ORIGINAL ARTICLE

Treadmill exercise induces murine cardiac allograft survival and generates regulatory T cell

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Summary

Exercise therapy has been associated with improvement in functional capacity and quality of life. The role of exercise therapy in heart transplant recipients is of great interest for the transplant society, although concerning the effect of exercise therapy, there is little knowledge at present. We analyzed the effects of exercise on alloimmune responses in murine cardiac allograft transplantation. CBA mice ($H2^k$) underwent transplantation of C57Bl/6 ($H2^b$) hearts and exercised on a treadmill. Untreated CBA recipients rejected C57Bl/6 cardiac grafts acutely (median survival time [MST], 7 days). CBA recipients treated with treadmill for 1 week after transplantation, and for 1 week both before and after transplantation prolonged allograft survivals (MSTs, 35 and 18 days, respectively). However, treadmill exercise recipients for 1 week before transplantation were not effective to allograft survival (MST, 8 days). Adoptive transfer of whole splenocytes and $CD4^+$ cells from treadmill exercise recipients significantly prolonged allograft survival in naive secondary recipients (MSTs, 30 and 52 days, respectively), suggesting that regulatory cells was generated after treadmill exercise. Moreover, flow cytometry studies showed that $CD4^+CD25^+Foxp3^+$ cell population increased in treadmill exercise recipients. Therefore, postoperative but not pre-operative exercise could induce prolongation of survival of fully allogeneic cardiac allografts and generate $CD4^+CD25^+Foxp3^+$ regulatory T cells.

Introduction

Activity is the best medicine. Over the past several decades, numerous epidemiological studies have shown that exercise has beneficial effects on human health such as a low risk of dementia [1], protection against metabolic disorders [2], and also quality of life [3]. In particular, moderate exercise has been reported to provoke various efficiencies to human health. In animal studies, many reports have demonstrated that exercise had the therapeutic potential to protection against virus infection [4], regression of tumor growth [5], and upregulation of humoral immune response [6]. *In vitro*, moderate exercise also had the effect of immunomodulation by an increase in $CD8^+$ cells [7] and suppressed natural killer (NK) cell cytotoxicity [4]. Besides,

voluntary moderate exercise with vaccination enhanced T cell responses [8]. On the other hand, stressful or intense exercise resulted in temporary immune depression [9], and possibility of immunosuppression because of exhaustive acute exercise was pointed out [7]. Therefore, effects of exercise on immune response seem to vary with the duration and intensity of the exercise regimen.

Transplantation of cells, tissues, and vascularized solid organs is a successful therapeutic intervention for many end-stage chronic diseases. Especially, heart transplantation is the gold standard of the crucial care for end-stage cardiac diseases such as heart failure and dilated cardiomyopathy for the past 30 years [10]. In some current study, heart transplantation continues to provide patients with end-stage cardiac diseases with approximately 9.3 years [11].

However, cardiac transplant patients keep to be at a risk for acute and chronic rejection in spite of improvement of immunosuppression protocol. Therefore, to induce the significant prolongation of allograft survival, facilitate graft tolerance, and avoid toxic immunosuppressive drugs, many studies such as targeting cosignaling [12] are currently in progress. However, the number of studies that deal with the relationship between heart transplantation and postoperative exercise is comparatively low.

Exercise immunology, correlation between physical exercise and immunology, has been investigated from various perspectives and by various measurements. However, little immunological research including cellular mechanisms has been done on whether exercise is effective to the immune system after solid organ transplantation. This study investigated the effects of exercise with treadmill on alloimmune response in a murine model of cardiac allograft transplantation.

Materials and methods

Mice

Male C57Bl/6 ($H2^b$ [B6]) and CBA ($H2^k$) mice that were 8–12 weeks of age were purchased from Sankyo Ltd (Tokyo, Japan), housed in conventional facilities at the Biomedical Services Unit of Teikyo University, were maintained on a 12:12 h light:dark cycle with free access to food and water, and used in accordance with the guidelines for animal experimentation approved by the Animal Use and Care Committee of the university and the "Principles of laboratory animal care" (NIH publication, vol 25, no. 28, revised 1996).

Heart transplantation

Heart transplantation was performed as described previously [13]. Postoperatively, cardiac graft function was assessed daily by palpating the heart for evidence of contraction. Rejection was defined as complete cessation of the heartbeat and confirmed by direct visualization and histologic examination of the graft.

