Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 1333–1340

Journal of Nutritional Biochemistry

Down-regulation of vascular HMGB1 and RAGE expression by n-3 polyunsaturated fatty acids is accompanied by amelioration of chronic vasculopathy of small bowel allografts☆

Wei Wei[a,b], Min Chen[c], Yanfei Zhu[b,d], Jian Wang[e], Ping Zhu[f], Yousheng Li[b,*], Jieshou Li[b]

[a]Department of Gastroenterological Surgery, Hangzhou First Hospital, School of Clinical Medicine, Nanjing Medical University, Hangzhou, 310006, China
[b]Research Institute of General Surgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing 210002, China
[c]Department of Gastroenterology, Nanjing Drum Tower Hospital, Affiliated Hospital of Nanjing University Medical School, Nanjing, 210008, China
[d]Department of General Surgery, Wuxi People's Hospital, School of Clinical Medicine, Nanjing Medical University, Wuxi, 214023, China
[e]Department of General Surgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, 210002, China
[f]Section of Epidemic Febrile Disease, School of Basic Medicine, Nanjing University of Chinese Medicine, Nanjing, 210046, China

Received 27 March 2011; received in revised form 31 July 2011; accepted 10 August 2011

Abstract

Chronic allograft rejection, which is manifested as chronic allograft vasculopathy (CAV), continues to refrain the long-term success of small bowel transplantation (SBTx). The pathway mediated by the receptor for advanced glycation end products (RAGE) and its ligand, high mobility group box-1 (HMGB1), may contribute to the pathogenesis of CAV, given that they were involved in the process of allograft rejection. n-3 polyunsaturated fatty acids (PUFAs), which have been discovered to attenuate CAV, may have potential impacts on this pathway. The present study investigated whether n-3 PUFAs attenuated CAV via the regulation of the HMGB1-RAGE pathway in a chronic rejection model of rat SBTx. We revealed that the expression of HMGB1 and RAGE was increased in CAV-bearing vessels as well as endothelial cells isolated from these vessels. Oral administration of fish oil with high levels of n-3 PUFAs following SBTx significantly reduced the HMGB1 and RAGE expression, which coincided with the amelioration of CAV. In contrast, feeding of corn oil that contained low levels of n-3 PUFAs had no favorable effects on CAV development and failed to decrease the HMGB1 and RAGE expression. These results indicate that protective effects of n-3 PUFAs on allograft vessels exist via down-regulation of the HMGB1-RAGE pathway.

Keywords: n-3 polyunsaturated fatty acids; Chronic allograft vasculopathy; Receptor for advanced glycation end-products; High mobility group box 1; Small bowel transplantation

1. Introduction

The introduction of new immunosuppressants, such as tacrolimus, has reduced the frequency of acute rejection episodes, enabling small bowel transplantation (SBTx) with a moderate success rate [1]. But they hardly control chronic allograft rejection [2], which is manifested as a progressively obliterative vasculopathy, commonly referred to as chronic allograft vasculopathy (CAV) that restricts blood supply to the graft and eventually leads to graft loss [3]. Thus, exploring alternative therapies has attracted great interest over the last few years. n-3 polyunsaturated fatty acids (PUFAs), a group of fatty acids that are typically recommended for the treatment of coronary heart disease, have been discovered to ameliorate CAV [4–7]. However, the links between n-3 PUFA treatment and pathways that may inhibit CAV remain poorly defined.

High mobility group box-1 (HMGB1) is a nonhistone nuclear protein and, if released from cells, takes proinflammatory effects through its interaction with two types of cell-surface receptors: toll-like receptor (TLR) and receptor for advanced glycation end-products (RAGE) [8]. RAGE is a member of the immunoglobulin superfamily and has multiple ligands including advanced glycation end products (AGEs), HMGB1, S100/calgranulins and β-amyloid peptides [9]. Ligation of RAGE by HMGB1 activates the innate immune response [8] and can also potentiate the adaptive immune response [10]. In murine models of transplantation of fully mismatched cardiac grafts, pharmacological blockade of RAGE or HMGB1 suppressed RAGE expression and HMGB1 release within the grafts, and delayed the onset of rejection [11,12]. These findings implicate the HMGB1-RAGE pathway as a positive regulator of allograft rejection and thereby suggest its contributory role in the pathogenesis of CAV.

An increasing amount of evidence has suggested that n-3 PUFAs can modulate the HMGB1-RAGE pathway in graft vessels. Endothelial RAGE is localized to caveolae, a specialized subset of membrane rafts

☆ Funding sources: National Natural Science Foundation of China (grant number: 30672060) and National Program on Key Basic Research Project (973 Program; grant number: 2009CB522405).
* Corresponding author. Tel.: +86 25 8086 0137; fax: +86 25 8480 3956.
E-mail address: liys@medmail.com.cn (Y. Li).

