Rapamycin Ameliorates Experimental Autoimmune Myocarditis

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SUMMARY

Myosin-induced autoimmune myocarditis in rats is a model of human dilated cardiomyopathy. Rapamycin is a potent immunosuppressant and specifically inactivates the mammalian target of rapamycin (mTOR).

To examine the role of mTOR in autoimmune myocarditis, we administered rapamycin to rats immunized with cardiac myosin. Phosphorylation of p70 ribosomal S6 kinase 1 (S6K1), a target of mTOR, was increased by 6.9 fold in the heart tissue of myosin immunized rats. Rapamycin (2 mg/kg/day) completely suppressed S6K1 and S6 phosphorylation. The amount of interleukin-1β, interferon-γ, interleukin-2, or tumor necrosis factor-α mRNA in the heart tissue was markedly increased in myosin-immunized rats, and rapamycin significantly attenuated the cytokine gene expressions. Rapamycin improved the survival of the rats and preserved cardiac function. The plasma level of brain natriuretic peptide increased by 4.7 fold in myosin-immunized rats, and rapamycin attenuated the increase in plasma brain natriuretic peptide. The heart weight/tibial length ratio of vehicle-treated myosin-immunized rats was increased by 1.81 ± 0.06 fold compared with vehicle-treated unimmunized rats, and rapamycin suppressed the increase in heart weight. Rapamycin decreased the cellular infiltration and fibrosis of the myocardium. The amount of phosphorylated S6 was increased in the infiltrating mononuclear cells in vehicle-treated myosin-immunized rats.

Rapamycin significantly ameliorated myocardial injury and preserved cardiac function in a rat model of autoimmune myocarditis. (Int Heart J 2005; 46: 513-530)

Key words: Drugs, Molecular biology, Myocarditis, Heart failure, Signal transduction

MYOCARDITIS is a major cause of sudden death in people younger than 40 years old1) and often follows viral infections.2) The pathogenesis of myocarditis is not fully understood, but there is substantial evidence suggesting that autoimmune responses to heart antigens, particularly cardiac myosin, following viral infection may contribute to the disease process.3) A novel experimental model of
autoimmune myocarditis (EAM) in Lewis rats produced by immunization with cardiac myosin is characterized by extremely severe myocardial lesions and multinucleated giant cells. It has been reported that the pathogenesis of the tissue damage in human giant cell myocarditis and viral myocarditis resembles that of EAM. This model of myocarditis has also been demonstrated to develop into postmyocarditic dilated cardiomyopathy (DCM) in the chronic phase, and the histopathological findings are similar to some forms of human idiopathic DCM.

Rapamycin, a lipophilic macrolide, is an immunosuppressant, and it effectively inhibits cytokine production and lymphocyte proliferation. Rapamycin effectively attenuated disease progression in animal models of autoimmune disease. Rapamycin has been used to control renal transplant rejection in clinical practice. The mammalian target of rapamycin (mTOR) is an intracellular effector of rapamycin. In response to growth factors and amino acids, the mammalian target of rapamycin (mTOR) controls the mammalian translational machinery. Rapamycin inhibits growth by forming a gain-of-function inhibitory complex with FKBP12 (FK506-binding protein, with a MW of 12 kD). This complex binds to mTOR, and decreases mTOR activity. mTOR controls a diverse set of downstream effectors which are important for cellular growth.

p70 ribosomal S6 kinase is a target of mTOR, and is a physiological kinase for the 40S ribosomal S6 protein. In mammals, 2 highly homologous genes, called p70 ribosomal S6 kinase 1 (S6K1) and p70 ribosomal S6 kinase 2 (S6K2), have been identified. S6K1 is activated by the phosphoinositide 3-kinase (PI3K) pathway. S6K1 is also activated by calcium or protein kinase C (PKC).

Rapamycin was shown to inhibit angiotensin II- and phenylephrine-induced increases in protein synthesis in cardiac myocytes in vitro. Recently, rapamycin has been shown to prevent pressure overload-induced cardiac hypertrophy in mice. Furthermore, rapamycin reverses established cardiac hypertrophy. Cardiac hypertrophy is an established risk factor for cardiac mobility and mortality. Proinflammatory responses are known to be involved in the pathogenesis of heart failure. Since rapamycin can attenuate proinflammatory response and hypertrophy, rapamycin may favorably modulate heart failure induced by myocarditis. In this study, we examined the effects of rapamycin on a rat model of heart failure induced by autoimmune myocarditis.