Treadmill exercise

CBA recipients were randomly assigned to one of six groups, which were a group of treadmill exercise on 1 post-operative day (POD), 1–3 POD and for 1 week before and/ or after the day of transplantation or a sedentary group (untreated group). In addition to above six groups, a group in which B6 donors were exposed to 1-week pretransplant treatment with treadmill exercise was added. Naïve CBA, B6, and CBA recipients of cardiac allografts exercised on a treadmill (Fig. 1a, Treadmill System; Melquest, Toyama, Japan). The exercise protocol was as fellows: The speed on 1 POD and 2–7 POD was 9.6 and 12.8 m/min, respectively. Besides, treadmill exercise continued for 1 h per day, and treadmill had angle of gradient 5°. All mice ran without electric stimulation. In case that the mice were caught into the treadmill machine, we prepared slightly electric stimulation behind the mice. All experiments were conducted in an environment in which a cycle of 12 h of light and 12 h of darkness and a room temperature of 24 °C were maintained.
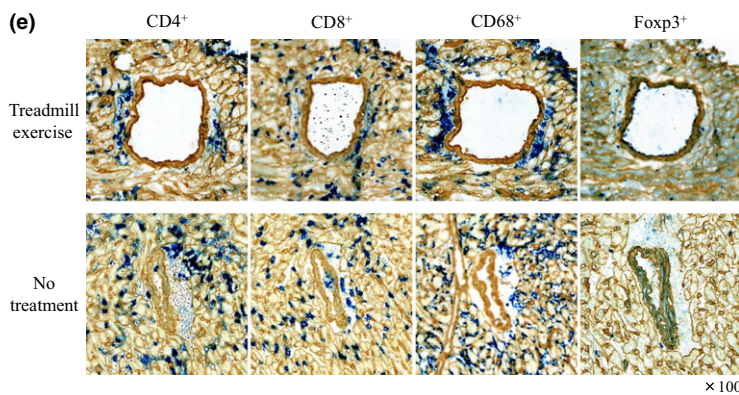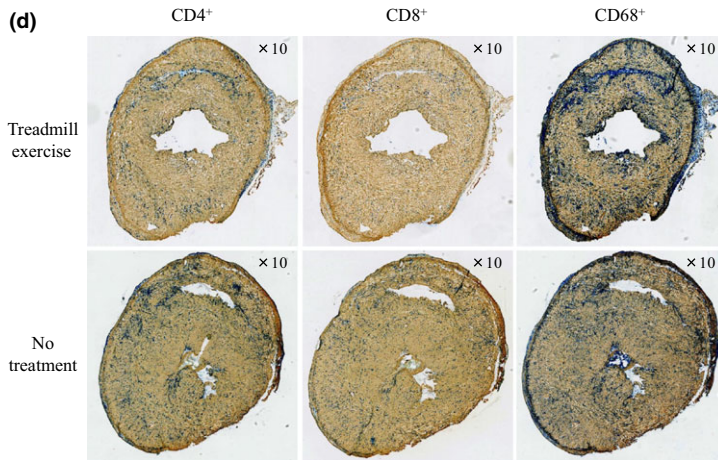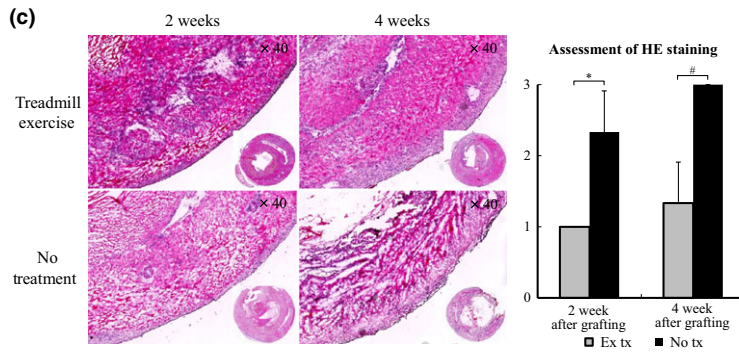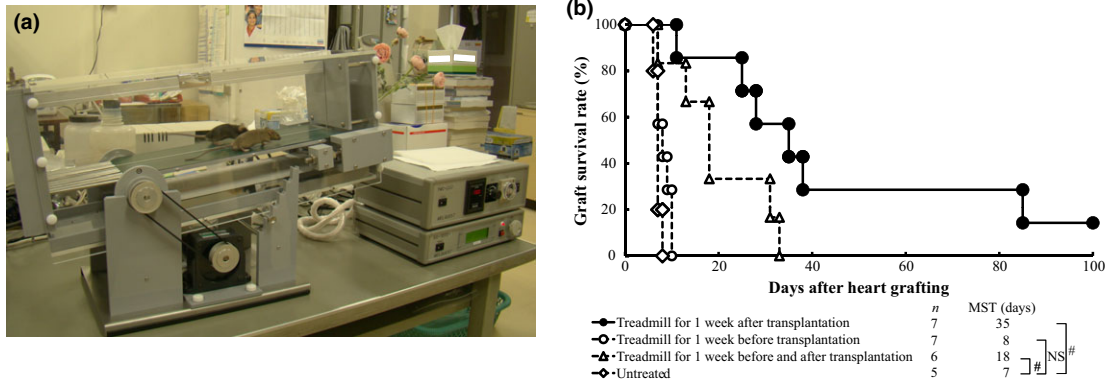
Adoptive transfer studies

Adoptive transfer studies were conducted to determine whether regulatory cells were generated in mice exercised on a treadmill. Thus, 30 days after transplantation of B6 hearts into primary CBA recipients exercised on a treadmill for 1 week after grafting, splenocytes (5.0×10^7) from primary recipients with functioning allografts were adoptively transferred into naïve secondary CBA recipients by means of intravenous injection into the penile vein. Immediately afterward, the secondary recipients underwent transplantation of a B6 heart. In some experiments, $CD4^+$ cells were purified from the spleens of primary transplant recipients by positive selection using a magnetically activated cell sorter (MACS) and CD4 microbeads (Miltenyi Biotec, Auburn, CA; purity >98%), and $CD4^+$ cells (2.0×10^7) were adoptively transferred into naïve secondary recipients, which then immediately underwent transplantation of a B6 heart (Fig. 2a).

