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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

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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

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* Corresponding author. Tel.: +86 25 8086 0137; fax: +86 25 8480 3956.
E-mail address: liys@medmail.com.cn (Y. Li).

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[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,

Abcam Inc., Cambridge, MA, USA) or rabbit anti-HMGB1 antibody (1:500 dilution for tissue sections, 1:200 dilution for coverslips; catalog no. ab18256, Abcam Inc., Cambridge, MA, USA) at 4°C overnight, followed by incubation with Alexa Fluor 594-conjugated goat anti-rabbit antibody (1:100 dilution; catalog no. A-11037, Molecular Probes, Invitrogen Inc., Carlsbad, CA, USA) for 1 h. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:5000 dilution; catalog no. D3571, Molecular Probes, Invitrogen Inc., Carlsbad, CA, USA). To detect RAGE expression in isolated VECs, the secondary antibody was replaced by Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:100 dilution; catalog no. A-11034, Molecular Probes, Invitrogen Inc., Carlsbad, CA, USA). Immunofluorescence was visualized using a fluorescence microscope (Axio Imager.A1, Carl Zeiss, Jena, Germany).

2.9. Western blotting

Cytoplasmic protein was extracted from graft mesentery vessels using a commercial kit (Beyotime Institute of Biotechnology, Nantong, China). Protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% gel) and electrically transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin for 1 h. Then, they were incubated with rabbit anti-RAGE (1:500 dilution) or rabbit anti-HMGB1 (1:1000 dilution) antibodies at 4°C overnight. A rabbit anti-tubulin antibody (1:100 dilution; catalog no. ab59680, Abcam Inc., Cambridge, MA, USA) served as the control for protein loading. Antibody binding was detected by incubating the membranes with horseradish-peroxidase-conjugated mouse anti-rabbit IgG (H+L) antibody (1:10,000 dilution; catalog no. 211-035-109, Jackson Immunoresearch Lab, West Grove, PA, USA) for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (ECL kit; Beyotime Institute of Biotechnology, Nantong, China); and images were acquired and analyzed by Quantity One V4.31 (Bio-Rad, USA).

2.10. Statistical analysis

Statistical analysis was performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±standard deviation and analyzed by Student's *t* test. Difference of *P* value<.05 was considered significant.

3. Results

3.1. Fish oil feeding attenuated CAV of rat small bowel allografts

Preliminary results established that the orthotopic SBTx model of F344 to LEW displayed typical CAV with few episodes of acute rejection when treated with an initial short course of low-dose cyclosporine A and that the substitution of cyclosporine A with tacrolimus promoted long-term F344 allograft survival [25]. This model was used to assess the long-term anti-CAV effects of n-3 PUFAs in the present study. In the PBS group, all the rats survived for more than 6 months after transplantation until being sacrificed on POD 190 for histology. The allogeneic mesenteric vessels were diffusely affected and showed a concentric neointimal layer inside internal elastic lamina that compromised the vessel lumen (Fig. 1B, F, J).

These lesions were accompanied by perivascular intense inflammatory infiltrates and mesenteric fibrosis. The vasculopathy was absent in LEW isografts (Fig. 1A, E, I) and resembled that observed from human CAV-bearing allografts.

In the FO group, a single daily dose of fish oil was given to supplement n-3 PUFAs after transplantation. The amount of oil given without dilution or emulsifying reagent was absorbed during passage through the small bowel allografts without causing digestive discomfort, like diarrhea or vomiting. The significant alterations of fatty acid composition in RBCs indicated increments in n-3 PUFA intake. The ratio of n-6 to n-3 PUFAs was reduced to nearly 1:1 in the FO group, significantly lower than approximately 10:1 in the PBS group ($P<.001$) (Table 2). The two groups had the same graft survival rate on POD 190, while the average body weight of the FO group was significantly greater than that of the PBS group after the 13th postoperative week ($P=.038$) (Fig. 1). Neointimal hyperplasia and perivascular inflammatory infiltrates were also alleviated in the FO group (Fig. 2D, H, L vs. Fig. 2B, F, J; CAV score 0.44±0.22 vs. 1.96±0.36; $P<.001$).

