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Original Article

Changes in Serum Cytokine Profile after AEB071 (Sotrastaurin) or Tacrolimus versus Their Combinations in Rat Heterotopic Cardiac Allografts

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Background: AEB071, an orally available PKC inhibitor, prevents organ rejection after transplantation in rodents and man. Furthermore, pro-inflammatory cytokines and inflammatory processes are important mediators of transplanted organ rejection. We therefore examined whether single or combination therapies of AEB071 and/or tacrolimus affect cytokine profiles in a rat cardiac allograft model.

Methods: AEB071 (60 mg/kg twice a day) and tacrolimus (0.6 or 1.2 mg/kg once a day) were orally administered daily after cardiac transplantation. Interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, and tumor necrosis factor (TNF)-α levels in serum were subsequently measured 5 days after cardiac transplantation using a multiplex protein assay system.

Results: All cytokine levels were significantly depressed in cardiac transplanted rats treated with AEB071, whereas tacrolimus only reduced IFN-γ, IL-2, IL-4, IL-6, and IL-10 levels. When administered in combination, AEB071 and low- or high-dose tacrolimus had additive effects on IFN-γ, IL-4, IL-6, and TNF-α.

Conclusions: These results suggest that AEB071 inhibits T cell activation by blocking the production of proinflammatory cytokines, and that tacrolimus combined with AEB071 can effectively regulate inflammatory cytokines in the transplantation setting.

Key Words: Sotrastaurin, Cytokines, Heart transplantation, Immunosuppression, Tacrolimus
중심 단어: 소트라스타우린, 사이토카인, 심장이식, 면역억제, 타크로리무스

Introduction

Several novel immunosuppressive agents that utilize different mechanisms from calcineurin inhibitor to suppress T cell activation have the potential to avoid nephrotoxicity(1). Protein kinase C (PKC) isoforms play key roles in the downstream signaling pathways of T cell receptor (signal 1) and CD28 (signal 2), and thus, targeting these molecules may provide novel approaches that block early T cell activation(2).

AEB071 (sotrastaurin) is a new low molecular weight compound that blocks early T cell activation by selectively inhibiting PKC(3). The PKC family contains 12 isoforms, and each isoform plays a unique role in the regulation of cellular functions(4). However, only PKCα, β, and θ have been reported to participate significantly in T and B cell signaling(5). PKCα-deficient mice exhibit a Th1 defect and much reduced interferon (IFN)-γ levels(6), and PKCβ knockout mice are immunodeficient due to reduced humoral and cellular responses(7). In one study, pretreatment of cardiac myocytes with either calphostin or PKCε inhibitory peptide prevented the serum-related redistribution of the PKCε isoform and inhibited the serum-related se-

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cretion of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 by cardiac myocytes(8). PKCθ is a specific isoform that can be translocated to the immunological synapse(9), and plays an important role in the activation of nuclear factor-κB, nuclear factor of activated T cells, and transcription factor activator protein-1, and in the production of IL-2 and development of Th2 and Th17 immune responses *in vivo*(10). AEB071 selectively inhibits the classical (α, β) and novel (δ, ε, η, and θ) PKC isoforms(11), and biochemical and pharmacological characterizations show that AEB071 inhibits IL-2 and IFN-γ, which is consistent with the phenotypes observed in PKCα and PKCθ single and double knockout mice(6).

In a previous study, we examined the effect of AEB071 on the prevention of acute rejection and on graft survival prolongation in a rat heart transplantation model(12). In the present study, we examined serum cytokine profiles in rats administered a single use of AEB071 and/or tacrolimus to characterize the anti-inflammatory and T cell-blocking effects of these drugs at different doses.

Materials and Methods

1) Animals

Adult male, 8- to 9-week-old, inbred Brown-Norway rats ($RT1^n$ haplotype) and Lewis rats ($RT1^1$ haplotype) were used as donors and recipients, respectively. Rats were housed in a specific pathogen-free room in a temperature controlled animal care facility under a set light-dark diurnal cycle. Rats had free access to water and food and were allowed to adapt to the local environment for at least one week prior to any procedure.

2) Experimental heart transplantation

Rat heterotopic abdominal heart transplantation was performed(12). Average cold ischemic time was less than 40 minutes. Graft function was assessed daily via abdominal palpation by a single investigator. Technical failure was assumed for rats that lost palpable contraction of the graft within 3 days post-operatively, and these animals were excluded from the analysis.

