Cytokine and Murine Coxsackievirus B3 Myocarditis

Interleukin-2 Suppressed Myocarditis in the Acute Stage But Enhanced the Condition in the Subsequent Stage

Chiharu Kishimoto, MD, PhD; Yoshito Kuroki, MD; Yuji Hiraoka, MD; Hiroshi Ochiai, MD, PhD; Masahiko Kurokawa, PhD; Shigetake Sasayama, MD, PhD

Background  It has been shown that the development of coxsackievirus B3 (CB3) myocarditis is regulated by T cells and not by B cells. Interleukin-2 (IL-2) is a T-cell–derived cytokine that stimulates the growth of T cells. This study was carried out to determine the effects of IL-2 on CB3-infected BALB/c mice.

Methods and Results  In two separate experiments, recombinant human IL-2 (5 x 10⁴ U) was administered subcutaneously to 30 mice early (days 0 to 7) and 30 mice late (days 7 to 14) after infection with CB3. Each experiment had a control group of infected animals that did not receive IL-2. On days 7 and 10, splenic natural killer (NK) cell activity determined by ⁵¹Cr release assay and the distribution of myocardial lymphocyte subsets were compared in the treated and untreated groups. In the early treatment experiment, survival at 7 days was higher in treated compared with control animals, myocardial virus titers were lower, inflammatory cell infiltration was less (as was the severity of necrosis at the time the mice were killed), and NK cell activity was higher. However, in the late treatment experiment, survival at 14 days was lower in treated compared with control animals, and there was more infiltration, more severe necrosis, and more T-cell infiltration, but the NK cell activity did not differ significantly. In a third experiment similar to the late experiment described above but involving infected athymic nude mice, we confirmed the lack of effect of late in vivo administration of IL-2 on outcome.

Conclusions  IL-2 has the capacity to limit CB3 myocarditis by enhancing NK cell activity in the acute viremic stage, resulting in a reduction of cardiac pathology. However, in the subacute aviremic stage, in contrast, IL-2 exacerbates the course and severity of the disease by increasing the number of T cells infiltrating the myocardium. That is, IL-2 has differential effects on acute CB3 myocarditis. IL-2 is beneficial if treatment is given early but not later in murine CB3 myocarditis. (Circulation. 1994;90:2836-2842.)

Key Words  • myocarditis • coxsackievirus B3 • T cells • interleukin-2 • cytokines • natural killer cells

It has been established that coxsackieviruses are the predominant cause of viral myocarditis in humans.¹⁻³ Virus-induced immunologic mechanisms have been implicated in the pathogenesis of human myocarditis.⁴⁻⁵ Indeed, we have previously demonstrated experimentally that T cells play a role in the development of coxsackievirus B3 (CB3) myocarditis.⁶⁻⁸ Interleukin-2 (IL-2), a T-cell–derived cytokine that stimulates the growth of the T cell,⁹⁻¹⁰ has been shown to play a significant role in supporting the growth of natural killer (NK) cells¹¹⁻¹⁵; it may also stimulate the cytotoxic activity of T-lymphocytes.¹³⁻¹⁵ Increased NK cell activity has been shown to occur early during the course of infections with various viruses.¹⁶⁻¹⁸ Many of these viral infections induce interferon, which mediates antiviral effects; interferon also activates NK cells.¹⁸ Numerous studies also have demonstrated that the in vitro incubation of lymphocytes with IL-2 leads to the generation of activated T cells that can lyse a wide variety of tumor cells.¹³⁻¹⁵ Furthermore, the direct in vivo administration of IL-2 has been shown to generate cytotoxic T cells in various tissues.¹⁹⁻²² In the present study, to clarify the function of IL-2 during the development of myocarditis and to reconfirm the underlying biphasic disease process in the pathogenesis of the condition, we studied the clinical course, cardiac pathology, myocardial lymphocyte subsets, and NK cell activity in murine CB3 myocarditis with and without IL-2 treatment.

