The immunological role of lipid transfer/metabolic proteins in liver transplantation tolerance


Abstract

Background: In a rat tolerogenic orthotopic liver transplantation (OLT) model, recipient serum after OLT (post-OLT serum) has been reported to prevent allograft rejection. A previous proteomic study indicated that apolipoprotein E (apo-E), which is an important factor for cholesterol transportation, is expressed at the latter tolerogenic phase after OLT. It has also been known that adipose tissue-derived adipokine, adiponectin, is an essential factor for fatty acid catabolism. This study aimed to characterize the role of lipid transfer/metabolic proteins in liver transplantation tolerance.

Methods: To identify the apo-E and adiponectin in post-OLT serum, Western analyses and enzyme-linked immunosorbent assay (ELISA) were performed, respectively. The immunosuppressive activities of those factors were evaluated by inhibition of the mixed lymphocyte reaction (MLR). Results: Western analyses showed that the mobility of apo-E was shifted at the latter tolerogenic phase after OLT in a natural tolerance model, and a similar phenomenon was confirmed in the serum of a drug-induced tolerance model (rejection model+cyclosporin A (CsA); 0 to 14 days) after cessation of CsA. Further study revealed that neutralization of modified apo-E in post-OLT serum reduced the immunosuppressive activity. Additionally, plasma adiponectin was significantly elevated at the latter phase after OLT, and possessed MLR-inhibitory activity.

Conclusions: These results suggest that the mobility shift of apo-E and/or the up-regulation of adiponectin may be necessary for overcoming the rejection, recovering the liver allograft function, and following tolerance induction in experimental OLT models, and may be useful as one indicator to surmise the prognosis after liver transplantation.

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1. Introduction

The immunobiology of liver transplantation is unique and never obeys the transplantation rule (Medawar’s rule of transplantation); e.g., all grafts are rejected between unrelated individuals. In a rat model of orthotopic liver transplantation (OLT) in which the DA strain liver is implanted into the PVG recipient, liver allografts are spontaneously accepted without any kind of immunosuppressive treatments [1,2]. Furthermore, recipient rats grafting the allogeneic liver can accept subsequent same donor hearts or skin grafts [3]. In clinical
cases, patients receiving a combined kidney and liver allograft experience significantly less rejection of the kidney than patients receiving a kidney allograft alone [4]. These observations suggest that a liver allograft can induce donor-specific tolerance.

The liver as an important hemopoietic organ gives rise to all leukocyte lineages, including extrathymic T cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells (DCs), and granulocytes [5]. This unique combination of leukocytes in the liver may be the major cause of liver tolerogenicity. In addition to these cell-mediated mechanisms, it has been known that the recipient serum after OLT (post-OLT serum) possesses immunosuppressive activity in a rat tolerogenic OLT model (DA liver into PVG) [6,7], raising the possibility that humoral immunosuppressive factors might also play a role in liver tolerogenicity. Several candidates for the immunosuppressive factors have been identified in the post-OLT serum. In the early phase after OLT, soluble, donor-specific MHC class I molecules and anti-histone H1 autoreactive antibodies (Abs) are transiently expressed in the post-OLT serum and possess immunosuppressive activity [8–11]. Additionally, in the latter tolerogenic phase after OLT, anti-donor MHC class II Abs and liver suppressor factor (LSF)-1 (40 kDa), which was purified from post-OLT serum at 60 days, are expressed and can suppress allograft rejection in rat heterotopic heart transplant and OLT models [12–14]. However, the search for novel potent immunosuppressive factors especially induced at the latter phase after OLT remains to be performed. Recently, Pan TL et al. performed proteomic analysis of post-OLT serum (day 60) by MALDI–TOF mass spectrometry and reported a serum proteome map which could provide guidance with respect to discovering potential protein targets in OLT tolerance [15]. Our previous proteomic study indicated that apolipoprotein E (apo-E) was induced at a latter tolerogenic phase after OLT [15,16]. It has been known that apo-E is one of the mediators for cholesterol transportation [17]. Similar to apo-E, adipose tissue-derived adipokine, adiponectin, possesses the fundamental role of glucose regulation and fatty acid catabolism [18,19]. Furthermore, recent reports pointed to a possible role of adipose tissue and adiponectin as potent regulators of inflammatory processes [20,21]. In clinical transplantation, the plasma adiponectin levels have been traced after kidney transplantation [22–24]. However, the evaluation of plasma adiponectin before/after OLT remains to be performed.

