MCI-186 (edaravone), a novel free radical scavenger, protects against acute autoimmune myocarditis in rats

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Nimata, Masaomi, Taka-aki Okabe, Miki Hattori, Zuyi Yuan, Keisuke Shioji, and Chiharu Kishimoto. MCI-186 (edaravone), a novel free radical scavenger, protects against acute autoimmune myocarditis in rats. Am J Physiol Heart Circ Physiol 289: H2514–H2518, 2005.—In this study, we tested the hypothesis that MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one; edaravone), a novel free radical scavenger, protects against acute experimental autoimmune myocarditis (EAM) in rats by the radical scavenging action associated with the suppression of cytotoxic myocardial injury. Recent evidence suggests that oxidative stress may play a role in myocarditis. We administered MCI-186 intraperitoneally at 1, 3, and 10 mg·kg\(^{-1}\)·day\(^{-1}\) to rats with EAM for 3 wk. The results were compared with untreated rats with EAM. MCI-186 treatment did not affect hemodynamics. MCI-186 treatment (3 and 10 mg·kg\(^{-1}\)·day\(^{-1}\)) reduced the severity of myocarditis as assessed by comparing the heart-to-body weight ratio and pathological scores. Myocardial interleukin-1β (IL-1β)-positive cells and myocardial oxidative stress overload with DNA damage in rats with EAM given MCI-186 treatment were significantly less compared with those of the untreated rats with EAM. In addition, MCI-186 treatment decreased not only the myocardial protein carbonyl contents but also the myocardial thiobarbituric acid reactive substance products in rats with EAM. The formation of hydroxyl radicals in MCI-186-treated heart homogenates was decreased compared with untreated heart homogenates. Furthermore, cytotoxic activities of lymphocytes of rats with EAM treated with MCI-186 were significantly lower compared with those of the untreated rats with EAM. Hydroxyl radicals may be involved in the development of myocarditis. MCI-186 protects against acute EAM in rats associated with scavenging hydroxyl free radicals, resulting in the suppression of autoimmune-mediated myocardial damage associated with reduced oxidative stress state.

lymphocytes; immune system; inflammation

IN PATIENTS WITH HEART FAILURE, reactive oxygen species (ROS) have been found to be elevated in plasma (2). Acute myocarditis is a potentially lethal disease and frequently precedes the development of acute and chronic heart failure. Two mechanisms to explain how myocarditis develops into heart failure have been proposed: one is a persistent viral or etiologic agent infection, and the other is a progressive autoimmune myocardial injury. The autoimmune giant cell myocarditis in rats mimics human fulminant myocarditis with heart failure (12).

Excess amount of cytokine induced by inflammatory stimuli contributes to the progression of myocardial damage in myocarditis (1, 5, 7, 15, 16, 22, 23). Also, recent reports indicated that myocardial injury was produced by ROS (18, 19, 29). Lipid peroxidation is also caused by ROS (20), resulting in cell membrane damage. As proposed, MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one; edaravone) is a novel free radical scavenger (8, 27, 28) and may be an effective agent for myocardial inflammation by inhibiting overproduced cytokines (24). The purpose of the present study was to examine the effects of MCI-186 on experimental autoimmune myocarditis (EAM), focusing on its inhibitory effects on the inflammatory cytokines and on free radical generations with the analysis of cytotoxic activities of lymphocytes.

MATERIALS AND METHODS

Immunization. Acute EAM was induced in 6-wk-old Lewis rats by subcutaneous injection of 0.2 ml of porcine cardiac myosin (10 mg/ml; Sigma), mixed with an equal volume of Freund’s complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37Ra (Difco) in the foot pads on days 1 and 8, as previously described (12, 24). Control rats were immunized with FCA alone. The animals were observed up to 21 days. The day of injection was designated day 1.

Medication experiment. To analyze the effects of MCI-186 on the rats with or without EAM, the animals were divided into four groups and treated with either 1) vehicle (saline, n = 17), 2) low-dose MCI-186 (1 mg·kg\(^{-1}\)·day\(^{-1}\), n = 14) (MCI-1), 3) middle-dose MCI-186 (3 mg·kg\(^{-1}\)·day\(^{-1}\), n = 14) (MCI-3), or 4) high-dose MCI-186 (10 mg·kg\(^{-1}\)·day\(^{-1}\), n = 14) (MCI-10) intraperitoneally daily for 3 wk. MCI-186 was kindly supplied by Mitsubishi Pharma (Tokyo).