Methods

Virus Preparation  The method used was similar to that described in previous reports.⁶⁻⁸ In brief, CB3 (Nancy strain, American Type Culture Collection) was used; the virus stock was prepared in cultures of VERO (African green monkey kidney) cells in Eagle’s minimum essential medium (EMEM). Virus suspensions were centrifuged after the cytopathic effect had developed. Virus stock had a titer of more than 10⁶ plaque-forming units (PFU) per 0.1 mL, determined by plaque assay. The suspension was stored at ~80°C until use.

Treatment of Mice  Four-week-old, inbred, certified virus-free BALB/c BYJ mice (nu/+ and nu/nu) were inoculated intraperitoneally with 0.2 mL of virus suspension containing 6 x 10⁶ PFU/0.2 mL.

Received October 14, 1993; revision accepted February 25, 1994.

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This work was presented in part at the 39th Annual Scientific Session of the American College of Cardiology, March 1990.

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They were maintained in laminar-flow isolation rooms throughout the study period. It has been reported that BALB/c BYJ mice, which belong to the Andervont family, develop CD4+ cell- and IL-2–dependent autoimmunity.23 Recombinant human IL-2 was kindly supplied by Shionogi Pharmaceutical Co., Ltd., Osaka, Japan. IL-2 was administered subcutaneously daily at 0.2 mL per mouse per day. 

**Experiment 1**

This experiment (early protocol) was carried out to determine the effects of IL-2 on acute viremic CB3 myocarditis. Sixty mice were randomized to group 1 (n=30, no IL-2 treatment) or group 2 (n=30, treatment with IL-2). Starting simultaneously with the virus inoculation, treatment was administered daily for 7 days. The mice were observed daily, and necropsy was performed immediately on mice that were found dead. Mice surviving until the end of the treatment period were killed by bleeding from the retro-orbital plexus and then were necropsied; the spleens were processed for NK cell activity study and the hearts for pathological and virologic studies. Other organs were processed for pathological study.

Additional groups of uninfected mice treated for 7 days with saline (n=3) or IL-2 (5×10^6 U per mouse per day) (n=3) also were prepared; the spleens were processed for the assay of NK cell activity and other organs for pathological study.

**Experiment 2**

This experiment (late protocol) was carried out to determine the effects of IL-2 on subacute viremic CB3 myocarditis. Altogether, 98 mice were inoculated with virus and randomized to the treatment or control groups. Before treatment began, 5 mice in each group were killed and processed for pathological study to confirm group homogeneity (data not presented). At 7 days when treatment began, only 48 mice were still alive: 18 in the control group (group 3, n=18; no IL-2 treatment) and 30 in the group to be treated (group 4, n=30; IL-2 treatment). Treatment was administered daily for 7 days until 14 days after virus inoculation, when surviving mice were killed and necropsied. The mice were observed daily, and necropsy was performed on all mice that died during the course of the study.

Analysis of splenic NK cell activity and the myocardial immunohistological examination were performed on day 10 in subsampled mice in both groups (each n=4). Thus, the numbers of the subsampled mice were subtracted from those in the mortality study in both groups.

**Experiment 3**

We investigated the changes of cardiac pathology in CB3-infected nude mice (BALB/c-nu/nu) with or without IL-2 treatment (nude mouse protocol) in parallel with experiment 2. Twenty-one 4-week-old, male, athymic nude mice were inoculated intraperitoneally with 6×10^3 PFU of CB3 on day 0. Sixteen mice that survived for 7 days after the virus inoculation were divided into groups and received the same treatment as in experiment 2 (starting on day 7); they served as the controls for the in vivo administration of IL-2 in T-cell–depleted mice. On day 14, the mice were killed, and their organs were processed for pathological study.

**NK Cell Assay**

NK cell assay was performed on day 7 in experiment 1 and on day 10 in experiment 2. YAC-1 cells (NK cell sensitive), a Moloney virus–induced lymphoma of A/Sn mice, were used for the assay of NK cell activity. The cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS).

**Spleen Cells**

Spleens were mechanically dissociated with a 23-gauge needle in RPMI-1640 medium. The cell suspension was collected, and erythrocytes were lysed with 0.85% NH₄Cl in Tris buffer (pH 7.2) followed by two washes with RPMI-1640 medium. To remove macrophages, spleen cells were incubated in RPMI-1640 medium with 10% silica suspension and 10% autologous fresh serum for 45 minutes at 37°C. The cell suspensions were carefully overlaid on Ficoll-Paque (Pharmacia, Inc.) and then centrifuged at 3000 rpm for 30 minutes. The cells remaining on the interface were collected, washed twice, and resuspended in RPMI-1640 medium with 10% FCS.