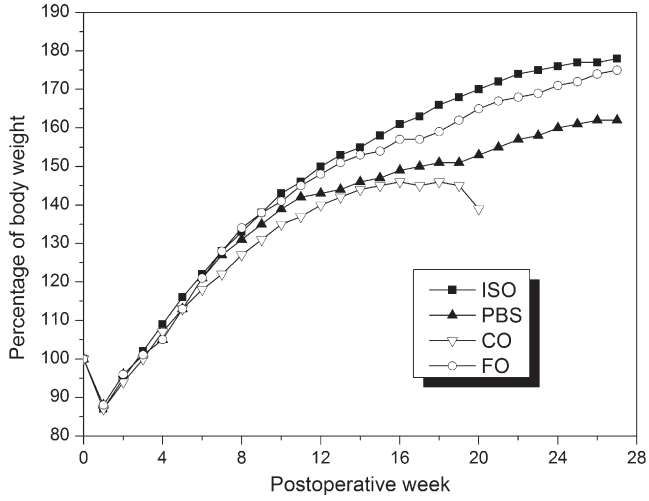

Fig. 1. Summary of postoperative average body weight changes. The postoperative body weight was expressed as a percentage of the preoperative body weight. Due to the ischemic injury of small bowel grafts [25], the average body weight of all groups decreased during the first postoperative week, followed by continuous weight gain throughout the experiment. However, a progressive reduction of body weight was observed in the CO group during the 17th–20th weeks postoperatively. The FO group had a significantly greater average body weight gain than the PBS group after the 13th postoperative week ($P=.038$).

3.2. Corn oil feeding failed to ameliorate CAV of rat small bowel allografts

To exclude the possibility that the histological improvements of FO group resulted from the increased calorie intake, the CO group was fed an equal amount of corn oil that contained predominantly n-6 PUFAs with an n-6: n-3 ratio of 58.8: 1.3. n-6 PUFAs exert opposing immunoregulatory effects via competitive inhibition of n-3 PUFAs and their potent bioactive products [33]. Like fish oil, corn oil feeding was well tolerated in the transplanted rats. The ratio of n-6 to n-3 fatty acids in RBCs was increased to approximately 16:1 in the CO group, significantly higher than that in the PBS and FO groups ($P<.001$)(Table 2). Their postoperative body weight continued to increase until declining on the 17th–20th weeks after transplantation (Fig. 1), concomitant with abdominal swelling, anorexia and constipation. Chronic rejection episodes were suspected; therefore, the rats were sacrificed for pathological examination. Consistent with our predictions, morphological features of severe chronic rejection were prominent throughout CO group (Fig. 2C, G, K). Fibrosis and retraction of graft mesentery led to distortion of intestinal loops with

Table 2
Fatty acid composition of erythrocyte membrane

PUFAs (%)	PBS	CO	FO
C18:3 n-3	<0.1	<0.1	0.84±0.22*,***
C20:5 n-3 (EPA)	<0.1	<0.1	6.52±0.56*,***
C22:5 n-3	<0.1	0.63±0.07**	3.93±0.51*,***
C22:6 n-3 (DHA)	0.60±0.13	1.70±0.23**	4.34±0.51*,***
∑ n-3 PUFAs	0.64±0.12	2.38±0.24**	15.64±1.31*,***
C18:2 n-6	0.63±0.12	12.00±0.93**	4.10±0.47*,***
C20:2 n-6	<0.1	0.35±0.09**	0.77±0.29*,***
C20:4 n-6	6.33±0.85	24.15±1.30**	11.22±0.85*,***
C22:4 n-6	<0.1	2.24±0.13**	1.11±0.31*,***
∑ n-6 PUFAs	6.96±0.90	38.74±2.39**	17.20±1.80*,***
$\frac{\sum n-6PUFAs}{\sum n-3PUFAs}$	11.16±1.83	16.54±2.67**	1.11±0.21*,***
Omega-3 index	0.63±0.13	1.73±0.21**	10.87±0.89*,***

$^{*}P<.01$, FO group vs. PBS group; $^{**}P<.01$, CO group vs. PBS group; $^{***}P<.01$, FO group vs. CO group.

