Transplant Immunology 31 (2014) 55–59

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Accelerated telomere reduction and hepatocyte senescence in tolerated human liver allografts ☆

Wulamujiang Aini [a], Aya Miyagawa-Hayashino [a,*], Munetaka Ozeki [b], Salah Adeeb [a], Masahiro Hirata [a], Keiji Tamaki [b], Shinji Uemoto [c], Hironori Haga [a]

[a] Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan
[b] Department of Forensic Medicine and Molecular Pathology, Kyoto University Graduate School of Medicine, Kyoto, Japan
[c] Department of Hepatobiliary Pancreatic Surgery and Transplantation, Kyoto University Hospital, Kyoto, Japan

ARTICLE INFO

Article history:
Received 20 April 2014
Received in revised form 21 June 2014
Accepted 23 June 2014
Available online 30 June 2014

Keywords:
Tolerance
Liver transplantation
Real-time PCR
Cyclin-dependent kinase inhibitor p21
Hepatocyte nuclear area

ABSTRACT

Background: In living donor liver transplantation, the biological organ age of the donated allograft is unknown in young patients who receive grafts from older donors. Few studies have focused on the effects of aging on allografts in the state of tolerance. The purpose of this study was to assess the biological organ age of liver grafts.
Methods: In 20 tolerated allografts over a 10-year post-transplant follow-up period, the relative telomere lengths were measured by multiplex quantitative polymerase chain reaction, and hepatocyte nuclear size and cell cycle phase markers were determined by immunohistochemistry. The results were compared with the same measurements that had been obtained prior to transplantation in the recipients' pre-implantation donor livers. Tolerance was defined strictly as a condition in which the allograft functioned normally and showed normal histology without any histological signs of rejection, fibrosis or inflammation in the absence of immunosuppression.
Results: First, telomere length correlated with chronological donor age (n = 41). Accelerated telomere reduction was seen in tolerated grafts compared with the predicted telomere length of each allograft calculated from the regression line of donor livers. Tolerated grafts were associated with higher hepatocyte p21 expression and greater nuclear area than in the donor livers prior to transplantation.
Conclusions: These findings suggest that allografts age more rapidly than in the normal population, and that grafts may reach the limit of proliferative capacity even in the state of tolerance.

1. Introduction

Telomeres are located on the ends of chromosomes and help maintain genomic integrity and stability. Telomeres consist of tandem (TTAGGG)n nucleotide repeats, and shorten with age [1]. In the setting of chronic liver disease (i.e., liver cirrhosis, viral hepatitis), telomere length has been shown to be significantly shorter than in normal livers of the same age [2–4]. Pediatric patients often receive grafts from their parents through living donor liver transplantation; the biological organ age of the donated allograft is unknown in young patients who receive grafts from older donors. We previously reported that the hepatocyte telomere signal intensity was significantly lower than that of the predicted decline according to age in the tolerated liver allograft as well as that in chronic rejection, as revealed by quantitative fluorescence in situ hybridization [5]. In a larger number of cases, we performed quantitative real-time polymerase chain reaction (PCR), and confirmed accelerated telomere shortening relative to the chronological graft age in tolerated grafts. Recently, it has been demonstrated that measurement of relative average telomere lengths can be accomplished by real-time PCR using a carefully designed pair of oligonucleotide primers [6]. It is possible that a significant proportion of liver transplantation recipients are tolerant [7–9]. Tolerance is a condition in which an allograft functions normally and lacks histological evidence of rejection in the absence of immunosuppression [10]. Tolerated grafts are good material for evaluating the biological organ age of grafts unaffected by inflammation and immunosuppression. Marked heterogeneity of hepatocyte nuclear area is a feature of aging as well as of advanced liver disease [11,12]. Increased hepatocyte nuclear area, telomere shortening, and p21 expression—all markers of aging or cellular senescence—have been described in hepatocytes in non-alcohol-related fatty liver disease [11–13].

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FFPE, formalin-fixed, paraffin-embedded; PCR, polymerase chain reaction; T-Bil, total bilirubin; T/S ratio, the relative ratio of telomere repeat signals (T) to single-copy gene signals (S).

☆ This work was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (23590424) to AM-H.

* Corresponding author at: Department of Diagnostic Pathology, Kyoto University Hospital, 54 Kawahara-cho, Sakyo-ku, Kyoto 606-8517, Japan. Tel.: +81 75 751 3488; fax: +81 75 751 3499.
E-mail address: ayam@kuhp.kyoto-u.ac.jp (A. Miyagawa-Hayashino).

http://dx.doi.org/10.1016/j.trim.2014.06.008
0966-3274/

2. Objective

The aim of the present study was to use quantitative real-time PCR to assess whether accelerated telomere reduction was seen in tolerated grafts compared with telomere length as predicted by the chronological age of each allograft calculated from the regression line of donor livers. In addition, hepatocyte nuclear area and cell cycle phase markers were assessed morphologically and immunohistochemically. Graft aging was evaluated in 20 tolerated grafts in which the grafts functioned normally and lacked histological signs of rejection with no fibrosis or inflammation in the absence of immunosuppression.

3. Materials and methods

3.1. Definition of tolerance state

Transplantation tolerance has long been clinically defined as graft acceptance without functional impairment together with sustained acceptance for years in the absence of immunosuppression [7,10]. This status has been called operational tolerance [14]. Analyses of protocol liver biopsies performed during long-term follow-up of liver transplant recipients who are tolerant have revealed a high frequency of graft fibrosis, albeit with the grafts showing normal liver function, compared with the grafts of patients on maintenance immunosuppression [15–17]. Thus, in the present study, tolerance was defined strictly as a condition in which the allograft functioned normally and showed normal histology without any histological sign of rejection, fibrosis or inflammation in the absence of immunosuppression.