**Cytotoxicity Assay**

Target cells (1×10⁶) were labeled with 3.7 MBq ⁵¹Cr for 60 minutes at 37°C in 5% CO₂. Labeled target cells were washed three times, and 1×10⁶ cells/well were added to flat-bottomed, 96-well microtiter plates (Corning) together with effector cells at effector–target cell (ET) ratios of 12.5:1, 25:1, and 50:1. After 4 hours of incubation at 37°C, the supernatant was collected using a supernatant-collecting system made by Skatron, and the amount of ⁵¹Cr released into the supernatant fluid was measured. The percent cytotoxicity was calculated with the formula

\[
\text{% Cytotoxicity} = \frac{(E-S)}{(M-S)} \times 100
\]

where E is the counts per minute (cpm) released in the presence of effector cells, S is the cpm released from target cells incubated alone in medium, and M is the cpm released from target cells incubated alone followed by 0.1N HCl treatment. All experimental points shown in the text are the mean of duplicate or triplicate samples.

**Pathologic Study**

Tissues (heart, thymus, spleen, lung, liver, kidney, psoas muscle, and pancreas) were processed by standard methods, embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin. Myocardial sections were graded by two of the authors, who were blinded to the respective treatment groups for the severity of infiltration and necrosis of the ventricles. The mean values are cited. The pathological criteria for grading the severity of infiltration and necrosis were grade 1 (mild), one or two small foci; grade 2 (slight), several small foci; grade 3 (moderate), multiple small foci or several large foci; and grade 4 (severe), multiple large foci, diffuse infiltration, or necrosis. The other organs were evaluated for evidence of viral or other pathological lesions. Immunohistological study was performed on day 7 in experiment 1 and on day 10 in experiment 2. The method was similar to that used in previous studies.8–8 In brief, cryostat heart tissue sections (6 μm) were incubated for 1 hour at 37°C with a series of rat anti-mouse monoclonal antibodies that recognized Thy 1.2 (CD3, Becton-Dickinson), L3T4 (CD4, Becton-Dickinson), or Lyt 2 (CD8, Becton-Dickinson)–positive cells, followed by visualization by an indirect immunoperoxidase method. For the quantitation of positive-stained lymphocyte subpopulations, we recorded the number of lymphocytes in each section that were stained by each monoclonal antibody recorded along with the total number of nucleated cells, and we calculated the percentage of stained lymphocytes calculated, as described before.8–8

**Virologic Study**

For the infectivity assays, the hearts were removed aseptically, weighed, and homogenized in 2 mL of phosphate-buffered saline. After centrifugation at 1500 rpm for 15
minutes, the resultant supernatants were inoculated into VERO cells, and plaque assays were performed.\textsuperscript{6-8}

Statistics

ANOVA for multiple sample comparisons (Bonferroni) was used to evaluate differences in pathological scores, NK cell activity, immunohistological data, and myocardial virus titers. Kaplan-Meier plots were made of the survival data, and differences between the control and treatment survival curves were evaluated by the Mantel-Cox log-rank test.\textsuperscript{25} \(\chi^2\) analysis with Yates’ correction was used to compare the incidence of congestive failure by gross appearance. A probability value of <.05 was considered statistically significant.

Results

Infection with CB3 produced a similar pathological picture to that reported previously.\textsuperscript{6-8} In brief, 3 days after the virus inoculation, the mice appeared ill; some developed coat ruffling, weakness, and irritability. In gross appearance, the myocardium had pale yellow patches that correlated with the infiltration, necrosis, and calcification seen microscopically. After day 7, myocardial necrosis and inflammatory cell infiltrations were more extensive (Fig 1). Pleural effusion, ascites, and congestion of the lungs and liver were noted thereafter.\textsuperscript{6-8}

Mortality

In experiment 1, the survival rate of the IL-2–treated group (group 2) was significantly higher (\(P<.01\)) than that of the untreated group (group 1). The survival rate on day 7 in each group was 33.3% for group 1 (10 of 30) and 80.0% for group 2 (24 of 30). In experiment 2, in contrast, the survival rate of the IL-2–treated group (group 4) was lower (\(P<.01\)) than that of the control group (group 3). The survival rate on day 14 in each group was 71.4% for group 3 (10 of 14) and 7.7% for group 4 (2 of 26).