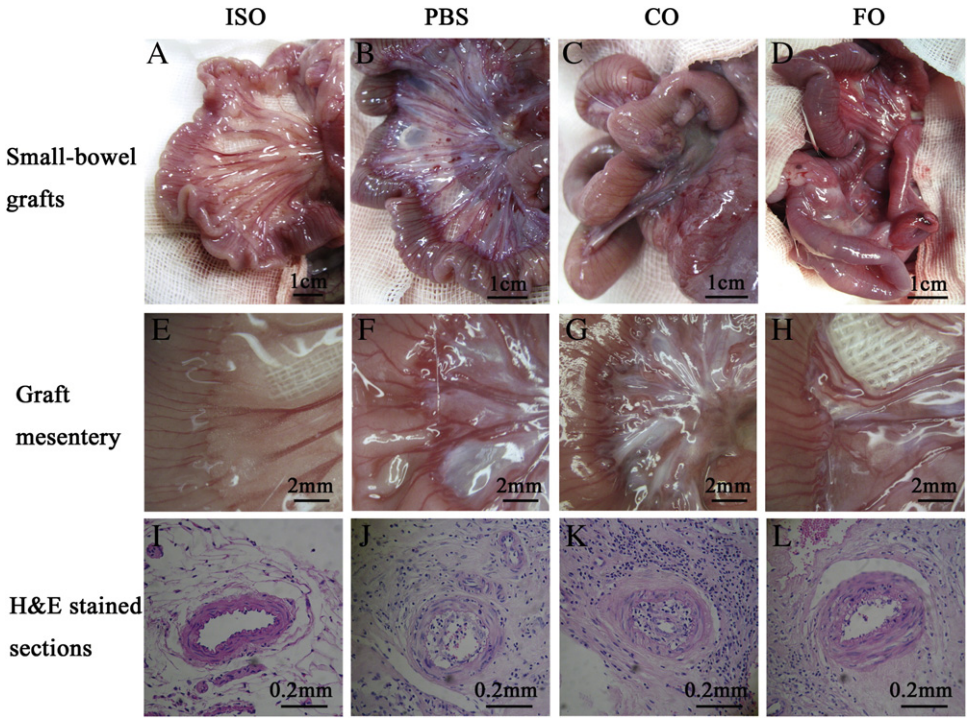

Fig. 2. Gross views and H&E-stained sections of graft mesentery vessels. Representative sections of graft mesentery vessels are shown in (I–L) (×200). Almost normal mesenteric vessels were observed throughout the ISO group (A, E, I). In contrast, fibrotic and poorly vascularized mesentery was macroscopically visible in other groups (B–D, F–H) and was predominant in the CO group (G), leading to retraction of graft mesentery and consequent distortion of intestinal loops (C). The FO group had minimal mesenteric fibrosis in comparison to the PBS and CO groups (H). The sections of allograft vessels demonstrated a concentric obliterative lesion in which a neointimal layer appeared inside the internal elastic lamina, with underlying perivascular inflammation and fibrosis (J–L). The FO group was less affected by the vasculopathy than the PBS and CO groups.

consequent intestinal obstruction (Fig. 2C, G). Further histological examination revealed the presence of diffuse neointimal hyperplasia in mesenteric vessels with perivascular inflammation and fibrosis, which were slightly more severe than that seen in the PBS group (Fig. 2G, K vs. Fig. 2F, J). Therefore, the CO group deserved a higher average CAV score than the PBS group, although this did not reach statistical significance (2.30±0.33 vs. 1.96±0.36, P=.073). Actually, the CO group showed an accelerated progression of CAV since such lesions appeared much earlier in the CO group than the PBS group.