Histologic and immunohistochemical studies of harvested cardiac grafts

Cardiac grafts transplanted into untreated mice and mice exercised on a treadmill for 1 week after grafting were removed 28 days after transplantation, and also cardiac

Figure 1 Treadmill, median survival times, and histological study. (a) All CBA recipients of cardiac allografts exercised on a treadmill system, (b) cardiac graft survival in CBA recipients of a B6 heart that were treated on a treadmill for 1 week after grafting, 1 week before grafting, and 1 week before and after grafting and untreated allograft recipients. (c) Histologic examination (hematoxylin–eosin (HE) staining) of cardiac allografts obtained 2 and 4 weeks after grafting from postoperative 1-week treadmill-exercised recipients and untreated recipients, and assessment of HE staining. (d, e) Results of double immunostaining of cardiac allografts obtained 4 weeks after transplantation from postoperative 1-week treadmill-exercised mice and untreated mice. Fresh 4-μm-thick graft cryosections were incubated with anti-CD4, CD8, and CD68 monoclonal and anti-Foxp3 polyclonal antibody (magnification ×10 or ×100). *MST* median survival time. *Ex Tx* exercise treatment. *No Tx* no treatment. *P < 0.05 and #P < 0.01 for difference between two groups.

(a)

(b)

	n	MST (days)
Treadmill for 1 week after transplantation	7	35
Treadmill for 1 week before transplantation	7	8
Treadmill for 1 week before and after transplantation	6	18
Untreated	5	7

(c) Assessment of HE staining

(d) CD4⁺ CD8⁺ CD68⁺
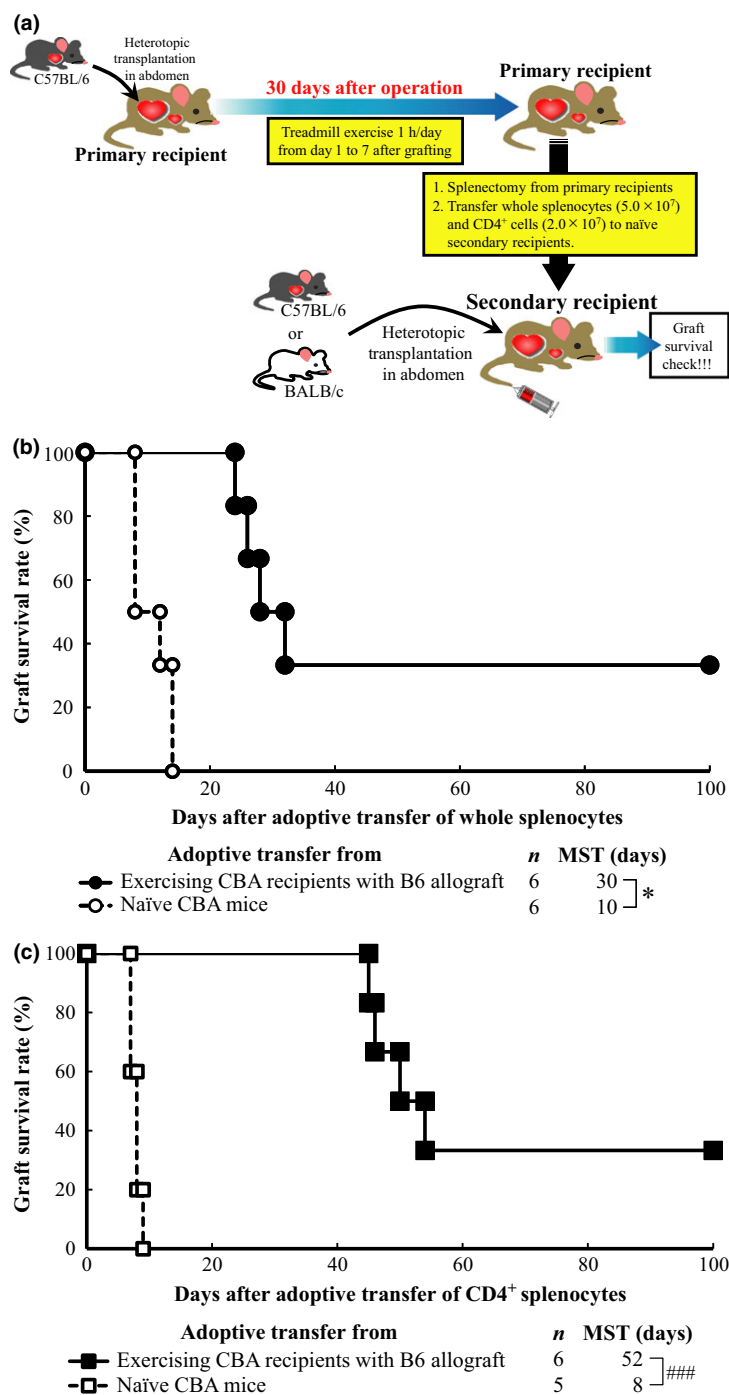
(e) CD4⁺ CD8⁺ CD68⁺ Foxp3⁺

