraviolet illumination photo version 99 (UVItec) and TotalLab software version 1.00 (Nonlinear USA, Inc., Durham, NC, USA). PCR products were titrated to establish standard curves for documenting linearity and permitting semiquantitative analysis. Levels of gene expression were expressed as the ratios of densities between PCR products and GAPDH in the same sample. All experiments were repeated three times.

2.6. Two-dimensional (2-D) electrophoresis

Approximately 5 μl of serum samples were solubilized in a rehydration buffer containing 8 M urea, 2% of 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate (CHAPS), 0.002% of bromophenol blue, 0.5% of immobilized pH gradient (IPG) buffer (pH 3–10 linear) and 18 mM dithiothreitol (DTT). The samples were then separated by 13 cm Immobiline DryStrip 3–10 linear on sn IPGphor Isoelectric Focusing System (Amersham Bioscience Corp., Piscataway, NJ, USA, and Uppsala, Sweden) in the first dimension. The running conditions of the IEF were as follows: 30 V for 12 h; 500 V for 1 h; 1000 V for 1 h; 8000 V for 2 h; up to 17 kV h. Before second-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the IPG strips were equilibrated for 15 min in an equilibration buffer containing 50 mM Tris–HCl (pH 8.8), 6 M urea, 2% of SDS, 30% of glycerol, 1% of DTT and a trace of bromophenol blue. A second equilibration was performed with 2.5% of iodoacetamide in the same buffer with displaced DTT. The IPG gel strips were embedded on top of the gels. SDS–PAGE was carried out on 10% acrylamide gels at 24 mA/gel until the bromophenol blue dye front reached the bottom of the gel. After approximately 5 h, all of the gels were visualized by a silver staining method (Amersham), and the gel profiles were visualized (photographed) with UVI gel documentation (UVItec) and analyzed

Table 1
Characteristics of primers and PCR conditions

mRNA	Sense primer	Antisense primer	Annealing temperature (°C)	Sample cDNA (bp)
GAPDH	5′-CCATGGAGAAGGCTGGGG-3′	5′-CAAAGTTGTCATGGATGACC-3′	55	196
IL-2	5′-ATGTACAGGATGCAACTCCTGTCTT-3′	5′-GTCAGTGTTGAGATGATGCTTTGAC3′	60	458
IL-4	5′-CAACTTTGTCCACGGACAC-3′	5′-TCCAACGTACTCTGGTTGG-3′	60	345
IFN-γ	5′-GCTGTTACTGCCAGGACCCA-3′	5′-GCGTTGGACATTCAAGTCAG-3′	60	332

Table 2
Concentration of IL-2 cytokines in the serum of patient A

Days	IL-2 concentration (pg/ml)
POD 3 [a]	33.5±2.3
POD 7	44.5±3.9
Weaned of IMS [b]	N.D. [c]

[a] POD: early postoperative period.
[b] IMS: immunosuppressant.
[c] N.D.: undetectable <23 pg/ml.

with UVI photo version 99 (UVItec). Protein isoelectric points (p*I* values) and molecular weights were assigned by p*I* calibration markers and molecular weight markers (Bio-Rad Laboratories, Inc., Burlington, MA, USA), respectively. Protein spots were quantified using the Image-Master 2D Elite software (Amersham). Each experiment was repeated at least three times.

2.7. In-gel tryptic digestion of target proteins and mass spectrometry

Protein spots of interest were excised from the two-dimensional gels and destained in 30 mM potassium ferricyanide/100 mM sodium thiosulfate for 15 min. They were then washed three times with 25 mM ammonium bicarbonate/50% acetonitrile for 10 min, reduced in 10 mM DTT/ 25 mM ammonium bicarbonate for 1 h and alkylated in 55 mM iodoacetic acid/ 25 mM ammonium bicarbonate at room temperature. After 45 min, the gel pieces were incubated with 100 μl of 25 mM ammonium bicarbonate for 15 min. The supernatant was removed with 25 mM ammonium bicarbonate/ 50% acetonitrile and the samples were washed in 100% acetonitrile. The gel was then dried using a SpeedVac concentrator. The dried gel pieces were swollen in 10 μl of 25 mM ammonium bicarbonate containing 0.1 μg trypsin (Promega) and then incubated at 37 °C for 16 h. Peptides were subsequently extracted with 50 μl of 5% formic acid/50% acetonitrile, and dried with the SpeedVac concentrator. The peptides or pellets were resuspended in 30 μl of 0.1% formic acid/5% acetonitrile/95% ddH_2O. The obtained peptides were stored at −20 °C until analysis.