3) Administration of immunosuppressive agents and study groups

AEB071, provided by Novartis (Basel, Switzerland), was dissolved in polyethylene glycol 400 and distilled water for oral administration. Tacrolimus solution (5 mg/mL) was obtained from CKD Pharmaceutical (Seoul, Korea) and diluted in distilled water for oral administration. All rats were treated by oral gavage using rat-feeding needles. Doses of immunosuppressive agents were adjusted for animal body weight on a daily basis.

Six groups of six animals per group (randomly assigned) were used in this study, as follows: the control group (animals received no treatment after transplantation), the AEB071 group (60 mg/kg twice a day), the low-dose tacrolimus group (0.6 mg/kg once a day), the high-dose tacrolimus group (1.2 mg/kg once a day), the AEB071/low-dose tacrolimus group (AEB071 60 mg/kg twice a day, tacrolimus 0.6 mg/kg once a day), and the AEB071/high-dose tacrolimus group (AEB071 60 mg/kg twice a day, and tacrolimus 1.2 mg/kg once a day) The data about graft survival and the pathologic effects of AEB071 and/or tacrolimus in a rat heterotopic heart transplantation model of these groups were in detail in our previous report(12,13).

4) Blood sampling and the quantification of serum cytokine levels

Blood samples were obtained from all six recipients per group on day 5 post-transplantation after confirming heart graft functioning. To determine the effects of AEB071 and/or tacrolimus on serum cytokine profile, we measured the serum levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α. Blood samples were obtained from the retro-orbital plexus of recipients using microhematocrit tubes. Serum IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α levels were measured using MILLIPLEX MAP Rat Cytokine Kits (Millipore Co., St. Charles, MO, USA) and the Luminex 200 Multiplex Protein Analysis System (Luminex, Austin, TX, USA), according to the manufacturer's instructions. Serum was diluted 1:5 for assays, and all samples were run in duplicate. The detection limit for each cytokine was

4.88 pg/mL.

5) Statistical analysis

Data are expressed as means±standard errors. One-way ANOVA with Bonferroni's post hoc analysis was used to compare groups. *P*-values were adjusted using Bonferroni's correction. Data were analyzed using SPSS for Windows ver. 14.0 (SPSS Inc., Chicago, IL, USA), and statistical significance was accepted for $P<0.05$.

Results

The AEB071 60 mg monotherapy group showed significant decreases in all serum cytokine levels versus the control group and a significant decrease in IL-4 versus the tacrolimus 0.6 mg monotherapy group. AEB071 60 mg monotherapy decreased the serum levels of IL-6 and TNF-α more so than tacrolimus at 0.6 or 1.2 mg (Fig. 1).

Combination treatments (groups 4 and 6) decreased IFN-γ more than tacrolimus monotherapy (groups 3 and 5), and low-dose tacrolimus (0.6 mg/kg once a day) plus AEB071 decreased IFN-γ more than high-dose tacrolimus (1.2 mg/kg once a day). However, AEB071 plus low-dose tacrolimus did not significantly decrease serum IL-1β as compared with tacrolimus monotherapy group. However, serum IL-1β was significantly decreased by AEB071 plus high-dose tacrolimus than by tacrolimus high-dose monotherapy (Fig. 2B). Furthermore, serum IL-2 levels were lower in all treatment groups, except the low-dose tacrolimus group, than in the control group (Fig. 1). Low-dose tacrolimus plus AEB071 significantly decreased serum IL-2 levels more so than low and high-dose tacrolimus (Fig. 2C). All doses of tacrolimus and/or AEB071 markedly decreased serum IL-4 and IL-6 as compared with the control, and AEB071 decreased serum IL-6 and TNF-α more than tacrolimus at any dose (Fig. 1). Low-dose tacrolimus and AEB071 significantly decreased serum IL-4, IL-6, and TNF-α levels versus high-dose tacrolimus (Fig. 2D, E, and G). Serum IL-10

