Original Communications

Dietary ω-3 Polyunsaturated Fatty Acids Can Inhibit Expression of Granzyme B, Perforin, and Cation-Independent Mannose 6-Phosphate/Insulin-Like Growth Factor Receptor in Rat Model of Small Bowel Transplant Chronic Rejection

Zhao Kun, MD; Zhang Haiyun, MD; Wang Meng, MD; Li Ning, MD; Li Yousheng, MD; and Li Jieshou, MD

From the Department of General Surgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China

ABSTRACT. Background: The aim of our work was to investigate the effects of ω-3 polyunsaturated fatty acids on apoptosis and granzyme B, perforin, and cation-independent mannose 6-phosphate/insulin-like growth factor receptor (CI-MPR) expression of intestinal epithelial cells of chronic rejection after small intestinal transplantation. Methods: Small bowel transplantation was performed in a rat combination of 3 groups: group 1, Lewis-to-Lewis; group 2, F344-to-Lewis, dietary corn oil; group 3, F344-to-Lewis, dietary fish oil. All recipients were killed at 16 weeks posttransplantation. The apoptosis rate of mucosal cells was evaluated by flow cytometry. The expression of granzyme B, perforin, and CI-MPR was analyzed by reverse transcriptase PCR. Results: A high apoptotic rate was observed when the allografts demonstrated 1 or more histologic features of chronic rejection. ω-3 Polyunsaturated fatty acids decreased in rate of the apoptosis, and it can inhibit the expression of granzyme B, perforin, and CI-MPR. Conclusions: ω-3 Polyunsaturated fatty acids can suppress the rejection to mucosal cells of allograft at the time of chronic rejection in small intestinal transplantation, which may be significant in increasing the surviving rate of allograft, delaying the chronic dysfunction, and prolonging the lifetime of both allograft and acceptor. (Journal of Parenteral and Enteral Nutrition 32:12–17, 2008)

Organ transplantation has become routine in many centers throughout the world. Although success rates for small bowel transplantation (SBT) are lower than other organs, it is still high enough to warrant this form of therapy for patients with short bowel syndrome or other untreatable bowel diseases.

However, SBT causes many injuries in the graft due to ischemia-reperfusion, interruption of lymphatic drainage and graft-vs-host disease, whereas immunosuppressive agents have reduced the early graft losses due to rejection. There is increasing evidence that apoptosis is involved in the mechanisms of transplant rejection and immunological tolerance of liver allografts. During allograft rejection, apoptosis occurs in both graft infiltrating lymphocytes and in graft parenchymal cells. The serine proteinase granzyme B is highly expressed in cytotoxic T cells (CTLs) and natural killer (NK) cells. It is stored in granules of activated CTLs and NK cells with a number of other cytotoxins, including the pore-forming protein perforin. The granzyme and perforin pathway is a primary mechanism through which the immune system targets and kills allogeneic cells. Upon stimulation by foreign antigen, T cells exocytose granzymes and perforin into the T-cell-target cell synapse. Perforin then facilitates the delivery of granzymes into the cytoplasm of target cells, where these cytotoxic serine proteases induce cell death. Granzyme B is the most extensively studied granzyme and it is the only granzyme that induces apoptosis of target cells through the indirect and direct activation of caspases. Granzyme B was recently demonstrated to enter cells in a perforin-independent manner, thus predicting the existence of cell surface receptors. Motyka et al presented the evidence that this receptor is the cation-independent mannose 6-phosphate/insulin-like growth factor receptor (CI-MPR).

The presence of apoptosis during acute allograft rejection has been investigated extensively in heart, renal, and liver allografts. However, only a few studies have investigated the occurrence and role of apoptosis in experimental models of chronic rejection after intestinal transplantation. ω-3 Polyunsaturated fatty acids (PUFA) have anti-inflammatory and immunomodulatory activities. Among the well-documented effects of ω-3 PUFA are reductions in proinflammatory eicosanoid and cytokine production, as well as T-cell proliferation. Our work investigates the effects of ω-3 PUFA on apoptosis of intestinal epithelial cells of chronic rejection after small intestinal transplantation.

MATERIALS AND METHODS

Experimental Animals

All experimental procedures using laboratory animals were approved by the Vitairiver Company, Beijing, China. Male F344 rats weighing 220–280 g were
used as donors, and female Lewis rats weighing 250–280 g, as recipients.

The transplanted rats included 3 groups: Lewis-to-Lewis group (n = 8); 2 groups of F344-to-Lewis (n = 10). Lewis-to-Lewis isogenic (ISO) transplants were included as controls for nonallogeneic injury. From 2 weeks pretransplantation to the 60th day posttransplantation, the Lewis rats had been supplemented by gavage with fish oil (FO) or corn oil (CO; both from Sigma Chemical, St. Louis, MO; product number F8020), 0.6% V/W (mL per 100 g body weight). The fish oil contained EPA (139 mg/mL) and DHA (136 mg/mL). The protocol was approved by the Animal Research Committee of Nanjing University. All procedures were carried out in accordance with Principles of Laboratory Animal Care (NIH publication No. 85–23, revised 1985).

