Eicosapentenoic Acid Attenuates Allograft Rejection in an HLA-B27/EGFP Transgenic Rat Cardiac Transplantation Model

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The development of an animal model bearing definite antigens is important to facilitate the evaluation and modulation of specific allo-antigen responses after transplantation. In the present study, heterotopic cardiac transplantation was performed from F344/EGFPTg and F344/HLA-B27Tg rats to F344 rats. The F344 recipients accepted the F344/EGFPTg transplants, whereas they rejected the cardiac tissue from the F344/HLA-B27Tg rats by 39.4 ± 6.5 days, due to high production of anti-HLA-B27 IgM- and IgG-specific antibodies. In addition, immunization of F344 rats with skin grafts from F344/HLA-B27Tg rats resulted in robust production of anti-HLA-B27 IgM and IgG antibodies and accelerated the rejection of a secondary cardiac allograft (7.4 ± 1.9 days). Of interest, the F344 recipients rejected cardiac grafts from double transgenic F344/HLA-B27&EGFPTg rats within 9.0 ± 3.2 days, and this was associated with a significant increase in the infiltration of lymphocytes by day 7, suggesting a role for cellular immune rejection. Eicosapentenoic acid (EPA), one of the ω -3 polyunsaturated fatty acids in fish oil, could attenuate the production of anti-HLA IgG antibodies and B-cell proliferation, significantly prolonging double transgenic F344HLA-B27&EGFPTg to F344 rat cardiac allograft survival (36.1 ± 13.6 days). Moreover, the mRNA expression in the grafts was assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR), revealing an increase in the expression of the HO-1, IL-10, TGF- β , IDO, and Foxp3 genes in the EPA-treated group. Hence, our data indicate that HLA-B27 and/or GFP transgenic proteins are useful for establishing a unique animal transplantation model to clarify the mechanism underlying the allogeneic cellular and humoral immune response, in which the transplant antigens are specifically presented. Furthermore, we also demonstrated that EPA was effective in the treatment of rat cardiac allograft rejection and may allow the development of novel immunomodulatory strategies for organ transplantation.

Key words: Allograft; GFP; HLA-B27; Regulatory T cell; Tolerance; Transgenic rat

INTRODUCTION

Current immunosuppressive therapies are broad based and nonspecific. Induction of peripheral tolerance in a transplant antigen-specific manner is an attractive but still unrealized clinical goal. The development of an animal model bearing definite antigens is important to facilitate the evaluation and modulation of the specific allo-antigen response after transplantation. In the present study, we first used human leukocyte antigen (HLA)-B27Tg rats in a transplantation study and found that F344 (Fischer) recipients rejected cardiac grafts from the F344/HLA-B27Tg rats via a process of chronic rejection, resulting from high

production of anti-HLA IgM- and IgG-specific antibodies. In contrast, the F344 recipients rejected cardiac grafts from double transgenic F344 /HLA-B27 and enhanced green fluorescent protein (EGFP)Tg rats through acute rejection, associated with a significant increase in the infiltration of lymphocytes, which indicated that the HLA-B27 and/or GFP transgenic proteins are useful for establishing a unique animal transplantation model to clarify the mechanism(s) underlying the allogeneic cellular and humoral immune responses.

Eicosapentenoic acid (EPA) is one of the ω -3 polyunsaturated fatty acids (PUFA) present in fish oil. Many studies have shown that ω -3 PUFA have immunomodulatory

Received January 31, 2011; final acceptance April 1, 2011. Online prepub date: May 8, 2012.

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effects on leukocytes, which are beneficial in patients with autoimmune diseases (1,2,18). In the current study, we found that purified EPA could significantly prolong the survival of double transgenic F344/HLA-B27&EGFPTg to F344 rat cardiac allografts by attenuating the production of anti-HLA IgG antibodies and B-cell proliferation, which may allow for the development of novel immunomodulatory strategies in organ transplantation.

MATERIALS AND METHODS

Rats

F344 (Fisher) rats were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), F344/HLA-B27Tg rats were purchased from Taconic Farms, Inc. (Hudson, NY), and F344/EGFPTg rats were produced by injecting the purified pCAG-EGFP plasmid DNA into F344 rat fertilized eggs (19). Double F344/HLA-B27&EGFPTg rats were the offspring of mating F344/HLA-B27 and F344/EGFPTg rats and were identified by PCR. All rats were maintained under standard conditions and fed rodent food and water, in accordance with the guidelines of the Animal Use and Care Committee of the National Research Institute for Child Health and Development, Tokyo, Japan.