Figure 2 Evidence of generation of regulatory cells in treadmill-exercised CBA allograft recipients. (a) Scheme on adoptive transfer study to confirm the generation of regulatory T cells. (b, c) Cardiac allograft survival after adoptive transfer of whole splenocytes (b) or CD4+ cells (c). (d–h) Results of double immunostaining of cardiac allografts obtained 4 weeks after transplantation from untreated mice and postoperative 1-week treadmill-exercised mice (d–g) and 100 days after adoptive transfer of CD4+ cell from longtime surviving secondary CBA recipients with B6 beating heart (h). Fresh 4-μm-thick graft cryosections were incubated with anti-CD4, CD8, and CD68 monoclonal antibody or anti-Foxp3 polyclonal antibody. In (d–g), the left-hand panels show samples obtained from mice exercising on a treadmill, and the right-hand panels show samples from untreated mice (magnification ×40). In (h), all panels show samples obtained from longtime surviving transplant recipients in CD4+ cell adoptive transfer groups (magnification ×100) (i) CD4, CD25, and Foxp3 expression in splenocytes as determined by flow cytometry 1, 2, and 4 weeks after transplantation. The right-hand graph shows the percentage of CD4+CD25+Foxp3+ cells in the CD4+ cells as determined by flow cytometry. Data are mean ± SD values (*n* = 5 mice in each group). *MST* median survival time. *P < 0.05 and ###*P* < 0.001 for difference between two groups. *NS* not significant.
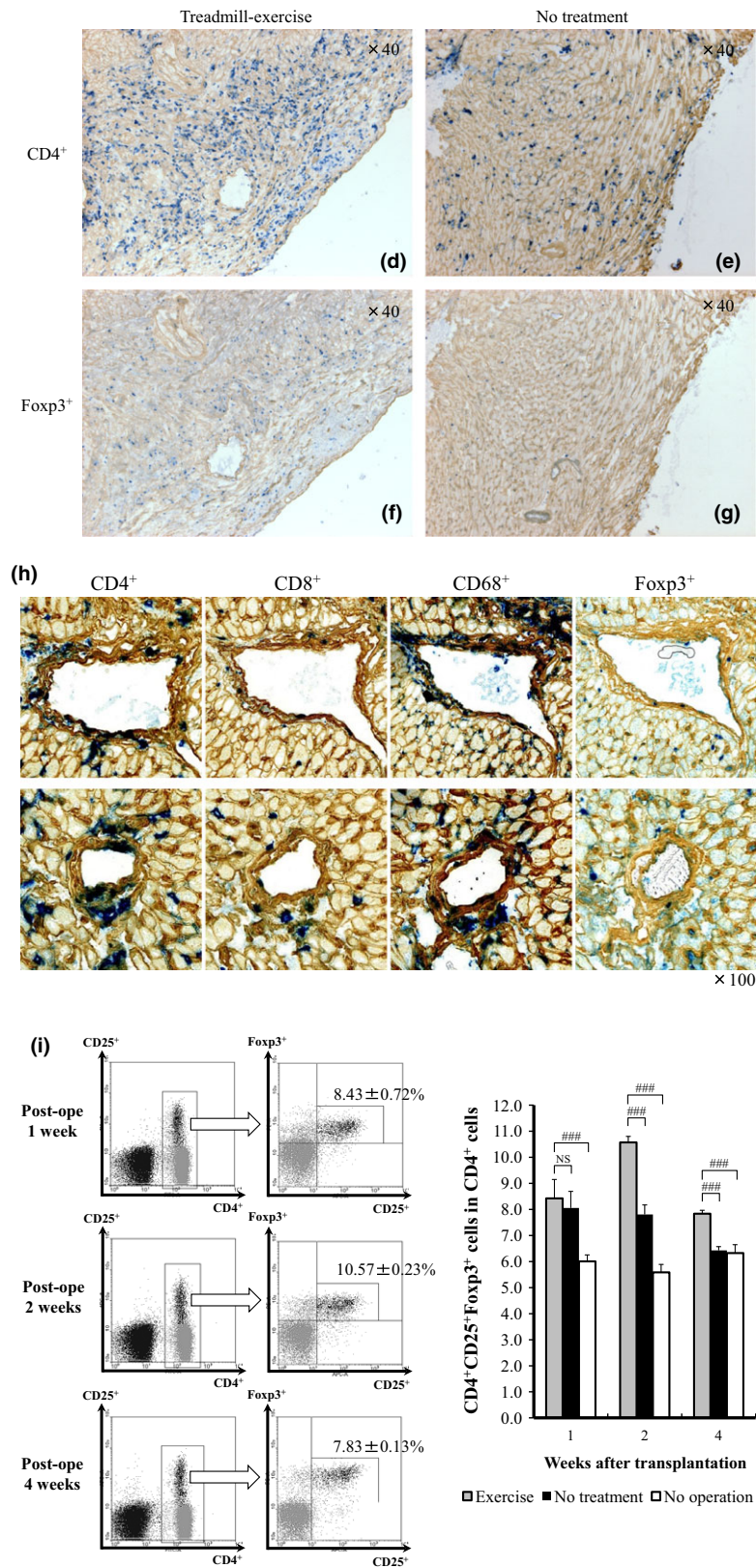
Figure 2 continued

