Yanfei Zhu MD¹
Jing Zhou MD²

¹ Department of General Surgery, Wuxi People's Hospital of Nanjing Medical University, Wuxi, China

² Department of Pathology, Wuxi People's Hospital of Nanjing Medical University, Wuxi, China

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.

Manuscript submitted 11th February, 2011
Manuscript accepted 19th April, 2011

Clin Invest Med 2011; 34 (3): E172-E178.

Correspondence to:

Dr. Yanfei Zhu
Department of General Surgery,
Wuxi People's Hospital of Nanjing Medical University,
299 Qingyang Road,
Wuxi 214023,
Jiangsu Province, China.
E-mail: zhuyanfei_2002@163.com

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 from both donor and recipient animals for 24 h prior to surgery. The protocol was approved by the Animal Research Committee of the Nanjing University, China. All procedures were carried out in accordance with 'Principles of laboratory animal care' (NIH publication NO. 85-23, revised 1985).

Animals were 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. FK506 was intramuscularly administered to recipients on postoperative days (POD) 0-13, 20 and 27 at a dose of 1.0 mg/kg/day [9].

Surgical procedures

OSBT was performed using standard microvascular techniques [10]. Donor and recipient surgery were performed under anesthesia induced with 100mg/kg ketamine intraperitoneally. The entire small bowel, from the ligament of Trietz to 1cm from the ileocecal valve, was removed on a vascular pedicle consisting of the superior mesenteric artery and the portal vein. The intestinal lumen was then flushed with saline to remove debris, and the vascular bed was flushed with cold 4°C heparinized saline.

Recipient was 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).

Postoperative care and observation

Rats were kept on a warming blanket and put under a heating lamp until recovery from anesthesia. All animals had access to regular rat chow and tap water *ad libitum*. Death within five days due to arterial anastomosis leakage or thrombus was considered as a technical failure and excluded. All rats were kept for a long-term observation until euthanized on POD 190. All animals were euthanized and grafts were harvested and used for histology and PCR. Blood samples were taken for cholesterol and triglyceride analysis.

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-

TABLE 1. Sequence of primers used for PCR

Gene	Primer sequence	Annealing temperature	Amplicon
NPC1L1	Forward: TTCCAGGTTGCGTTCTC Reverse: GACTCCACCGCTACTCG	54°C	161
β -actin	Forward: GACAGGATGCAGAAGGAGATTACT Reverse: TGATCCACATCTGCTGGAAGGT	56°C	142

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].

Serum Total Cholesterol and Triglyceride Measurements

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). PrimeScript™ 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, Denmark) on 2-mm formalin-fixed, paraffin-embedded sections. Antigen retrieval was performed by incubation of the slides in 10 mM sodium citrate buffer (pH6.0) 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 $\pm$ 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 levers. 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 ($P < 0.001$).

FIGURE 1. Macroscopic appearance of grafts and pathological changes of mesentery arteries. (A) Isografts showed normal appearance. (B) Thickened bowel wall, feces retention, mesenteric fibrosis and indiscernible mesenteric vessels were observed in allografts. (C) Mesenteric artery in isografts showed normal intimal ($\times 200$). (D) Mesenteric artery in allografts displayed severely concentric intimal hyperplasia, narrow lumen and inflammatory infiltration ($\times 200$).

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 dysfunction³. 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

FIGURE 2. *Fibrosis of grafts (Masson stain $\times 200$).* (A) Low-power view showed no fibrosis of mesentery in the isograft. (B) Diffusing and severe fibrosis of mesentery in the allograft was observed. (C) No fibrosis of muscularis in the isograft was manifested. (D) Severe fibrosis of muscularis in the allograft was displayed.

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 2. Histological mean score of grafts

Grafts	Villous blunting	Epithelial apoptotic body	Muscularis fibrosis	Mesenteric fibrosis	Intimal thickening
Isografts	0	0	0	0	0
Allografts	$1.91 \pm 0.28^*$	$2.35 \pm 0.42^*$	$2.03 \pm 0.39^*$	$2.18 \pm 0.35^*$	$1.96 \pm 0.36^*$

* vs isografts, $P < 0.001$

TABLE 3. Serum levels of cholesterol and triglycerides

Group	Cholesterol mmol/L	Triglycerides mmol/L
Allogenic group	1.85 ± 0.19	0.98 ± 0.24
Syngenic group	$1.63 \pm 0.16^\dagger$	0.95 ± 0.21

† vs allogenic group, $p=0.034$

FIGURE 3. NPC1L1 expression of grafts evaluated using RT-PCR. The gray density of NPC1L1 in the allogenic group was significantly higher than in the syngenic group (0.92 ± 0.13 and 0.66 ± 0.08 , $p=0.004$).

nant. 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 successfully in a proceeding study⁹ that was designed to evaluate the changes in cholesterol absorption and the role of long term intragastric supplementation with fish oil in the development of TA. During first week after surgery, intragastric 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 intragastric 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

FIGURE 4. NPC1L1 expression of grafts evaluated by immunohistochemistry ($\times 200$). (A) NPC1L1 mainly expressed in brush border membrane and the expression notably increased in allografts. (B) The expression of NPC1L1 in isografts was much smaller.