**METHODS**

**Animals:** Female Lewis rats (8 weeks old) were purchased from Charles River (Kanagawa, Japan). Animal care and experimentation were approved by the Institutional Animal Care and Use Committee of Kitasato University.
Immunization: Antigen was prepared from porcine hearts by a previously described procedure. The cardiac myosin thus obtained was emulsified with an equal volume of complete Freund's adjuvant supplemented with Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) to a final concentration of 5 mg/mL. The emulsified solution (0.25 mL) or adjuvant alone (0.25 mL) was subcutaneously injected into the footpads of the rats. The day of myosin injection was defined as day 0.

Experimental protocols:

Protocol 1. To examine the phosphorylation of S6K1, S6 protein, Akt, and extracellular signal-regulated kinase (ERK), myosin-immunized or unimmunized rats were sacrificed 5, 9, 14, and 19 days after immunization.

Protocol 2. To examine the effect of rapamycin on the phosphorylation of S6K1, S6 protein, Akt, and ERK, rapamycin or vehicle was administered to immunized or unimmunized rats sacrificed 14 days after immunization. Rapamycin (2 mg/kg/day) was given daily from 3 days before the immunization to 14 days after the immunization. The dose of rapamycin was determined based on the results when rapamycin was administered to aortic-banded mice. Rapamycin was administered three days before the immunization to test the effect of the drug after it had accumulated to a sufficiently high degree in the tissues.

Protocol 3. To examine the effect of rapamycin on the disease course of EAM rats, we treated myosin-immunized and unimmunized rats with rapamycin or vehicle for 19 days after immunization. Rapamycin or vehicle was orally administered to the animals from three days before to 19 days after the immunization. Survival was monitored, echocardiography was performed 19 days after immunization, and the rats were sacrificed. Body weight, heart weight, lung weight, and tibial length were measured. Heart tissue was used for analysis.

Hemodynamics: Systolic blood pressure (SBP) and heart rate (HR) were recorded by the tail cuff method (Softron BP-98A, Tokyo, Japan). For measuring SBP, rats were placed in a plexiglas restrainer for 10 minutes in a warm chamber maintained at 40°C. The tail cuff device was placed around the tail; three pressure measurements were recorded for each rat, and the average SBP was calculated.

Echocardiography: Nineteen days after immunization, transthoracic echocardiographic analysis was performed using a ProSound SSD-4000 (ALOKA, Tokyo, Japan) echocardiograph with a 7.5-MHz imaging transducer. 2-2-2 Tribromoethanol (Aldrich, 0.4-0.6 mg/kg) was used for anesthesia. The M-mode echocardiogram was performed at the papillary muscle level, and left ventricular end-diastolic dimension (LVDd), end-systolic dimension (LVDs), and LV posterior wall thickness (LVWT) were measured, and fractional shortening (FS) was calculated as described previously.
**Western-blotting:** Hearts were removed and immediately frozen in liquid nitrogen. Heart lysates were obtained by homogenization in ice-cold buffer as described. The protein concentration was determined by the BCA method (PIERCe). Cardiac tissue lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corporation, Bedford, MA) as described. For analysis of p70 S6K phosphorylation, the blots were probed with anti-phospho-p70S6K (Thr 389) antibody (1:200; Cell Signaling, Beverly, MA) or anti-p70S6K antibody (1:200; Santa Cruz, CA). For analysis of S6 phosphorylation, the blots were probed with anti-phospho-S6 protein (Ser 235/236) antibody (1:500; Cell Signaling), or anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH; 1:5000; Research Diagnostics, Flanders, NJ). The blots were probed with anti-phospho-Akt (Ser 473) antibody (1:500; Cell Signaling) or anti-Akt antibody (1:500; Cell Signaling) to analyze Akt phosphorylation. For analysis of ERK phosphorylation, the blots were probed with anti-phospho-ERK antibody (1:200; Santa Cruz) or anti-ERK2 antibody (1:200; Transduction Lab., Lexington, KY).