2.8. Liquid chromatography–mass spectrometry

The dimethylated tryptic peptides were analyzed by a Q-TOF micro spectrometer (Micromass, Manchester, UK) equipped with a nanoflow high performance liquid chromatography (HPLC) system (LC Packings, Amsterdam, Netherlands). Briefly, a tryptic digest solution (10 μl) was injected onto a column (NAN75-15-03-C18-PM; 75 μm×15 cm) packed with C18 beads (3 μm, 100 Å pore size, PepMap). Mobile-phase buffer A consisted of 5% acetonitrile in 0.1% formic acid; mobile-phase B was 95% acetonitrile in 0.1% formic acid. The peptides were first separated using buffer A for 5 min, and subsequently using a linear gradient of 0–50% solvent B over 55 min at a flow rate of 200 nl/min. For sequencing, MS/MS spectra were obtained by a survey scan and automated data-dependent mass spectrometry (MS) analysis was carried out using the dynamic exclusion feature built into the MS acquisition software. Each MS scan was followed by four MS/MS scans of the first four most intense peptide mass peaks to obtain as many collision-induced dissociation (CID) spectra as possible. The peptide sequences were identified using Mascot Search (www.matrixscience.com); identified proteins were categorized based on functions as described in the Swiss-Prot database.

2.9. Immunoblotting of transthyretin

The serum samples from liver transplantation recipients, which included approximately 30 μg total proteins, were added into each well for SDS–PAGE. The proteins were separated in 10% SDS–PAGE gels and transferred to immune-Blot PVDF (polyvinylidene difluoride) membranes (Bio-Rad) using a Mini Trans-Blot system (Bio-Rad) in transfer buffer (39 mM glycine, 48 mM Tris, 20% methanol) at 0.12 mA for 90 min. The membranes were blocked with 5% skimmed milk in PBS/0.05% Tween-20 (PBST) (1 h, 37 °C), and all the washing steps were performed with PBST (3×5 min). The membranes were incubated with diluted prealbumin rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500 in PBST/2% milk) overnight at 4 °C, and washed and further incubated with anti-rabbit IgG coupled to horseradish peroxidase-linked secondary antibody (1:5000 in PBS-T/2% milk) for 1 h at room temperature. Color development was performed with ECL Plus™ detection reagents (Santa Cruz Biotechnology).

2.10. Statistical analysis

Descriptive statistics, mean, standard deviation and range were used where appropriate. For comparison of groups the one-way ANOVA and Duncan post hoc test were used where appropriate. Results are given as mean values ±standard deviation of the mean. A $p<0.05$ was considered to indicate statistical significance.

3. Results

3.1. IL-2 level in the serum of a weaned patient

IL-2, a product of activated T-helper type 1 lymphocytes, is thought to be one of the main cytokines responsible for acute allograft rejection. Therefore, we investigated whether IL-2 concentrations increase after weaning off immunosuppressants. During immunosuppressant treatment in our patient, the

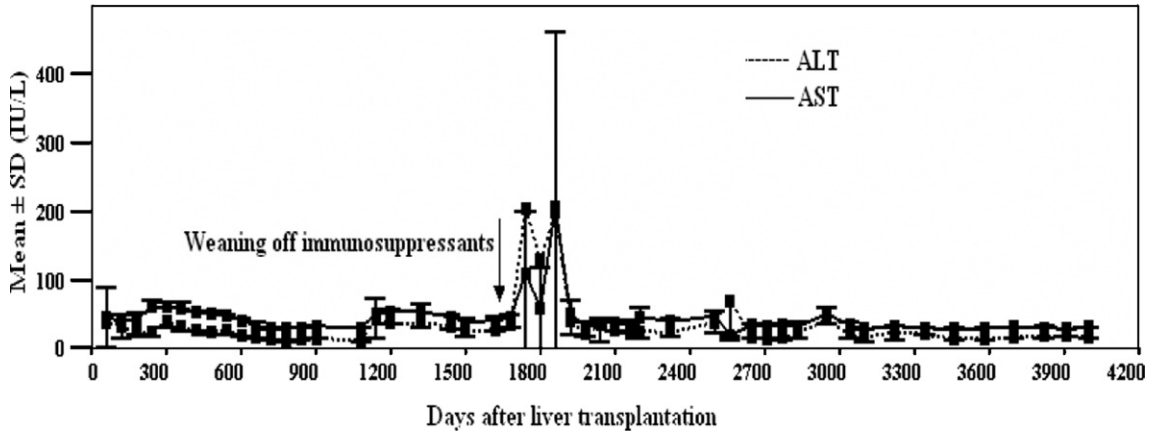

Fig. 1. Physiological changes in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in patient A before and after the cessation of immunosuppression.