grafts from secondary CBA recipients on 100 days after adoptive transfer of CD4$^+$ cells were removed to investigate vascular rejection. All grafts were studied immunohistochemically with use of double immunostaining. Fresh 4-μm-thick graft cryosections were incubated with anti-CD4 (RM4-5; BD Biosciences, San Jose, CA), anti-CD8 (53-6.7; BD Biosciences), and anti-CD68 (ab53444, abcam, Tokyo, Japan) monoclonal antibody (mAb) or anti-Foxp3 (kindly provided by Professor Kenjiro Matsuno, Dokkyo Medical University, Tochigi, Japan) polyclonal antibody, incubated with alkaline phosphatase (ALP)-conjugated anti-rat Ig (712-055-153; Jackson ImmunoResearch Laboratories, West Grove, PA) for anti-CD4, CD8, and CD68 and with ALP-conjugated anti-rabbit Ig (712-055-152; Jackson ImmunoResearch Laboratories) for anti-Foxp3, and developed blue with Vector Blue (Vector Laboratories, Burlingame, CA). Cryosections were then incubated with rabbit anti-mouse type IV collagen polyclonal antibody (LB1403; Cosmo Bio, Tokyo) and peroxidase-conjugated anti-rabbit Ig (55693; Mitsubishi Chemical, Tokyo) and then developed brown with diaminobenzidine (Vector Laboratories). The assessment of the infiltrate on immunohistochemical (IHC) study was based subjectively.

Cardiac allografts in untreated mice and mice exercised on a treadmill for 1 week after grafting were removed 14 and 28 days after transplantation and studied histologically. Frozen sections (4-μm thick) were cut, mounted on silane-coated slides, and stained with hematoxylin–eosin (HE). HE staining was assessed by grading with a semiquantitative scale for the amount of mononuclear cell infiltration (0, no infiltration; 1, faint and limited infiltration; 2, moderate infiltration; 3, severe infiltration) [14,15]. All graft heart slides were assessed blindly by unrelated three researchers.

Flow cytometry analysis of CD4, CD25, and Foxp3 expression

Splenocytes were obtained from naïve CBA mice and from postoperative 1-week treadmill-exercised and untreated cardiac allograft recipients 1, 2, and 4 weeks after transplantation. The cells were stained with fluorochrome-conjugated anti-CD4 or anti-CD25 mAb (RM4-5 and PC61, respectively; BD Biosciences) or anti-mouse Foxp3 mAb (FJK-16s; eBioscience, San Diego, CA), as well as their isotype controls (eBioscience). The stained cells were analyzed using a FACS Canto2 system (BD Biosciences). The percentage of CD4$^+$CD25$^+$Foxp3$^+$ in CD4$^+$ cells was determined.

Mixed leukocyte cultures

In other mixed leukocyte culture (MLC) studies [16], the responder cells were splenocytes from naïve CBA mice or

from untreated or postoperative 1-week treadmill-exercised CBA mice that had undergone transplantation of a B6 heart 14 days earlier. The stimulator cells were B6 (allogeneic) or CBA (syngeneic) splenocytes treated with 100 μg/ml mitomycin C (MMC; Kyowa Hakko, Osaka, Japan) for 30 min at 37 °C. The responder cells (2.5×10^6/ml) were cocultured with the stimulator cells (5×10^6/ml) in complete medium in a humidified 5% CO_2 atmosphere (CH-16M; Hitachi, Tokyo) at 37 °C in 96-well, flat-bottomed tissue culture plates (Iwaki Scitech Division, Tokyo) for 4 days. Maximum proliferation of naïve CBA splenocytes (responder cells) against B6 splenocytes (stimulator cells) treated with MMC occurred on the fourth day of MLCs. Proliferation was assessed using an enzyme-linked immunosorbent assay (ELISA) for BrdU incorporation (Biotrak, version 2; Amersham, Little Chalfont, UK) according to the manufacturer's instructions [17].

Cytokine assays

ELISAs were also performed to assess levels of interleukin (IL)-2, IL-4, IL-10, and interferon (IFN)-γ in the supernatant of the MLCs on day 4 and in the serum of CBA recipients with/without postoperateive 1-week treadmill exercise on day 14. The capture mAb (JES5-2A5), detection mAb (JES5-16E3), and recombinant standard for IL-10 were from BD Biosciences. The capture and detection mAbs for IL-2 (JES6-1A12 and JES6-5H4, respectively), IL-4 (BVD-1D11 and BVD-24G2), and IFN-γ (R4-6A2 and XMG1.2) were from Caltag Laboratories (Burlingame, CA). Recombinant standards for IL-2, IL-4, and IFN-γ were from PeproTech (London, UK).

Statistical analysis

Cardiac allograft survival in two experimental groups was compared using Mann–Whitney testing. In the cell proliferation, cytokine, and flow cytometry studies, the difference between two groups was assessed using unpaired Student *t*-tests. A *P*-value <0.05 was considered significant.

Results

Survival of fully mismatched cardiac allografts

CBA recipients of B6 cardiac allografts that were given either no treatment (*n* = 5) or treadmill exercise for 1 week before transplantation (*n* = 7) rejected their grafts acutely [median survival times (MSTs), 7 and 8 days; standard errors (SEs), 0.32 and 0.52, respectively; Fig. 1b]. CBA recipients exercising on 1 POD and 1–3 POD also had no significant prolongation of their grafts (MSTs, 9 and 8 days; SEs, 5.74 and 1.46, respectively). Additionally, CBA recipients rejected cardiac grafts from B6 exposed to 1-week

pretransplant treadmill exercise (MST, 9 days; SE, 0.33). In contrast, CBA allograft recipients exercised on a treadmill for 1 week ($n = 7$) after and for 1 week before and after the day of transplantation ($n = 6$) had significantly prolonged survival of their B6 grafts (MSTs, 35 and 18 days; SEs, 12.54 and 4.15, respectively; $P < 0.01$ and $P < 0.01$ compared with the no treatment group; Fig. 1b). These results indicate that postoperative exercise on a treadmill may induce hyporesponsiveness to cardiac allografts.