There were no deaths throughout the treatment period in the uninfected (IL-2–treated or untreated) groups. (See Fig 2.)
Cardiac Pathology

The incidence of myocarditis was 100% in each infected group in both experiments. In experiment 1, both cellular infiltration and myocardial necrosis were significantly less severe in the IL-2-treated group (group 2) than in the untreated group (group 1). However, in experiment 2, both cellular infiltration and myocardial necrosis were significantly more severe in group 4 (IL-2 treated, n=30) than in group 3 (IL-2 untreated, n=18).

Other Organ Involvement in Experiments 1 and 2

Overall, pancreatic lesions, likely of viral origin, were noted in some mice in each infected group. No viral lesions were noted in the lungs, liver, kidney, thymus, spleen, or muscle. Although there was no difference in the incidence of congestive heart failure in experiment 1 (3 of 30 in the untreated group vs group 1 and 7 of 30 in the treated group vs group 2), pleural effusion, ascites, and congestion of the lungs and liver were noted in more mice in group 4 (24 of 30; P<.01) than in group 3 (5 of 18) in experiment 2.

Myocardial Virus Titers

The myocardial virus titers of the IL-2-treated mice (group 2) were lower than those of the untreated mice (group 1) on day 7 in experiment 1. There were no viruses isolated on day 10 in either group in experiment 2. (See Table 1.)

NK Cell Activity

NK cell activity at various ET ratios in IL-2-treated (groups 2 and 4) and untreated (groups 1 and 3) mice is shown in Fig 4. In experiment 1, NK cell activity in IL-2-treated mice (70.7±19.1% and 42.7±16.7% at ET ratios of 50:1 and 25:1, respectively) was significantly higher than that in untreated mice (30.4±7.2% and 15.8±5.1%). In experiment 2, the NK cell activity did not differ significantly in groups 3 and 4 (12.5±9.4% and 9.4±13.3% at ET ratios of 25:1 and 12.5:1, respectively, in group 3 versus 20.5±6.9% and 16.1±9.1% in group 4).

NK cell activity in uninfected mice was 66.8±17.0% at an ET ratio of 50:1 (P<.01 versus untreated, NS versus group 2), 42.2±18.9% at an ET ratio of 25:1 (P<.05 versus untreated, NS versus group 2) in the IL-2-treated group (n=3), and 3.8±1.2% (P<.001 versus group 1) and 2.2±1.8% (P<.01 versus group 1) in the untreated group (n=3).

Immunohistological Study

Immunohistological study of the heart confirmed the results of previous studies: most of the stained cells on days 7 and 10 were CD3, CD4, and CD8 positive. The percentage of CD8-positive cells exceeded that of CD4-positive cells. Although there were no significant differences between the two groups in experiment 1, the

Table 1. Myocardial Virus Titers in Experiments 1 and 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>IL-2 Treatment</th>
<th>PFU/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>No</td>
<td>1.0±0.7x10^3 (n=6)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Yes</td>
<td>1.9±1.8x10^4 (n=6)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>No</td>
<td>ND (n=4)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Yes</td>
<td>ND (n=4)</td>
</tr>
</tbody>
</table>

IL indicates interleukin; PFU, plaque-forming units; and ND, not detected.

The table shows the PFU/g tissue levels of myocardial virus titers in experiments 1 and 2.
percentage of T-series lymphocytes in the diseased myocardium on day 10 in IL-2–treated mice was significantly higher than that in IL-2–untreated mice in experiment 2. This finding indicated that the in vivo administration of IL-2 possibly increased the accumulation of T lymphocytes in the inflamed myocardium in subacute (immune phase) CB3 myocarditis. (See Table 2.)