Polymerase Chain Reaction (PCR)

We extracted total genomic DNA from rat ear skin by using CellEase® Mouse Tail (Kanto Chemical, Tokyo, Japan) according to the protocol described. We performed PCR amplification in a 20- μ l reaction mixture containing 10 μ l AmpliTaq Gold® PCR Master Mix (Life Technologies, NY), 2 μ l Betain and 5 pmol of each primer (as specified by Taconic for genotyping): HLA-B27 (272 bp), forward: GTGAGTGACCCCGGCCCG and reverse: GCCCGCCCCGACCAAC; α -actin (313 bp), forward: CACCACACCTTCTACAATGAGCTG and reverse: TCATCAGGTAGTCAGTGAGGTCGC. We used the ASTEC Thermal Cycler PCR system (Fukuoka, Japan) and the "hot start" technique (94°C for 60 s) to increase specificity. The thermal cycling parameters were denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension for 30 s at 72°C (35 cycles). PCR products (8 μ l) and 6xDNA loading dye (2 μ l, TaKaRa, Shiga, Japan) were analyzed on 2% Tris/EDTA/borate (TBE) agarose gels. We visualized prominent bands of the correct size with ethidium bromide staining.

Heterotopic Cardiac Transplantation and Skin Graft Sensitization

Heterotopic cardiac transplantation was performed from sex-matched F344/HLA-B27, F344/EGFP, and F344/HLA-B27&EGFPTg donors into F344 recipients by the cuff techniques. The cervical heterotopic rat cardiac transplantation was performed as previously

described (7,9). In brief, after thoracotomy under inhalation anesthesia, the donor heart was harvested. The heart graft was rapidly cooled and flushed with 5 ml of physiological saline (4°C) containing 200 U/ml heparin, which was infused via the aorta and pulmonary artery. The heart graft was preserved in physiological saline at 4°C.

The cuff was prepared from an intravenous cannula and was then anastomosed in the cervical region of the recipient with arterial and venous cuffs. The total ischemic time for the donor operation and vascular anastomoses did not exceed 30–35 min. All procedures were performed under clean but not aseptic conditions. Cardiac graft survival was determined by daily palpation from the skin above the cervical grafted heart. Rejection was considered complete at the time of cessation of a palpable heart beat and confirmed visually by laparotomy.

In addition, skin graft sensitization to the HLA-B27 antigen was performed in F344 rats transplanted from skin grafts of F344/HLA-B27Tg rats as described (12). Two weeks later, cervical heterotopic cardiac transplantation was performed from sex-matched F344/HLA-B27Tg donors to the skin graft-sensitized F344 recipients. The skin graft was observed daily, and rejection was defined as >50% necrosis of the skin allograft. The survival of the cardiac allograft was also observed at the same time.

Lymphocyte Proliferation Assays

The mixed lymphocyte reaction (MLR) was performed with F344 rat nylon wool column (Wako)-enriched T cells (1×10^5 /well) as responders and 20-Gy irradiated F344, F344/EGFP, F344/HLA-B27, and F344/HLA-B27&EGFPTg rat splenocytes (1×10^5 /well) as stimulators, incubated in a flat-bottomed 96-well white plate (Costar; Corning, NY) at a final volume of 200 μ l/well of the GIT medium containing 50 μ M 2-mercaptoethanol (Wako) in a humidified atmosphere at 37°C for 5 days. The proliferation of T cells was measured with cell proliferation ELISA kits (Roche Diagnostics GmbH, Penzberg, Germany).

Briefly, the cells were labeled with 5-bromo-2-deoxyuridine (BrdU) solutions at 10 μ l/well and incubated for an additional 2 h at 37°C. After centrifugation, the supernatant was removed, 200 μ l/well of fixation and DNA denaturation (FixDenat) solution was added to the cells, and they were incubated for another 30 min at 15–25°C. The cells were cultured for 90 min with an anti-BrdU–peroxidase solution and subsequently washed three times. After adding the substrate solution at 100 μ l/well, the BrdU incorporation was measured with a chemiluminescence reader (Wallac ARVO™ SX; PerkinElmer, Inc., Wellesley, MA), and the data were processed using the Wallac1420 manager software program (PerkinElmer).