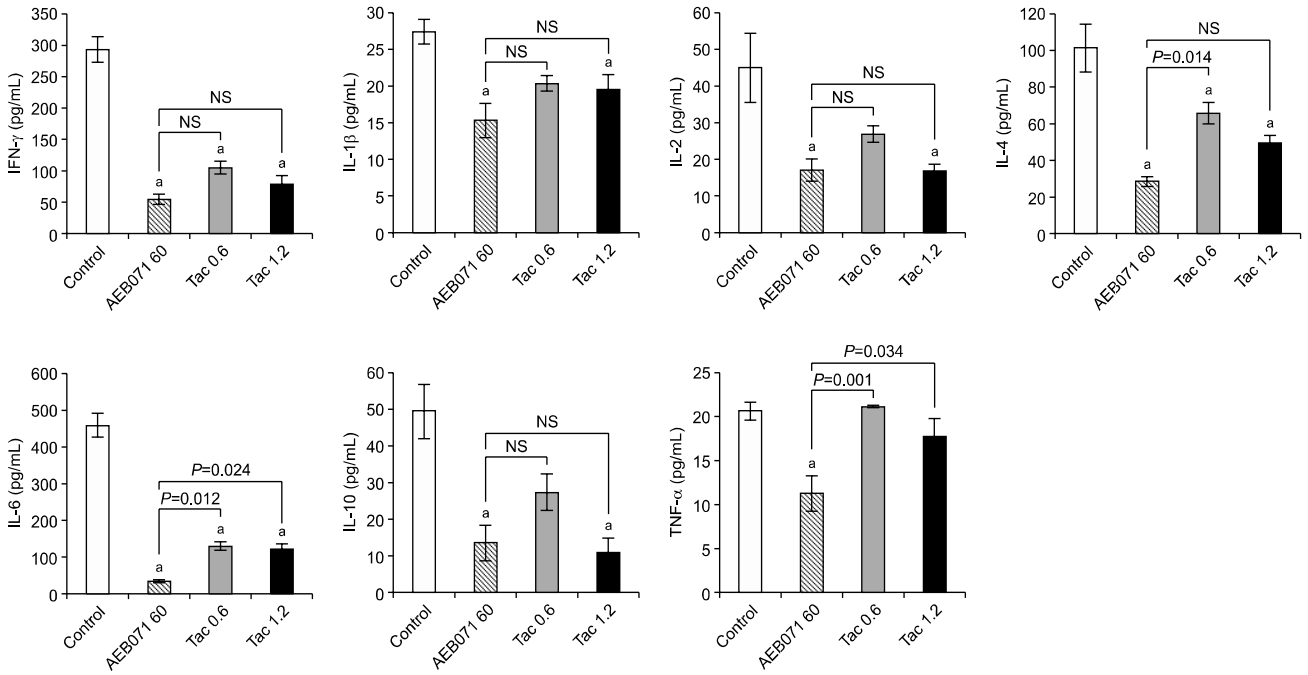

Fig. 1. Serum levels of cytokines after AEB071 or tacrolimus monotherapies. The AEB071 60 mg monotherapy group had significantly lower serum levels of all cytokines than the untreated controls and significantly lower interleukin (IL)-4 levels than tacrolimus at 0.6 mg daily. Furthermore, the AEB071 60 mg group had lower serum levels of IL-6 and tumor necrosis factor (TNF)-α than either the tacrolimus 0.6 or 1.2 mg groups. Data are presented as mean±standard errors. Abbreviations: Tac, tacrolimus; IFN-γ, interferon-gamma; NS, not significant. $^{a}P<0.05$ compared with the untreated control group.