SBT

Heterotopic SBT was performed using a standard microvascular technique.12 Donor and recipient operations were performed under anesthesia induced with 100 mg/kg ketamine. The abdomen was opened by a midline incision under ketamine anesthesia. The donor small bowel (jejunum-ileum) was removed after intraluminal and intravascular irrigation with cold heparinized saline solution (4°C). The bowel was excised with its intact vascular pedicle, consisting of the superior mesenteric artery with an aortic cuff and the portal vein. The graft was kept in cooled lactated Ringer's solution for 25 minutes. The recipient abdomen was opened and the graft was reperused by anastomosing the aortic cuff to the recipient infrarenal aorta, although the portal vein was anastomosed to the recipient inferior vena cava. The proximal and distal ends of the bowel were exteriorized as stomas. Animals surviving <24 hours were considered technical failures and excluded. All of the recipients were treated with cyclosporine A (Sandimune; Novartis Pharmacy Ltd, Basel, Switzerland), 5 mg/kg/d for POD 0–13, to prevent acute rejection.

Histopathology

Sequential protocol biopsies were performed every 2 weeks from 2 to 8 weeks after transplantation. All recipients were killed at 16 weeks posttransplantation as ending. Sequential biopsies were performed under ether anesthesia. Stoma was placed at a midline incision convenient for biopsy and to alleviate the incision scar. When biopsied, the segment of intestinal open stoma, about 2 cm length, was excised with the corresponding mesentery. Paraffin sections 2–4 μm thick were stained with hematoxylin and eosin stain and evaluated in a blinded manner by 2 observers.

Flow Cytometry

Epithelial cells were isolated from the recipients. In brief, the small intestine was resected, dissected open, cut into 2- to 3-mm pieces, and rinsed clean in 50 mL of Ca2+ and Mg2+-free HBSS at room temperature. Tissue was then diced into <1- to 3-mm pieces, incubated with 20 mL of enzyme solution (0.1 mg/mL dispass type I and 300 U/mL collagenase type XI; Sigma-Aldrich, St. Louis, MO) for 30 minutes with agitation at 80 cycles/min. The cell suspension was pipetted vigorously for 3 minutes and left to sediment under gravity for 1 minute. The supernatant was collected and added to 10 mL of DMEM +2.5% FCS with 2% sorbitol and centrifuged at 1000 × g for 2 minutes. This process was repeated until the supernatant was clear and the pellet was well defined.

Phosphatidylserine (PS)

Rat small intestine mucosa cells were stained with propidium iodide (PI) and FITC-labeled annexin V by using the Annexin-V-APOP kit (Alexis Co, Switzerland) to assess cellular integrity and the externalization of PS. Cells were washed twice in PBS and suspended in modified annexin binding buffer (10 mmol/L HEPES/NaOH, pH 7.4; 40 mmol/L NaCl; 50 mmol/L CaCl2; 1.2 M sorbitol). Annexin V binding assays were performed according to the protocol of Monchik et al12 in modified annexin binding buffer containing 20 μL/mL annexin reagent and 20 μg/mL PI. We measured exposed PS by treating red cells with commercially available FITC-labeled annexin V in the presence of calcium, washing with a calcium-containing buffer, and measuring cell-associated fluorescence in a Becton, Dickinson FACScan flow cytometer (Ex = 488 nm; Em = 530 nm). Acquisition and data analysis were performed by using CELLQUEST software (Becton, Dickinson, Franklin Lakes, NJ). Ten thousand events per sample were acquired to minimize sampling errors. Fluorescent channels and light scatter were set at log gain. The FSC setting was at E-1 with a threshold of 36. The SSC PMT voltage was set at 250 and the FL1 PMT voltage, at 750.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Expression of specific gene transcripts identified within biopsy tissue was quantified by RT-PCR. RT was performed for 50 minutes at 42°C with Superscript II (Invitrogen, Carlsbad, CA). PCR primers were based on conserved sequences of rat perforin 1 (NM_017330), granzyme B (NM_138517), and CI-MPR (RNU59809). Primer design for RT-PCR was carried out with the software Primer Express (Applied Biosystems, Foster City, CA). Amplification of the universally expressed β-actin gene (V01217) served to confirm successful RNA isolation and reverse transcription. Primers were designed as follows:

- perforin 1
  - forward primer: CCTAAACCGGAAGCAGACTGTGCA
  - reverse primer: TGTACATGCGACACTCCACTGTGT