2. Objective

This study aimed to characterize the humoral factors, especially related to cholesterol transportation, glucose regulation, and fatty acid catabolism, before/after OLT. In this study, we provide evidence that apo-E and adiponectin are induced after OLT in a rat tolerogenic OLT model and a drug-induced tolerance model, and that the mobility shift of apo-E and/or the up-regulation of adiponectin may be related with recovery of the liver allograft function and the subsequent tolerance induction.

3. Materials and methods

3.1. Animals, orthotopic liver transplantation (OLT), and recipient serum after OLT (post-OLT serum)

Male DA (MHC haplotype RT1a) and PVG (RT1c) rats were obtained from Japan SLC (Hamamatsu, Japan) and the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Kyoto, Japan), respectively.

Fig. 1. Mobility shift of apo-E after OLT. 0.25 μl of post-OLT serum (A: DA–PVG natural tolerance model; B: DA–LEW acute rejection model and DA–LEW+CsA drug-induced tolerance model) and control serum were run on SDS-PAGE and then subjected to Western blot analysis. Protein bands were electronically transferred onto an Immobilon PVDF transfer membrane and the signals were visualized by a Western Blotting Luminol Reagent. Dotted line shows the mobility of the control apo-E (34 kDa).
Male LEW rats (RT1l) were obtained from the National Animal Breeding Center (Taipei, Taiwan). All animals were maintained in specific pathogen-free animal facilities with water and commercial rat food provided ad libitum.

OLT was carried out in the combination of DA to PVG (DA-PVG); natural tolerance model, DA to LEW (DA-LEW); acute rejection model, and DA-LEW with Cyclosporin A (CsA, 15 mg/kg/day, 0 to 14 days after OLT); drug-induced tolerance model by a previously described technique [25].

Post-OLT serum was obtained from the recipients from 7 to 86 days after OLT. All serum samples were stored at −70 °C until further analysis.

3.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 2-dimensional (2-D) electrophoresis, and Western blot analyses

Post-OLT serum or control serum was mixed with three times the volume of Laemmli’s loading buffer [26], and boiled for 5 min. The samples were then run on a 12.5% SDS-PAGE gel using a mini gel apparatus (BIO-RAD, Burlington, MA), and the protein bands were visualized by Coomassie brilliant blue R-250 (CBB) staining or GelCode® Glycoprotein Staining Kit (PIERCE, Rockford, IL).

2-D electrophoresis was performed according to the manufacturer’s protocol. Briefly, Immobiline DryStrips (pH 3–10, 13 cm long; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) were rehydrated with a rehydration buffer (8 M urea, 2% CHAPS, 18 mM dithiothreitol (DTT), 2% IPG buffer (pI 3–10, GE Healthcare Bio-Sciences Corp.), 0.002% bromophenol blue) containing 5 µl of post-OLT serum. First-dimensional isoelectric focusing was performed at 20 °C for 18 kV h using an IPGphor Isoelectric Focusing System (GE Healthcare Bio-Sciences Corp.). Before the 2-D electrophoresis, the strips were equilibrated for 10 min in an equilibration solution [50 mM Tris–HCl (pH 6.8), 30% glycerol, 6 M urea, 1% SDS] containing 0.25% DTT, and then for 10 min in an equilibration solution containing 240 mM iodoacetamide and a small amount of bromophenol blue. 2-D SDS-PAGE was performed in 10% SDS-PAGE gel using a PROTEAN II xi Cell (BIO-RAD). The proteins on the 2-D gels were visualized by CBB staining or glycoprotein staining.

To detect the serum apolipoprotein E (apo-E), fractionated proteins were electronically transferred onto an Immobilon transfer membrane (Millipore, Bedford, MA). The membrane was blocked using 5% skim milk at room temperature for 1 h and immunoprobed with goat anti-rat apo-E polyclonal antibody (Ab) (∗ 5000; Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-conjugated donkey anti-goat IgG (∗ 5000; Santa Cruz Biotechnology). Signals were visualized by a Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

3.3. Enzyme-linked immunosorbent assay (ELISA)

Plasma adiponectin was measured by using an Adiponectin (rat, mouse) ELISA Kit (Phoenix Pharmaceuticals, Inc., Belmont, CA) according to the manufacturer’s protocol. Briefly, control serum (n=3) and pooled post-OLT serum (100 µl, ×1111 dilution) were added to the rabbit anti-mouse adiponectin polyclonal Ab coated wells and incubated at room temperature for 1 h. Then secondary Ab (biotinylated rabbit anti-mouse adiponectin Ab, 100 µl) was added and incubated at room temperature for 1 h, followed by the addition of peroxidase-conjugated streptavidin and substrate solution. The absorbance at 450 nm was then measured using an MRX Microplate Reader (Dynex Technologies, Chantilly, VA).

