Effect of Adiponectin on Cardiac Allograft Vasculopathy

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Background: The role of adiponectin (APN), an adipose tissue-specific secretory protein, on chronic rejection after cardiac transplantation in APN-sense transgenic mice (APN-SE) was evaluated.

Methods and Results: Heterotopic cardiac transplantation in major histocompatibility complex class II-mismatched mice was performed. B6.C-H-2\textsuperscript{m13}KtEg (Bm12) hearts were transplanted into APN-SE, and allografts were harvested at 8 weeks after transplantation. Quantitative polymerase chain reaction (PCR) and immunohistochemical staining showed that the expression of both AdipoR1 and AdipoR2 was induced in APN-SE recipients. Neointimal hyperplasia was significantly decreased in allografts transplanted into APN-SE (luminal occlusion, 8.9±2.2%) compared to those transplanted into controls (49.4±10.5%; P=0.011). APN-SE showed significantly reduced mRNA levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-6, and monocyte chemoattractant protein-1 (MCP-1) by quantitative PCR. Western blot analysis revealed that the protein levels of IFN-γ and MCP-1 were reduced in APN-SE recipients. Proliferation of smooth muscle cells stimulated with activated T cells was suppressed by APN addition, and this effect was canceled by treatment with an adenosine monophosphate-activated protein kinase (AMPK) inhibitor.

Conclusions: APN plays a critical role in the attenuation of chronic rejection by suppressing inflammatory cytokine and chemokine expression and enhancing APN receptor expression. APN plays a beneficial role in reducing the progression of cardiac allograft vasculopathy through the AMPK pathway. (Circ J 2011; 75: 2005–2012)

Key Words: Adiponectin; AMPK; Cardiac transplantation
and the 3' flanking sequence of the rabbit $\beta$-globin gene of the pCAGGS expression vector. A Basic Local Alignment Search Tool (BLAST) analysis (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA) of the GenBank nucleotide database indicated that this antisense sequence showed no significant homology to any other mouse gene. The transgenic mice were derived from a C57BL/6 (B6) background. In the APN-sense transgenic mice (APN-SE), sense APN mRNA was detected in the heart, liver, kidney, and intestine, in addition to the white adipose tissue and brown adipose tissue. The expressions of APN protein in the adipose tissues were measured by western blot analysis. In APN-SE, the amount of APN protein in mesenteric, subcutaneous, and abdominal white adipose tissue had increased approximately 1.6-, 3.1-, and 1.9-fold, respectively, compared to that in wild-type mice. 

Male B6 mice, aged 6–8 weeks, were obtained from Japan Clea (Tokyo, Japan), B6.C-H.2m12KhEg (Bm12) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).

All animals were maintained at our animal facility and weighed 20–25 g. This study protocol was approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University.

Cardiac Transplantation
Donor hearts were heterotopically transplanted into recipient mice, as described previously. The aorta and pulmonary artery of donor hearts were anastomosed to the recipient abdominal aorta and inferior vena cava, respectively. In this chronic rejection model, Bm12 murine hearts were transplanted into APN-SE, which represented a class II-mismatch combination. Allografts were harvested 8 weeks after transplantation. After harvest, allografts were sectioned transverse-ly into 2 parts. The basal section was fixed in 10% formalin every day after transplantation for 7 days at a dose of 10 mg/kg (ip, injection volume 0.2 ml per 20 g animal body weight). Allografts were harvested at 7 days after transplantation and total RNA was isolated from allografts.

Histological Evaluation
Grafts and arteries were analyzed after hematoxylin and eosin (HE) and Elastica van Gieson (EVG) staining. The areas within the internal elastic lamina (IEL), the external elastic lamina, and the lumen were carefully traced, and planimetric areas were measured using an image analysis system (Scion Image Beta 4.02; Scion Corp, Frederick, MD, USA). The cross-sectional area of luminal stenosis was calculated as follows: luminal occlusion = ([IEL area–luminal area]/IEL area)× 100 (%).

Quantification of Total Plasma APN
Plasma APN concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems, Inc, Minneapolis, MN, USA) in accordance with the manufacturer’s instructions.