Histologic features of cardiac grafts

Histologic examinations of cardiac allografts obtained 2 and 4 weeks after transplantation showed preserved graft structure in postoperative 1-week treadmill-exercised recipients, whereas allografts from untreated recipients showed myocyte damage, edema, and more aggressive inflammatory infiltrate in a process of acute rejection (Fig. 1c). Moreover, in each section of HE staining, there was a significant difference by grading with a semi-quantitative scale ($P < 0.05$ and $P < 0.01$ compared with no treatment group; Fig. 1c).

IHC study showed that there was no significant difference on the degree of macrophage infiltration in both groups on 4 weeks after grafting; however, the degree of CD4$^+$ and CD8$^+$ cell infiltration in postoperative 1-week treadmill-treated mice was little lower than that in no treatment mice (Fig. 1d). Additionally, small arteries in the myocardium of both groups had no significant differences on the intimal thickening, multilayering of this elastic fibers, and obfuscation of the internal elastic lamia. However, perivascular infiltration of Foxp3$^+$ cells in the postoperative 1-week treadmill group seems to be a little stronger than that in no treatment group (Fig. 1e). On 100 days after adoptive transfer of CD4$^+$ cells, longtime surviving transplant CBA recipients had the preserved vascular structure in their myocardium (Fig. 2h).

Generation of regulatory T cells

We previously found that some anti-inflammatory or immunomodulatory agents induce hyporesponsiveness to fully allogeneic grafts by means of generation of regulatory cells [18–20]. In the current investigation, adoptive transfer studies were conducted to determine whether generation of regulatory cells was involved in the induction of hyporesponsiveness in the postoperative treadmill-exercised mice. We found that naïve secondary CBA allograft recipients given adoptive transfer of splenocytes ($n = 6$) or CD4$^+$ cells ($n = 6$) from postoperative 1-week treadmill-exercised primary CBA recipients 30 days after heart transplantation had significantly prolonged survival of B6 hearts (MSTs, 30 and 52 days; SEs, 15.32 and 10.88, respectively; $P < 0.05$

and $P < 0.001$ compared with transfer of splenocytes or CD4$^+$ cells from naïve CBA mice, respectively; Fig. 2b and c). In contrast, naïve secondary CBA recipients given adoptive transfer of splenocytes ($n = 6$) or CD4$^+$ cells ($n = 5$) from naïve CBA mice rejected B6 hearts acutely (MSTs, 10 and 8 days; SEs, 1.23 and 0.37, respectively). These data indicate that exercise on a treadmill generated regulatory cells in the primary allograft recipients and that one of the regulatory populations consisted of CD4$^+$ cells.

IHC studies showed that cardiac allografts from untreated transplant recipients had severe myocardial damage and aggressive infiltration of CD4$^+$ cells, whereas allografts from postoperative 1-week treadmill-exercised recipients had only slight myocardial damage and also more CD4$^+$Foxp3$^+$ cell infiltration (Fig. 2d–g), indicating that treadmill exercise may induce more CD4$^+$Foxp3$^+$ cells, not CD4$^+$ effector cells. Besides, flow cytometry studies showed that the population of CD4$^+$CD25$^+$Foxp3$^+$ cells was increased in the spleens of postoperative 1-week treadmill-exercised recipients after 2 and 4 weeks, compared with those of untreated and naïve CBA mice (Fig. 2i). These data suggest that the CD4$^+$ regulatory cells contained a population that was CD4$^+$CD25$^+$Foxp3$^+$.

Cell proliferation and cytokine production

Proliferation of splenocytes from CBA transplant recipients exercised on a treadmill for 1 week after grafting was markedly suppressed compared with that of splenocytes from untreated recipients or naïve CBA mice (Fig. 3a).

Levels of IL-4 (Fig. 3b) and IL-10 (Fig. 3c) in splenocytes from CBA mice exercised on a treadmill for 1 week after grafting were significantly higher than those in splenocytes from untreated or naïve syngeneic mice ($P < 0.05$ compared with untreated CBA mice). On the other hand, levels of IL-2 (Fig. 3d) and IFN-γ (Fig. 3e) were considerably decreased in postoperative 1-week treadmill-exercised recipients compared with untreated recipients ($P < 0.005$ compared with untreated CBA mice). Additionally, level of IL-10 (Fig. 3f) in the serum of CBA recipients with postoperative 1-week treadmill exercise was significantly higher than that of CBA recipients without treadmill exercise ($P < 0.001$ compared with untreated CBA mice). The other cytokines had no significant differences (data not shown).