3.3. Fish oil feeding reduced the expression of HMGB1 and RAGE at CAV Lesions

To investigate whether vascular HMGB1-RAGE pathway played a role in CAV, the expression of HMGB1 and RAGE was examined at the CAV lesions. As demonstrated by immunofluorescence microscopy, HMGB1 was located primarily within the nuclei of isograft vascular cells; and therefore, positive staining of HMGB1 was invisible when the nuclei were counterstained with DAPI (Fig. 3E). But a significant fraction of HMGB1 was localized in the cytoplasm of allograft vascular cells (Fig. 3F). The translocation of nuclear HMGB1 to the cytoplasm indicated that cells actively secreted HMGB1 into the extracellular milieu. Similar results were obtained in that RAGE had differential expression at CAV lesions vs. normal tissue (Fig. 3B vs. Fig. 3A). The number of RAGE-positive staining cells was greater in allograft vessels than isograft vessels. These observations were in accord with the experiments by Western blotting that showed that the cytoplasmic expression of HMGB1 and RAGE was increased in allograft vessels (Fig. 3I).

Comparison of the allograft groups revealed that HMGB1 and RAGE shared similar alteration pattern of expression that correlated with CAV scores. As compared to the PBS group, HMGB1 and RAGE expression was significantly decreased in the FO group, with a lower average CAV score (Fig. 3D, H vs. Fig. 3B, F). But there was no significant difference between the PBS and CO groups in terms of HMGB1 and RAGE expression as well as average CAV scores (Fig. 3C, G vs. Fig. 3B, F). On the other hand, feeding of fish oil with high n-3 PUFA levels significantly decreased the expression of HMGB1 and RAGE in the allograft vessels (as shown in the FO group vs. the PBS group), whereas their expression was almost unchanged after feeding of corn oil containing high levels of n-6 PUFAs (as shown in the CO group vs. the PBS group).

3.4. Fish oil feeding reduced the expression of HMGB1 and RAGE in VECs isolated from CAV-bearing graft vessels

Activation of VECs plays a substantial role in the pathogenesis of CAV. To explore whether the HMGB1-RAGE pathway was involved in endothelial activation at CAV lesions, the VECs of graft vessels were isolated to assess the expression of the two molecules by immunofluorescence. The numbers of individual grafts, from which VECs were successfully isolated, were 5, 4, 4 and 5 for the ISO, PBS, CO and FO groups, respectively. Approximately $1–2\times10^5$ viable VECs were yielded per graft, with a purity of greater than 70%. Few VECs from isograft vessels expressed detectable HMGB1 and RAGE in cytoplasm, whereas a great number of VECs strongly expressing HMGB1 and RAGE were isolated from allograft vessels (Fig. 4B, F vs. Fig. 4A, E). When comparing between allograft groups, we found that the FO group with a minimal average CAV score had the smallest population of VECs positively stained for HMGB1 and RAGE (Fig. 4D, H). The high percentage of positive-staining VECs was not significantly different between the PBS and CO groups that both obtained similarly high average CAV scores (Fig. 4C, G vs. Fig. 4B, F). On the other side, differences between HMGB1 and RAGE expression were closely