3.4. Mixed lymphocyte reaction (MLR) and evaluation of immunosuppressive activity

A mixed lymphocyte reaction (MLR) was performed as described [9]. Briefly, responder LEW cells (2 ×10^5 cells in 100 µl) were mixed with mitomycin C (MMC)-treated stimulator DA cells (2 ×10^5 cells in 100 µl) in 96-well round-bottom plates (Nalge Nunc International, Roskilde, Denmark), and incubated at 37 °C for 84 h in a humidified atmosphere of 5% CO2/95% air. The allogeneic T-cell response was determined using a Cell Proliferation ELISA, BrdU (Roche Diagnostics, Mannheim, Germany) with an MRX Microplate Reader (Dynex Technologies). To evaluate the immunosuppressive activity of...
post-OLT serum and recombinant rat adiponectin (Phoenix Pharmaceuticals, Inc.), the samples were added in the MLR culture at a final concentration of 1% or up to 10 μg/ml. To investigate whether modified apo-E in post-OLT serum possesses MLR-inhibitory activity, anti-rat apo-E polyclonal Ab (Santa Cruz Biotechnology) was added in the MLR culture at up to 10 μg/ml to neutralize the apo-E activity in post-OLT serum. Anti-rat apo-E Ab was used after desalting treatment to remove sodium azide. Stimulatory Index=[BrdU incorporation of allogeneic combination (Stimulator/Responder=DA/LEW)]/[BrdU incorporation of syngeneic combination (LEW/LEW)].

3.5. Preparation of liver extracts

To detect the expression level of apo-E and adiponectin in liver after OLT, naïve DA or PVG liver and liver graft at tolerogenic phase after OLT were manually homogenized with T-PER® Tissue Protein Extraction Reagent (PIERCE) supplemented with protease inhibitor complete (Roche Diagnostics). After centrifugation, liver extracts were run on a 10% SDS-PAGE gel using a mini gel apparatus (BIO-RAD), and the protein bands were visualized by CBB staining or immunostaining using an anti-apo-E polyclonal Ab (Santa Cruz Biotechnology) and a rabbit anti-human adiponectin precursor (Phoenix Pharmaceuticals, Inc.) as described above.

3.6. Statistical analysis

Statistical analysis was performed using Student’s t test. A confidence level of P<0.05 was evaluated as significant. Each sample was tested in triplicate, and data were indicated as mean±standard deviation (SD). The cut-off for seropositivity was determined as the mean+3SD of the levels of the negative control group.

4. Results

4.1. Identification of apo-E in post-OLT serum

To identify the apo-E expression both in the rejection model (DA–LEW) and in the drug-induced tolerance model (DA–LEW+CsA). As shown in Fig. 1B, we could not confirm the mobility shift of apo-E in the rejection model (days 7 to 14), whereas the mobility shift of apo-E (34 to 37 kDa) was confirmed after cesation of CsA (>14 days after OLT) in the drug-induced tolerance model similar to those in the natural tolerance model (Fig. 1A).

4.2. Glycosylation of apo-E after OLT

It has been known that the amino acid sequence of apo-E possesses a glycosylation site [27–29]. To explore whether the modification of apo-E is related with post-translational glycosylation, we next used a GelCode® Glycoprotein Staining Kit (PIERCE) and detected the glycoproteins in post-OLT serum. As shown in Fig. 1B, the mobility shift of apo-E (34 to 37 kDa) was confirmed at the latter phase (tolerance induction phase) after OLT (28–49 days), and during the maintenance phase (>81 days), apo-E level was recovered to control level.

We next tried to identify the apo-E expression both in the rejection model (DA–LEW) and in the drug-induced tolerance model (DA–LEW+CsA). As shown in Fig. 1B, we could not confirm the mobility shift of apo-E in the rejection model (days 7 to 14), whereas the mobility shift of apo-E (34 to 37 kDa) was confirmed after cesation of CsA (>14 days after OLT) in the drug-induced tolerance model similar to those in the natural tolerance model (Fig. 1A).