Discussion

Sports and exercise researches in the form of various animal- and human-associated studies have significantly contributed to our appreciation of physiology and immunology, and there seems to be promising potentials for newer discovery. In the 1990s, however, the effects of exercise were limited, skeptical, and controversial except
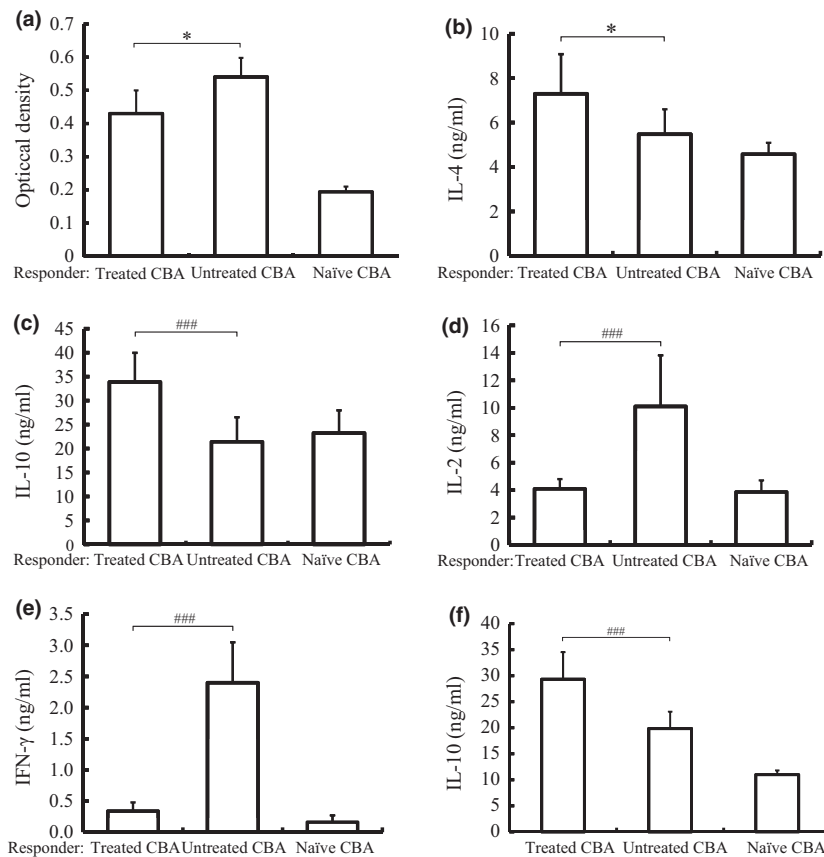
Figure 3 Evidence of induction of alloproliferative hyporesponsiveness by treadmill exercise. (a) Results of cell proliferation assays in mixed leukocyte cultures (MLCs). (b–e) MLC studies of cytokines. Levels of interleukin (IL)-4 (b), IL-10 (c), IL-2 (d), and interferon-γ (e) in MLCs were assessed by enzyme-linked immunosorbent assays (ELISA). (f) Serum concentration of IL-10. Levels of IL-10 in the serum of CBA recipients with/without postoperative 1-week treadmill exercise and naïve CBA mice were assessed by ELISA. The data shown are mean ± SD values obtained in one representative experiment because similar results were achieved in three independent experiments. *$P < 0.05$ and ###$P < 0.001$ for difference between two groups.

for the field of immunology [21,22]. After that, an increasing number of scientific studies using various animal models to assess the physiologic effects of exercise have been conducted, mainly in the field of oncology [23–25], cardiology [3,26,27], and also immunology [4–7,28–30]. For example, data in animal-tumor model demonstrated that physical exercise attenuated the development of solid leukemia tumor [24] and delayed allogeneic tumor growth with reduction of intratumoral inflammation and vascularization [25]. On cardiology, exercise training is associated with improvement in quality of life [3], and Tabet *et al.* [27] reported that there was a strong correlation between unrecovered exercised capacity after exercise program and adverse cardiac events in patients with chronic heart failure. On immunology, moderate exercise showed the modulation of immune system such as increasing cell proliferation [7], reduction of NK cell cytotoxicity [4], and augmentation of macrophage tumoricidal function [5]. In particular, Yeh *et al.* [31,32] have reported that one specific exercise, Tai chi chuan exercise, improve impaired glucose tolerance

and increase CD4$^+$CD25$^+$ regulatory T cell in type 2 diabetic patients. The reports suggested that exercise stimulation with any methods such as Tai chi chuan or on a treadmill may affect the immune response and generate regulatory T cells as a feature of the response. However, the possible effect of exercise on the immune response in murine heart transplantation has not previously been explored. In our current study, exercise on a treadmill for 1 week after grafting apparently induced generation of CD4$^+$ regulatory T cell with suppressive function in allograft recipients and changed the balance of helper T cell (Th)-1 and Th-2 cytokines, resulting in the significant prolongation of allograft survival, while CBA mice with no treadmill exercise rejected acutely. On the basis of these findings, we hypothesized that the correlation between exercise stimulation and autoimmune response included the generation of regulatory T cells.

There are several possible mechanisms by which treadmill exercise might have induced increased allograft survival in our model. One is that the exercise resulted in

generation of regulatory T cells. Acquisition of hyporesponsiveness to an allograft is a dynamic, multistep process involving many mechanisms, including immune regulation, deletion, anergy, and ignorance [33]. Among these, immune regulation—control of alloimmune responses by regulatory cells—is considered one of the most important. Active suppression by regulatory T cells is involved in the induction and maintenance of self-tolerance [34] and unresponsiveness to allografts [35]. In our adoptive transfer studies, most naïve secondary CBA transplant recipients given splenocytes from treated primary CBA recipients with functioning B6 cardiac allografts had significantly prolonged survival of their allografts (MST, 30 days). Furthermore, adoptive transfer of CD4$^+$ cells from treated primary transplant recipients resulted in longer or indefinite prolongation of allograft survival in secondary recipients (MSTs, >50 days), and the longtime surviving transplant CBA recipients after adoptive transfer of CD4$^+$ cells had the preserved vascular structure in their myocardium. These data suggest that exercise on a treadmill generated CD4$^+$ regulatory T cells in the primary recipients and that the regulatory population contained CD4$^+$ cells. In addition, flow cytometry and IHC studies showed that the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells in CD4$^+$ cells and the number of CD4$^+$Foxp3$^+$ cells were increased in the primary allograft recipients, respectively.