**Analysis of cytokine mRNA:** The heart tissue was quickly removed, frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated by the acid guanidinium-phenol-chloroform method. The RNAs (1 µg) were reverse-transcribed into cDNAs using random hexamers (TaqMan Gold RT-PCR Kit; Applied Biosystems, Foster City, CA).

The amounts of interleukin (IL)-1β, interferon (IFN)-γ, IL-2, and tumor necrosis factor (TNF)-α mRNAs were measured by a real-time PCR method. Real-time PCR was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). A reaction included: 1 µL of cDNA sample, 10 µL of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µL of IL-1β, IFN-γ, IL-2, or TNF-α primer and a probe (FAM labeled, purchased from Applied Biosystems), 1 µL of eukaryotic 18S rRNA primer and a probe (internal control gene, VIC-labeled probe, Applied Biosystems), and 7 µL of RNase-free water. For each reaction, the polymerase was activated by pre-incubating at 95°C for 10 minutes, after which amplification was performed by switching between 95°C for 15 seconds and 60°C for 60 seconds. The amplification cycle was 40. The signals of IL-1β, IFN-γ, IL-2, or TNF-α were normalized using the 18S rRNA signal.

**Histopathology:** The rats were sacrificed on day 19, and the hearts were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. The heart sections were stained by hematoxylin-eosin or the Mallory-Azan method. Macroscopic findings were expressed in terms of infiltration and fibrosis scores. The extent of inflammatory cell infiltration and myocardial necrosis was estimated using hema-
toxylin-eosin staining. The degree of fibrosis was estimated using Mallory-Azan staining. The areas of the entire heart and of regions affected by myocarditis were determined using MacSCOPE image processing software (Mitani Corp., Tokyo, Japan) and the area ratio (affected area/total area in percent) was calculated.

**Plasma brain natriuretic peptide:** The concentration of brain natriuretic peptide (BNP) was determined by enzyme-linked immunoassay (BNP-32 Rat RIA Kit, Peninsula Laboratories, San Carlos, CA).

**Immunohistochemical analysis of S6 phosphorylation:** Immunohistochemical analysis was performed as described. Heart sections were embedded in O.C.T. compound tissue medium, snap-frozen on dry ice, and stored at -70°C. Tissues were sectioned on a cryostat at 4 to 10 μm. Tissue sections were fixed for 10 minutes in acetone at 4°C. Anti-phospho-S6 protein (Ser 235/236) antibody (1:100; Cell Signaling) was used. All dilutions were performed using PBS containing 1% bovine serum albumin. Incubation with primary antibody was carried out at 4°C overnight. Biotinylated goat anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) diluted 1:300 was used as secondary antibody. Incubations with secondary antibodies were carried out at room temperature for 30 minutes. After incubation with avidin-biotin-horseradish peroxidase complexes (Vector Laboratories, Burlingame, CA), peroxidase was visualized by 3',3'-diaminobenzidine followed by incubation with diaminobenzidine enhancing solution (Vector Laboratories). Counterstaining was performed with hematoxylin. Omission of the primary antibody was used as controls.

**Statistical analysis:** All values are expressed as the mean ± SEM. Differences between the groups were compared using the Student t-test. The survival of the animals was analyzed using the Kaplan-Meier method. P < 0.05 was considered to be significant.