Measurement of Serum Anti-HLA-B27 IgM and IgG Antibodies

The titers of anti-HLA-B27 IgG or IgM in rat sera were determined by flow cytometry. Sera (1:10 diluted) were incubated with F344 thymocytes for 1 h. Cells were washed and incubated with goat anti-rat IgM or IgG antibodies (SouthernBiotech, Birmingham, AL), and then stained cells were analyzed with a BD FACSCalibur flow cytometer and analyzed using the CellQuest software program (BD Biosciences, San Jose, CA).

Histological Analysis

Cardiac graft specimens were fixed in 10% buffered formalin and embedded in paraffin. The sections were cut (1 μ m thick) and stained with hematoxylin and eosin. A light microscopic analysis was performed to assess the overall cellularity and myocardial damage.

EPA Treatment

On the day of transplantation, some F344 recipients who received cardiac allografts from F344/HLA-B27&EGFPTg donors were given one intraperitoneal injection of 0.1 g/kg purified EPA (supplied by Mochida Pharma, Inc., Tokyo, Japan), and then the survival of the cardiac allografts was determined by daily palpation.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Splenocytes were obtained from F344/HLA-B27&EGFPTg rat heart grafted F344 recipients with or without EPA treatment on postoperative day 7 (POD7) and suspended in PBS and then incubated at 4°C for 30 min with an optimal concentration of fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated anti-rat CD45RA, CD3, CD4, CD8, and CD25 mAb (BD Pharmingen) diluted with PBS containing 0.5% fetal calf serum. The stained cells were analyzed with flow cytometry and analyzed using the CellQuest software program (BD Biosciences).

RNA Isolation and Quantitative Real-Time RT-PCR

The total RNA was extracted from the cardiac grafts and spleens using the RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol. The concentration of total RNA was determined by measuring the optical density at 260 nm. Each 600-ng sample of RNA was reverse transcribed to cDNA using oligo (dT) primers and Super Script™ reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Quantitative RT-PCR was performed using a TaqMan system on the Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers amplifying the rat mRNAs and specific Taqman probe were designed with Primer Express software program

(Applied Biosystems): heme oxygenase-1 (HO-1), forward: CAGGGTGACAGAAGAGGCTAAGAC, reverse: TCTTTGTGTTCTCTGTCAGCAGT, probe: TCCTGCTCAACATTGAGCTGTTTGAGGA; interleukin-10 (IL-10), forward: GAGAAGCTGAAGACCCTCTGGA, reverse: TTCACCTGCTCCACTGCCT, probe: TGCGACGCTGTCATCGATTCTCC; indoleamine 2,3-dioxygenase (IDO), forward: AGACTGCGTCCTGGCAAAC, reverse: AGGAAACGAGAACAGAATGTCCAT, probe: AGGACCCCAATGGGCCCATGA; transforming growth factor- β (TGF- β), forward: AGAAGTCACCCGCTGTGCT, reverse: CGAATGTCTGACGTATTGAAGACA, probe: TGGACCGCAACAACGCAATCTATGA; forkhead box P3 (Foxp3), forward: TGGGAAGATGGCA TTGACAA, reverse: GGCTCGAGGCAGCAGGA, probe: AGCTCCACCTGTGGCCTCAGTGGGA; 18s, forward: ATGAGTCCACTTTAAATCCTTTAA CGA, reverse: CTTTAATATACGCTATTGGAGCTGGAA, probe: ATCCATTGGAGGGCAAGTCTGGTGC. The data were expressed as the comparative cycle thresholds (Ct). The normalized Ct value of each gene was obtained by subtracting the Ct value of 18s rRNA. The fold change versus one sample of the control group was calculated as described previously.

Statistical Analysis

Student's *t* tests were used to compare the paired and unpaired analyses. A statistical evaluation of mouse survival was performed using the Kaplan–Meier test. Values of $p < 0.05$ were considered statistically significant. All in vitro experimental data were representative of three independent experiments and represented the mean ratio of triplicate results for each experiment.

