Increased NPC1L1 and serum cholesterol in a chronic rejection rat

Abstract

Purpose: To measure serum cholesterol and triglyceride levels and NPC1L1 mRNA and protein as an index of cholesterol absorption during the development of chronic rejection (CR) in a rat model of intestinal transplantation.

Methods: Rats were randomly divided into two groups: Group 1 (n=20) underwent syngenic Lewis-to-Lewis transplantation and Group 2 (n=20) underwent allogenic F344-to-Lewis transplantation as well as treatment with FK506. Blood samples and intestinal tissue were procured on the 190th day after operation. Histological changes were analyzed and the semiquantitative scores of histological parameters were compared. The serum levels of cholesterol and triglyceride were determined. The expression of Niemann-Pick C1 Like 1 (NPC1L1) mRNA and protein were analyzed by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and immunohistochemistry, respectively.

Results: All the animals survived for the 190 days. The appearance and histology of isografts were almost normal whereas the allografts displayed thickened bowel wall and mesenteric fibrosis, concentric intimal thickening and interstitial fibrosis and inflammatory infiltration. The histology scores displayed a significant difference between the allografts and isografts (P<0.001). No differences were observed for triglycerides for the two groups. The serum cholesterol levels increased significantly in the allogenic group in comparison with the syngenic group (P=0.034), while no difference was observed for triglyceride levels between groups. RT-PCR showed that the expression of NPC1L1 of allografts increased significantly (P=0.004). Immunohistochemistry confirmed RT-PCR findings.

Conclusions: Neointima formation and mesenteric fibrosis were the dominant pathological features. The increased expression of NPC1L1 might contribute to hypercholesterolemia, which may be involved in the pathogenesis of transplant arteriosclerosis.

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Small bowel transplantation has evolved over the last decades from being considered an experimental procedure to become a clinically accepted therapy for patients with chronic, irreversible intestinal failure [1]. Chronic rejection (CR), a silent but progressive form of graft injury that eventually results in graft failure, remains a major concern. Transplant arteriosclerosis (TA) in the graft is the main pathological characteristic of CR [2]. Hypercholesterolemia occurs in up to 80% of patients after transplantation, with significant increases in the ratio of total cholesterol to high density lipoprotein cholesterol creating an atherogenic milieu, and is implicated in the development of graft arteriosclerosis [3-5].Immunosuppression has been regarded as an important causative agent in hypercholesterolemia, resulting in adverse cardiovascular events and atherosclerosis of large caliber vessels. Nevertheless, the pathogenic mechanisms of altered cholesterol absorption and metabolism are still unknown.

Identification and characterization of Niemann-Pick C1 Like 1 (NPC1L1) protein has established it as essential in the intestinal cholesterol absorption process. NPC1L1 is the molecular target of ezetimibe, which is a cholesterol absorption inhibitor [6,7]. Lack of NPC1L1 causes nearly complete protection from the development of atherosclerosis in apoE-/- mice, which provides new target for the treatment for atherosclerosis [8].

The aim of this study was to observe the changes in serum cholesterol levels, which reflect intestinal absorption, in a rat orthotopic small bowel transplantation (OSBT) model of CR, and to evaluate the possible role of NPC1L1 in the development of TA.

Materials and Methods

Animals

Inbred adult male F344 and Lewis rats, weighing 220-300g, were obtained from Vitalriver Company, Beijing, China. The donors were 30-70g smaller than the recipients. All rats were housed individually in standard animal facilities, maintained on 12-h light/dark cycles, and fed with rat chow and tap water ad libitum for one week before surgery. Food was withheld on 12-h light/dark cycles, and fed with rat chow and tap water during the surgical procedures.

Recipient were anesthetized in a similar fashion. After mobilization of the inferior vena cava and the aorta from surrounding connective tissue, transplantation was performed by anastomosing the graft superior mesenteric artery to the recipient infrarenal aorta and the graft portal vein to the recipient infrarenal inferior vena cava reperfusion, the recipient’s own small bowel, with its mesentery, was resected from the ligament of Treitz to the ileocecal valve and replaced with the graft by performing two primary end-to-end small bowel anastomosis. (The cold ischemia time 35±6 minutes).

Histology

Three to four micrometer tissue sections were stained with hematoxylin and eosin, as well as Trichrome Masson’s stain. They were examined by two pathologists in a blind manner. The histological scale was from 0 to 3, where 0 indicated no pathological changes and 3 indicated extreme changes. These parameters included villous blunting, epithelial apoptotic body, muscularis and mesenteric fibrosis [11]. Intimal thickening was graded as follows: grade 0, normal artery; grade 1, intimal thickening up to approximately 50% of the perimeter of the lumen, with <20% luminal compromise; grade 2, intimal thickening involv-
ing between 50% and 100%, with <20% luminal compromise; grade 3, 20–50% luminal compromise; grade 4, 50–80% luminal compromise; grade 5, >80% luminal compromise [11,12].