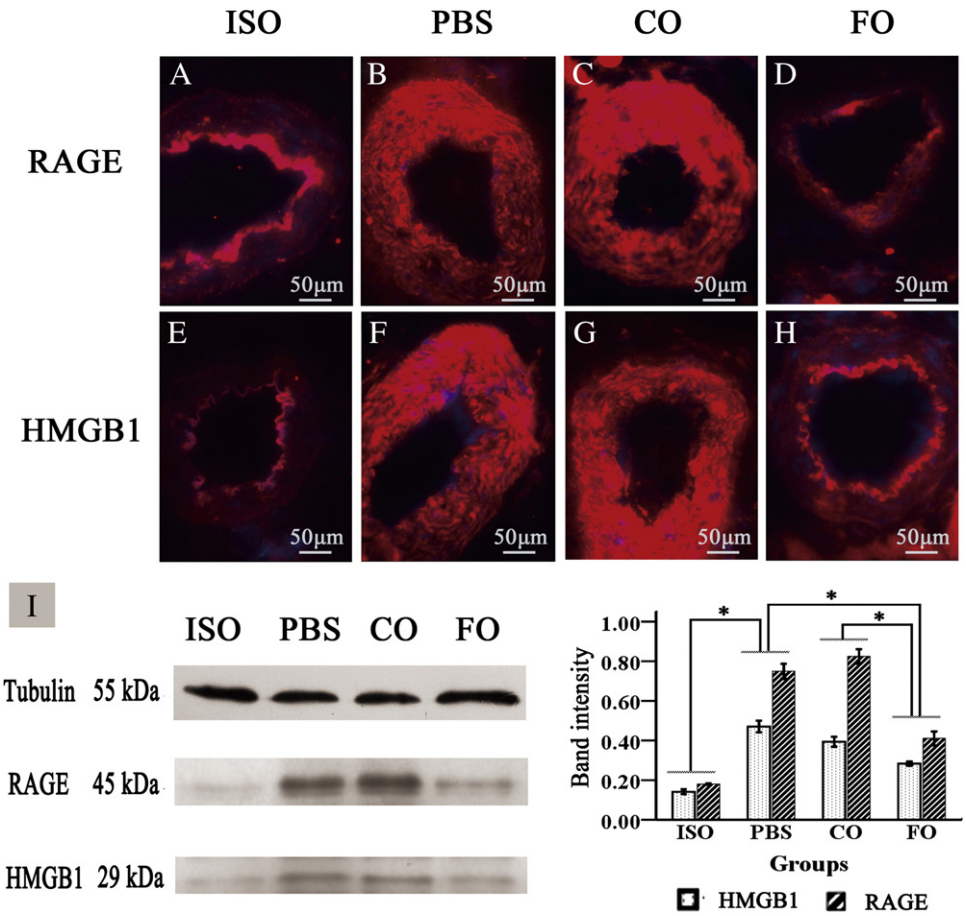

Fig. 3. Expression of HMGB1 and RAGE in graft mesentery vessels. Representative sections of graft mesentery vessels immunostained for HMGB1 and RAGE (both stained in red color) are shown in (A–H) (×400). Immunofluorescence microscopy revealed that isograft vessels expressed fairly low levels of HMGB1 and RAGE (A, E), whereas a great number of vascular cells strongly expressing HMGB1 and RAGE were detected in the allograft groups, except the FO group (B–D, F–H). In Western blotting analysis, the expression of HMGB1 and RAGE was reported as the intensity of their bands relative to that of the tubulin band in the same lane. Consistent with the immunofluorescence experiment, the expression of HMGB1 and RAGE was increased in the allograft groups as compared to the ISO group (I). The FO group had the lowest expression of HMGB1 and RAGE among the allograft groups (I). *P<.01.

related to the feeding supplements. Feeding of fish oil rich in n-3 PUFAs greatly reduced the number of VECs positively stained for HMGB1 and RAGE (as shown in the FO group vs. the PBS group), while feeding of corn oil with high n-6 PUFA levels had no significant effects (as shown in the CO group vs. the PBS group).

4. Discussion

Accelerated CAV represents one of the most serious long-term complications of SBTx as well as transplantation of other vascularized organs [3,34,35]. The precise mechanism that contributes to the development of CAV is complex, but the immunologic events, involving alloreactive T cells and the humoral immune system, are thought to constitute the principal stimuli [36]. Unfortunately, conventional immunosuppressive protocols have little impact on CAV [2] and indeed may themselves be implicated in the vasculopathy [37]. Administration of n-3 PUFAs may offer a viable therapeutic option, which has been discovered to attenuate CAV in rodent transplantation experiments [4–7]. But most of these studies focused on the outcomes of n-3 PUFA supplementation over a short period of time, and few studies used an orthotopic SBTx model, although it was more clinically relevant [27]. The present study investigated the long-term effects of n-3 PUFAs in a model of rat orthotopic SBTx. It was demonstrated that feeding fish oil for 6 months to the transplanted rats slowed down the process of neointima formation and reduced the persistence of inflammation and fibrosis in the vicinity of the allograft vasculature (Fig. 2). A high level of n-3 PUFAs in fish oil presumably conferred the effects. The contribution of n-3 PUFAs was confirmed by the experiment that feeding corn oil containing less n-3 PUFAs and more n-6 PUFAs caused no amelioration of CAV (Fig. 2, Table 1). It might be queried whether the increased energy intake from fish oil was responsible for the body weight gain and the histological improvements of the FO group. This was most unlikely since the CO group that was fed an equal amount of corn oil with the same calories had progressive weight loss during the 17th–20th weeks postoperatively (Fig. 1). We also found that moderate levels of neointima hyperplasia resembling what was observed in the PBS control group occurred much earlier after corn oil was fed (Fig. 2, Table 1). The mechanistic underpinnings for this observation were likely to be complex and associated with the proinflammatory properties of n-6 PUFAs.

