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Prospective highlights of serum glycoproteins in spontaneous tolerance after orthotopic liver transplantation

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ABSTRACT

Background: We examined the effects and the mechanisms of serum glycoproteins on spontaneous tolerance after orthotopic liver transplantation (OLT) between DA (RT-1^a) and PVG (RT-1^c) rats.
Methods: A functional proteome analysis was introduced to investigate differently expressed proteins involved in overcoming major histocompatibility complex (MHC) barriers and lectin blotting was applied to survey the levels of fucosylated proteins.
Results: Two-dimensional difference gel electrophoresis (2D-DIGE) coupled with mass spectrometry revealed statistically significant changes in the intensity of 19 proteins at 14 and 60 post-OLT days which respectively corresponded to rejection and tolerance. An interaction network analysis of the identified proteins indicated that the interleukin (IL)-6 signaling pathway might be correlated with the immune events in this model. The peak of IL-6 expression occurred during the recovery period might play a role in the remarkable shifts in the immune response towards spontaneous tolerance. Furthermore, increased levels of IL-6-modulated fucosylation of Igh-6 were also observed during the rejection response while fucosylated hemopexin and haptoglobin were obviously upregulated in the tolerogenic period.
Conclusions: These findings suggest that IL-6 and glycoproteins may play critical roles in this spontaneous tolerogenic DA/PVG OLT model and shed light on prolonging hepatic allograft survival in the future.

1. Introduction

Liver transplantation is generally accepted as the most effective treatment for end-stage liver disease and acute liver failure; however, transplant rejection between genetically diverse individuals is still a common cause of operation failure and morbidity [1]. In addition, long-term use of immunosuppressive agents was found to cause severe side effects such as hypertension, hyperglycemia, peptic ulcers, liver damage and kidney injury [2]. Therefore, inducement of donor-specific transplant tolerance was suggested as one of the potential solutions to prevent immune rejection in organ transplantation [3]. An interesting observation of spontaneous occurrence of tolerance was previously reported upon orthotopic liver transplantation (OLT) between different MHC haplotype inbred rat strains: a Dark Agouti (DA, MHC haplotype RT-1^a) donor and a Piebald Virol Glaxo (PVG, RT-1^c) recipient [4]. It is generally believed that the tolerogenic DA/PVG OLT combination serves as an important model to investigate related immunological responses and potential immunosuppressive factors in order to overcome allograft rejection after OLT [5]. Although several possible mechanisms were proposed, the exact mechanism involved in the induction of this unique tolerance remains unclear [6,7].

Agents targeting serum proteins with proteomics technologies were used to monitor the changes reflecting immune responses in the animal models. However, serum proteins have a very wide range of concentrations and usually occur in multiple isoforms that need to be separately quantified [8]. Thus, serum proteins from the OLT rat model were dissected with 2D-DIGE upon Cy-dye-labeled proteins. The method can overcome the disadvantages of traditional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis such as inter-gel variation, low sensitivity, and poor linearity [9,10]. Functional proteomics which integrated 2D-PAGE, mass spectrometry (MS) and bioinformatics analytical tools was introduced to analyze protein alterations involved in multiple signaling networks in response to spontaneous tolerance post-OLT. Herein, differently expressed proteins between the rejection and tolerance stages of the DA/PVG OLT model

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were identified by MS. A bioinformatic analysis based on differently expressed proteins revealed that IL-6 signaling network which induces the expression of fucosylation-related genes such as those of fucosyltransferases, GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (FX), and GDP-mannose-4,6-dehydratase (GMD) should be closely linked to allograft rejection through regulating the fucosylation of serum proteins [11].

Moreover, fucose-containing glycans play important roles in an extensive range of biological responses such as protein folding and antigen processing [12]. Previous reports demonstrated that the synthesis of glycoproteins appears to be affected by experimental inflammation, indicating that levels of glycans can be easily altered by the physiological conditions of cells [13]. *Aleuria aurantia* lectin (AAL) is widely used to evaluate the extent of α1, 6-fucosylated oligosaccharides and to fractionate glycoproteins associated with the pathogenesis of various diseases [14]. In the current study, we determined the glycosylation status changes in serum glycoproteins with respect to specific time points post-OLT by lectin blotting method.

Taken together, the primary goal of this study is to comprehensively delineate the profiles and glycosylation of rat serum proteins between rejection and tolerance OLT regimens in the hope that it would promote effective, drug-free acceptance of allografts and enhance the survival rate after transplantation.

2. Materials and methods

2.1. Orthotopic liver transplantation

Inbred strains of male rats, DA (RT-1^a) and PVG (RT-1^c) weighing 200–250 g, were housed at the specific pathogen-free (SPF) animal facility at Chang Gung Memorial Hospital Kaohsiung, and allowed free access to water and standard rat chow. OLT was performed under ether anesthesia using Kamada's method with some modifications [4,15]. A combination of DA and PVG rats was used as a rejection-tolerant model without immunosuppressive treatments. The Committee on Research Involving Animal Subjects of the Chang Gung Memorial Hospital in Kaohsiung, Taiwan, approved the study.