RESULTS

F344 Recipients Rejected Cardiac Allografts From F344/HLA-B27 and F344 /HLA-B27&EGFPTg Donors

Heterotopic cardiac transplantation was performed first from F344/EGFP, F344 /HLA-B27, and F344/HLA-B27&EGFPTg donor rats into the F344 recipient rats. We found that F344 recipients accepted cardiac grafts from F344/EGFPt donors [$n=8$, median survival time (MST) >100 days], whereas they rejected cardiac grafts from F344/HLA-B27Tg donors at 39.4 ± 6.54 days ($n=5$). Of interest, we found that F344 recipients rejected cardiac grafts from double transgenic F344/HLA-B27&EGFPTg donors via acute rejection ($n=8$, MST: 9.0 ± 3.16 days, $p < 0.001$; Fig. 1). In addition, immunization of F344 rats with skin grafts from F344/HLA-B27Tg rats 2 weeks prior to cardiac transplantation resulted in an accelerated rejection of the secondary cardiac allograft ($n=5$, MST: 7.4 ± 1.95 days) compared with the mice not preimmunized with the skin grafts ($n=5$, MST: 39.4 ± 6.54 days, $p < 0.001$; Fig. 2).

Figure 1. F344 recipients rejected cardiac allografts from F344/HLA-B27Tg and F344/HLA-B27&EGFPTg donors. Heterotopic cardiac transplantation was performed, in which Fischer F344 rats were recipients and sex-matched F344/enhanced green fluorescent protein (EGFP)Tg, F344/human leukocyte antigen (HLA)-B27Tg, or F344/HLA-B27&EGFPTg rats were donors. MST, median survival time. The results are shown as the MST±SD. ** $p < 0.001$ for the difference between two groups.

Figure 2. Skin priming accelerated the rejection of a secondary cardiac allograft from F344/HLA-B27Tg rats. Heterotopic cardiac transplantation was performed, in which F344/HLA-B27Tg rats were donors and sex-matched naive F344 rats or F344 rats received skin grafts from F344/HLA-B27Tg rats 2 weeks before receiving the cardiac grafts. MST, median survival time. The results are shown as the MST±SD. ** $p < 0.001$ for the difference between two groups.

Then a MLR assay was performed to assess the effects of the HLA-B27 and/or EGFP transgenes on the allo-antigen response in vitro. We found that the splenic T cells obtained from naive F344 rats showed the same proliferative response to irradiated F344 naive and F344/EGFPTg spleen cells; in the case of irradiated spleen cells obtained from F344/HLA-B27 and F344/HLA-B27&EGFPTg rats, however, the proliferative response was significantly enhanced ($p=0.014$ and 0.012 ; Fig. 3).

Vigorous Production of Anti-HLA-B27 IgG and IgM Antibodies Following Allogenic Sensitization

Allo-antibodies, whose main targets are major histocompatibility complex (MHC) molecules, can also contribute to acute and chronic graft rejection, and so we analyzed the serum concentration of anti-HLA-B27 IgG and IgM antibodies by flow cytometry at different time points after cardiac transplantation. As shown in Figure 4A, the total IgG titers steadily increased on POD7 and 14 and peaked at POD21 in F344 recipients that received cardiac grafts from F344/HLA-B27 and F344/HLA-B27&EGFPTg donors. In contrast, the total IgM titers showed the reverse tendency, with a gradual decrease posttransplantation in F344 recipients with cardiac grafts from F344/HLA-B27 and F344 /HLA-B27&EGFPTg donors. In comparison, the IgG and IgM titers showed no difference between F344 recipients who were transplanted with cardiac grafts from isograft F344 or F344/EGFPTg donors at different time points posttransplantation (Fig. 4A).

In addition, the sensitization of F344 rats with skin grafts from F344/HLA-B27Tg rats 2 weeks before cardiac transplantation resulted in robust production of anti-HLA-B27 IgG antibodies on POD7, and the IgG and IgM titers

showed the same tendencies as with the F344/HLA-B27 or F344/HLA-B27&EGFPTg transplants on POD14, 21, and 28 (Fig. 4B).

Histopathological Features

We then compared the histopathological features of the allograft between these four groups. The F344/EGFPTg cardiac grafts were free of myocardial injury and had markedly reduced inflammatory cell infiltration even on POD100, while the histopathological features of F344/HLA-B27Tg cardiac grafts on POD40 showed the features of chronic rejection, including mild interstitial infiltration of inflammatory cells, hemorrhage, and fibrosis. In contrast, classic signs of acute rejection could be seen in the F344/HLA-B27&EGFPTg cardiac grafts and F344/HLA-B27Tg cardiac grafts from skin graft-primed rats on POD5, including strong interstitial infiltration of inflammatory cells, severe hemorrhage, edema, and necrosis (Fig. 5).