Coculture of SMCs With Splenocytes
Primary SMCs were obtained from the thoracic aortas of Bm12 mice by the explant technique described previously. Cells were grown in Dulbecco’s modified Eagle’s medium (Sigma Chemical Co, St Louis, MO, USA) containing 50 μg/ml streptomycin, 50 IU/ml penicillin, and 10% fetal bovine serum at 37°C and 5% CO₂. Cultured SMCs were identified by the typical hill-and-valley morphology and by immunostaining with a monoclonal antibody to smooth muscle actin. All experiments were performed with cells that were between passages 3 and 8. SMCs were trypsinized and seeded into 96-well plates. At confluence, SMCs were arrested in the medium containing 0.4% fetal bovine serum for 5 days. Mitomycin-C-inactivated splenocytes from B6 mouse controls after transplantation were washed with phosphate-buffered saline and placed into selected wells with high molecular weight APN purified from human plasma (50 ng/ml) and/or an AMPK inhibitor (0.5 mg/ml). After 4 days of stimulation by splenocytes, SMC proliferation was assessed with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan with Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cell proliferation is expressed as optical density.
Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from murine hearts 8 weeks after cardiac transplantation and from control hearts harvested from B6 mice. We also isolated total RNA from murine hearts 7 days after cardiac transplantation with or without AMPK inhibitor injection. cDNA was prepared using the RT-PCR Kit (Applied Biosystems). TaqMan-based real-time PCR using a StepOne Real-Time PCR system (Applied Biosystems) was used to determine the mRNA expression of monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2, IL-6, IL-10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. The real-time PCR protocol consisted of an initial step at 95°C for 20 s followed by 50 cycles of 95°C for 1 s and annealing at 60°C for 20 s. cDNA preparation was run in duplicate. Quantitative data were calculated using the comparative CT (delta delta CT) method, and mRNA expressions of MCP-1, TNF-α, IFN-γ, IL-2, IL-6, and IL-10 were normalized to native GAPDH.
Figure 3. (A) Expression of cytokines in allografts harvested 8 weeks after transplantation. mRNA expression of IFN-γ (**P=0.007, n=4), TNF-α (**P=0.002, n=4), IL-2 (**P=0.016, n=4), IL-6 (**P=0.005, n=4), and MCP-1 (*P=0.027, n=4) was significantly reduced in allografts transplanted into APN-SE. Expression of IL-10 (P=0.123, n=4) mRNA did not differ significantly between these 2 groups. (B) Protein levels of IFN-γ (*P<0.01, n=4) and MCP-1 (*P<0.01, n=4) were examined by western blot analysis. The protein levels of IFN-γ and MCP-1 were significantly reduced in recipient APN-SE. IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; MCP-1, monocyte chemotactic protein-1; APN-SE, adiponectin-sense transgenic mice.
Heart sections were homogenized in extraction buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EGTA, 10 mmol/L EDTA, 100 mmol/L NaF, 1 mmol/L Na3VO4, 2 mmol/L NaF, 100 μg/ml phenylmethysulfon fluoride, and a cocktail of protease inhibitor tablets (Roche, Basel, Switzerland). After centrifugation, the supernatant was stored. The protein concentration of each sample was measured using a Bio-Rad Protein Assay Kit (Bio-Rad, Milan, Italy). Protein concentrations of all samples were equal in subsequent experiments. Proteins were separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with the respective primary antibodies at 4°C overnight. The membranes were then incubated with the appropriate secondary antibody for 2 h and were developed with an enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). Enhanced chemiluminescence was detected using an LAS-1000 Gel Documentation System (Fujifilm, Tokyo, Japan). The levels of MCP-1 and IFN-γ proteins were normalized to that of actin.

Statistical Analysis
All values are reported as mean ± standard deviation. Differences between groups for western blot data, luminal occlusion, and SMC proliferation were analyzed with one-way analysis of variance, and post-hoc analysis was performed with the Bonferroni test, which was included in the commercially available SPSS software package (SPSS Japan, Inc, Tokyo, Japan). Differences were considered significant at P<0.05.