2.2. Sample preparation and pathological analysis

Blood samples (1 ml) were taken from each of the animals at various days after orthotopic liver transplantation (OLT), and OLT rats receiving syngenic transplant combination (a DA liver to a DA rat) were used as controls. Serum was obtained from the blood by centrifugation at 3000×*g* for 10 min and then stored at −80 °C until used. Resected liver specimens were fixed in 5% buffered formalin and embedded in paraffin. Sections were cut at 4 μm and stained with hematoxylin and eosin (H&E). For each liver sample, three different sites were examined and evaluated under light microscopy (Olympus BX51, Tokyo, Japan). The digital photomicrographs were then processed with DP-72 (Olympus). The data shown in the figures are representative data since reproducible results were obtained.

2.3. 2D-DIGE and image analysis

CyDye™ DIGE Fluor saturation dyes were reconstituted and stored according to the manufacturer's protocol (GE Healthcare Bio-Sciences, Amersham, UK). The labeling reaction was optimized according to the manufacturer's protocol. For the differential analysis, 5 μg of serum proteins was mixed with 2 nmol Tris-2-carboxyethyl- phosphine hydrochloride (TCEP), and incubated at 37 °C for 1 h in the dark. Then the serum sample was respectively labeled with CyDye DIGE Fluor Cy3 or Cy5 saturation dye (GE Healthcare, Uppsala, Sweden) by mixing with 4 nmol saturation dye and incubating at 37 °C for 30 min in the dark. Cy3- and Cy5-labeled protein samples were mixed together with lysis sample buffer, and then 2D-PAGE was performed. The CyDye-labeled DIGE gels were scanned on a Typhoon 9400 image scanner (GE Healthcare) using excitation and emission filters specified by GE Healthcare (Cy3 and Cy5 excitation at 540 and 620 nm, and emission at 590 and 680 nm, respectively). The photomultiplier voltage was set to 600 V (pH 4–7). Remaining parameters were set according to the manufacturer's protocol. In order to alleviate the software-based spot matching, border regions of the images with no spots were cropped. DeCyder differential analysis software ver. 6.0 was used in this study. Spot matching of the gel images was manually corrected.

2.4. Protein identification using MS

The protein spots were manually excised from the 2D-PAGE gels and cut into pieces. The pieces were washed with 50% acetonitrile in 25 mM ammonium bicarbonate (pH 8.5) for 15 min twice, and dehydrated in acetonitrile for 10 min, vacuum dried, and rehydrated with a total of 0.1 μg of modified trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate (pH 8.5), at 37 °C for 16 h. Following digestion, tryptic peptides were extracted with 50% acetonitrile containing 1% trifluoroacetic acid (TFA) for 15 min twice with moderate sonication. The extracted solutions were pooled and vacuum-dried. The tryptic peptides were then subjected to concerted MALDI peptide mass fingerprinting (PMF) and CID MS/MS analysis for protein identification using a dedicated Q-TOF Ultima™ MALDI instrument (Micromass, Manchester, UK). For the MALDI MS and MS/MS analyses, samples were premixed 1:1 with matrix solution (5 mg/ml CHCA in 50% acetonitrile, 0.1% v/v TFA, and 2% w/v ammonium citrate) and spotted onto the 96-well-format MALDI sample stage. Data-directed acquisition on the Q-TOF Ultima™ MALDI instrument was fully automated with a predefined probe motion pattern and peak intensity threshold for switching over from the MS survey scan to MS/MS, and from one MS/MS to another. The PMF and individual MS/MS ion data were output as Mascot-searchable .txt and .pkl files for independent searches against the Swiss-Prot database, and PMF was performed using the Mascot program (http://www.matrixscience.com/), provided by the European Molecular Biology Laboratory (EMBL).

2.5. Western blot analysis

Serum protein was transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% (w/v) non-fat milk in TBST for 2 h at room temperature. The membrane was washed with TBST 4 times for 15 min each, and then incubated with specific antibodies of (1) ceruloplasmin (Abcam, Cambridge, UK), (2) haptoglobin (Dako-Cytomation, Glostrup, Denmark), and (3) T-kininogen (Santa Cruz Biotechnology, Santa Cruz, CA, USA), (4) C-reactive Protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA), in TBST containing 5% (w/v) non-fat milk overnight at 4 °C. The membrane was washed with TBST four times for 15 min each and finally incubated with anti-rabbit or goat immunoglobulin G (IgG)-HRP (Santa Cruz Biotechnology) in TBST containing 5% (w/v) non-fat milk. The membrane was finally washed with TBST four times for 15 min each, and then the immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection system by film exposure (GE Healthcare).

2.6. Bioinformatic analysis using MetaCore™

Network analyses of differentially expressed proteins identified by MS were performed using MetaCore™ software (ver. 5.3 build 18499, GeneGo, St. Joseph, MI). The results of different expression protein lists were uploaded into MetaCore as a dataset, scored, and ranked based on the GeneGo Process Networks. The statistical relevance of the ontological matches was calculated as the *p* value, which is defined as the probability that a given number of proteins from the