A second mechanism for exercise-induced hyporesponsiveness is to change the balance of Th-1 cytokines (IL-2 and IFN-gamma) and Th-2 cytokines (IL-4 and IL-10). In particular, our ELISA finding of upregulation of IL-10 production by splenocytes and higher level of serum IL-10 concentration in exercise-treated allograft recipients suggests that IL-10 contributed to the generation of regulatory cells. IL-10 has anti-inflammatory and suppressive effects on most hematopoietic cells and plays a crucial role not only in the function of regulatory cells but also in their generation [36]. We previously demonstrated the requirement of IL-10 in generating regulatory cells in our murine cardiac transplantation model [12,37,38]. Thus, it is probable that in the current study, it was through the upregulation of IL-10 that treadmill exercise resulted in induction of CD4$^+$CD25$^+$ regulatory T cells. Also, an anti-inflammatory effect may be induced through the regulatory cells. Our histologic studies of allografts obtained from postoperative 1-week treadmill-exercising mice on 2 and 4 weeks after grafting showed only minimal leukocyte infiltration and had significant difference between two groups. In addition, our IHC studies showed lower infiltration of CD4$^+$ and CD8$^+$ cells in their myocardium and stronger accumulation of Foxp3$^+$ cells around coronary arteries in cardiac graft from postoperative 1-week treadmill-treated mice. In the light of these findings, it appears possible that exercise-induced regulatory T cells and subsequent upregulation of

IL-10 may inhibit immune responses against cardiac allografts.

A third possible mechanism for the exercise-induced hyporesponsiveness is the effects on temporary immunosuppression induced by the treadmill exercise itself. Recently, You *et al.* [39] have showed anti-inflammatory effects of exercise training to patients with obesity. Also, the data on correlation between the intensity of exercise and immune responses were reported in murine model, which demonstrated that exhaustive exercise training itself had a potential of immunosuppression such as reduction of IFN-α and tumor necrosis factor (TNF)-α [9,40] and attenuation of NK cell cytotoxicity [4], although it might be temporary. As shown above, most reports demonstrated that intense exercise suppressed the immune responses; on the other hand, moderate exercise modulated them. However, little is known about the definition of the intensity of exercise in various models, and boundary of moderate and exhaustive has not been well determined. According to current reviews [7,41], physical activity is classified as sedentary, moderate, or vigorous and its effects depend on the total amount of time spent in each type. Exercise can also be classified "acute," which means a single bout of high-intensity exercise, or as "chronic," which means regular training or a moderate-intensity exercise. In this study, our exercise protocol in which the running speed is 9.6–12.8 m/min seems to be more moderate, compared with the previous protocol for exhaustive exercise in which the running speed is 9–17 m/min and continues to be raised until exhaustion [9]. Indeed, CBA transplant recipient exposed to treadmill exercise on 1 POD and 1–3 POD did not prolong the graft. Therefore, these findings suggest that the amount of treadmill exercise for 1 week after grafting can be at the minimum required to prolong the graft survival and the possibility of exercise-induced immunosuppression was indicated.

The context of exercise stimulation may have an important role on induction of prolonged allograft survival in our transplant model. In our protocol, CBA mice were assigned to three groups as pre-operative exercise, postoperative exercise and both exercise group for 1 week, and two titration groups. The MSTs showed that prolongation of cardiac allograft survival needed postexercise stimulation for 1 week, not the pre-operative exercise. Additionally, the load of the same extent as pre-operative exercise may attenuate the effect of postoperative exercise. Also, FACS study showed an intriguing results that the rate of CD25$^+$Foxp3$^+$ cells in CD4$^+$ cells from day 1, 2 and 4 weeks after heart transplantation gradually increased on 1 week, peaked on 2 weeks, but returned to the 1-week level by 4 weeks. Indeed, the data of flow cytometry on 4 weeks after heart transplantation clearly had the difference between exercise group and untreated group, but it was on a decreasing

trend compared to that on 2 weeks. It might suggest that the postoperative exercise stimulation for 1 week was not permanent but temporary. Therefore, postoperative and the daily exercise may be important to prolong the graft survival and may be to be kept to maintain the generation of Treg.

In conclusion, in a murine cardiac transplantation model, not pre-operative but postoperative exercise on a treadmill could induce immunomodulatory effects that resulted in prolonged survival of fully allogeneic grafts and generation of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells. Mandatory exercise on a treadmill may have an important efficiency to peripheral immune responses such as generation of regulatory cells and upregulation of anti-inflammatory cytokines, which resulted in significant prolongation of allograft survival. Subsequent researches on the degree of the most effective exercise in suppressing the rejection reaction in organ transplantation, including the terms and intensity of exercise that are most effective, must include studies in large animals.

Authorship

Funding

Acknowledgements

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