3.2. Study population

From 1990 to December 2012, 798 pediatric patients (≤18 years of age at liver transplantation) underwent living donor liver transplantation at Kyoto University Hospital using donor livers from their parents. There were 393 patients who were followed for more than 10 years after liver transplantation; 227 of those patients underwent a total of 598 biopsies at more than 10 years post-transplant. Of these, 70 patients showed normal histology, with 28 patients off immunosuppression. Thus, 12% (28/227) of pediatric patients at more than 10 years of follow-up were able to withdraw from immunosuppression, resulting in a state of tolerance.

For 6 of the 28 tolerant patients, DNA from paraffin-embedded sections of their donor livers was highly degraded, resulting in poor PCR amplification; thus, no further analysis could be performed. For another 2 patients, preserved samples of their donor livers at the time of transplantation were not available; therefore, those 2 patients were excluded from analysis. The remaining 20 patients were subjects for telomere length analysis.

This study was approved by the Kyoto University Institutional Review Board (G553).

3.3. Histological analysis

Histological analysis was performed on liver needle biopsy samples obtained at last follow-up. The donor livers (time zero biopsies) served as controls; these had been fixed in formalin and embedded in paraffin. All specimens were interpreted by pathologists (AM-H and HH) on routine hematoxylin and eosin (HE) staining, Masson's trichrome for evaluation of fibrosis, and immunohistochemistry for cytokeratin 7 (OV-TL12/30 DakoCytomation, Glostrup, Denmark; 1:300) in the bile duct epithelium.

3.4. Laboratory analysis

Postoperative clinical data were collected retrospectively. Laboratory data at the time of protocol biopsies for tolerant patients included the following variables: serum aspartate aminotransferase (AST, normal range, 13–29 IU/L), alanine aminotransferase (ALT, 8–28 IU/L) and total bilirubin (T-Bil, 0.2–1.0 mg/dL). The incidence of biopsy-proven acute rejection, other complications such as biliary and vascular complications during the follow-up period, and the amount of time off immunosuppression were also recorded.

3.5. Telomere length analysis by quantitative real-time PCR

Genomic DNA was extracted from the archived paraffin-embedded liver tissue using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Venlo, Netherlands). Telomere length was measured using a multiplexed quantitative real-time PCR method previously described by Cawthon on a real-time PCR cycler (Rotor-Gene Q, QIAGEN). In brief, telomere length value was determined by the relative ratio of telomere repeat signals (T) to single-copy gene signals (S) in experimental samples compared with a reference DNA sample (T/S ratio). The single copy gene used in the study was *36B4*, which encodes acidic ribosomal phosphoprotein PO located on chromosome 12. The telomere PCR experiments and the *36B4* PCR experiments were performed in triplicate [18]. The comparative threshold cycle method (ΔΔCt) was employed as the method of choice to quantify relative gene expression. The quantification result was transformed to an exponential value $2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle.

3.6. Immunohistochemistry and hepatocyte nuclear area measurement

Monoclonal antibody for p21 was used as a marker of cell cycle arrest (SX118, DakoCytomation; 1:50). The BenchMark ULTRA Slide Staining System (Roche Diagnostics Japan, Tokyo, Japan) was used for the performance of automated immunohistochemistry. Hepatocyte nuclear p21 reactivity was assessed in a quantitative manner. The number of positive hepatocyte nuclei divided by the total number of hepatocytes counted on a biopsy specimen equaled the calculated index (%).

Hepatocyte nuclear area was recorded simultaneously on p21 staining slides for which the nuclear counter stain was hematoxylin. NIS-Elements D Microscope Imaging Software (Nikon Instruments Inc., Tokyo, Japan) was used to measure the nuclear area of hepatocytes. For each slide, at least 1000 hepatocytes were measured for nuclear size.

3.7. Statistical analysis

For analysis of clinical data, a *t*-test or U-test was performed. Regression analysis was used to test relationships between quantitative variables. A *P*-value <0.05 was considered significant. For statistical analysis, JMP Start Statistics version 9 was used (Statistical Discovery Software SAS Institute, Cary, NC, USA).

4. Results

4.1. Clinical profiles of tolerated grafts

The clinical characteristics of 20 tolerant patients included in the study are summarized in Table 1. The median age at the time of liver transplantation was 1 year (range, 0–15). The original diseases in the 20 study patients were biliary atresia in 16, liver cirrhosis from unknown cause in 1, Budd–Chiari syndrome in 1, fulminant hepatic failure of unknown etiology in 1, and congenital biliary dilatation in 1. Seven of the patients received left or lateral grafts from their father, and 13 received grafts from their mother. The graft was ABO-blood-type identical in 17 patients, compatible in 2, and incompatible in 1.

The study patients were followed at a median of 13 years after transplantation (range, 10–20). All received tacrolimus as baseline immunosuppression. Weaning was intentionally performed in 14 patients in a gradual manner. Two of the patients were noncompliant with respect to their immunosuppressant medication, and 4 had stopped immunosuppression due to infection. The median time off immunosuppression was 6 years (range, 2–18). Similarly, the duration of on immunosuppression was a median of 8 years (range, 2–17 years). Laboratory data for the patients who were tolerant at the time of the last biopsy revealed a median AST of 23 IU/L (range, 15–22), ALT 19 IU/L (range, 12–33), and T-Bil 0.7 mg/dL (0.4–1.4).