EPA Treatment Significantly Prolonged Double Transgenic F344/HLA-B27&EGFPTg Rat Cardiac Allograft Survival

As shown in Figure 6, a single intraperitoneal injection of 0.1g/kg purified EPA could significantly prolong the survival of double transgenic F344/HLA-B27&EGFPTg to F344 rat cardiac allografts ($n=9$; MST: 36.1 ± 13.6 days), compared to those in animals not treated with EPA ($n=8$, MST: 9.0 ± 3.16 days, $p<0.001$).

We also compared the histopathological features of the allografts harvested on POD7 between F344 recipients that received cardiac grafts from F344/HLA-B27&EGFPTg donors with and without EPA treatment. The classic signs of acute rejection could be seen in the allografts obtained from the F344 recipients without EPA treatment, including

Figure 3. The allo-antigen response was significantly enhanced by HLA-B27 and HLA-B27&EGFP, but not by the EGFP transgene. Mixed lymphocyte reaction (MLR) was performed with F344 rat nylon wool column-enriched T cells as responders and irradiated F344/EGFP, F344/HLA-B27, or F344/HLA-B27&EGFPTg rat splenocytes as stimulators. The results are shown as the means $\pm$ SD. * $p=0.014$, ** $p=0.012$, the difference between F344, F344/EGFP, F344/HLA-B27, and F344/HLA-B27&EGFPTg, respectively.

Figure 4. There is vigorous production of specific anti-HLA-B27 IgG and IgM antibodies following allogeneic sensitization. Fluorescence-activated cell sorting (FACS) measurement of serum IgG (A) and IgM (B) at different time points post cardiac transplantation, showing that the total serum IgG increased steadily while the IgM decreased gradually in F344 recipients transplanted with cardiac grafts from F344/HLA-B27 or F344/HLA-B27&EGFPTg donors. Furthermore, immunization of F344 rats with skin grafts from F344/HLA-B27Tg rats 2 weeks before cardiac transplantation resulted in robust production of anti-HLA-B27 IgG antibodies on postoperative day 7 (POD7). In comparison, the IgG and IgM titers showed no difference between F344 recipients who were transplanted with cardiac grafts from isograft F344 or F344/EGFPTg donors at different time points post cardiac transplantation.

strong interstitial infiltration of inflammatory cells, severe hemorrhage, edema, and necrosis. In contrast, the allografts harvested from the F344 recipients treated with EPA were free of myocardial injury and had markedly reduced inflammatory cell infiltration (Fig. 7).

EPA Treatment Attenuated Anti-HLA-B27 IgG Antibody Production by Decreasing the B-Cell Population

Because the F344 recipients rejected cardiac grafts from F344/HLA-B27 and F344/HLA-B27&EGFPTg donors Due to high production of anti-HLA-B27 IgM- and

Figure 5. Histopathological features of cardiac allografts between F344/EGFP, F344/HLA-B27, and F344/HLA-B27&EGFP rats and skin-primed F344/HLA-B27Tg rats. The F344/EGFP cardiac grafts were free of myocardial injury and had markedly reduced inflammatory cell infiltration even on POD100 (A, B), while the histopathological features of F344/HLA-B27Tg cardiac grafts on POD40 (C, D) showed chronic rejection, including mild interstitial infiltration of inflammatory cells, hemorrhage, and fibrosis. In contrast, classic signs of acute rejection could be seen in the F344/HLA-B27&EGFP (E, F) cardiac grafts and cardiac grafts implanted into skin-primed rats (G, H) on POD5, including strong interstitial infiltration of inflammatory cells, severe hemorrhage, edema, and necrosis. Scale bars: 100 and 200 μ m.

Figure 6. EPA treatment could significantly prolong the survival of double transgenic F344/HLA-B27&EGFP Tg to F344 rat cardiac allografts. Heterotopic cardiac transplantation was performed from F344/HLA-B27&EGFP Tg donors to F344 recipients. On the day of transplantation, F344 recipients were given one intraperitoneal injection of 0.1 g/kg purified eicosapentenoic acid (EPA), then the survival time of the cardiac allograft was determined by daily palpation and compared with that in rats without EPA treatment. MST, median survival time. The results are shown as the means ± SD. ** $p < 0.001$ for the difference between two groups.

