below 20% (day 9; Figure 2A). Days 4 and 9 were therefore chosen as the sacrifice days in experiment 2.

WY14643-early and -late treatment had similar effects on the BW of KKAy mice, with the BW decreasing significantly after the injection of WY14643 to reach statistical significance on day 9 (Figure 2B).

BW and HW in experiment 2: After 3 days of prior treatment, the HW/BW ratio was lower in the WY14643-early group than in the control group, although the difference was not statistically significant. The HW in the two WY14643 treatment groups was markedly lower than in the vehicle group on day 4, although statistical significance was only found between the WY14643-early and vehicle groups after normalization by BW. The HW on day 9 was significantly lower in the two treatment groups than in the vehicle group, whereas HW/BW showed no significant differences between the 3 groups (Table I).

Pathological findings: Myocardial lesions and inflammatory cell infiltration were present in EMCv-inoculated KKAy mice sacrificed on days 4 and 9 (Figure 3). The pathological scores (Figure 4A) and number of infiltrating cells (Figure 4B) showed that treatment with WY14643 reduced the severity of the inflammation on both days, and was statistically significant on day 9.

As shown in Table II, LW, LC, and their ratio (LW/LC) were lower in the WY14643-early group than in control, with the latter two reaching statistical significance. The LW was smaller and the LC larger in the WY14643 treatment groups on day 4 than those in vehicle, with the comparison between the WY14643-late group and vehicle groups reaching statistical significance. The LW/LC ratio was therefore lower in the WY14643 treatment groups than in vehicle on day 4. The LC in the vehicle group on day 9 was markedly higher than for either the WY14643-early group or -late group, which means that the LW/LC ratio in the vehicle group was significantly smaller than for either of the other two groups. No significant difference was found between the WY14643-early and -late groups as regards LW, LC, and LW/LC on either day 4 or day 9.

The LV myocardial fiber diameters were smaller in the WY14643-early group than in the control on day 0, although the difference was not statistically significant. The diameters in the WY14643-late group on days 4 and 9 were significantly lower than those in vehicle ($P < 0.01$), and this significance also existed between the vehicle and WY14643-early groups on day 4, although not on day 9. The myocardial diameters in the WY14643-late group were significantly lower than those in the WY14643-early group

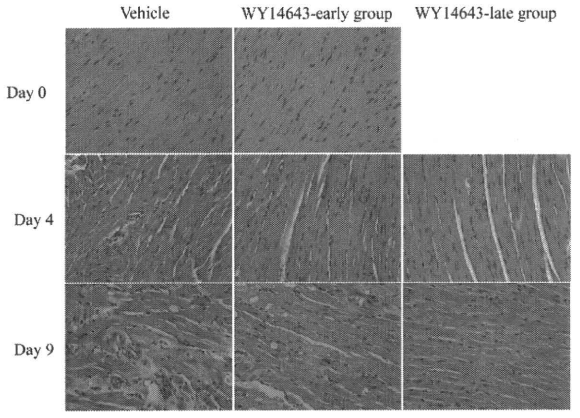

Figure 3. Cardiac pathological findings for KKAy mice on days 0, 4, and 9. Myocardial necrosis with inflammatory cell infiltration was found after viral inoculation. Treatment with WY14643 reduced the inflammation in heart tissue.

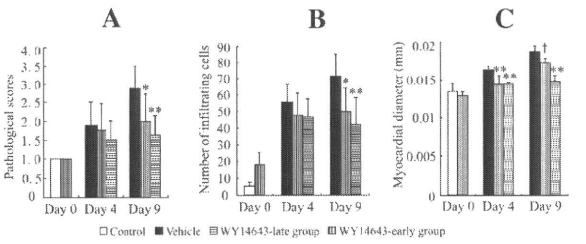

Figure 4. A: The pathological scores for heart tissue; **B:** the number of infiltration cells at high magnification (400 ×); **C:** the diameter of myocardial fibers in KKAy mice. Myocardial necrosis, pathological score, and the number of infiltration cells on day 9 were lower in the two WY14643 treatment groups, especially in the late treatment group. LV myocardial fiber diameters were greater in the vehicle group than in the two WY14643 treatment groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\dagger}P > 0.05$ with respect to vehicle.