**RESULTS**

**Phosphorylation of S6 K1 and S6 in EAM rats:** First, we examined whether S6K1, a target of mTOR, was activated in response to experimental autoimmune myocarditis (Protocol 1). Myosin-immunized or unimmunized rats were sacrificed 5, 9, 14, and 19 days after the immunization. At 14 and 19 days after immunization, S6K1 phosphorylation in the hearts from myosin-immunized rats had increased compared with that from unimmunized rats (Figure 1A). S6K1 phosphorylation was the highest at 14 days (Figure 1A). The phosphorylation of S6 was measured to confirm the activation of S6K1 in heart tissue in response to myocarditis (Figure 1B). The amount of phosphorylated S6 had increased 19 days after the immunization compared with that of unimmunized rats. The phosphorylation of Akt, a well-characterized target of PI3K, was not remarkably
The phosphorylation of Akt, S6K1, S6 protein, or ERK was analyzed by Western blotting. A: Phosphorylation of S6K1. The amount of phosphorylated S6K1 was analyzed using a phospho-specific antibody for p70-S6K1 (upper panel). Protein loading was normalized by reprobing the membrane with S6K (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of S6K peaked 14 days after the immunization. B: Phosphorylation of S6 protein. The amount of phosphorylated S6 protein was analyzed using a phospho-specific antibody for S6 protein (upper panel). Protein loading was normalized by reprobing the membrane with GAPDH (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of S6 protein was increased 14 and 19 days after the immunization. C: Phosphorylation of Akt. The amount of phosphorylated Akt was analyzed using a phospho-specific antibody for Akt (upper panel). Protein loading was normalized by reprobing the membrane with Akt (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of Akt was not changed at the time points examined. D: Phosphorylation of ERK. The amount of phosphorylated ERK was analyzed using a phospho-specific antibody for ERK (upper panel). Protein loading was normalized by reprobing the membrane with ERK2 (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of ERK was not changed at the time points examined. Control (C) untreated rats (n = 3); unimmunized rats on days 5, 9, 14, and 19 (n = 3 for each); myosin-immunized rats on days 5, 9, and 14 (n = 5 for each); and myosin-immunized rats on day 19 (n = 8) were used for the analysis. The mean value of control rats was defined as 1 unit. *P < 0.05 versus control rats. †P < 0.05 versus unimmunized rats on the same day.

Figure 1. Phosphorylation of Akt, S6K1, S6 protein, or ERK in experimental autoimmune myocarditis. Rats were sacrificed 5, 9, 14, and 19 days after immunization. Phosphorylation of Akt, S6K, S6 protein, or ERK was analyzed by Western blotting. A: Phosphorylation of S6K1. The amount of phosphorylated S6K1 was analyzed using a phospho-specific antibody for p70-S6K1 (upper panel). Protein loading was normalized by reprobing the membrane with S6K (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of S6K peaked 14 days after the immunization. B: Phosphorylation of S6 protein. The amount of phosphorylated S6 protein was analyzed using a phospho-specific antibody for S6 protein (upper panel). Protein loading was normalized by reprobing the membrane with GAPDH (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of S6 protein was increased 14 and 19 days after the immunization. C: Phosphorylation of Akt. The amount of phosphorylated Akt was analyzed using a phospho-specific antibody for Akt (upper panel). Protein loading was normalized by reprobing the membrane with Akt (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of Akt was not changed at the time points examined. D: Phosphorylation of ERK. The amount of phosphorylated ERK was analyzed using a phospho-specific antibody for ERK (upper panel). Protein loading was normalized by reprobing the membrane with ERK2 (middle panel). Results of quantitative densitometry analysis are shown (lower panel). Phosphorylation of ERK was not changed at the time points examined. Control (C) untreated rats (n = 3); unimmunized rats on days 5, 9, 14, and 19 (n = 3 for each); myosin-immunized rats on days 5, 9, and 14 (n = 5 for each); and myosin-immunized rats on day 19 (n = 8) were used for the analysis. The mean value of control rats was defined as 1 unit. *P < 0.05 versus control rats. †P < 0.05 versus unimmunized rats on the same day.

changed (Figure 1C). The phosphorylation of ERK, which is not regulated by PI3K, was not significantly changed (Figure 1D).