- granzyme B
  - forward primer: AGCGCCAGTGAAAGTTCAAACAG
  - reverse primer: TTACAGCAGGGAGGCTTCCAGA

- CI-MPR (mannose 6-phosphate/insulin-like growth factor II receptor)
  - forward primer: ACAGGGCTTGCTGTAACAGATGA
  - reverse primer: TGTACATGCGACACTCCACTGTGT

- β-actin
  - forward primer: AGAGAAGCTGCTGATGATGCTGCCCT
  - reverse primer: ACTCCTGCTTGCTGACATCT

- annexin V
  - forward primer: CCTAAACCGGAAGCAGACTGTGCA
  - reverse primer: TGTACATGCGACACTCCACTGTGT
PCR was performed in a 50-μL volume containing 2 units of Taq polymerase, 1.5 mmol/L MgCl₂, and 1–2 μL of cDNA from the RT reaction. The initial denaturation step was at 94°C for 3 minutes, followed by 30 cycles of denaturation for 25 seconds at 94°C, annealing for 40 seconds at 60°C, and extension for 1 minute at 72°C. Each PCR reaction was replicated a minimum of 3 times. β-Actin expression was determined for each sample to equalize cDNA loading and permit semiquantitative comparisons between samples. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed in UV light.

Statistical Analysis

Results are expressed as mean ± SD, using SPSS 11.5 for Windows. Statistical analyses were carried out with 1-way ANOVA. Values were considered significant at \( p < .05 \).

RESULTS

Body Weight and Histology

Animals undergoing transplantation lost weight during the first 3–5 days posttransplant and then began a slow recovery and increase in body weight. They regained weight and continued growing after 7–10 days posttransplantation. The difference of the mean body weights of different experimental groups was not obvious.\(^13\)

We have previously described that all of the allografts demonstrated 1 or more histologic features of chronic rejection at 16 weeks. The intestine wall of allografts appeared thickened and stiff, with some peritoneal adhesion. Mesenteries showed a milky whitish surface and appeared fibrotic. Lengths of mesenteries were moderate and mildly shortened. The significant characteristics of allografts on histology were changes of villous architecture, interstitial fibrosis, leukocyte infiltration, and arteriopathy.\(^13\)

Annexin V Analysis

Apoptosis occurred inevitably in allograft mucosal cells after small intestinal transplantation. Although no rejection was detected in the Lewis-to-Lewis group, apoptosis of mucosal cells was triggered due to the injury of ischemia/reperfusion mechanism and the disuse atrophy. Obviously, chronic rejection raised the rate of mucosal cells apoptosis when chronic rejection of the allograft took place. It suggested a remarkable increase of CI-MPR activation in mucosal cells when chronic rejection of the allograft took place. The value of the FO group was 0.201 ± 0.021 (mean ± SD; \( n = 10 \)), which was much less than that of the CO group, \( p < .05 \), suggesting that ω-3 PUFA inhibited the expression of granzyme B in mucosal cells.

The perforin RT-PCR result was 0.144 ± 0.010 (mean ± SD; \( n = 8 \)) and 0.345 ± 0.043 (mean ± SD; \( n = 10 \)), \( p < .05 \), in the ISO group and CO group, respectively. It suggested a remarkable increase of perforin activation in mucosal cells when chronic rejection of the allograft took place. The value of the FO group was 0.0413 ± 0.006 (mean ± SD; \( n = 10 \)), which was much less than that of the CO group, \( p < .05 \), suggesting that ω-3 PUFA inhibited the expression of CI-MPR in mucosal cells.

DISCUSSION

Clinical and experimental evidence showed that ω-3 PUFAs have anti-inflammatory actions in a number of inflammation-associated disease states, including atherosclerosis, autoimmune disorders, malignancy, and sepsis. Study on ω-3 PUFA and apoptosis focused on the field of tumor cells, and ω-3 PUFA was generally reported to promote the apoptosis of tumor cells. ω-3 PUFA was demonstrated to inhibit the apoptosis of mucosal cells in allograft small intestine in our study. Apoptosis plays a critical role in mucosal restitution after injury. Apoptosis prevents the release of cellular material (eg, lysosomal proteases) that occurs after cellular necrosis, which may lead to mucosal injury. However, exaggerated apoptosis is observed in inflamed/ulcerated areas of colonic mucosa in inflammatory bowel disease and this may impair mucosal restitution.\(^13\)
Fayyazi et al.\textsuperscript{15} first demonstrated the presence of a high number of apoptotic enterocytes in a rat model of allogenic small intestinal transplantation without immunosuppression. The authors described a positive correlation between histologic findings of rejection and the number of apoptotic cells detected in allografts. It was shown in our study that the apoptosis rate of mucosal cells was as high as 31% in allograft with chronic rejection, demonstrated by pathologic evaluation, when immunosuppression was used. The rate decreased to 14.8% in the fish oil group with administration of rich ω-3 PUFA. It suggested that ω-3 PUFA depressed the rejection to allograft small intestine, reduced the dosage of immunosuppression, and minimized the risk of infection.