Most previous studies have revealed that the positive effects of n-3 PUFAs are through the regulation of allogeneic responses [4–7]. However, few researches have focused on the regulation of the innate immune system because the innate immunity has been traditionally considered not to participate in the immune recognition of solid organ allografts. In recent years, this idea has been challenged by the discovery of TLR, RAGE and one of their endogenous ligands — HMGB1 [10,38]. Two individual studies showed that the expression of HMGB1 and RAGE was increased in murine rejecting cardiac

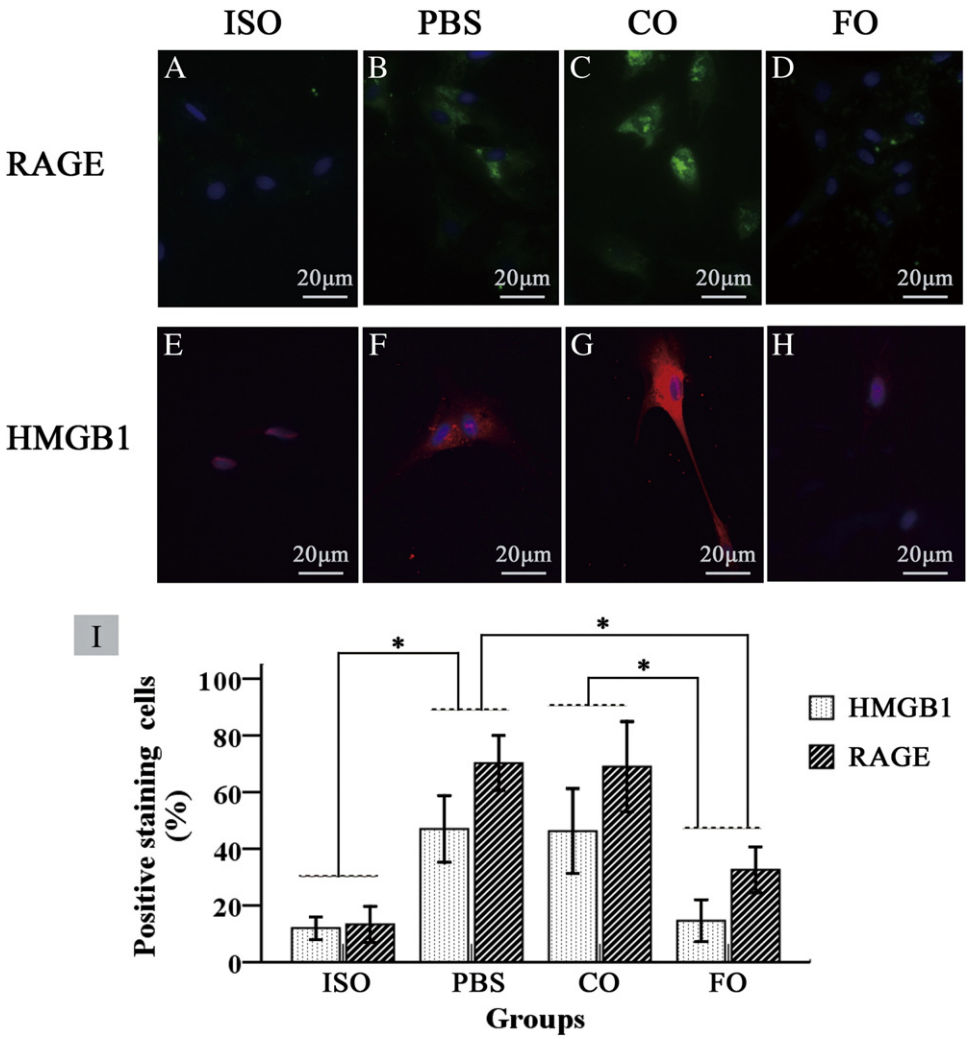

Fig. 4. Expression of HMGB1 and RAGE in graft VECs. Representative views of graft VECs immunostained for HMGB1 (stained in red color) and RAGE (stained in green color) are shown in (A–H) (×400). As shown by immunofluorescence microscopy, VECs isolated from isograft vessels expressed such low levels of HMGB1 and RAGE that positive staining for HMGB1 and RAGE was not easily detectable (A, E). In contrast, a great number of VECs from the allograft groups except the FO group strongly expressed HMGB1 and RAGE (B–D, F–H). Numbers of positive staining cells are expressed as a percentage of total cells. The FO group showed a smaller population of VECs that positively stained for HMGB1 and RAGE compared with the PBS and CO groups (I). *P<.01.

allografts. Inhibition of their expression delayed the progression of allograft rejection and prolonged graft survival [11,12]. It has been revealed that the interaction of RAGE with HMGB1 produces a continuous cycle of cell activation, receptor up-regulation and further cell activation, and also leads to increased release of HMGB1 to extracellular milieu [8]. Once the HMGB1-RAGE pathway is initiated, it creates a vicious circle that perpetuates immunological responses. According to the mechanistic model established by Obhrai and Goldstein [38], extracellular HMGB1 activates TLR on dendritic cells (DCs) and induces DC maturation, which in turn promotes T cell priming and recruitment to allografts. Other studies have revealed that the HMGB1-RAGE pathway is also responsible for maturation and migration of DCs as well as priming and differentiation of T cells [39–42]. These findings indicate that the HMGB1-RAGE pathway plays an essential role in initiation and persistence of adaptive immune responses to alloantigens and thereby may contribute to the development of CAV. The current