Results
Circulating Levels of APN Increased After Transplantation
We measured serum APN levels before and after transplantation (Figure 1). Serum APN levels were increased after transplantation both in wild-type B6 and APN-SE mice, but the concentration was significantly higher in APN-SE recipients.

APN Attenuates Graft Vasculopathy
To investigate the effect of APN on chronic rejection, we performed cardiac transplantation with wild-type B6 and APN-SE recipients and Bm12 donors. In this combination of major histocompatibility complex class II-mismatch, neointimal hyperplasia developed in recipient wild-type mice (n=6), whereas neointimal thickening was significantly reduced in APN-SE recipients (n=6; Figure 2A). The degree of luminal occlusion was significantly lower in allografts in the APN-SE recipient group (8.9±2.2%) than in the wild-type B6 recipient group (49.4±10.5%) (P=0.011; Figure 2B). We also performed cardiac transplantation using Bm12 recipients and APN-SE donors. The degree of luminal occlusion of this combination was 10.05±6.05% (n=6). These results showed that neointimal thickening was significantly reduced in APN-SE as donors (P=0.004, Figure 2B).

APN Suppresses Expression of IFN-γ, TNF-α, IL-2, IL-6, and MCP-1
We examined whether APN could modulate the expression of cytokines and chemokines in allografts at 8 weeks after transplantation. The mRNA expression of IFN-γ, TNF-α, IL-2, IL-6, and MCP-1 was significantly reduced in allografts transplanted into APN-SE recipients (n=6, Figure 3A). The expression level of IL-10 mRNA did not differ significantly between these 2 groups.

APN Suppresses the Proliferation of SMCs Induced by Activated Splenocytes
The SMC proliferation assay showed that SMCs proliferated in response to activated T cells. APN, at a concentration of 50 ng/ml, significantly reduced SMC proliferation (Figure 4). The effect of the suppression of SMC proliferation due to...
APN was canceled when we used the AMPK inhibitor.

**AdipoR1 and AdipoR2 Are Induced by APN**

Immunohistochemistry of AdipoR1 and AdipoR2 was performed using the allografts harvested 8 weeks after transplantation. In the APN-SE recipient group, expression of both AdipoR1 and AdipoR2 was enhanced after cardiac transplantation, in comparison with the wild-type recipient group (Figures 5A, B). The immunohistochemistry results suggested that these AdipoR1- and AdipoR2-positive cells mainly consisted of macrophages and other infiltrating inflammatory cells. Results of the quantitative PCR for AdipoR1 and AdipoR2 also supported this result. The mRNA expression of AdipoR1 and AdipoR2 was strongly induced in the APN-SE recipient group, in comparison with the wild-type recipients. (Figures 5A, B). The immunohistochemistry results suggested that these AdipoR1- and AdipoR2-positive cells mainly consisted of macrophages and other infiltrating inflammatory cells. Results of the quantitative PCR for AdipoR1 and AdipoR2 also supported this result. The mRNA expression of AdipoR1 and AdipoR2 was strongly induced in the APN-SE recipient group, in comparison with the wild-type recipients.
AMPK Inhibitor Suppressed the Effects of APN for Cytokines
The mRNA expression of MCP-1 was significantly reduced in allografts transplanted into APN-SE on the 7th day after transplantation. This effect of APN was canceled by treatment with the AMPK inhibitor (Figure 6).

Discussion
Many clinical studies have shown that hypoadiponectinemia is associated with endothelial dysfunction, greater carotid intima-media thickness, and coronary artery disease, mostly in diabetic patients. Okamoto et al reported that allografts transplanted into APN-deficient mice showed severe acute rejection relative to transplantations in wild-type mice, and provision of APN by adenovirus in APN-deficient mice reversed these exacerbated responses to allografting. However, their study did not show a difference in cardiac allograft survival in APN-deficient mice. The effect of higher plasma levels of APN on allograft rejection after cardiac transplantation has not been examined.