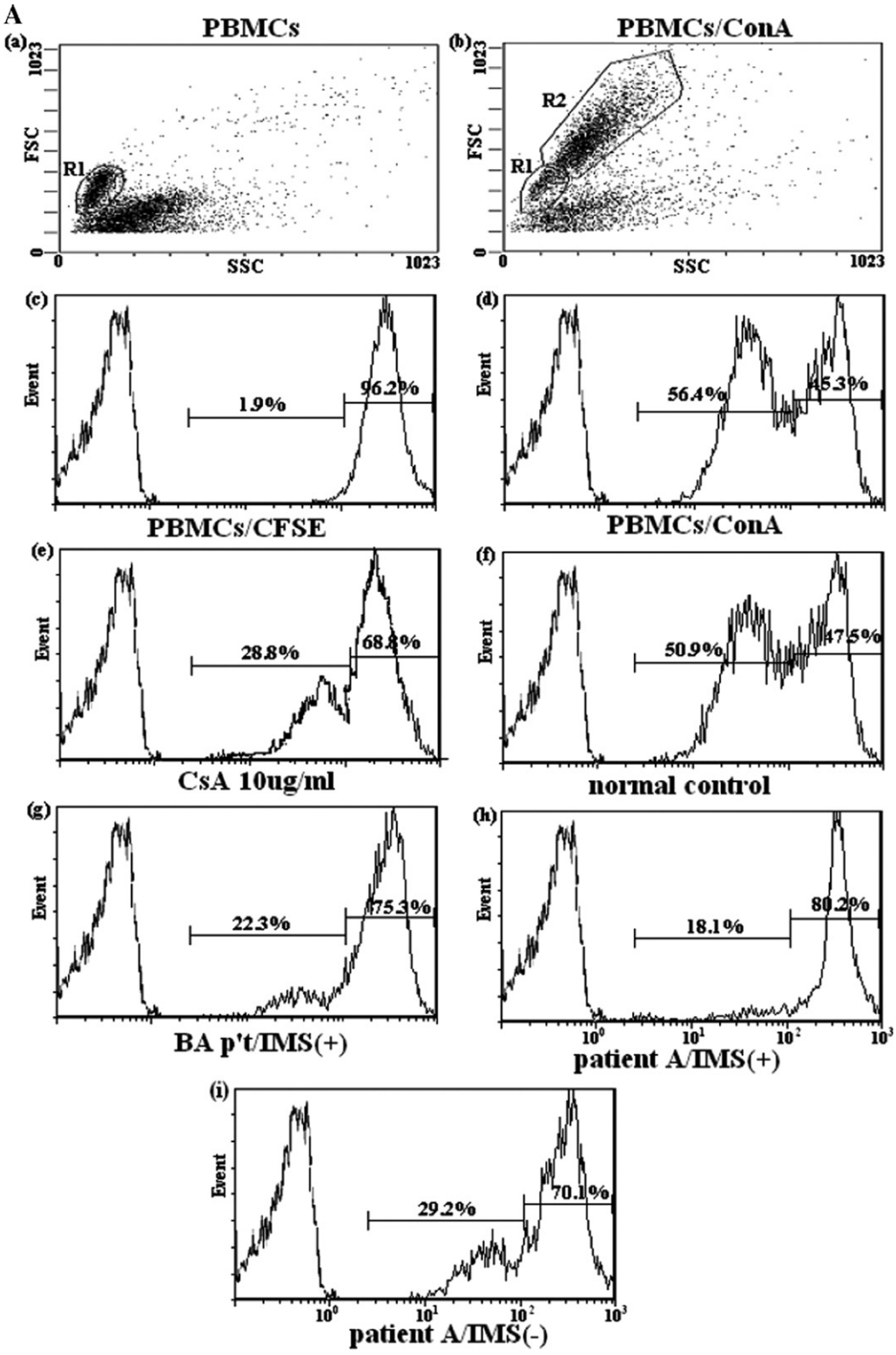

Fig. 2. Immunosuppressive activity of sera from patients. (A) Histogram plots (a, b) show the cell proliferation after ConA stimulation (R1: resting lymphocytes; R2: ConA blast). Histograms (c–h) gated by R1 and R2 show the cell division associated with CFSE labeling of human PBMCs: (c) PBMCs alone; (d) ConA blast; (e) ConA blast mixed with CsA (10 μg/ml); (f) ConA blast mixed with control serum; (g) ConA blast mixed with the serum of a BA patient (p′t); (h) ConA blast mixed with serum from patient A, who received immunosuppressants; and (i) ConA blast mixed with serum from patient A after weaning off immunosuppressants. The data are representative of three independent experiments with essentially similar characteristics. (B) Sera of patient A after the cessation of immunosuppressants still possess immunosuppressive activity. **Significantly suppressed as compared with normal control serum. IMS (+): immunosuppressants therapy; IMS (−): cessation of immunosuppressants.

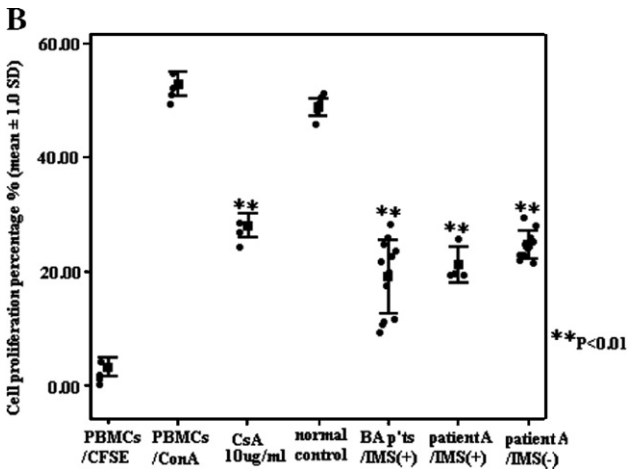

Fig. 2. (*continued*).