Nude Mouse Study (Experiment 3)

The survival rates of IL-2–treated and untreated groups did not differ significantly. The pathological study showed that the severity of myocarditis was very mild in both groups of mice (IL-2 treated and untreated) and that there were no significant differences in the pathological scores of the IL-2–treated and untreated groups; these results strongly indicated that IL-2, administered in vivo, acts via T lymphocytes. (See Table 3.)

Discussion

This study clearly demonstrates that IL-2 has the capacity to limit myocardial virus replication by enhancing NK cell activity in the acute, viremic stage of CB3 myocarditis, resulting in a reduction of cardiac pathology; however, in the later stage, in contrast, IL-2 exacerbated the course and severity of the disease by increasing the number of T cells infiltrating the myocardium. In other words, IL-2 has differential effects on acute CB3 myocarditis. This finding may be of great value in increasing our understanding of the pathogenesis of this condition.

The murine model of CB3-induced myocarditis has been shown to closely mimic the pathology of human myocarditis.1,3 The myocardial lesions are focal, consisting of necrotic myocytes within an infiltration of mononuclear and polymorphonuclear cells. Most animal studies suggest that the heart lesions result not only from virus-induced direct myocytolysis but also from immune responses to virus-infected cells in the heart.26–29 A cell-mediated reaction to virus-induced neantigen in heart tissues of infected mice has been strongly implicated in the pathogenesis of CB3-induced myocarditis,26–29 and cytotoxic T lymphocytes have been proposed to be the major effector cells involved in the immunopathology.6–8,26–29

In experiment 1, there was a correlation between virus titers and NK cell activity, suggesting that a primary role of NK cells in this model is to reduce CB3 titers in heart tissues early in the course of infection. Presumably, NK cell activity also reduces some of the subsequent pathology. Godney and Gaunt30,31 also found evidence that NK cells played a role in limiting virus replication in the heart tissues of CB3-inoculated mice, which in turn ameliorated the severity of the myocarditis. CB3 infection per se stimulated NK cell activity, which was further stimulated by IL-2 administration.

Experiment 2 provides evidence that the in vivo administration of IL-2 increased the number of T cells infiltrating the myocardium, thus exacerbating the severity of myocardial damage. In vivo–stimulated T cells might damage both CB3-induced inflamed and intact myocytes. In vivo–generated T cells have been shown to kill fresh autologous, syngeneic, and allogeneic tumor cells (probably by the recognition of tumor antigenicity).19–22 Our findings demonstrated that the in vivo–stimulated T cells derived from the aviremic stage, when immune or autoimmune mechanisms may operate in the development of myocarditis, increased the severity of myocarditis. Stimulated T cells induced by IL-2 administration that might include a major population of cytotoxic T cells caused the severe myocardial damage. Regarding the survival rate, especially in experiment 2, it is conceivable that, when congestive heart failure caused by severe myocardial damage developed, IL-2 side effects, that is, peripheral and pulmonary edema, hypotension, and tachycardia,32 may have been superimposed.

### Table 2. Myocardial Lymphocyte Subsets in Animals With and Without Interleukin-2 Treatment

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1 (Day 7, n=4)</th>
<th>Experiment 2 (Day 10, n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 (IL-2 untreated)</td>
<td>Group 2 (IL-2 treated)</td>
</tr>
<tr>
<td>CD3, %</td>
<td>21.3±5.4</td>
<td>22.3±3.9</td>
</tr>
<tr>
<td>CD4, %</td>
<td>8.5±4.7</td>
<td>10.0±3.3</td>
</tr>
<tr>
<td>CD8, %</td>
<td>13.3±7.0</td>
<td>13.5±3.1</td>
</tr>
</tbody>
</table>

IL indicates interleukin.

Myocardial lymphocyte subsets in animals infected with coxsackievirus B3 were determined on day 7 in experiment 1 and on day 10 in experiment 2. Cryostat sections were stained with monoclonal antibodies and by the horseradish immunoperoxidase technique. Percentage of positively stained cells was determined in a blind manner.

*P<.05 vs group 3. Values are mean±SD.