The granule-exocytosis model has evolved since its original enunciation by Henkart.\textsuperscript{16} Initially, one of the granule proteins, perforin, was thought to be the major effector of CTL-induced death. However, it then became obvious that target cells were dying through an apoptotic mechanism that involved damage of DNA.\textsuperscript{17} The factor responsible was purified and identified as a known cytotoxic cell protease, now referred to as granzyme B.\textsuperscript{18} In a complementary approach, it was shown that cells were endowed with the ability to induce membrane damage and DNA fragmentation when they

**Figure 1.** Annexin V assessment of apoptosis rate of mucosal cells by flow cytometry. The Annexin V/PI assay can discriminate normal cells, apoptotic cells, and necrotic cells quite well. The value of LR described the rate of apoptosis.
were transfected to express granzyme B and perforin.\(^\text{19}\)

The CI-MPR is a receptor for granzyme B on the target cell surface, and this recognition is necessary for the efficient apoptosis of target cells mediated by granule-purified granzyme B or by CTL. The modulation of the CI-MPR on the target cell surface would thus have profound repercussions on the ability of CTL to induce apoptosis by the granzyme B–mediated pathway.\(^\text{5}\)

The study on granzyme/perforin pathway in apoptosis suggested that the granzyme and perforin were activated when chronic rejection to allograft small intestine occurred, which correlated with the increasing activity and amount of the T cell that released granzyme and perforin toward target cells in immunological rejection. Through perforin introduction, granzyme induced the apoptosis of the target cells. \(\omega\)-3 PUFA inhibited the expression of granzyme and perforin to suppress the apoptosis, which may be associated with its effect on the activity and amount of T cells.

CI-MPR, expressed on the surface of cells, was of great importance in granzyme-triggered apoptosis by introducing granzyme into the target cells, in place of perforin. The regulation and signal transference pathway remain to be well documented. Our study found that the CI-MPR expression increased on mucosal cells significantly at the time of chronic rejection, which induced granzyme into cells and accelerated the apoptosis. In the \(\omega\)-3 PUFA group, however, RT-PCR demonstrated the reduction of CI-MPR expression markedly, which may inhibit the granzyme in cells and the apoptosis finally.

The decreasing apoptosis rate retained the mucosal barrier and prevented the bacteria translocation. Granzyme and perforin were inhibited in \(\omega\)-3 PUFA group; so was CI-MPR expression, which induced granzyme into cells without perforin.

Apoptosis induced by granzyme/perforin and CI-MPR correlated with immunological rejection in organ allograft, and \(\omega\)-3 PUFA inhibited this progres-

---

**FIGURE 2.** The apoptosis rate of intestinal mucosal cell. The mean rate of apoptotic cell in the isogenous group was the lowest of the 3 groups. The mean rate of CO group was the highest.

**FIGURE 3.** Granzyme B expression of mucosal cells evaluated using RT-PCR. The gray density of granzyme B in the ISO group was the lowest of the 3 groups. The density of FO group was lower than CO group.

**FIGURE 4.** Perforin expression of mucosal cells evaluated using RT-PCR. The gray density of perforin in the ISO group was the lowest of the 3 groups. The density of FO group was lower than CO group.

**FIGURE 5.** CI-MPR expression of mucosal cells evaluated using RT-PCR. The specific value of gray density of perforin in the CO group was the highest of the 3 groups. The density of ISO group was the lowest.

Isogenous (ISO): 3.684±2.04; corn oil (CO): 31.137±5.71; fish oil (FO): 14.831±3.75 p<.05

Isogenous (ISO): 0.144±0.010; corn oil (CO): 0.345±0.043; fish oil (FO): 0.201±0.021 p<.05

Isogenous (ISO): 0.024±0.005; corn oil (CO): 0.087±0.007; fish oil (FO): 0.041±0.006 p<.05

Isogenous (ISO): 0.009±0.009; corn oil (CO): 0.645±0.057; fish oil (FO): 0.281±0.042 p<.05
sion. It suggested in some studies that the apoptotic rate of mucosal cell of the intestine correlated with the level of rejection. It suggested that ω-3 PUFA depressed the rejection to allograft small intestine, reduced the dosage of immunosuppression, and minimized the risk of infection. Our study suggested that ω-3 PUFA suppressed the rejection to mucosal cells of allograft at the time of chronic rejection in small intestinal transplantation, which may be significant in increasing the surviving rate of allograft, delaying the chronic dysfunction, and prolonging the lifetime of both allograft and acceptor.

ACKNOWLEDGMENTS

This work was funded by the National Basic Research Program of China (No. 2003CB515502).

REFERENCES