Effect of rapamycin on the phosphorylation of S6K1 and S6: Next, we examined the effects of rapamycin on the phosphorylation of S6K1, S6 protein, Akt, and ERK (Protocol 2). Since S6K1 phosphorylation reached a maximum 14 days after immunization, we examined S6K1 phosphorylation in hearts from rapamycin-treated rats at this time point. Rapamycin (2 mg/kg/day) completely sup-
pressed S6K1 phosphorylation (Figure 2A) and completely inhibited S6 phosphorylation (Figure 2B). We then examined Akt phosphorylation at this time point. Akt was not significantly phosphorylated in the EAM rats. Furthermore, rapamycin treatment did not affect the phosphorylation of Akt (Figure 2C). We also examined the effect of rapamycin on other signaling molecules in response to myocarditis as well as the phosphorylation of ERKs. ERKs were not significantly affected by rapamycin treatment at this time point (Figure 2D).
Effect of rapamycin on the survival and heart weight of EAM rats: To examine whether rapamycin attenuates myosin-induced myocarditis, we treated myosin-immunized and unimmunized rats with rapamycin or vehicle for 19 days after immunization (Protocol 3, Figure 3). Rapamycin improved the mortality of myocarditis (0/10 vehicle-treated unimmunized rats, 19/46 vehicle-treated myosin-immunized rats, 0/15 rapamycin-treated unimmunized rats, and 0/19 rapamycin-
treated myosin-immunized rats died within 19 days after immunization). The rats were sacrificed 19 days after immunization. The heart weight/tibial length ratio of myosin-immunized rats was increased $1.81 \pm 0.06$ fold compared with vehicle-treated unimmunized rats (Table I). In contrast, the heart weight/tibial length ratio of rapamycin-treated myosin-immunized rats was increased $1.09 \pm 0.04$ fold compared with rapamycin-treated unimmunized rats. Thus, rapamycin almost completely suppressed the increase in heart weight.

**Effect of rapamycin on hemodynamic parameters:** Blood pressure was measured using the tail-cuff method to examine the effect of rapamycin on hemodynamics in EAM. The peak systolic pressure of vehicle-treated myosin-immunized rats

| Table II. Blood Pressure and Heart Rate of EAM Rats Treated With Rapamycin |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | Unimmunized                 | Myosin-immunized            |                             |                             |
|                             | Vehicle         | Rapamycin     | Vehicle         | Rapamycin     |
| Number of Animals           | 3                          | 3                          | 4                          | 5                          |
| Heart rate (bpm)            | $355 \pm 14$              | $395 \pm 17$              | $404 \pm 27$              | $401 \pm 14$              |
| Systolic body pressure (mmHg)| $116.1 \pm 7.2$          | $131.6 \pm 8.6$          | $91.1 \pm 5.4^*$          | $134.1 \pm 3.9^+$          |
| Diastolic body pressure (mmHg) | $65.1 \pm 2.3$         | $82.0 \pm 8.7$          | $63.0 \pm 4.6$           | $94.5 \pm 5.9^+$           |

*P < 0.05 versus unimmunized rats treated with vehicle or rapamycin. †P < 0.05 versus vehicle-treated myosin-immunized rats.

![Figure 4. Macroscopic analysis of EAM rats treated with rapamycin. (A) Vehicle-treated unimmunized rat, (B) vehicle-treated myosin-immunized rat, (C) rapamycin-treated unimmunized rat, and (D) rapamycin-treated myosin-immunized rat. The left ventricle was dilated and wall thickness was increased in vehicle-treated myosin-immunized rats. Rapamycin attenuated the cardiac hypertrophy. The bar represents 1 mm.](image-url)
decreased compared with that of vehicle-treated myosin-immunized rats (Table II). Rapamycin prevented the decrease in peak systolic pressure.

**Effect of rapamycin on myocardial inflammation and fibrosis:** Rapamycin or vehicle was administered to myosin-immunized or unimmunized rats (Figure 4) and then the rats were sacrificed 19 days after the immunization. The heart sections

**Figure 5.** Effect of rapamycin on cellular infiltration and fibrosis. **A:** Effect of rapamycin on cellular infiltration. Representative pictures of (1) vehicle-treated unimmunized rats, (2) vehicle-treated myosin-immunized rats, (3) rapamycin-treated unimmunized rats, and (4) rapamycin-treated myosin-immunized rats are shown. Rapamycin attenuated cellular infiltration. **B:** Effect of rapamycin on fibrosis. Representative pictures of (1) vehicle-treated unimmunized rats, (2) vehicle-treated myosin-immunized rats, (3) rapamycin-treated unimmunized rats, and (4) rapamycin-treated myosin-immunized rats are shown. Rapamycin attenuated fibrosis. Bars represent 50 µm.
were stained with hematoxylin-eosin or the Mallory-Azan method (Figure 5). The extent of inflammatory cell infiltration was estimated using hematoxylin-eosin staining. The cell infiltration area of vehicle-treated myosin-immunized rats was 27.7 ± 4.0%. The infiltration area of hearts from rats treated with rapamycin was 9.4 ± 1.6% (Table IIIA). The fibrosis area of vehicle-treated myosin-immunized rats was 32.8 ± 2.1%, and the fibrosis area of hearts from rapamycin-treated rats was 12.6 ± 1.4% (Table IIIB). Collectively, rapamycin attenuated the inflammation and fibrosis of the myocardium of EAM rats.