study supported the assumption and demonstrated that the expression of HMGB1 and RAGE was increased in allograft vessels as compared to the isograft control (Fig. 3). Furthermore, our study also revealed that the expression of HMGB1 and RAGE in the VECs isolated from CAV-bearing vessels was up-regulated (Fig. 4), which suggested activation of the endothelial HMGB1-RAGE pathway during the development of CAV. It has been reported that phenotypic changes induced by activation of the HMGB1-RAGE pathway vary depending on cell types. This pathway mediates the expression of leukocyte adhesion molecules and tissue factor in VECs, whereas its activation enhances SMC migration, proliferation and production of extracellular matrix [43]. Leukocyte adhesion molecules expressed on activated VECs are responsible for leukocyte recruitment to allograft vessels [44]. The procoagulant activity of tissue factor abrogates VEC anticoagulant properties and increases the risk of graft vessel thrombosis [44]. Whether the activation of the HMGB1-RAGE pathway produces similar effects in allograft VECs remains to be clarified.

The present study demonstrated that the expression of HMGB1 and RAGE was down-regulated in allograft vessels and VECs by increased intake of n-3 PUFAs (Figs. 3 and 4) and that the reduction of their expression coincided with the histological improvement of CAV in small bowel allografts (Fig. 2). These observations suggest that protection of allografts against CAV by n-3 PUFA treatment is associated with the regulation of the HMGB1-RAGE pathway. There are two possible mechanisms for modulation of this pathway by n-3

PUFAs. Firstly, many protective effects of n-3 PUFAs on cardiovascular system occur via modification of cell membrane structure and function [45]. Caveolae, a subset of membrane microdomains, are potentially modifiable by n-3 PUFAs [46,47]. RAGE is localized in caveolae [13,14], and its physiological function depends on the integrity of caveolae [15,48]. Therefore, the HMGB1-RAGE pathway may be regulated by n-3 PUFAs via the modification of caveolea. Secondly, previous studies has revealed that activation of PPARγ, a nuclear receptor of regulating gene expression, inhibits neointimal formation through down-regulation of RAGE expression [22–24]. Since n-3 PUFAs and their metabolites are generally considered as agonists of PPARγ [17–20], it is likely that n-3 PUFAs may down-regulate the HMGB1-RAGE pathway by activating PPARγ.

