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貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：**研究報告書**

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2．研究テーマ

既存抗体陽性移植の克服および臓器移植後新規抗体産生機序の解明とその制御に関する研究

3．成果の概要

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. 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. 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.

4．研究業績

（1）学会における発表　　無・㊒（学会名・演題）

Z. Liu, K. Kato, N. Hatayama, L. Xie, Y. Nagahara, X-K.Li. Eicosapentaenoic acid attenuate allograft rejection in HLA-B27/EGFPtransgenic rat cardiac transplantation model. 第37回日本臓器保存生物医学会. 新潟. 2010.11.19-20.

（2）発表した論文　　㊚・有（雑誌名・題名）

—日中医学協会助成事業—

臓器移植後抗体産生機序の解明に関する研究

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Abstract

The development of an animal model bearing definite antigens is important to facilitate the evaluation and modulation of specific alloantigen 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. 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.

Key words：Allograft, GFP, HLA-B27, regulatory T-cell, tolerance, transgenic rat

Introduction

The development of an animal model bearing definite antigens is important to facilitate the evaluation and modulation of the specific alloantigen response after transplantation. In the present study, we first used HLA-B27Tg rats in a transplantation study, and found that F344 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&EGFPTg 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.

Materials & 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[1]. 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.

Heterotopic cardiac transplantation

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 [2, 3]. In brief, after thoracotomy under inhalation anesthesia, the donor heart was harvested. The heart graft was rapidly cooled and flushed with 5 ml 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. 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.

Lymphocyte proliferation assays

The mixed lymphocyte reaction (MLR) was performed with F344 rat nylon-wool column (Wako) enriched T cells (1x105/well) as responders, and 20-Gy irradiated F344, F344/EGFP, F344/HLA-B27 and F344/HLA-B27&EGFPTg rat splenocytes (1x105/well) as stimulators, incubated in a flat-bottom 96-well white plate (Costar; Corning, NY) at a final volume of 200 μl/well of the GIT medium containing 50μM 2-mercaptoethanol (Wako) in a humidified atmosphere at 37˚C for five days. The proliferation of T cells was measured with cell-proliferation ELISA kits (Roche Diagnostics Gmbh, Penzberg, Germany).

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 1hr. Cells were washed and incubated with goat anti-rat IgM or IgM antibodies (SouthernBiotech, Birmingham, AL), 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 (1μm-thick) and stained with hematoxylin and eosin. A light microscopic analysis was performed to assess the overall cellularity and myocardial damage.

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. P values <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/EGFPTg donors (n=8, MST>100days), 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<.001; Fig1)

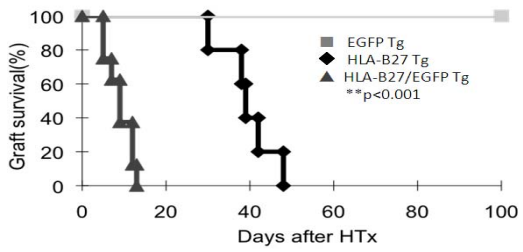

Figure 1. F344 recipients rejected cardiac allografts from F344/HLA-B27Tg and F344/HLA-B27&EGFPTg donors. Heterotopic cardiac transplantation was performed, in which F344 rats were recipients and sex-matched F344/EGFPTg, F344/HLA-B27Tg or F344/HLA-B27&EGFPTg rats were donors. MST= median survival time. The results are shown as the MST ± SD. **p < .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 alloantigen 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=.014 and .012; Fig. 2).

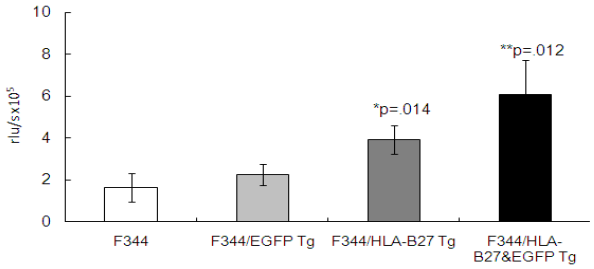

Figure 2. The alloantigen response was significantly enhanced by HLA-B27 and HLA-B27&EGFP, but not by the EGFP transgene. MLR was performed with F344 rat nylon-wool column enriched T cells as responders, and irradiated F344/EGFP, F344/HLA-B27 or F344/HLA-B27&EGFPTg rat splenocytes as stimulators. The results are shown as the means ± SD. *p=.014, **p=.012, the difference between F344 and F344/EGFP, F344/HLA-B27, and F344/HLA-B27&EGFPTg, respectively.