### Table 3. Results of Experiment 3

<table>
<thead>
<tr>
<th>IL-2 Treatment</th>
<th>Survivors (n)</th>
<th>Cardiac Pathology</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infiltration</td>
<td>Necrosis</td>
<td></td>
</tr>
<tr>
<td>No (n=8)</td>
<td>6</td>
<td>1.4±0.5</td>
<td>1.6±0.5</td>
<td></td>
</tr>
<tr>
<td>Yes (n=8)</td>
<td>5</td>
<td>1.5±0.8</td>
<td>1.4±0.5</td>
<td></td>
</tr>
</tbody>
</table>

IL indicates interleukin.

This study was conducted to determine the effects of IL-2 administration on T-cell–depleted mice. For this study, we used the protocol used in experiment 2. There was no significant difference between IL-2–treated and untreated groups in cardiac pathological scores. For myocardial lesions, cellular infiltration and myocardial necrosis were scored blindly, on a scale of 1+ to 4+ in terms of severity.
In experiment 3, the lack of effect of IL-2 administration on nude mice was confirmed. Estrin and Huber et al.\textsuperscript{33,34} clearly demonstrated that two cytotoxic T-lymphocyte populations were recognized in CB3-infected BALB/c mice, one population belonging to the Lyt 2\textsuperscript{*} T-lymphocyte subset (CD8) and reacting specifically with intact myocytes (autoreactive T) and the other belonging to the L3T4\textsuperscript{*} T-lymphocyte subset (CD4) and reacting with damaged myocytes. Taking our findings together with those of Huber et al, we can postulate that in vivo-stimulated T cells induced by IL-2 may damage both inflamed and intact myocardium, since they may consist of both Lyt 2\textsuperscript{*} and L3T4\textsuperscript{*} T cells.\textsuperscript{9-11} More recently, Zhang et al.\textsuperscript{35} reported the cardiotoxicity of IL-2 in a morphological study. They found that the myocardial damage caused by the in vivo administration of IL-2 was triggered by IL-2-activated lymphocytes; these lymphocytes had a cytolytic effect on endothelial cells and cardiac myocytes, producing lesions that involved both the cardiac microcirculation and the muscle cells.\textsuperscript{35}

It is considered that a biphasic disease process results when mice are infected with CB3.\textsuperscript{1,3} During the acute phase, viral replication in the myocardium results in acute myocardial necrosis with inflammation. The results of experiment 1 strongly support this evidence. After the virus has been eliminated from the myocardium, a subacute or chronic inflammatory reaction\textsuperscript{36} results in progressive damage and hypertrophy, ventricular dilation, and heart failure. So-called persistent infection also has been documented.\textsuperscript{36} The present study appears to confirm the underlying biphasic disease process in the pathogenesis of myocarditis; in CB3 myocarditis, primary myocyte damage is a direct result of virus replication as noted above, and subsequent damage is T-cell dependent.

It has been suggested that other cytokines also exert important effects on inflammatory myocardial disease. For example, several studies have suggested that the blockade of leukocyte adhesion is important in limiting inflammatory injury.\textsuperscript{37,38} Both tumor necrosis factor (TNF) and IL-1 inhibit the response of isolated myocytes to \( \beta \)-adrenergic stimuli.\textsuperscript{39} Furthermore, TNF is an important mediator and interferon gamma is an important regulator early in the pathogenesis of myocardial inflammation in myosin-induced myocarditis.\textsuperscript{40}

In conclusion, our findings show that IL-2 has differential effects on acute experimental CB3 myocarditis. Namely, in the acute, viremic stage, IL-2 limits myocardial virus replication by enhancing NK cell activity, resulting in a reduction of cardiac pathology. In the subacute, aviremic stage, in contrast, IL-2 exacerbates the course and the severity of the disease by increasing the number of T cells infiltrating the myocardium. IL-2 is beneficial if treatment is given early but not later in murine CB3 myocarditis.

Acknowledgments
This work was supported in part by research grants from the Japanese Heart Foundation, Education Fellowship of Toyama Medicine and Pharmaceutical University, Iwaki Fellowship, Japanese Education of Science and Welfare (Nos. 01570478 and 03670445), and Uehara Memorial Foundation.

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_Circulation_. 1994;89:2836-2842
doi: 10.1161/01.CIR.89.6.2836

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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