The protocol was approved by the Institutional Animal Research Committee of Kyoto University.

Histopathology. When animals were killed, microscopic findings of myocardial damage and cellular infiltration were graded on a scale of 0–4, as previously described (19). We used an immunoperoxidase technique to perform immunohistochemistry for interleukin-1β (IL-1β) staining, as previously described (24). To analyze the myocardial oxidative stress overload, immunohistochemistry for thioredoxin (TRX), which is a redox-active protein and considered as a marker of oxidative stress, was performed as previously described (18, 19, 29). Also, to analyze the myocardial DNA damage, 8-hydroxy-2'-deoxyguanosine (8-OHdG) staining was performed, as previously described (11, 19, 25, 26).

Detection of myocardial oxidized proteins. Oxidative inactivation of enzymes and oxidative modification of protein by metal-catalyzed oxidation reactions are accompanied by the generation of protein carbonyl derivatives. Thus oxidized protein was detected using an oxidized protein detection kit (OxyBlot, Oncor) as described previously (18). The OxyBlot provides reagents for sensitive immunodetection of carbonyl groups, which is a hallmark of the oxidation status of all proteins.

Electron spin resonance spectroscopy. The hearts from rats with and without EAM were homogenized in cold PBS (0.2 g hearts/ml) and then added to 0.05 ml of 9.0 ml of 5,5′-dimethyl-1-pyrroline-1-

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oxide (DMPO) (Labotec). The generation of hydroxyl radicals (·OH) was observed as DMPO·OH adduct on a JEL-390 spectrometer, as previously described (18). Quantification of the DMPO signal intensity was performed by comparing the observed signal to a standard Mn2+ marker; the hydroxyl radical signal relative to the internal standard of manganese ion was calculated.

Thiobarbituric acid reactive substance assay. Lipid peroxide formation in the myocardium was determined using a modified thiobarbituric acid reactive substance (TBARS) method for estimating malondialdehyde as described previously (9).

Cytotoxicity assays. Lewis rats were immunized with cardiac myosin, treated with high-dose MCI-186 or PBS, and killed on day 22. Lymph node cells from rats treated with high-dose MCI-186 or PBS were used as effector cells. Neutonal cardiomyocytes (2 × 10^4/well) plated in 96-microwell plates were labeled with sodium chromatates at 1 µCi/well (51Cr, Amersham International) for 1 h as previously described (18, 29). After labeled target cells had been washed with PBS three times, lymph node cells were incubated at an effector-to-target ratio (E/T) of 50:1, 100:1, and 200:1 for 4 h. The supernatant was collected, and the radioactivity of 51Cr released into the supernatant was measured by a gamma counter. The percentage of cytotoxicity was calculated using the formula

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

where E is the counts per minute (cpm) released in the presence of effector cells, S is the spontaneous cpm released from target cells incubated in the medium, and M is the maximal cpm released from target cells incubated with 2% Triton X-100.

Statistics. All values were expressed as means ± SD. One-way ANOVA followed by Fisher’s protected least significant difference test and χ² test were performed. A value of P < 0.05 was considered statistically significant.

RESULTS

There were no significant pathological changes in the myocardium of rats without EAM (control rats) by the MCI-186 treatment (0, 1, 3, and 10 mg·kg^-1·day^-1) (data not shown).

There were no significant changes of heart rate (HR) or blood pressure (BP) by the MCI-186 treatment (0, 1, 3, and 10 mg·kg^-1·day^-1) among the four groups with or without EAM (data not shown).

Histopathology and heart-to-body weight ratio in rats with acute EAM. On day 22 at time of death, the hearts showed severe and diffuse discolored myocarditis in rats immunized with cardiac myosin. Extensive injuries to myocytes with inflammatory changes and giant cells (arrows, Fig. 1) were observed. Treatment with MCI-186 at 3 and 10 mg·kg^-1·day^-1 but not with MCI-186 at 1 mg·kg^-1·day^-1 reduced the severity of the disease, as assessed by measuring heart weight-to-body weight ratio and microscopic scores (Table 1 and Fig. 1).

Myocardial IL-1β, TRX, and 8-OHdG expression. Immunohistochemistry showed that IL-1β-positive cells were localized mainly in infiltrating inflammatory cells (Fig. 1). MCI-186 treatment markedly reduced the number of IL-1β-positive cells, the intensity of TRX stain, and 8-OHdG-positive cells in the inflammatory lesions compared with rats with EAM treated with vehicle (Fig. 1). These findings obtained by immunohistochemical studies were constant.