**Rapamycin preserved cardiac function of EAM rats:** The effect of rapamycin on cardiac function was examined using echocardiography 19 days after immunization (Table IV). In vehicle-treated myosin-immunized rats, LVDd was increased 1.4 fold and LVWT was increased 1.6 fold compared with vehicle-treated unimmunized rats. Rapamycin treatment significantly decreased the LVDd and LVWT of myosin-immunized rats. Cardiac contractility was assessed by fractional shortening (FS). In vehicle-treated myosin-immunized rats, FS had decreased to 79% of vehicle-treated unimmunized rats. Rapamycin significantly maintained FS. FS was not different between vehicle-treated and rapamycin-treated unimmunized animals. Thus, rapamycin treatment attenuated left ventricular dilatation and preserved cardiac function.

**Table IIIA.** Cellular Infiltration in the Myocardium of EAM Rats Treated With Rapamycin

<table>
<thead>
<tr>
<th></th>
<th>Unimmunized</th>
<th>Myosin-immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>Number of animals</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Cellular infiltration area ratio (%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*P < 0.05 versus unimmunized rats treated with vehicle or rapamycin. †P < 0.05 versus vehicle-treated myosin-immunized rats.

**Table IIIB.** Fibrosis in the Myocardium of EAM Rats Treated With Rapamycin

<table>
<thead>
<tr>
<th></th>
<th>Unimmunized</th>
<th>Myosin-immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>Number of animals</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Fibrosis area ratio (%)</td>
<td>2.0 ± 0.3</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

*P < 0.05 versus unimmunized rats treated with vehicle or rapamycin. †P < 0.05 versus vehicle-treated myosin-immunized rats.
Effect of rapamycin on proinflammatory cytokine expression in EAM rats:

Cytokines play a major role in the pathogenesis of EAM. In EAM, mRNA expression of IL-1β, IFN-γ, IL-2, and TNF-α increased from the onset of the disease. We examined cytokine expression 14 days after immunization by

Table IV. Echocardiographic Data of EAM Rats Treated With Rapamycin

<table>
<thead>
<tr>
<th></th>
<th>Unimmunized</th>
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<th>Myosin-immunized</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Rapamycin</td>
<td>Vehicle</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>Number of animals</td>
<td>4</td>
<td>6</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>311 ± 14</td>
<td>301 ± 13</td>
<td>284 ± 9</td>
<td>322 ± 8</td>
</tr>
<tr>
<td>Diastolic anterior wall thickness (mm)</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.1*</td>
<td>1.4 ± 0.1†</td>
</tr>
<tr>
<td>Diastolic posterior wall thickness (mm)</td>
<td>1.5 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>2.5 ± 0.1*</td>
<td>1.8 ± 0.1†</td>
</tr>
<tr>
<td>LV diastolic diameter (mm)</td>
<td>3.5 ± 0.2</td>
<td>3.3 ± 0.1</td>
<td>4.9 ± 0.2*</td>
<td>4.3 ± 0.1*†</td>
</tr>
<tr>
<td>LV systolic diameter (mm)</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>2.6 ± 0.1*</td>
<td>1.7 ± 0.1*†</td>
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<tr>
<td>Fractional shortening (%)</td>
<td>58 ± 3</td>
<td>59 ± 2</td>
<td>46.1 ± 1.7*</td>
<td>60 ± 2†</td>
</tr>
</tbody>
</table>

LV = left ventricle. Results are presented as mean ± SEM.

*P < 0.05 versus unimmunized rats treated with vehicle or rapamycin. †P < 0.05 versus vehicle-treated myosin-immunized rats.