It might be argued that some of the n-3 PUFA effects can also be induced by n-6 PUFAs. For example, PPARγ can also be activated by n-6 PUFAs and their derivatives [49]. In that case, it is likely that n-6 and n-3 PUFAs would have similar effects on the HMGB1-RAGE pathway as well as CAV, which, however, contradicts the present study. The argument neglects the generally opposing effects of n-3 and n-6 PUFAs [33]. n-6 PUFA metabolism generates proinflammatory eicosanoids that are agonists of cellular functions with inflammatory, atherogenic and prothrombotic effects. In contrast, n-3 PUFAs inhibit synthesis of these eicosanoids from n-6 PUFAs and promote production of anti-inflammatory autacoids from n-3 PUFAs. Therefore, the use of n-3 PUFAs is more beneficial overall than the use of n-6 PUFAs. And it is likely that the harmful effects of n-6 PUFAs far outweigh their benefits under certain circumstances, particularly (not exclusively) in CAV, given that an accelerated process of neointimal formation was observed after corn oil supplementation in the current study (Fig. 2).

Although n-3 PUFAs have found many successful applications in rodent transplantation models, current evidence for these fatty acids having beneficial effects in humans is equivocal [50]. The paradoxical findings may possibly be ascribed to differences in experimental conditions. Researchers typically control all aspects of dietary nutrient intake in most animal experiments, whereas little or no control of background diet is taken in most clinical trials [51]. In that case, naturally occurring variations in intake of n-6 PUFAs may unfavorably affect responsiveness to n-3 PUFA supplementation. Thus, the effects of n-3 PUFAs may fail to be elicited in humans. On the other hand, n-3 PUFA actions are likely to be unduly magnified in animal models. Rodent diets are often designed to provide many-fold levels of n-3 PUFA intake that exceed current clinical recommendations, and some are even beyond what is achievable in humans with supplementation. The excessive levels of n-3 PUFA enrichment in animal models may lead to experimental results that may not be reproducible in humans. Therefore, precise estimation of PUFAs intake may help explain the discrepant results between human and animal experimentation and, more importantly, extrapolate rodent experimental data to explain the effects of n-3 PUFAs in humans. The present study measured fatty acid content of RBC membrane for this purpose. This method provides a reliable access to long-term n-3 PUFA consumption, and the omega-3 index, a summation of RBC EPA and DHA levels, is recommended for clinical and epidemiological studies [29]. We found that the omega-3 index rose dramatically to 10.87%±0.89% in the FO group, falling in the range of ≥8%. By contrast, in the PBS and CO groups, the rats consumed inadequate n-3 PUFAs from rodent chow and corn oil, and subsequently had undesirable omega-3 indexes of ≤4%. Our findings were in accord with the clinical evidence that an omega-3 index of ≥8% afforded the greatest protection against cardiovascular diseases, whereas levels of ≤4% were least protective [29]. Moreover, the omega-3 index demonstrated that levels of n-3 PUFA intake by the transplanted rats seemed achievable in clinical settings. It was revealed that oral supplementation of 1.0 g and 2.0 g of EPA and DHA to healthy adults for 6 months raised omega-3 index to 9.9%±2.9% and 11.6%±2.4%, respectively [29], which were comparable to that of the FO group. However, in the PBS and CO control groups, the omega-3 index remained at very low levels that were far from the minimal range ever reported for humans [29]. This was largely due to the paucity of n-3 PUFAs in rodent chow as compared to human daily diets. Therefore, this study may be more convincing and clinically relevant if the rats were fed diets containing modest amounts of PUFAs to mimic human diets in most clinical trials.

In general, the present study confirmed the long-term efficacy of n-3 PUFA treatment for CAV in rat SBTx and provided a potentially important link between their beneficial effects and suppression of the vascular HMGB1-RAGE pathway.

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