We performed cardiac transplantation using APN-SE recipients and BALB/c donors, a total allotransplantation match, to investigate the effect of APN on acute rejection. We found that the survival time of cardiac allografts in APN-SE did not differ significantly (8.8±1.5 days; n=6) from that of B6 wild-type recipients (7.7±0.5 days; n=6; P=0.097).

In our experiments with the combination of major histocompatibility complex class II-mismatch, neointimal thickening was significantly attenuated in the APN-SE recipient group compared to the wild-type B6 recipient group. Neointimal formation was associated with proliferation of SMCs. We previously reported that coculture of SMCs and T cells induced SMC proliferation in an in vitro assay. In the present study, we investigated whether APN suppressed SMC proliferation, which was induced by the interaction of SMCs with splenocytes. We verified that SMC proliferation was increased by the interaction of SMCs with splenocytes and that APN suppressed the proliferation of SMCs. There are 2 major pathways that are activated by APN through AdipoR1 and AdipoR2. One of these pathways is the AMPK pathway, which is mainly activated by AdipoR1. We showed that the suppression of SMC proliferation induced by APN was canceled when we used an AMPK inhibitor. This result indicates that the observed effects of APN involved the AMPK pathway.

In western blotting analyses, the expression of phosphorylated-AMPK-α (p-AMPK-α) in allografts significantly decreased after transplantation (data not shown). Therefore, it is reasonable to assume that this disadvantage that occurred during allograft rejection could be overcome by the increase in APN levels.

Overexpression of cytokines and chemokines contributes to cardiac allograft vasculopathy. Compared with the controls, the APN-SE recipient group showed significantly reduced expressions of IFN-γ, TNF-α, IL-2, IL-6, and MCP-1 in the allografts. These cytokines and chemokines are considered atherogenic. In this study, the expression of IL-10 mRNA did not differ significantly between these 2 groups. The effect of IL-10 on the development of cardiac allograft vasculopathy is controversial at present. Furukawa et al reported that blockade of IL-10 activity did not attenuate neointimal thickening after transplantation. We previously reported that IFN-γ is associated with a decrease in graft-infiltrating cells and that suppression of MCP-1 expression is associated with infiltration of mononuclear cells. Suppression of cytokine expression could be associated with the regulation of inflammation as well as with the decrease in the number of graft-infiltrating cells. Therefore, it is reasonable to assume that APN suppresses T cell inflammatory responses and proliferation of SMCs.

Kadowaki and Yamauchi reported that hypoadiponectinemia downregulates AdipoR1 and AdipoR2, thereby reducing APN sensitivity. In the present study, AdipoR1 and AdipoR2 were induced by the higher plasma level of APN in the allograft after transplantation. The higher plasma levels of APN upregulated these receptors, and this upregulation is one of the mechanisms by which APN has an effect on cardiac allografts.

It is known that AdipoR1 primarily activates the AMPK signaling pathway. In contrast, AdipoR2 mainly activates the PPAR-α signaling pathway. Both of these pathways suppress nuclear factor κB (NFκB) activity. APN also suppresses the activity of NFκB. The results of our study suggest that one of the mechanisms by which APN prevents chronic rejection is by suppressing the activity of NFκB, which promotes inflammation and atherosclerosis, that is, reducing NFκB-dependent cytokine promotion.

In our study, the approximate 20% transgene-mediated increase in APN levels resulted in a significant reduction in histological vasculopathy, cytokine productions, and upregulation of AdipoR1 and AdipoR2. A study suggests that caloric restriction increases serum APN levels by approximately 20% in mice. In a human study, the serum APN level was decreased by approximately 20% by oral glucose or fat ingestion. It is known that caloric restriction has a protective effect against cardiovascular disease in association with...
with increased APN levels. Therefore, it is reasonable that a 20% increase in APN levels would result in a significant reduction in vasculopathy in our mice model.

Conclusion

APN plays a critical role in inhibiting the pathogenesis of chronic rejection through suppressed expression of inflammatory cytokines and chemokines and enhanced expression of AdipoR1 and AdipoR2. APN might provide the basis for a novel strategy for managing chronic rejection in clinical cardiac transplantation.

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References