Figure 6. Effect of rapamycin on cytokine gene expression. The mRNA levels of (A) IL-1β, (B) IFN-γ, (C) IL-2 and (D) TNF-α were measured by quantitative RT-PCR. The expression of IL-1β, IFN-γ, IL-2 or TNF-α was increased in vehicle-treated myosin-immunized rats, and rapamycin attenuated the cytokine gene expression. *P < 0.05 versus unimmunized rats treated with vehicle or rapamycin. †P < 0.05 versus vehicle-treated myosin-immunized rats. Vehicle-treated unimmunized rats (n = 4), rapamycin-treated unimmunized rats (n = 4), vehicle-treated myosin-immunized rats (n = 9), and rapamycin-treated myosin-immunized rats (n = 6) were used for the analysis.
quantitative RT-PCR. The mRNA expression of IL-1β, IFN-γ, IL-2, and TNF-α was markedly increased in vehicle-treated myosin-immunized rats. Rapamycin significantly attenuated the mRNA expression of proinflammatory cytokines (Figure 6).

Figure 7. Effect of rapamycin on level of serum brain natriuretic peptide (BNP). The serum levels of BNP in EAM rats were increased, and rapamycin attenuated the increase. Vehicle-treated unimmunized rats (n = 3), rapamycin-treated unimmunized rats (n = 8), vehicle-treated myosin-immunized rats (n = 5), and rapamycin-treated myosin-immunized rats (n = 10) were used for the analysis. *P < 0.05 versus unimmunized rats treated with vehicle or rapamycin. †P < 0.05 versus vehicle-treated myosin-immunized rats.

Table V. Concentration of Serum Biochemical Constituents of EAM Rats Treated With Rapamycin

<table>
<thead>
<tr>
<th></th>
<th>Unimmunized</th>
<th>Rapamycin</th>
<th>Myosin-immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td>15.2 ± 0.7</td>
<td>15.5 ± 0.7</td>
<td>18.8 ± 1.5</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.24 ± 0.01</td>
<td>0.24 ± 0.02</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>73.8 ± 2.5</td>
<td>86.6 ± 6.4</td>
<td>63.1 ± 2.8*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>46.3 ± 10.0</td>
<td>56.8 ± 10.7</td>
<td>75.6 ± 12.6</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>167.9 ± 12.1</td>
<td>213.2 ± 14.7#</td>
<td>180.5 ± 10.3</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST, IU/L)</td>
<td>58.8 ± 3.0</td>
<td>65.7 ± 8.3</td>
<td>154.0 ± 18.4*</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT, IU/L)</td>
<td>26.1 ± 2.1</td>
<td>25.4 ± 1.7</td>
<td>23.4 ± 2.1</td>
</tr>
</tbody>
</table>

*P < 0.05 versus unimmunized rats treated with vehicle or rapamycin. †P < 0.05 versus vehicle-treated myosin-immunized rats. #P < 0.05 versus unimmunized vehicle-treated rats.
Effect of rapamycin on brain natriuretic peptide in EAM rats: Brain natriuretic peptide (BNP) is a cardiac neurohormone secreted from the cardiac ventricles as a response to ventricular volume expansion and pressure overload.29,30) BNP levels have been shown to be elevated in patients with LV dysfunction and are correlated to New York Heart Association class as well as prognosis.31) We examined plasma BNP levels using ELISA (Figure 7). BNP levels of vehicle-treated myosin-immunized rats were increased 4.7 fold compared with vehicle-treated unimmunized rats. Rapamycin attenuated the increase 2.1 fold. Thus, rapamycin prevented heart failure caused by myocarditis.

Blood chemical analysis: The serum concentrations of serum creatinine, blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Cho), and triglycerides (TG) and the blood glucose level were measured (Table V). Serum creatinine was slightly but significantly increased in rapamycin-treated myosin-immunized rats, compared with vehicle-treated myosin-immunized rats (0.303 ± 0.017 versus 0.253 ± 0.012 mg/dL). Total cholesterol decreased in myosin-immunized rats and rapamycin preserved serum total cholesterol. Rapamycin increased serum glucose in unim-

Figure 8. Immunohistochemical analysis of S6 phosphorylation. Representative immunohistochemical analysis of (A) vehicle-treated unimmunized rats, (B) vehicle-treated myosin-immunized rats, (C) rapamycin-treated unimmunized rats, and (D) rapamycin-treated myosin-immunized rats are shown. Phosphorylated S6 was increased in infiltrating mononuclear cells of vehicle-treated myosin-immunized rats (arrows). The bar represents 25 µm.
munized rats. AST increased in vehicle-treated myosin-immunized rats, possibly due to liver congestion, compared with vehicle-treated unimmunized rats. Rapamycin treatment attenuated the increase in AST.