Blood was obtained from the inferior vena cava at the time of euthanasia. Serum samples were analyzed for total cholesterol (TC) and triglyceride (TG) levels.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
Total RNA was extracted from frozen graft bowel samples with Trizol reagent (Invitrogen, Carlsbad, California) and then measured by spectrophotometer (BioPhotometer, Eppendorf, Hamburg, German). PrimeScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) and GoTaq Colorless Master Mix (Promega, Madison, WI, USA) were used for first strand cDNA synthesis and PCR. The transcriptions of NPC1L1 were detected with the primers in Table 1. The transcription of β-actin was used for normalization. The PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide staining. Images were obtained and the gray values of all the products were measured by ImageJ.

Immunohistochemistry
Immunohistochemical study was performed using the EnVision method (Dako, Glostrup, Danmark) on 2-mm formalin-fixed, paraffin-embedded sections. Antigen retrieval was performed by incubation of the slides in 10 mM sodium citrate buffer (pH 6.0) for 10 min at 95°C. Goat polyclonal antibody against ratNPC1L1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was incubated overnight in a 1:100 dilution.

Statistical Analysis
Results are expressed as mean values ± SD. Student’s t test was used to determine the significance of group differences. P<0.05 was considered statistically significant.

Results
Morphology and histology of grafts
Isografts revealed normal macroscopic appearance and the mesenteric arteries showed normal structure. Allografts had thickened bowel wall, bowel enlargement, feces retention, abundant mesenteric adhesions, severe mesenteric fibrosis and indiscernible mesenteric vessels and the mesenteric arteries in allografts manifested numerous inflammatory infiltration and severe intimal thickening, and the diameter of lumen decreased significantly (Figure 1).

Allografts demonstrated fibrosis at different levels. Severe mesenteric fibrosis and mild muscularis fibrosis were observed via Masson stain, while fibrosis was hardly observed in isografts (Figure 2).

Histologic evaluation of isografts and allografts was summarized in Table 2. Isografts scored 0 in all features analyzed. Compared with isografts, the scores of allografts displayed a significant difference (P<0.001).
Levels of serum TC and TG

The serum TC level in the allogenic group was significantly higher than in the syngenic group (1.85±0.19 mmol/L vs 1.63±0.16 mmol/L, p=0.034); however, the serum TG levels in two groups were similar (Table 3).

The expression of NPC1L1

The expression of NPC1L1 by RT-PCR (relative to β-actin) result was 0.66±0.08 and 0.92±0.13, p=0.004, in isografts and allografts, respectively, suggesting a significant increase of NPC1L1 with the development of chronic rejection (Fig 3). The NPC1L1 protein mainly expressed in the brush border membrane of the enterocyte. Compared with the syngenic group, a significant increase of NPC1L1 in allografts was also observed (Fig 4).

Discussion

Development of CR, as characterized by TA, is now recognized as the predominant cause of allograft loss long-term following solid organ transplantation. TA affects up to 50% of patients 5 years after transplantation, as diagnosed by angiography. Pathogenesis of TA seems to be multifactorial but precise mechanisms involved in the development of this remodeling process still remain obscure [2].

Hypercholesterolemia and abnormalities in lipid metabolism probably contribute to the chronic allograft dysfunction. Hyperlipidemia is common after cardiac transplantation affecting 60 - 80% of patients [13,14]. An altered lipid profile has been reported to occur in up to 80% of renal transplant recipients, and persists long after transplantation. Post-transplant lipid profile is characterized by a significant increase in TC, rendering as many as 77% of patients hypercholesterolemic [15,16]. Many causes have been shown to contribute to hypercholesterolemia in both animal models and clinical recipients after intestinal transplantation; CR and immunosuppression both played important roles. Currently, there was no definite research to demonstrate their roles separately. In our study, compared with syngenic group, hypercholesterolemia occurred in all recipients of the allogenic group, while no difference was observed in TG levels between allogenic and syngenic groups. Besides, allografts displayed typical histological changes of TA. Histological scores confirmed significant differences between isografts and allografts, and demonstrated that intimal thickening and mesenteric fibrosis were predomi-

<table>
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<tr>
<th>Grafts</th>
<th>Villous blunting</th>
<th>Epithelial apoptotic body</th>
<th>Muscularis fibrosis</th>
<th>Mesenteric fibrosis</th>
<th>Intimal thickening</th>
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<tbody>
<tr>
<td>Isografts</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Allografts</td>
<td>1.91±0.28*</td>
<td>2.35±0.42*</td>
<td>2.03±0.39*</td>
<td>2.18±0.35*</td>
<td>1.96±0.36*</td>
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* vs isografts, P<0.001

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<tr>
<th>Group</th>
<th>Cholesterol mmol/L</th>
<th>Triglycerides mmol/L</th>
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<tr>
<td>Allogenic group</td>
<td>1.85±0.19</td>
<td>0.98±0.24</td>
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<tr>
<td>Syngenic group</td>
<td>1.63±0.16†</td>
<td>0.95±0.21</td>
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† vs allogenic group, p=0.034
The results suggested that high cholesterol levels may accelerate the development of CR.