IL-2 levels in serum taken after OLT were 33.5 pg/ml±2.3 on day 3 and 44.5 pg/ml±3.9 on day 7. Interestingly, withdrawal of immunosuppression decreased IL-2 levels to undetectable levels (Table 2), suggesting that serum after weaning off immunosuppressants following OLT includes some factors which suppress IL-2 production. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were maintained in normal ranges after withdrawal of immunosuppression (Fig. 1).

3.2. Suppression of the lymphocyte proliferative response by the serum of a weaned patient

Sera from various groups including normal controls, BA patients after OLT, and patient A before and after weaning off immunosuppressant therapy following OLT were tested for their ability to suppress the in vitro proliferative response of ConA stimulation. CFSE-labeled cells were stimulated by ConA, 2% and 4% (data was not shown) of the sera of various groups were added to the ConA stimulation at the initiation of culturing. CFSE fluorescence was detected by flow cytometry. Two percent of the sera obtained from post-transplant BA patients or from patient A before and after weaning off immunosuppressants was found to be able to inhibit lymphocyte proliferation compared to healthy controls (Fig. 2A and B). The averages of trough levels of CsA in OLT patients are very high during immunosuppressive treatment. After the withdrawal of immunosuppression, the agent's CsA level is zero (Table 3). It is well know that jaundice suppress the proliferation and activity of lymphocytes, the total bilirubin (T-BIL) levels of this patient was within normal ranges (Table 3). These results indicate that the serum of an immunosuppression-weaned patient still contains immunosuppressive factors.

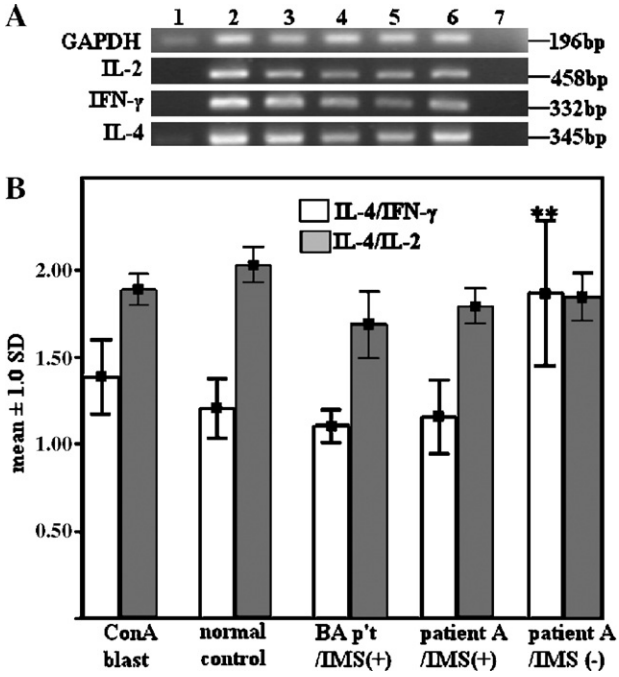

Fig. 3. Cytokine gene expression in ConA blast cultured with or without various sera. mRNA was isolated from PBMCs blasted by ConA with or without sera from control subjects, BA patients, or patient A. The mRNA was used to obtain first-strand cDNA, which was then used as a template in reverse transcriptase–polymerase chain reaction (RT–PCR) according to the protocols described in Materials and methods. (A) Th_1 and Th_2 cytokine gene expression (IL-2, IFN-γ and IL-4). Lane 1: marker; Lane 2: ConA blast; Lane 3: ConA blast cultured with control serum; Lane 4: ConA blast cultured with BA p′t serum; Lane 5: ConA blast cultured with patient A serum before cessation of immunosuppression; Lane 6: ConA blast cultured with patient A serum after cessation of immunosuppression; Lane 7: negative control. (B) The ratio of IL-4/IL-2 and IL-4/IFN-γ in ConA blast cultured with various sera. The relative gene expression was quantified by densitometric analysis. The bars indicate the average density values from three independent experiments. IMS (+): immunosuppressants therapy; IMS (−): cessation of immunosuppressants.

3.3. Cytokine profiles in ConA blast cultured with serum of a weaned patient

RNA was extracted from ConA blast co-cultured with diverse sera. The IL-4/IL-2 ratio of ConA blasts co-cultured with the sera of post-transplant BA patients or an immunosuppressant-weaned patient was not significantly different from that of ConA blasts co-cultured with the sera of control volunteers. In contrast, the IL-4/IFN-γ ratio of ConA blast co-cultured with

Table 3
Profiles of the clinical populations in this study

Group	Number of individuals (*N*)	Age (±S.D.)	Age at Tx (±S.D.)	Before Tx T-Bil[a] (mg/dl)	During 1 month after Tx T-Bil (mg/dl)	Past 5 years[b]		Weaning off IMS[c]	
						T-Bil (mg/dl)	CsA[d] (ng/ml)	T-Bil (mg/dl)	CsA (ng/ml)
(A) Normal	10	38 (2.5)	–	–	–	–	–	–	–
(B) Patient A	1	14.9	3 years/10 months	20.2	3.6±2.1	0.64±0.2	272.0±130.7	0.72±0.2	N.D.[e]
(C) Biliary atresia	14	5.9 (0.6)	1.5±0.7	25.3±6.6	3.3±2.9	0.75±0.3	711.3±487.0	–	–

[a] T-Bil: Total bilirubin (mean±S.D.).
[b] T-Bil and CsA levels in biliary atresia patients were mean of serum in this studied.
[c] IMS: immunosuppressant.
[d] Cyclosporin A, CsA levels in serum.
[e] N.D.: undetectable.

the serum of an immunosuppressant-weaned patient was higher than that of other groups (Fig. 3).