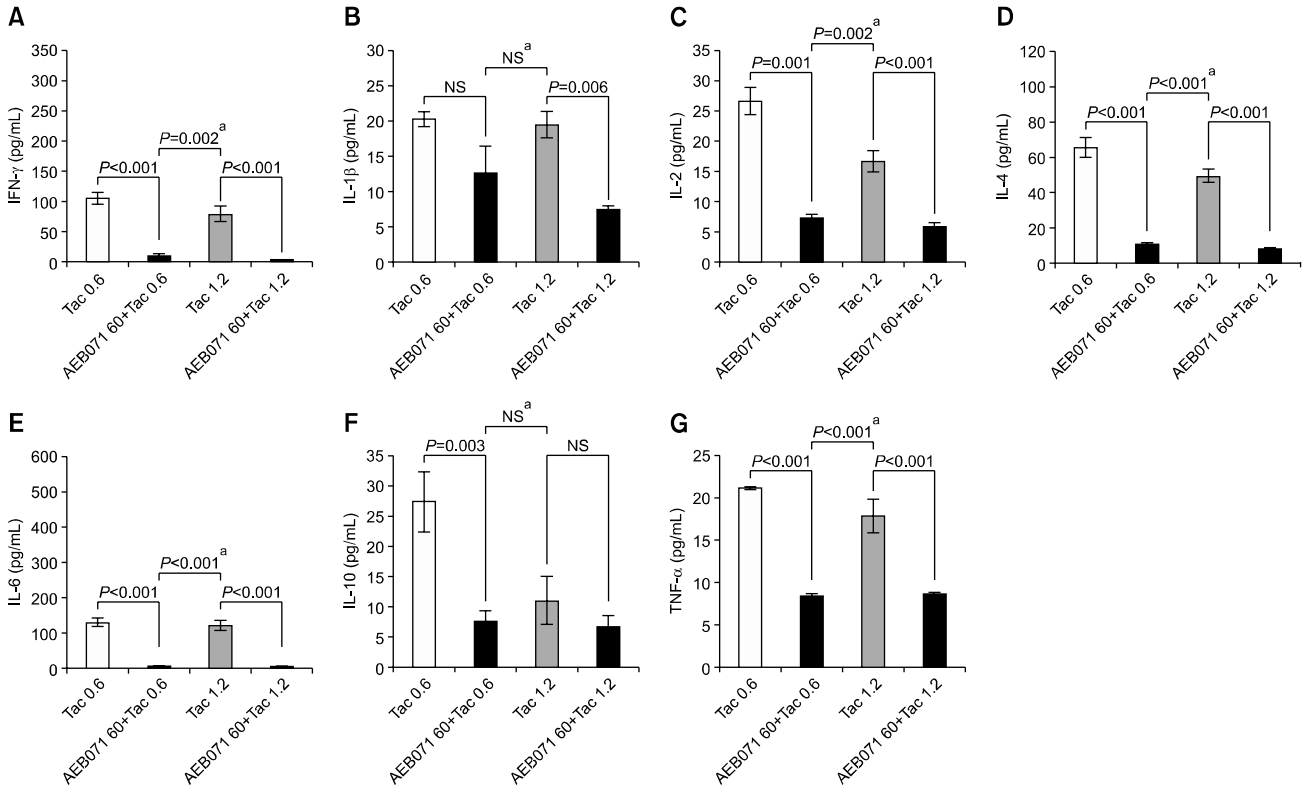

Fig. 2. (A-G) Serum levels of cytokines after tacrolimus monotherapy and after tacrolimus/AEB071 60 mg combination therapy. Data are presented as mean±standard errors. Abbreviations: IFN-γ, interferon-gamma; Tac, tacrolimus; IL, interleukin; NS, not significant; TNF, tumor necrosis factor. [a]Characters present comparisons between low-dose (0.6 mg) tacrolimus plus AEB071 60 mg and high-dose (1.2 mg) tacrolimus.

levels were decreased in all treated groups, but no significant difference was observed between treatment groups. Tacrolimus had no effect on serum TNF-α, but AEB071 monotherapies and AEB071/tacrolimus combination therapies significantly decreased serum TNF-α levels (Fig. 2G). The serum levels of all cytokines, except IL-1β and TNF-α, were significantly decreased by low-dose tacrolimus plus AEB071 as compared with high-dose tacrolimus.

We previously reported survival times and pathologic changes for AEB071 monotherapy and AEB071/tacrolimus combination therapy(12,13). In the previous study, AEB071 monotherapy prolonged allograft mean survival time (MST) versus untreated controls. Also, AEB071 plus tacrolimus prolonged MST versus high-dose tacrolimus monotherapy (Fig. 3). In terms of cardiac graft histology, AEB071 combined with tacrolimus 0.6 mg/kg/day significantly decreased rejection grades as indicated by reduced inflammatory cell infiltration in grafts (data not shown).