Changes in the oxidized proteins in myocardium. In rats with acute EAM, the myocardial carbonyl contents were markedly increased (Fig. 2). It was thus suggested that cellular protein oxidative damage was increased in acute EAM. The increased protein carbonyl contents were reduced by MCI-186 treatment compared with the untreated rats, suggesting that MCI-186 could prevent cellular proteins from oxidative damage (Fig. 2).

Electron spin resonance spectrometric analyses. The formation of hydroxyl radical was detected in the myocarditic heart homogenates and was decreased in the MCI-186-treated myocarditic heart homogenates compared with the untreated ones (Table 2). The hydroxyl radical signals relative to the internal standard of manganese ion in the MCI-186-treated hearts were lower compared with those in the untreated ones (Table 2).

TBARS products in myocardium. The myocardial TBARS contents in myocarditis were significantly higher than in controls (Table 2). The myocardial TBARS contents were significantly lower in the MCI-186 groups compared with untreated hearts (myocarditis) (Table 2).

MCI-186 suppressed the cytotoxic activity of lymphocytes in rats with EAM. The generation and significance of the so-called cytotoxic lymphocytes in this animal model were already reported (12, 28).

The cytotoxic activities of lymphocytes against cardiomyocytes were examined. At any E/T, the cytotoxic activities of lymphocytes in rats with EAM were higher compared with control (unimmunized) rats (Fig. 3). At an E/T of 200:1, the cytotoxic activities of lymphocytes of rats with EAM treated with MCI-186 were significantly suppressed compared with those of rats with EAM treated with saline (Fig. 3). These findings suggest that MCI-186 protects against myocarditis associated with suppression of the cytotoxic activities of immune effector cells.

DISCUSSION

The present findings clearly demonstrated that MCI-186, a novel free radical scavenger, reduced the severity of acute EAM in rats and that the cardioprotection of MCI-186 may be due not only to the suppression of inflammatory cytokines but to scavenging action for hydroxyl radicals, possibly by the modifications of oxidative stress, associated with the suppression of cytotoxic activities of lymphocytes.

Several clinical studies have described the participation of proinflammatory cytokines in the pathogenesis of cardiac disease (1, 7, 15, 16, 22, 23). In the present study, MCI-186 treatment suppressed the severity of acute EAM. Immunohistochemical study showed that the numbers of IL-1β-positive myocardial cells, the intensity of myocardial TRX stain, and 8-OHdG-positive cells were reduced by MCI-186 treatment. It was already established that the degree of myocardial TRX staining was inversely correlated with that of oxidative stress overload (18, 19, 29). 8-OHdG, one of the major DNA base-modified products, is an established marker for DNA damage, is induced either by hydroxyl radicals, singlet oxygen, or photodynamic action, and is known to be mutagenic in its pairing with adenine as well as cytosine, leading to G·C to T·A transversion at DNA replication (26). Accordingly, MCI-186 treatment for rats with EAM protects against myocardial DNA damage by reducing oxidative stress.

Why did MCI-186, a free radical scavenger, reduce the myocardial damage in murine autoimmune myocarditis? The beneficial effects of MCI-186 in EAM may be at least partly due to the suppression of inflammatory cytokines as well as oxidative stress. In another model, it was reported that liver...
necrosis was modified with MCI-186 treatment by suppressing inflammatory cytokines (3). Consistent with this report, the present study showed that MCI-186 suppressed the expression of IL-1β-positive cells in the inflammatory lesions. Next, MCI-186 scavenged the generation of hydroxyl radicals in heart homogenates ex vivo. As a result, it was demonstrated in OxyBlot and TBARS studies that MCI-186 treatment protected against cellular protein oxidative damage in vivo. It was demonstrated that free radicals, especially hydroxyl radicals, play an important role in the development of heart failure in various animal models (3, 6, 13, 17, 18). Accordingly, MCI-186 may act for scavenging pathognomonic radicals, resulting in less severe myocardial damage in this animal model.

In addition, MCI-186 suppressed not only myocardial protein oxidation but also the so-called cytotoxic activity of lymphocytes in rats with EAM. Recent evidence suggests that ROS are involved in the antigen-presenting function of dendritic cells and immune effector cells and that antioxidants...
suppress the activation these cells (14). In an animal model of EAM, it was already reported that dendritic cells play a crucial role in the autoimmune process for the development of disease (12, 20). Accordingly, MCI-186 treatment resulted in the reduction of autoimmune mechanisms by suppressing antigen presentation of dendritic cells, which may reflect the suppression of the cytotoxic activities of lymphocytes in this study.