3.4. Serum protein profiling of the serum of a liver transplant patient before and after complete withdrawal of immunosuppression

The mechanisms involved in graft acceptance in a liver transplant patient following cessation of immunosuppressive drugs remain largely unclear. We hypothesize that some serum proteins may be exerted in the cascades when drug-free tolerance is induced. First, we carried out conventional comprehensive 2-DE analyses of serum proteins from a liver transplant patient before (Fig. 4A(a)) and after complete withdrawal of immunosuppression (Fig. 4A(b)). After separation by 2-DE, the differentiated protein spots were identified by LC quadrupole time-of-flight MS (LC/Q-TOF MS). A total of 27 spots displayed a significant in abundance in each gel when compared between groups (Fig. 4). Fifteen spots, which appeared in sera taken before the withdrawal of immunosuppression, were extremely reduced or absent in sera taken after the cessation of immunosuppression. Conversely, the remaining 12 spots were found to be more abundant in quantity after the complete withdrawal of immunosuppression (Fig. 4A(b)) but were markedly decreased or absent in serum samples taken before the complete withdrawal (Fig. 4A(a)).

3.5. Sequential identification of sera proteins

In order to determine the protein factors which might be specific to OLT drug-free tolerance, 27 target protein spots were selected and identified with a combination of peptide mass fingerprinting (PMF) and peptide C-terminal sequencing (Fig. 5). The spots were then classified into two categories according to volume expression before and after the cessation of immunosuppressants. In the 27 spots we identified 13 different proteins: haptoglobin, haptoglobin precursor, complement component 3 precursor, complement component C4A, ceruloplasmin, immunoglobulin gamma-1, apolipoprotein A-IV precursor, Ig heavy chain V–III region, hemopexin precursor, transthyretin, alpha-1-