IgG-specific antibodies, we next analyzed the serum concentration of anti-HLA-B27 IgM and IgG antibodies at different time points posttransplantation between the F344 recipients that received cardiac grafts from F344/HLA-B27&EGFP Tg donors with and without EPA treatment. We found that EPA treatment could significantly attenuate the production of anti-HLA IgG antibodies on POD7, 14, 21 and 28 ($p = 0.004, 0.034, 0.037$, and 0.040 , respectively; Fig. 8A), but no difference was seen in the production of anti-HLA-B27 IgM antibodies at any of the time points between the F344 recipients with and without EPA treatment (Fig. 8B).

We also analyzed the T and B cells in F344 recipients that received cardiac graft from F344/HLA-B27&EGFP Tg donors with and without EPA treatment by a FACS analysis on POD7. No difference in the T cells was seen; however, EPA treatment could significantly decrease the B-cell population on POD7 compared with the rats not treated with EPA ($p = 0.005$; Fig. 9). Furthermore, EPA treatment slightly increased the CD4⁺CD25⁺ T-cell population, but there were no changes in the CD4⁺ and CD8⁺ T-cell populations.

The Effects of EPA Treatment on Cytokine mRNA Expression

We further compared the mRNA expression levels of various cytokines in the spleen and cardiac grafts on

POD7 between F344 recipients that received cardiac grafts from F344/HLA-B27&EGFP Tg donors with and without EPA treatment by quantitative real-time RT-PCR analysis and found that the mRNA expression of HO-1, IL-10, TGF- β , IDO, and Foxp3 were increased in the EPA-treated group in both the cardiac grafts (Fig. 10A) and in the spleen (Fig. 10B).

DISCUSSION

We herein described a unique and useful animal transplantation model in which grafts from F344/HLA-B27 Tg rats mainly provoke a humoral immune response and grafts from F344/HLA-B27&EGFP Tg rats represent a typical cellular immune response. T cells are essential for allograft rejection, but allo-antibodies, whose main targets are MHC molecules, can also contribute to acute and chronic graft rejection (4). Clinical studies have shown a correlation between the presence of anti-HLA antibodies, complement C4d deposition, and graft failure (4,6). The removal of allo-antibodies by intravenous immunoglobulin (IVIg) or plasmapheresis or by the depletion of B cells can improve the longevity of transplants (14). We found that F344 recipients rejected cardiac grafts from F344/HLA-B27 Tg donors via the process of chronic rejection (Fig. 1), due to high production of anti-HLA-B27 IgM- and IgG-specific antibodies (Fig. 4). In addition, the preimmunization of F344 rats with skin grafts from

Figure 7. Histopathological features of cardiac grafts harvested on POD7 from F344/HLA-B27&EGFPg rats with and without EPA treatment. The classic signs of acute rejection could be seen in the allografts of rats without EPA treatment, including strong interstitial infiltration of inflammatory cells, severe hemorrhage, edema, and necrosis (A, B). In contrast, the histopathological features of the allografts in rats that were treated with EPA were free of myocardial injury and had markedly reduced inflammatory cell infiltration (C, D). Scale bars: 100 or 200 μm .

F344/HLA-B27Tg rats resulted in robust production of anti-HLA-B27 IgM and IgG antibodies (Fig. 4) and the accelerated rejection of secondary cardiac allografts from F344/HLA-B27Tg rats (Fig. 2). These data show that F344 rats reject F344/HLA-B27Tg cardiac allografts via cellular immunity.

Green fluorescent protein (GFP) is an intracellular reporter molecule widely used to assess gene transfer and expression (3,16). Enhanced green fluorescent protein (EGFP) is a red-shifted GFP variant which fluoresces about 35 times more intensely than wild type GFP (5,17) and can be readily detected by using fluorescence microscopy, flow cytometry, or macroscopic imaging. The double transgenic F344/HLA-B27&EGFPg rats we used were the offspring of mating F344/HLA-B27Tg and F344/EGFPg rats. We found that F344 recipients rejected cardiac grafts from double transgenic F344/HLA-B27&EGFPg rats via acute rejection (Fig. 1), associated with a significant increase in the infiltration lymphocytes (Fig. 5), thus suggesting a role for cellular immune rejection. All of these data revealed that F344/HLA-B27Tg and F344/HLA-B27&EGFPg rats represent a unique and very useful animal transplantation model in which the transplant antigens are specifically presented.