4.3. Immunosuppressive activity of apo-E in post-OLT serum

To evaluate the significance of apo-E modification, we neutralized the apo-E from post-OLT serum (tolerance induction phase after OLT) by using an anti-rat apo-E neutralized Ab. As shown in Fig. 3, the MLR-inhibitory activity of post-OLT serum (day 49) was reduced by the effect of neutralized Ab in a dose-dependent manner (P=0.0452), whereas apo-E neutralized serum still possessed immunosuppressive activity (P<0.05) due to the effects of other immunosuppressive factors expressed at the latter phase after OLT (e.g., anti-MHC class II Ab, LSF-1, haptoglobin) [12–15].

4.4. Identification of adiponectin in post-OLT serum

It has been known that adiponectin is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism [18]. Adiponectin is exclusively secreted from adipose tissue into the bloodstream and is very abundant in plasma relative to many hormones [19]. However, the essential role of adiponectin in liver transplantation and the subsequent tolerance induction is still uncertain. Therefore, we next attempted to evaluate the plasma adiponectin level at various time points after OLT. As shown in Fig. 4A, the adiponectin level was gradually up-regulated after OLT in a natural tolerance model (DA–PVG). We also found that the adiponectin level was elevated after the withdrawal of CsA in a drug-induced tolerance model, whereas no such responses were confirmed in a rat acute rejection model and in a drug-induced tolerance model during immunosuppressive therapy (Fig. 4B). These results suggest that the induction of adiponectin may be important for recovering the liver function and the subsequent tolerance induction.

4.5. Immunosuppressive activity of recombinant rat adiponectin

To evaluate the immunological role of plasma adiponectin in a host immune system, recombinant rat adiponectin was added in the MLR culture at up to 10 μg/ml. As shown in Fig. 5, the adiponectin possessed MLR-inhibitory activity in a dose-dependent manner (P<0.01).

4.6. Expression of apo-E and adiponectin in liver graft after OLT

We next tried to identify the apo-E and adiponectin in naïve DA or PVG liver and DA liver graft after OLT in a tolerogenic OLT model (DA–PVG). As shown in Fig. 6, the apo-E and adiponectin expression were down-regulated after OLT. The results of blotting and immunostaining suggested that the expression of apo-E and adiponectin were suppressed at the latter phase after OLT.

*Statistical analysis was performed using Student’s t test. A confidence level of P<0.05 was evaluated as significant. Each sample was tested in triplicate, and data were indicated as mean±standard deviation (SD). The cut-off for seropositivity was determined as the mean+3SD of the levels of the negative control group. The results suggest that the induction of adiponectin may be important for recovering the liver function and the subsequent tolerance induction.
in Fig. 6, the liver graft after OLT strongly expressed high molecular weight apo-E (apo-E complexes) compared with naïve rats. On the other hand, the expression level of adiponectin in liver graft was lower than naïve rats. These results suggest that the liver grafts may actively express apo-E for activation of lipid metabolism after OLT and may conduct adipose tissues to secrete adiponectin.

5. Discussion

In this paper, we demonstrated that modified apo-E and adiponectin are induced after OLT in a rat tolerogenic OLT model (DA–PVG) and a drug-induced tolerance model (DA–LEW+CsA), and possessed immunosuppressive activity in vitro.

Apo-E, which includes three major isoforms (apo-E2, -E3, and -E4), has emerged as an important molecule in several biological processes not directly related to its lipid transport function, including Alzheimer’s disease and cognitive function, immunoregulation, and possibly even infectious diseases [30]. Apo-E is synthesized by the liver and several peripheral tissues.

**Fig. 4.** Plasma adiponectin levels in post-OLT serum (A: DA–PVG natural tolerance model; B: DA–LEW+CsA drug-induced tolerance and DA–LEW acute rejection models). Dotted line shows the cut-off line (mean+3SD of the levels of the naïve control serum). *Significantly up-regulated as compared with naïve control serum (P<0.01).

**Fig. 5.** Recombinant rat adiponectin possesses immunosuppressive activity in vitro. Recombinant rat adiponectin was added in MLR culture at up to 10 μg/ml to evaluate the MLR-inhibitory activity. Stimulatory Index=[BrdU incorporation of allogeneic combination (Stimulator/Responder=DA/LEW)]/[BrdU incorporation of syngeneic combination (LEW/LEW)]. *Significantly inhibited as compared with adiponectin (0 μg/ml) (P<0.01).