This present study uses the histological score criteria described by Orloff and coworkers, who demonstrated severe rejection in the absence of physiological intraluminal exposure to nutrients in their model of heterotopic intestinal transplantation [11]. In order to better represent the physiologic state of small intestinal function and to overcome the severe toxicity of Cyclosporine A, FK506 was used in the present study. FK506 had already been used successful in a proceeding study [9] that was designed to evaluate the changes in cholesterol absorption and the role of long term intragastrical supplementation with fish oil in the development of TA. During first week after surgery, intragastrical supplementation was shown to lead to severe diarrhea because of reperfusion injury, so a later time-point was selected for the present study (first week + half a year intragastrical supplementation).

Allograft vasculopathy may begin because of endothelial damage induced by ischemia at the time of transplantation and accelerate due to hyperlipidemia or other risk factors, as seen in other models of accelerated atherosclerosis [17]. Autopsy studies have shown that the cholesterol content in transplant coronary arteries is 10-fold greater than in native vessels, and clinical studies have associated increased cholesterol levels with a greater risk of TA [2]. Hypercholesterolemia contributes to the pathology of chronic and acute rejection. Histologically, there is arteriosclerosis of the graft vessels, with thickening of the intimal layer resulting in progressive lumen occlusion. Hypercholesterolemia has been found to accelerate the development of these vascular lesions, and is a risk factor for decline in graft function and CR [2,3,17].

Absorption of cholesterol by the proximal small intestine represents a major pathway for entry of cholesterol into the body pools. In adult humans, typically several hundred milligrams of cholesterol reach the liver from the intestine daily, with the potential to impact the plasma TC concentration [18]. Identification and characterization of NPC1L1 has established it as an essential protein in the intestinal cholesterol absorption process. While otherwise phenotypically normal, NPC1L1 null mice exhibit a significant reduction in the intestinal uptake and absorption of cholesterol [6,19]. NPC1L1 null mice are resistant to diet-induced hypercholesterolemia, and when crossed with apoE null mice, were completely resistant to the development of atherosclerosis [8]. Characterization of the NPC1L1 pathway revealed that ezetimibe specifically binds to NPC1L1 and inhibits its sterol transport function, and it is now widely used in combination therapy with statins for management of hypercholesterolemia in the general population [20]. In liver, cardiac and kidney transplant patients, hypercholesterolemia could be effectively treated with ezetimibe with few side effects [21-23]. The present study suggests that the significantly increased expression of NPC1L1 might promote intestinal cholesterol absorption, which might contribute to hypercholesterolemia involved in the development of CR; however, other important aspects of cholesterol metabolism were not studied simultaneously.

Immunosuppression has additional severe adverse effects on lipid metabolism. Although tacrolimus has been shown to have a less detrimental effect on plasma lipids than either cyclosporine orsirolimus, the assessment of interactions with immunosuppressants will be an important component of further studies. Another syngenic group, treated with FK506 to con-
clusively evaluate FK506 effects, or allogeneic transplants plus ezetimibe, to determine if ezetimide can reverse hypercholesterolemia, need to be investigated in future research. The current study focused only on intestinal cholesterol absorption, while the changes in cholesterol synthesis, transport and excretion need to be evaluated in future studies.

Conclusion

In the present study, we found that the expression of NPC1L1 was activated significantly, which likely promoted intestinal cholesterol absorption and increased serum cholesterol level. The changes of metabolic pathways for cholesterol mentioned above might be involved in the pathogenesis of CR, which suggests a possible therapeutic target.

Acknowledgments

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List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CR</td>
<td>chronic rejection</td>
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<tr>
<td>OSBT</td>
<td>orthotopic small bowel transplantation</td>
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<td>TA</td>
<td>transplant arteriosclerosis</td>
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<td>NPC1L1</td>
<td>Niemann-Pick C1 Like 1</td>
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<td>TC</td>
<td>total cholesterol</td>
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<td>TG</td>
<td>triglyceride</td>
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<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<td>POD</td>
<td>postoperative days</td>
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References