Vigorous production of anti-HLA-B27 IgG and IgM antibodies following allogenic sensitization

Because allo-antibodies, whose main targets are MHC molecules, can also contribute to acute and chronic graft rejection, 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 Fig. 3A, the total IgG titers steadily increased on postoperative day (POD) 7 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 post-transplantation 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 post-transplantation (Fig. 3B).

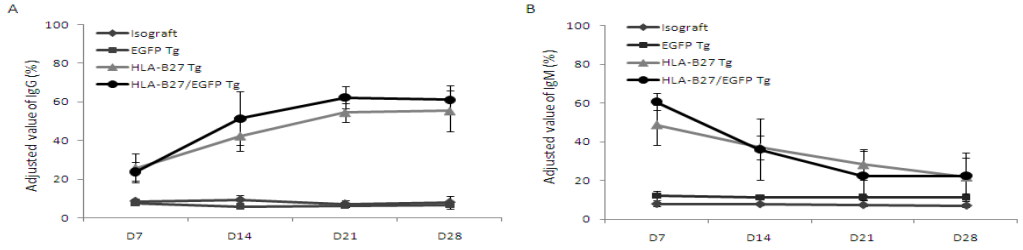

Figure 3. There is vigorous production of specific anti-HLA-B27 IgG and IgM antibodies following allogenic sensitization. FACS measurement of serum IgG (A) and IgM (B) at different time points post cardiac transplantation, showing that the total serum IgG increased steadily while the IgM decreased gradually in F344 recipients transplanted with cardiac grafts from F344/HLA-B27 or F344/HLA-B27&EGFPTg donors. 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.

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 cells 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. 4).

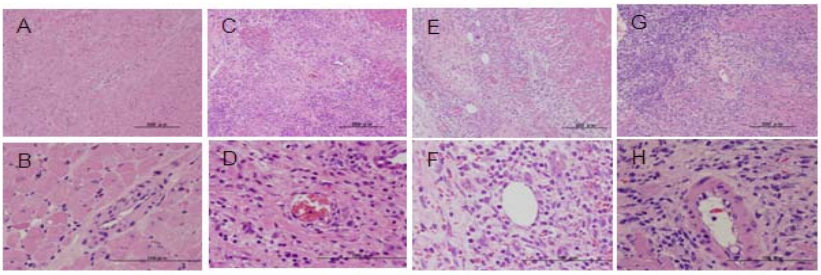

Figure 4. Histopathological features of cardiac allografts between F344/EGFP, F344/HLA-B27, and F344/HLA-B27&EGFPTg rats and skin-primed F344/HLA-B27Tg rats. The F344/EGFPTg cardiac grafts were free of myocardial injury and had markedly reduced inflammatory cell infiltration even on POD100 (A, B), while 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&EGFPTg (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 represent 100, 200mm.

Discussion

We herein described a unique and useful animal transplantation model in which grafts from F344/HLA-B27Tg rats mainly provoke a humoral immune response and grafts from F344/HLA-B27&EGFPTg 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, 5]. The removal of alloantibodies by IVIg or plasmapheresis, or by the depletion of B cells, can improve the longevity of transplants [6]. We found that F344 recipients rejected cardiac grafts from F344/HLA-B27Tg donors via the process of chronic rejection (Fig. 1), due to high production of anti-HLA-B27 IgM- and IgG-specific antibodies (Fig. 3). These data showed 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 [7, 8]. Enhanced green fluorescent protein (EGFP) is a red-shifted GFP variant which fluoresces about 35 times more intensely than wild-type GFP [9, 10], and can be readily detected by using fluorescence microscopy, flow cytometry, or

macroscopic imaging. The double transgenic F344/HLA-B27&EGFPTg rats we used were the offspring of mating F344/HLA-B27Tg and F344/EGFPTg rats. We found that F344 recipients rejected cardiac grafts from double transgenic F344/HLA-B27&EGFPTg rats via acute rejection (Fig. 1), associated with a significant increase in the infiltration lymphocytes (Fig. 4), thus suggesting a role for cellular immune rejection. All of these data revealed that F344/HLA-B27Tg and F344HLA-B27&EGFPTg rats represent a unique and very useful animal transplantation model in which the transplant antigens are specifically presented.

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