**Immunohistochemical analysis of S6 phosphorylation:** To identify cell types in which S6K1 is activated, immunohistochemical analysis was performed using a phosphor S6 antibody. Phosphorylated S6 was increased in infiltrating mononuclear cells of vehicle-treated myosin-immunized rats (Figure 8).

**DISCUSSION**

In this study, the phosphorylation of S6K1 and S6 protein was increased in the heart tissue of myosin-immunized rats, and rapamycin completely inhibited the phosphorylation. Rapamycin significantly suppressed cytokine gene expression in EAM rats. Rapamycin improved the survival of the animals, maintained cardiac function, and suppressed the increase in plasma BNP concentrations. Rapamycin ameliorated myocardial inflammation and fibrosis, and inhibited an increase in heart weight.

Rapamycin is an immunosuppressant, and it effectively inhibits cytokine production and lymphocyte proliferation. Rapamycin has been found to effectively attenuate disease progression in animal models of autoimmune disease. The expressions of IL-2 and IFN-γ, which are mainly produced by lymphocytes, were effectively attenuated by rapamycin in this study. S6 phosphorylation was mainly increased in the infiltrating mononuclear cells, and rapamycin decreased the amount of phosphorylated S6. Thus, it is likely that rapamycin ameliorates autoimmune myocarditis, at least in part, by inhibiting lymphocyte proliferation and cytokine production.

Animal models of autoimmune myocarditis resemble human DCM. A significant amount of research has been undertaken to elucidate the pathogenesis of autoimmune myocarditis. However, little is known about the intracellular signaling mechanism causing autoimmune myocarditis. In this study, S6K1 was activated whereas the phosphorylation of Akt, a well-characterized target of PI3K, or ERK was not increased. S6K1 is also activated by PKC or calcium. It is likely that S6K1 was activated in a PI3K-independent manner in this model.

Rapamycin is known to be a potent immunosuppressant. In this study, rapamycin effectively suppressed myocardial inflammation. However, we observed significant cellular infiltration in the rapamycin-treated animals (27.7 ± 4.0% in vehicle-treated myosin-immunized rats, 9.4 ± 1.6% in rapamycin-treated myosin-immunized rats). Rapamycin suppresses immune responses with other immunosuppressants in a synergistic manner. Thus, it might be useful to use rapamycin with other immunosuppressants to treat autoimmune myocarditis.
The serum creatinine concentration of rapamycin-treated myosin-immunized rats was higher than that of vehicle-treated myosin-immunized rats. It has been reported that rapamycin is less nephrotoxic than cyclosporine in animals. However, rapamycin nephrotoxicity has been reported in patients and animals. Serum glucose level was higher in rapamycin-treated unimmunized rats than in vehicle-treated unimmunized rats. Deletion of the S6K1 gene has been reported to induce diabetes.

It is still not known whether immunosuppression is effective against myocarditis in human patients. Several immunosuppressants effectively ameliorate myocardial injury in animal models of autoimmune myocarditis. However, some immunosuppressants, such as cyclosporine and prednisolone, have deleterious effects on myocardial injury in animal models of viral myocarditis. Furthermore, additional studies are needed to determine if rapamycin is also effective when given after the onset of myocarditis. Thus, further studies are needed before rapamycin can be considered a therapeutic modality for myocarditis.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Japan Society for the Promotion of Science (15390252), the Japan Heart Foundation, the Japan Foundation of Cardiovascular Research, the NOVARTIS Foundation for the Promotion of Science, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Takeda Medical Research Foundation, the Kanagawa Nanbyo Foundation, the Takeda Science Foundation (to T.S.), and grants from the Japan Society for the Promotion of Science (16590713) and the Postgraduate Research Project of Kitasato University (to T.I.).

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