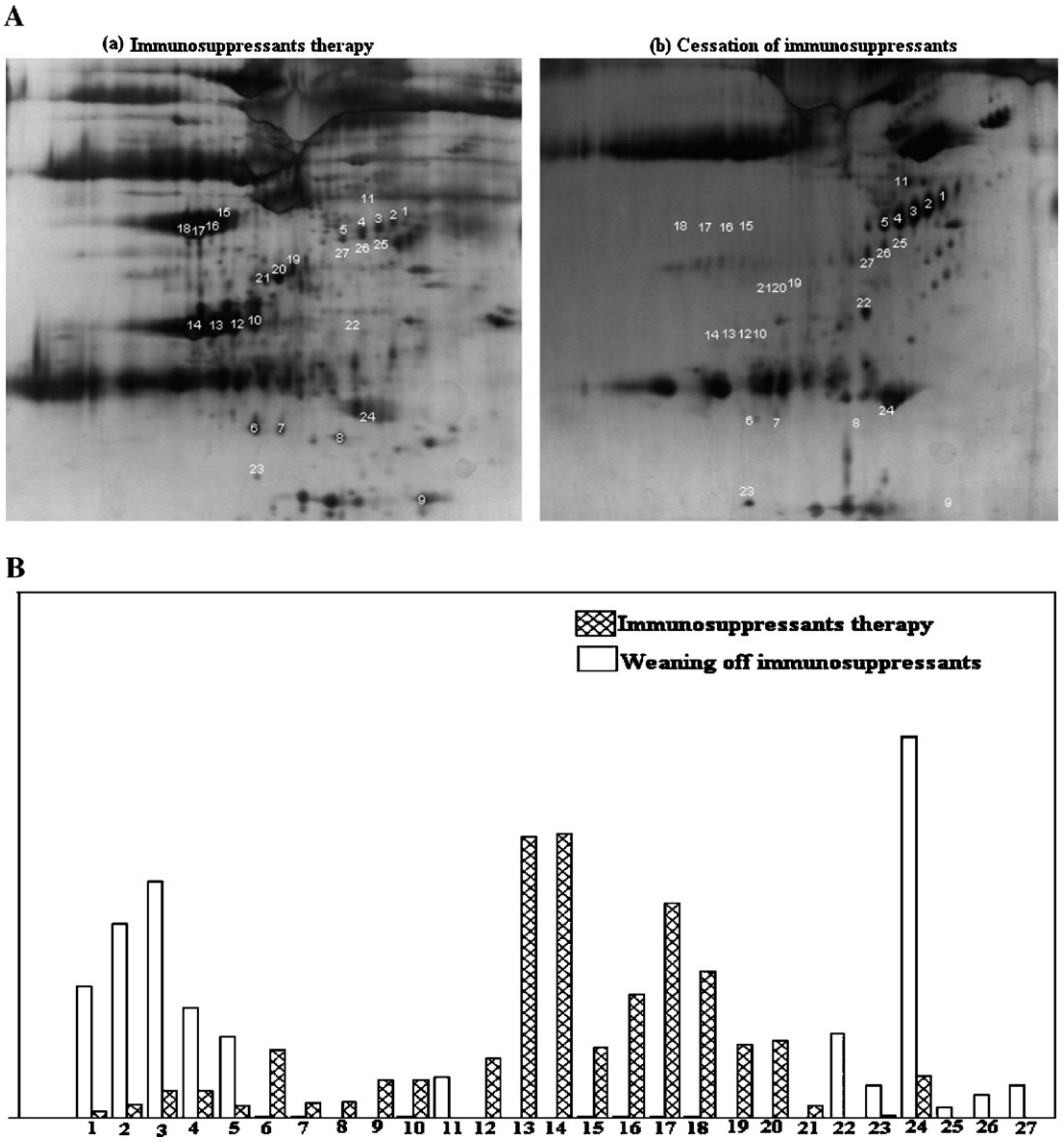

Fig. 4. 2-D analysis of serum from patient A before and after the cessation of immunosuppressants. (A) Sera (5 μl) were placed onto a linear 3–10 IPG, and second-dimensional SDS–PAGE was then performed in 10% SDS–PAGE. The protein patterns were visualized by silver staining. (B) Changes in the levels of protein expression before and after the cessation of immunosuppression. Each spot volume was determined and quantified from the intensity of spots derived from the silver-stained 2-DE (ImageMaster 2D Elite software). Solid bar: expression of protein in the serum from patient A with immunosuppressant treatment; open bar: expression of protein in the serum from patient A's after cessation of immunosuppressant therapy.

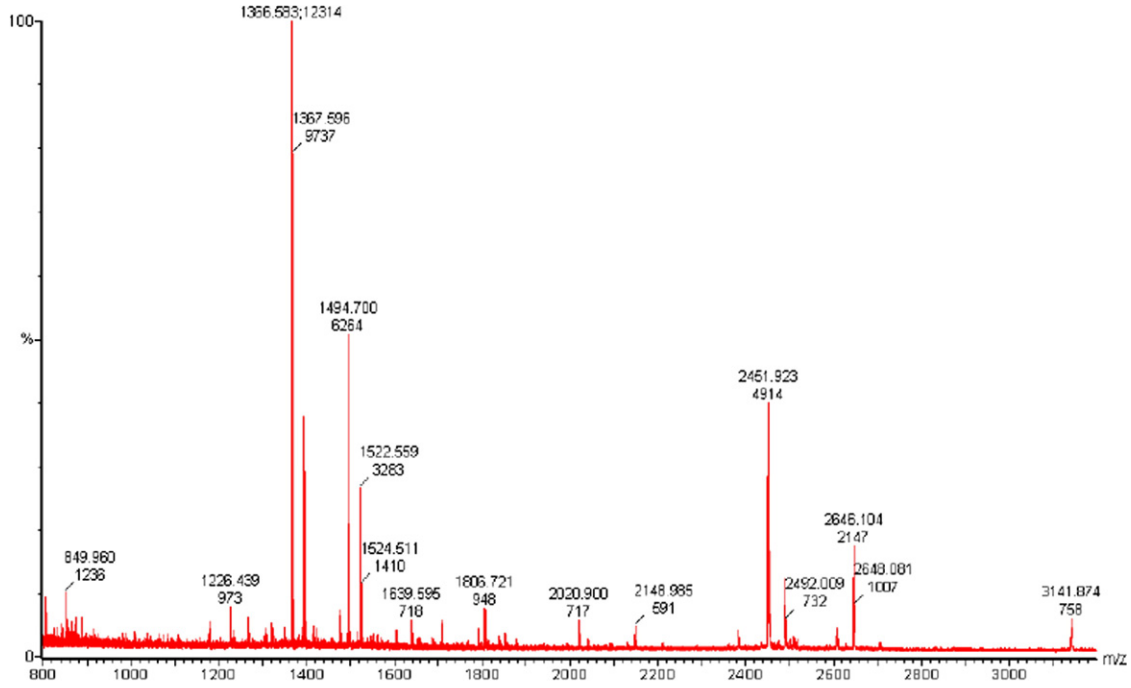

Fig. 5. Identification of transthyretin. A LC/MS spectrum obtained by in-gel trypsin digestion of the spot marked 22 in Table 3. By means of Mascot 9 peptides with *m/z*-values could be matched to Accession No. gi339685 human transthyretin from the Swiss-Prot database.

antitrypsin, proapolipoprotein and apolipoprotein AI (Table 4). The 27 mass spectrometry identifications each had a Mascot score over 82.