Moreover, in our study, we found that EPA treatment could significantly prolong the survival of double transgenic F344/HLA-B27&EGFPg to F344 rat cardiac allografts. EPA is one of the ω -3 PUFA, which is rich in fish oil. This finding may provide the rationale for the development of novel immunomodulatory strategies in organ transplantation.

The first possible mechanism by which EPA treatment may prolong the survival of F344/HLA-B27&EGFPg allografts in F344 rats is through attenuation of the production of anti-HLA-B27 IgG antibodies and B-cell proliferation. Allo-antibodies, whose main targets are MHC molecules, can contribute to acute and chronic graft rejection (11). Some studies have shown that the removal of allo-antibodies by the depletion of B cells can improve the longevity of transplants (14). In our study, we found that EPA treatment could significantly attenuate the production of anti-HLA-B27 IgG antibodies (Fig. 8) and B-cell proliferation (Fig. 9), but it had no effect on the production of anti-HLA-B27 IgM antibodies (Fig. 8).

The second possible mechanism by which EPA treatment prolongs the survival of F344/HLA-B27&EGFPg to F344 rat cardiac allografts might be through the induction of Treg cells. Treg cells play a crucial role in

Figure 8. EPA treatment could attenuate the production of anti-HLA-B27 IgG, but not IgM, antibodies. FACS measurement of the serum IgG (A) and IgM (B) levels at different time points post cardiac transplantation between F344 recipients that received cardiac grafts from F344/HLA-B27&EGFP Tg donors with and without EPA treatment, showing that EPA treatment could significantly attenuate the production of anti-HLA-B27 IgG antibodies on POD7, 14, 21, and 28 (* $p=0.004$, ** $p=0.034$, *** $p=0.037$, **** $p=0.040$, respectively), no difference was seen in the production of anti-HLA IgM antibodies.

Figure 9. EPA treatment could significantly decrease the B-cell population as determined by FACS analysis. Splenocytes were obtained from F344/HLA-B27&EGFP Tg heart grafted F344 recipients with or without EPA treatment on POD7 and stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated anti-rat CD45RA, CD3, CD4, CD8, or CD25 mAb. EPA treatment significantly decreased the B-cell population compared with the untreated rats on POD7 (* $p=0.005$). No differences in the CD3⁺, CD4⁺, or CD8⁺ T-cell populations were seen. Furthermore, EPA treatment slightly increased the CD4⁺CD25⁺ T-cell population.

Figure 10. EPA treatment up-regulated the mRNA expression of HO-1, IL-10, IDO, TGF- β , and Foxp3 in the graft and spleens of the recipients. Cardiac grafts (A) and spleens (B) were harvested on POD7 from F344 recipients that received cardiac grafts from F344/HLA-B27&EGFP Tg donors with and without EPA and were used for measurement of mRNA expression by quantitative real-time RT-PCR. The changes in mRNA expression vs. one sample of the control group were calculated. The relative quantity is presented as the ratio of the comparative cycle threshold (Ct) of the target genes against those of the housekeeping gene 18s. The data are representative of three independent experiments and indicate the mean ratio of triplicate results for each experiment.

controlling immune responses and in inducing tolerance to allografts (8,10,13,15). Iwami et al. (9) have shown that purified EPA could induce significantly prolonged survival of fully mismatched mouse cardiac allografts and could lead to the generation of regulatory cells. In our study, we found that EPA treatment slightly increased the CD4⁺CD25⁺ T-cell population by a FACS analysis (Fig. 9) and that the mRNA expression of Foxp3 was also significantly increased both in cardiac grafts (Fig. 10A) and the spleen (Fig. 10B) by a quantitative real-time RT-PCR analysis.

Taken together, our data indicate that HLA-B27 and/or EGFP transgenic proteins are useful for establishing a unique animal transplantation model to clarify the mechanism(s) responsible for the allogeneic cellular and humoral immune responses, in which the transplant antigens are presented specifically. Furthermore, regardless of which mechanism(s) proves to be most important, we have also provided evidence that EPA was effective for

the treatment of rat cardiac allograft rejection and may allow the development of novel immunomodulatory strategies for organ transplantation.

ACKNOWLEDGMENTS: The authors acknowledge Dr. Hiromitsu Kimura for his scientific comments and discussion. This study was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grants-in-aid 20390349, 21659310, 2109739). The authors declare no conflict of interest.

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