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2011.08.002

[13,14], and their integrity is required for RAGE-mediated inflammatory gene expression and migratory response of vascular smooth muscle cells (SMCs) [15]. n-3 PUFAs and other fatty acids are capable of incorporating into caveolae and altering the function of receptors in caveolae [16]. Another possible mechanism involves the activation of peroxisome proliferator-activated receptor-γ (PPARγ) [4,5,17–20], a nuclear hormone receptor that regulates gene expression and exhibits anti-inflammatory and antiatherogenic properties [21]. PPARγ agonists have been shown to reduce the RAGE expression and subsequently block the downstream signaling pathways, thus limiting the cells' susceptibility toward proinflammatory effects of RAGE's ligands [22–24].

Put together, these data suggest that the favorable effects of n-3 PUFAs on CAV are possibly achieved by the suppression of the vascular HMGB1-RAGE pathway. Here, we examined the hypothesis in a chronic rejection model of rat SBTx.

2. Materials and methods

2.1. Animals

Male inbred Lewis (LEW) rats ($RT1^{l}$, RT6.1) and Fisher 344 (F344) rats ($RT1^{lv1}$, RT6.2), weighing 240–290 g and 200–230 g, respectively, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). LEW and F344 rats served as donors of small bowel isografts and allografts, respectively, and the LEW rats served as naive recipients in all experiments [25]. All rats were maintained in specific pathogenic-free animal care facilities at 20°C±2°C with a 12-h light/dark cycle and were provided free access to distilled water and a rodent chow diet containing <0.1% PUFAs (Nanjing Animal Technology Co., Ltd., Nanjing, China). All animals received humane care in compliance with *The Principles of Laboratory Animal Care* formulated by the National Society of Medical Research and *The Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (Publication no. 85-23, revised 1996). The experimental protocol was approved by the Animal Research Committee of Nanjing University.

2.2. Small bowel transplantation

One-step orthotopic SBTx was performed as previously described [26]. Briefly, after induction of general anesthesia, the entire small bowel graft was removed from the donor based on a vascular pedicle consisting of superior mesenteric artery and portal vein, and subsequently was stored in heparinized Ringer's solution at 4°C. The end-to-side vascular anastomosis was performed between graft superior mesenteric artery and host infrarenal aorta, and between graft portal vein and host infrarenal vena cava. Then, the segment of host small bowel between ligament of Treitz and ileocecal valve was removed and replaced by the graft. After operation, rats were kept warm in individual cages and fed with water and food *ad libitum*. Those surviving for less than 1 week were considered technical failures and excluded from the study.

2.3. Medication

The experimental animals were divided into four groups, each containing at least eight animals (Table 1). The ISO group was composed of syngeneic animals without immunosuppressive treatment. The other groups consisted of allogeneic animals, which received intramuscular tacrolimus (Astellas Ireland Co., Ltd., Ireland) at 1.0 mg/kg/day on postoperative day (POD) 0–13, 20 and 27 [25] and one of three oral supplements (via gavage): phosphate-buffered saline, corn oil or fish oil, all at 6.0 ml/kg/day [6] from POD 7 to death. These groups were designated as the PBS, CO and FO groups, respectively, according to the fed supplements. The content of n-3 and n-6 PUFAs was 28% and 3% in the fish oil (catalog no. F8020, Sigma-Aldrich Inc., St. Louis, MO, USA) and 1.3% and 58.8% in the corn oil (catalog no. C8267, Sigma-Aldrich Inc., St. Louis, MO, USA).

Table 1
Experimental groups, treatment protocols and CAV scores

Group	Donor strain	Medication	Graft harvest date	CAV score
ISO (n=8)	LEW	–	190×8	0
PBS (n=8)	F344	Tac[a]+PBS[b]	190×8	1.96±0.36
CO (n=8)	F344	Tac[a]+CO[c]	119, 127, 132, 138×3, 141, 143	2.30±0.33
FO (n=9)	F344	Tac[a]+FO[d]	190×9	0.44±0.22*,**

*P<.01, FO group vs. PBS group; **P<.01, FO group vs. CO group.
[a] Tac: tacrolimus, intramuscular, 1.0 mg/kg/day on POD 0–13, 20, 27.
[b] PBS: phosphate-buffered saline, per so, 6.0 ml/kg/day from POD 7 onwards.
[c] CO: corn oil, per os, 6.0 ml/kg/day from POD 7 onwards.
[d] FO: fish oil, per os, 6.0 ml/kg/day from POD 7 onwards.