3.6. Immunoblotting of transthyretin expression in the OLT serum of an immunosuppressant-weaned patient

To view the 2-D gel profile, after immunosuppressant withdrawal the data showed that transthyretin protein increased compared to immunosuppressant treatment. To the explored transthyretin expression, sera of OLT patients and healthy controls were collected. After immunosuppressant stop in the drug-free patient, transthyretin protein was shown to have high levels of expression in the serum more than the healthy, BA patients when they accepted the immunosuppressant (Fig. 6).

4. Discussion

With respect to rejection, cytokines also play a crucial role in acute rejection. Several studies have examined the relationship between various cytokines and rejection in attempts to predict failure of immunotherapy after OLT [5–7]. One of the Th_1 cytokines, IL-2, is thought to be one of the main cytokines responsible for acute allograft rejection. The central role of IL-2 in T-cell activation and proliferation has been well documented [8–10]. In liver transplantation, Roayaie et al. have also reported that IL-2 levels in stable

Table 4
Proteins identified from patient A before and after withdrawal from immunosuppressant therapy

Spot no.	Protein name	Mascot score (NCBInr No.)	Spot no.	Protein name	Mascot score (NCBInr No.)
Before cessation of immunosuppressants			Cessation of immunosuppressants		
6–7	Complement component 3 precursor	>190 (gi4557385)	1–3	Haptoglobin	>152 (gi1212947)
8	Complement component C4A	82 (gi44367)	4–5	Haptoglobin precursor	>166 (gi67586)
9	Ceruloplasmin	148 (gi180249)	11	Apolipoprotein A–IV precursor	379 (gi28762)
10, 12–18	Immunoglobulin gamma-1	>144 (gi19717684)	22	Transthyretin	163 (gi339685)
19	Ig heavy chain V–III region	54 (gi106482)	23	alpha-1-antitrypsin	151 (gi177827)
20–21	Hemopexin precursor	188 (gi386789)	24	Apo-AI	250 (gi253362)
			25–27	Haptoglobin	>123 (gi1212947)

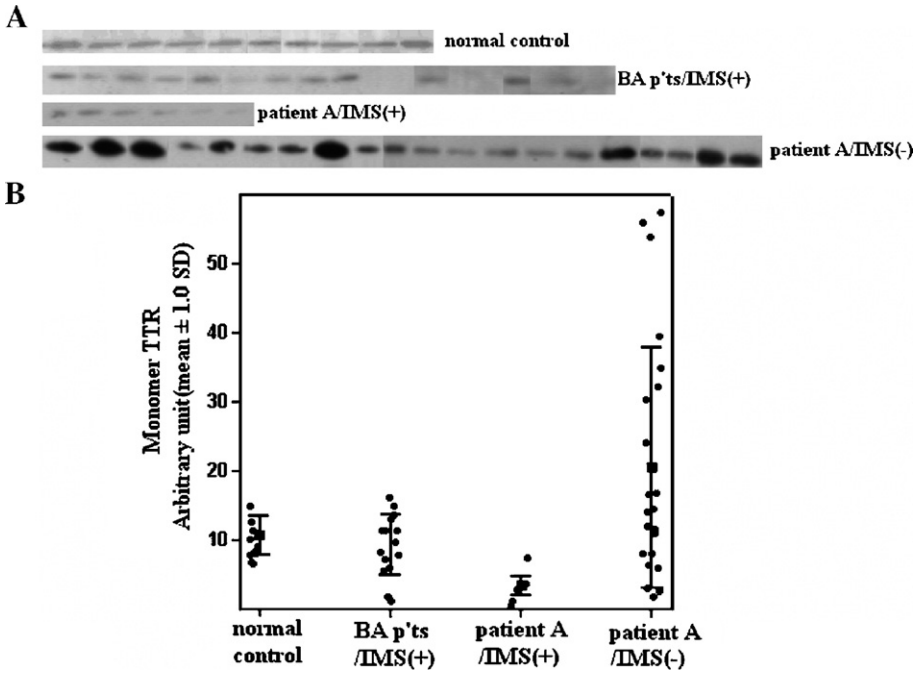

Fig. 6. (A) Western blot analysis using an anti-prealbumin antibody. Control sera ($n=10$), BA p′t sera ($n=14$), and patient A serum under immunosuppressant therapy (6 different time points) or after the cessation of immunosuppressants (20 different time points) were run on a 10% SDS–PAGE gel using a mini gel apparatus, and fractionated proteins were electronically transferred onto a transfer membrane. Anti-prealbumin antibody and anti-rabbit IgG–HRP were used as the first and second antibodies, respectively. (B) The expression of transthyretin (prealbumin) was quantified by densitometric analysis. IMS (+): immunosuppressants therapy; IMS (−): cessation of immunosuppressants.

recipients treated with immunosuppressive drugs are significantly lower than during the acute rejection phase [6,9]. In the present study, we have demonstrated that the IL-2 level of patient A was undetectable after weaning off immunosuppressants, suggesting that the rejection reaction of this patient may have been inhibited by other immunosuppressive factors after the cessation of immunosuppression. This speculation was well supported by showing the inhibitory effect of the serum (up to 2%) of patient A on ConA blast formation, as compared to that of the sera of normal volunteers (data not shown). In the cytokine paradigm of ConA blast added to serum in recipients of liver allografts, only patient A showed an increased IL-4/IFN-γ ratio after immunosuppressant withdrawal, suggesting that the serum of this patient after weaning included some factors capable of inducing Th_2-type responses and of down-regulating natural killer (NK) cell activity [11]. In the present study, proteomic analysis demonstrated that 27 spots were significantly different before and after immunosuppressant cessation (Table 4).