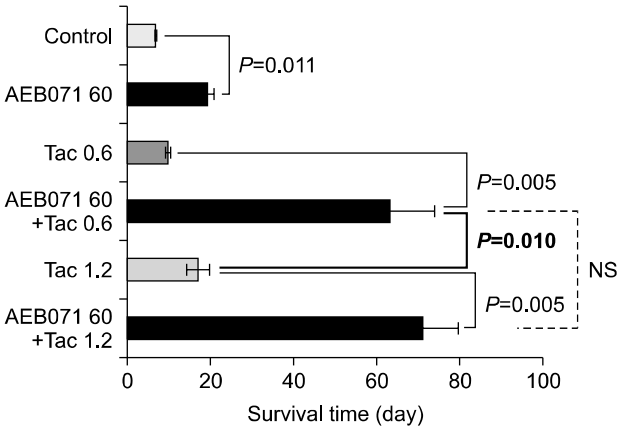

Fig. 3. Heart allograft survival time in response to AEB071 monotherapy or to tacrolimus/AEB701 combinatorial therapies with different tacrolimus doses. AEB071/low dose tacrolimus (Tac) enhanced heart allograft survival more so than high dose Tac monotherapy in the Brown-Norway-to-Lewis rat heterotopic cardiac transplant model. When AEB071 60 mg/kg twice a day was administered in combination with either low-dose or high-dose tacrolimus allograft survivals were similar. Adapted from Fig. 1 of reference [13]. Data are presented as mean survival times±standard errors. Abbreviation: NS, not significant.

Discussion

AEB071 monotherapy and combination therapies, involving a variety of immunosuppressive agents, prolong survival in the settings of rat heterotopic heart transplantation, rat islet transplantation, and cynomolgus monkey renal allografts(3,14). We previously demonstrated that AEB071 prevents acute rejection and prolongs graft survival in a rat heart transplantation model(12,13). In the current study, we utilized the same model and added a combination therapy group to monitor levels of serum cytokines, including proinflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-6, and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10), in rats treated with AEB071 and/or tacrolimus after receiving an cardiac allograft. We found AEB071 effectively reduced serum cytokine levels, and that its potency was similar to or greater than that of tacrolimus.

In the current study, we did not determine lymphocyte proportions or cytokine levels in graft tissues or draining lymph nodes. However, serum cytokines are implicated in the activation and regulation of Th1 and Th2 subsets of T cells. Th1 cells, which produce IL-2 and IFN-γ, are involved in the cellular immune response mediated by cytotoxic cells, macrophages, and natural killer cells(15,16). In contrast, Th2 cells produce IL-4, IL-5, IL-6, and IL-10, all of which predominantly stimulate antibody-mediated humoral immune response(17). In particular, it has been reported that serum cytokines are involved in acute rejection after kidney transplantation(18).

In the present study, AEB071 monotherapy suppressed IL-1β and TNF-α levels more effectively than low or high dose tacrolimus monotherapy. In addition, AEB071 decreased IL-4 more so than low-dose tacrolimus. IL-4 activates B cells in addition to acting as an anti-inflammatory or counter-regulatory cytokine with IL-10 and IL-13. Furthermore, IL-4 inhibits the secretion of pro-inflammatory cytokines by macrophages, but promotes the growth and differentiation of B cells and directs the differentiation of $CD4^+$ T cells into Th2 cells(19,20). These findings imply that AEB071 might exert its immunosuppressive effects by inhibiting the differentiations of B and T cells. TNF-α has important roles both as an apoptosis initiator and an inflammation mediator(21), and can promote nearly all types of humoral and cellular immune responses regulated by IL-1(22). In addition, TNF-α has been reported to be a marker of acute rejection after kidney transplantation(23).

The main limitation of this study was that no lower dosages of AEB071 were used and compared with tacrolimus combinations, and thus, we could not explore synergism between tacrolimus and lower doses of AEB071. Furthermore, we did not monitor serum cytokine changes after transplantation, and we did not evaluate changes in B and T lymphocytes numbers in grafts or lymph nodes. Additional studies are warranted.

Nevertheless, low-dose tacrolimus combined with AEB071 more effectively decreased serum inflammatory cytokine levels (IFN-γ, IL-4, IL-6, and TNF-α) than high-dose tacrolimus. Based on the finding of this and our previous studies(12,13), we suggest that the AEB071/tacrolimus combination is more potent than tacrolimus alone.

Conclusion

Summarizing, this study shows that AEB071 has unique anti-inflammatory activities. In particular, it more effectively suppressed the productions of IL-1β and TNF-α than tacrolimus. Furthermore, our findings show that AEB071 blocks T cell activation and proinflammatory cytokine production after solid organ transplantation, and suggest that the anti-inflammatory effects of the tacrolimus/AEB071 combination be considered in a clinical setting.

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