2.4. Postoperative observation

The recipient rats were weighed daily, and their overall health status was recorded. Typically, a clinical rejection episode was manifested as progressive weight loss and decreased physical activities [27]. Grafts were removed at the time of clinical rejection or, if continuing to appear normal, on POD 190 for histological and biochemical analysis.

2.5. Pathological assessment

Specimens of small bowel grafts were formalin-fixed, embedded in paraffin, cut at 5-μm intervals and stained with hematoxylin–eosin (H&E). The sections were inspected by a pathologist blinded as to experimental groups and were assigned a CAV score for individual grafts. This score was the average score for graft vessels in sections. At least 20 vessels were examined for each graft to insure uniformity of the results and unbiased comparison of outcomes. Each vessel was examined for neointimal hyperplasia and was subject to a 6-point grading scale from 0 to 5: 0 for unaltered vessel, 1 for lesions affecting up to 50% of the vessel luminal circumference with less than 20% luminal occlusion, 2 for lesions affecting more than 50% of the vessel luminal circumference with less than 20% luminal occlusion, 3 for 20%–50% luminal occlusion, 4 for 50%–80% luminal occlusion and 5 for more than 80% luminal occlusion [28]. The average CAV score of each experimental group was calculated from scores of all individual grafts.

2.6. Fatty acid analysis of red blood cell membrane

Fatty acid composition of red blood cell (RBC) membrane was analyzed by gas chromatography to reflect long-term fatty acid intake [29]. For extraction and derivatization of fatty acids, all solvents contained 50 μg/ml butylated hydroxytoluene as antioxidant. First, RBCs were separated from blood samples by centrifugation and hemolyzed to obtain cell membrane. After being freeze-dried, the membrane was ready for lipid extraction by the Folch method [30]. Fatty acids were prepared from lipid saponification and transformed into methyl esters by 14% methanolic boron trifluoride (catalog no. B1252, Sigma-Aldrich Inc., St. Louis, MO, USA) [31]. Fatty acid methyl esters were analyzed using a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) equipped with a capillary column (CP-Sil 88 for FAME, 50 m×0.25 mm×0.20 μm, Varian, Palo Alto, CA, USA) containing a nonpolar stationary phase (5% phenyl-methyl/95% siloxane) [31]. The oven temperature was programmed to increase from 160°C to 220°C at 6°C/min and maintained for 30 min at 220°C. The detector temperature was maintained at 280°C. The high pure nitrogen was used as the carrier gas with its pressure maintained at 80 kPa. Individual fatty acids were identified and quantified by comparison of their retention times and peak areas with those of authentic standards and standard mixtures of known quantity (Sigma-Aldrich Inc., St Louis, MO, USA), and their content was expressed as a percentage of the total fatty acids by weight.

2.7. Isolation of mesenteric endothelial cells

Vascular endothelial cells (VECs) were isolated and purified from graft mesentery vessels according to the method previously described [32]. Briefly, graft mesentery was freshly harvested, cut into small pieces and incubated with digestion solution containing 0.1% collagenase type II (catalog no. C6885, Sigma-Aldrich Inc., St. Louis, MO, USA) at 37°C for 50 min with agitation. Then, medium 199 (Gibco, Invitrogen Inc., Grand Island, NY, USA) was added to the digestion solution, followed by centrifugation at 180*g* for 10 min. The pellet was left and resuspended in medium 199 containing 20% fetal bovine serum (Gibco, Invitrogen Inc., Grand Island, NY, USA) and 70 μg/ml endothelial cell growth supplement (Macgene Tech, Beijing, China). The supplement was an extract of bovine pituitary glands that mainly contained VEC growth-promoting factors. The cell suspension was transferred to six-well culture plates containing gelatin-coated 20×20×1-mm^3 glass coverslips and incubated at 37°C in a 5% CO_2 incubator (Thermo Forma 3110, Waltham, MA, USA) for 4 h to allow VEC to be attached to the coverslips. Then, the supernatant containing nonadherent cells was gently aspirated off, and fresh medium was added to each well. The cells were cultured at 37°C in a 5% CO_2 incubator for 12 h. Viable cells were counted by trypan blue exclusion. VECs were identified by anti-von Willebrand factor antibody (ab6994, Abcam Inc., Cambridge, MA, USA) before they were used for immunofluorescence experiments to detect HMGB1 and RAGE.

2.8. Immunofluorescence

Specimens of graft mesentery were embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA) and cut into 5-μm sections. The sections and glass coverslips containing isolated VECs were fixed with ice-cold acetone for 10 min and blocked with 10% normal goat serum (Boster Biotech Inc., Wuhan, China) at room temperature for 30 min. They were incubated with rabbit anti-RAGE antibody (1:500 dilution for tissue sections, 1:200 dilution for coverslips; catalog no. ab3611,