Although most of the identified proteins are not specific to tolerance, we are particularly interested in haptoglobin, of which level after withdrawal of the immunosuppressants was higher than that during immunosuppressant treatment. Haptoglobin has also been differentiated as an immunosuppressive protein in serum in a rat tolerogenic OLT (DA-PVG) model through the proteome approach [12]. In previous studies, we and other researchers have ascribed anti-inflammatory and immunosuppressive activities to haptoglobin [12–14]. For example, haptoglobin has been suggested to play a role in maternal–fetal tolerance induction known to reduce leukocyte activity by competitive binding to CD11b/CD18 [15]. Additionally, it reduces adhesion and invasion of the leukocytes to extracellular matrices; it may serve as an early protection against maternal immune cells; and may further induce immunological tolerance [13,16]. In view of the results of the present study, haptoglobin may play an important role in maintaining drug-free tolerance as a natural immunological suppressor during immunosuppressant cessation. Additionally, haptoglobin specifically interacts with both resting and activated $CD4^+$ and $CD8^+$ T cells, and plays a modulating role in the Th_1/Th_2 balance by promoting a dominant Th_1 cellular response, resulting in the strong suppression of induced T-cell proliferation [15,17]. We also demonstrates that this protein is down-regulated when immunosuppressive agents. Noel et al. have shown that the introduction of CsA to kidney transplanted patients results in a decrease in a 25% decrease in haptoglobin levels [18]. This may explain why the haptoglobin level in our OLT patient was low during immunosuppressant treatment. Intriguingly, haptoglobin affinity to CD11b, which is expressed on the majority of lymph node dendritic cell (DC) subsets (myeloid and plasmacytoid DCs), could prevent the maturation of this DC subset for regulation of Langerhans cell function, resulting in T-cell skewing or in the induction of regulatory T-cells [15,17,19,20]. Itano et al. also demonstrated a role of haptoglobin as a mediator in nasal tolerance induction [21], which suggests that haptoglobin may qualify as a (bio)marker for effective immunotherapy. It may be part of a natural feedback regulation, rather than being associated with inflammatory conditions. To the best of our knowledge,

haptoglobin, which is an acute-phase plasma protein associated with inflammatory disorders [22–24], is the first candidate to be involved in clinical and experimental liver allograft tolerance.

We also found that α_1-antitrypsin appeared in the serum of patient A after cessation of immunosuppressants. Some properties of α_1-antitrypsin play a specific role in the modulation of the immune system, including the inhibition of neutrophil respiratory burst [25]. Funding et al. have shown that α_1-antitrypsin significantly increases on 2-DE gels of aqueous humour from patients with corneal rejection, and suggest that the primary role of α_1-antitrypsin is to protect the cornea from degradation by neutrophil elastase during inflammation [26] and from the immunological rejection process [27]. α_1-Antitrypsin may also be produced as a natural immunosuppressant in the serum of a patient after withdrawal of immunosuppressants following OLT.

In addition, a plasma protein (transthyretin) was identified from serum sample of LDLR by 2-D gel electrophoresis and LC–MS. In normal human serum, transthyretin, also known as prealbumin, exists as a 54 kDa tetramer of four identical subunits arranged to form a cylindrical channel [28]. It also formed a complex with the retinol-binding protein to transport vitamin A indirectly. Transthyretin dissociation and misfolding resulting in insoluble amyloid fibrils has been implicated with familial amyloid polyneuropathy and senile systemic amyloidosis [29]. It was proposed that transthyretin could self-assemble into amyloid-like fibrils in acidic environments, such as the lysosome [30]. Our results have shown that transthyretin could not inhibit the cell proliferation in either mixed leukocyte reaction (MLR) or ConA blasted (data not shown). No other reports discussed the role of transthyretin in the immunology field. Therefore, further studies are necessary to fully explain the up-regulation of this molecule in this drug-free OLT patient.

We need protocols to successfully achieve an immunosuppression-free state with benefits derived from the return of natural immunity and a reduction of drug-related toxicity. Our study has enabled the detection of several proteins associated with a drug-free OLT patient. Although proteomic studies of single individuals involve inter-individual variation in proteomes and further investigation with more clinical cases is required, in conjunction with proteome studies regarding rat tolerogenic OLT, haptoglobin seems to be a significant potential marker for liver allograft tolerance. Our experiences and further proteomic analysis will allow us to develop a novel weaning protocol not only for patients who suffer PTLD or drug-related toxicity but also for OLT patients who survive for a long time after transplantation with no major complications.

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