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 or sirolimus, 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 ezetimibe 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

This work was supported by a grant from the National Natural Science Foundation of China (No. 30672060) and National Program on Key Basic Research Project (973 Program; No. 2009CB522405).

List of Abbreviations

CR	chronic rejection
OSBT	orthotopic small bowel transplantation
TA	transplant arteriosclerosis
NPC1L1	Niemann-Pick C1 Like 1
TC	total cholesterol
TG	triglyceride
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
POD	postoperative days

References

- Braun F, Broering D, Faendrich F. Small intestine transplantation today. *Langenbecks Arch Surg* 2007;392:227-238.
- Hillebrands JL, Rozing J. Chronic transplant dysfunction and transplant arteriosclerosis: new insights into underlying mechanisms. *Expert Rev Mol Med* 2003;5:1-23.
- Zawaideh MA, Ghishan FK, Molmenti EP. Regulation of cholesterol homeostasis in solid organ transplantation. *Transplantation* 2006;81:316-317.
- Schonder KS, McKaveny TP, Lynch KJ. Retrospective analysis of hyperlipidemia management in a transplant population. *Pharmacotherapy* 2005;25:918-923.
- Tse KC, Lam MF, Yip PS, Li FK, Lai KN, Chan TM. A long-term study on hyperlipidemia in stable renal transplant recipients. *Clin Transplant* 2004;18:274-280.
- Altmann SW, Davis HR Jr, Zhu LJ. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* 2004;303:1201-1204.
- Chang TY, Chang C. Ezetimibe blocks internalization of the NPC1L1/cholesterol complex. *Cell Metab* 2008;7:469-471.
- Davis HR Jr, Hoos LM, Tetzloff G, et al. Deficiency of Niemann-Pick C1 Like 1 prevents atherosclerosis in ApoE^{-/-} mice. *Arterioscler Thromb Vasc Biol* 2007;27:841-849.
- Zhu Y, Wei W, Li Y. FK506 treatment in a Long-term chronic rejection rat model of small bowel transplantation. *Clin Invest Med* 2010;33:168-173.
- Murase N, Ye Q, Nalesnik MA, et al. Immunomodulation for intestinal transplantation by allograft irradiation, adjunct donor bone marrow infusion, or both. *Transplantation* 2000;70:1632-1641.
- Orloff SL, Yin Q, Corless CL, Loomis CB, Rabkin JM, Wagner CR. A rat small bowel transplant model of chronic rejection: histopathologic characteristics. *Transplantation* 1999;68:766-779.
- Ma H, Wang J, Wang J, Li Y, Li J. Features of chronic allograft rejection on rat small intestine transplantation. *Pediatr Transplant* 2007;11:165-172.
- Siirtola A, Ketomaki A, Miettinen TA, et al. Cholesterol absorption and synthesis in pediatric kidney, liver, and heart transplant recipients. *Transplantation* 2006;81:327-334.
- Le VV, Racine N, Pelletier GB, Carrier M, Cossette M, White M. Impact of ezetimibe on cholesterol subfractions in dyslipidemic cardiac transplant recipients receiving statin therapy. *Clin Transplant* 2009;23:249-255.
- Wissing KM, Unger P, Ghisdal L, et al. Effect of atorvastatin therapy and conversion to tacrolimus on hypercholesterolemia and endothelial dysfunction after renal transplantation. *Transplantation* 2006;82:771-778.
- Booth JC, Joseph JT, Jindal RM. Influence of hypercholesterolemia on patient and graft survival in recipients of kidney transplants. *Clin Transplant* 2003;17:101-107.
- Perrault LP, Mahlberg F, Breugnot C, et al. Hypercholesterolemia increases coronary endothelial dysfunction, lipid content, and accelerated atherosclerosis after heart transplantation. *Arterioscler Thromb Vasc Biol* 2000;20:728-736.
- Sane AT, Sinnett D, Delvin E, et al. Localization and role of NPC1L1 in cholesterol absorption in human intestine. *J Lipid Res* 2006;47:2112-2120.
- Yu L. The structure and function of Niemann-Pick C1-like 1 protein. *Curr Opin Lipidol* 2008;19:263-269.
- Garcia CM, Lisnock J, Bull HG, et al. The target of ezetimibe is Niemann-Pick C1 Like 1 (NPC1L1). *Proc Natl Acad Sci USA* 2005;102:8132-8137.
- Bilchick KC, Henrikson CA, Skojec D, Kasper EK, Blumenthal RS. Treatment of hyperlipidemia in cardiac transplant recipients. *Am Heart J* 2004;148:200-210.

22. Almutairi F, Peterson TC, Molinari M, Walsh MJ, Alwayn I, Peltekian KM. Safety and effectiveness of ezetimibe in liver transplant recipients with hypercholesterolemia. *Liver Transpl* 2009;15:504-508.
23. Patel AR, Ambrose MS, Duffy GA, Cote H, DeNofrio D. Treatment of hypercholesterolemia with ezetimibe in cardiac transplant recipients. *J Heart Lung Transplant* 2007;26:281-284.