Recently, there is increasing evidence supporting the critical role of free radicals in the development of heart failure (4, 9, 21). The rationale for prescribing MCI-186 in patients with heart failure is based on the radical-scavenging action and on the capacity of the drug to reduce free radicals as well as to prevent progression of myocardial dysfunction. In our study, MCI-186 treatment for 3 wk ameliorated myocarditis by comparing heart-to-body weight ratio and microscopic scores. Thus the results of the current study may add another potential utility of antioxidants for the treatment of heart failure caused by autoimmune mechanisms.

In conclusion, hydroxyl radicals may be involved in the development of myocarditis. MCI-186 protects against acute EAM in rats by scavenging hydroxyl free radicals, resulting in the suppression of autoimmune-mediated myocardial damage associated with reduced oxidative stress state.

Table 1. Results of MCI-186 treatment on rats with EAM

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>HW/BW, mg/g</th>
<th>Microscopic Scores (0 to +4)</th>
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<tr>
<td>Myocarditis</td>
<td>17</td>
<td>4.54±0.81</td>
<td>2.12±1.41</td>
</tr>
<tr>
<td>MCI-1</td>
<td>14</td>
<td>4.53±0.93</td>
<td>2.14±1.29</td>
</tr>
<tr>
<td>MCI-3</td>
<td>14</td>
<td>4.08±0.52</td>
<td>1.86±1.23*</td>
</tr>
<tr>
<td>MCI-10</td>
<td>14</td>
<td>3.84±0.68*</td>
<td>0.79±1.05†</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of rats. Myocarditis, rats with myocarditis; MCI-1, MCI-3, and MCI-10 refer to MCI-186 at 1, 3, and 10 mg·kg⁻¹·day⁻¹, respectively; HW/BW, heart-to-body weight ratio; EAM, experimental autoimmune myocarditis. *P < 0.05, †P < 0.01 vs. myocarditis.

Table 2. ESR spectrometric analysis and myocardial TBARS

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hydroxyl Radical Signal Relative to Internal Standard</th>
<th>TBARS, nmol/g heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>45.8±12.6 (n=5)</td>
</tr>
<tr>
<td>Myocarditis</td>
<td>0.98±0.05 (n=8)</td>
<td>294.3±40.7 (n=10)</td>
</tr>
<tr>
<td>MCI-1</td>
<td>0.90±0.12 (n=8)</td>
<td>240.7±31.6* (n=10)</td>
</tr>
<tr>
<td>MCI-3</td>
<td>0.85±0.09* (n=8)</td>
<td>120.4±23.8† (n=10)</td>
</tr>
<tr>
<td>MCI-10</td>
<td>0.72±0.08† (n=8)</td>
<td>95.8±21.0† (n=10)</td>
</tr>
</tbody>
</table>

Values are means ± SD. ESR, electron spin resonance; TBARS, thiobarbituric acid reactive substance; ND, not detected. *P < 0.05, †P < 0.01 vs. myocarditis.

Fig. 2. Changes in myocardial protein carbonyl contents. A: Oxyblot analysis; 10 μg of each protein sample were loaded. Control, normal rat; myocarditis, untreated rat with experimental autoimmune myocarditis (EAM); MCI-1, MCI-186 (1 mg·kg⁻¹·day⁻¹)-treated rat with EAM; MCI-3, MCI-186 (3 mg·kg⁻¹·day⁻¹)-treated rat with EAM; MCI-10, MCI-186 (10 mg·kg⁻¹·day⁻¹)-treated rat with EAM. MW, molecular weight markers. B: densitometric analysis of relative protein carbonyl contents (each n = 4). In rats with myocarditis, protein carbonyl contents were increased and were decreased by MCI-186 treatment. †P < 0.01 vs. control. *P < 0.01 vs. myocarditis.

Fig. 3. Cytotoxic activities of lymphocytes against myocytes. At an effector/target ratio of 200:1, the cytotoxic activities of lymphocytes of rats with EAM treated with MCI-186 (10 mg·kg⁻¹·day⁻¹) were significantly suppressed compared with those of rats with EAM treated with saline (each trial n = 6). *P < 0.01 vs. myocarditis.
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GRANTS

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