**Fig. 6.** The expression of apo-E and adiponectin in liver. Liver extracts were run on SDS-PAGE and then subjected to Western blot analysis. Protein bands were visualized by CBB staining or immunostaining. OLT: DA liver allograft after OLT (tolerogenic phase). Arrowheads show high molecular weight apo-E (apo-E complexes).
and cell types including macrophages, and is involved in the efficient hepatic uptake of lipoprotein particles, stimulation of cholesterol efflux from macrophage foam cells in the atherosclerotic lesion, and the regulation of immune and inflammatory responses [31]. Grainger DJ et al. reported that apo-E deficiency results in impaired clearance of apoptotic cell remnants and a functionally relevant systemic proinflammatory condition in apo-E deficient mice [32]. Additionally, Tenger C et al. described that apo-E itself inhibits T-cell activation by reducing the density of co-stimulatory surface proteins CD40 and CD80 on antigen-presenting cells [33]. Taken together, we speculate that apo-E expression is necessary to induce a tolerance status in experimental liver transplantation models (DA–PVG natural tolerance and DA–LEW + CsA drug-induced tolerance model). However, we could not confirm the prolongation of the allograft survival of the DA rat heart when we intramuscularly injected recipient LEW rats with 300 μg of recombinant apo-E (data not shown). Therefore, post-translational modification (possibly glycosylation) of apo-E after OLT (34 kDa to 37 kDa) may be more important for tolerance induction. Further investigation into the role and functional analysis of apo-E modification and the mobility shift will be ongoing.

Adiponectin, which is exclusively secreted from adipose tissue into the bloodstream, is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism [18,19]. Although the association of adiponectin with several diseases remains controversial, many clinical studies have demonstrated that low plasma concentrations of adiponectin are closely associated with obesity-related diseases, including atherosclerotic cardiovascular diseases, Type II diabetes mellitus, hypertension and dyslipidaemia [34]. In non-alcoholic fatty liver disease patients, the plasma adiponectin levels are significantly lower with elevated liver enzymes [35,36]. Recent reports pointed to a possible role of adipose tissue and adiponectin as potent regulators of inflammatory processes [20,21]. Recently, Wolf AM et al. reported that adiponectin is expressed by endothelial cells and hepatocytes in healthy and inflamed liver tissue [37]. However, we could not confirm the strong expression of adiponectin in liver graft after OLT compared with naïve rats. In contrast, plasma adiponectin level was gradually up-regulated after OLT both in a rat tolerogenic OLT model (DA–PVG) and in a drug-induced tolerance model (DA–LEW + CsA). These results suggest that the liver grafts may regulate the plasma level of adiponectin mostly secreted by adipose tissues. In the case of pregnancy, interestingly, it has been known that adiponectin is secreted in the human placenta [38] and that the plasma levels of adiponectin peak in midpregnancy, with the lowest levels being seen in late pregnancy [39]. These findings and our data remind us that adiponectin is one of the important factors protecting the allograft or fetus from rejection in the host or maternal immune system.

To date, random trials to withdraw immunosuppressive drugs are currently being performed for long-time surviving patients who have not had a rejection episode for over 2 years [40]. However, as far as we know, there are no unified standards in post-OLT serum to determine when the immunosuppressive therapy can be terminated. In the case of a clinical drug-free patient in Kaohsiung Chang Gung Memorial Hospital, the apo-E molecules were dramatically increased after OLT, whereas no such expression was found in the serum of other transplanted patients (unpublished data). Although this is just one case and it is thus too early to draw conclusions, the expression of apo-E and adiponectin may possibly be a diagnostic marker showing when the transplanted liver will recover its metabolizing function and/or when we should reduce (or stop) the immunosuppressive drugs. Further comparative analysis of an experimental OLT model (natural tolerance and drug-induced tolerance model) and clinical drug-free patient serum before/after liver transplantation or before/after reduction (or cessation) of immunosuppressive treatment needs to be conducted in order to gain an understanding of the essential mechanisms of transplant immunology and tolerance induction. We believe that the experience and knowledge we have obtained from this experimental OLT model will encourage us to develop a novel weaning protocol which could help alleviate the physiological, mental, and financial anguish related to liver transplantation therapy.

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