Table II. Left Ventricular Wall Thickness (LW) and Cavity Dimension (LC), and Their Ratios

	n	LW (mm)	LC (mm)	LW/LC
Day 0				
Control	8	2.19 ± 0.45	1.21 ± 0.53	1.83 ± 0.36
WY14643-early group	8	2.01 ± 0.65	1.51 ± 0.07$^{\#}$	1.33 ± 0.49$^{\#}$
Day 4				
Vehicle	8	2.23 ± 0.28	0.57 ± 0.21	3.84 ± 2.12
WY14643-early group	8	1.97 ± 0.46	0.84 ± 0.31	2.33 ± 0.66
WY14643-late group	8	1.96 ± 0.44*	0.93 ± 0.12*	2.11 ± 0.27*
Day 9				
Vehicle	7	2.07 ± 0.22	1.51 ± 0.29	1.37 ± 0.25
WY14643-early group	9	1.95 ± 0.35	1.35 ± 0.46*	1.45 ± 0.63*
WY14643-late group	8	1.89 ± 0.53*	1.26 ± 0.44**	1.50 ± 0.55**

$^{\#}P < 0.05$ with respect to control; $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to vehicle on the corresponding day.

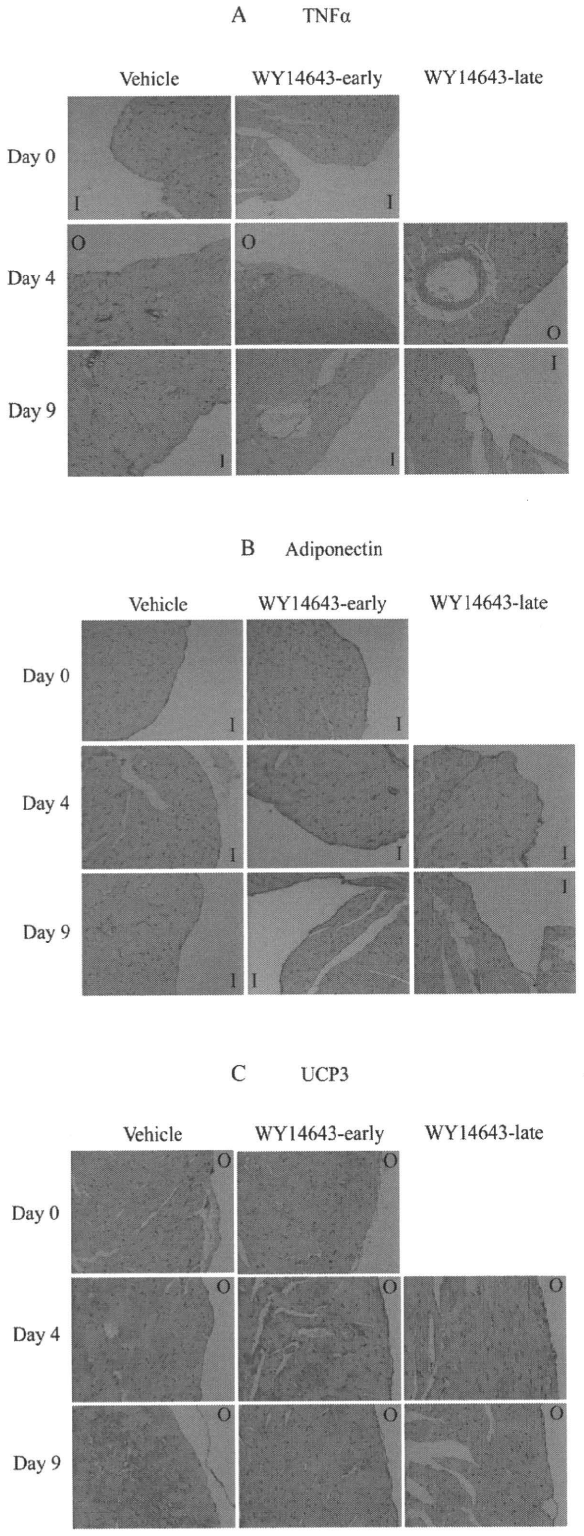

Figure 5. Immunohistochemical findings. Cardiac expression of TNF-α, adiponectin, and UCP3 is shown in A, B, and C respectively (magnification: 400 ×). I represents inner heart and O represents outer heart.

on day 9 (Figure 4C).

BG and plasma FFA: As shown in Table I, WY14643 treatment significantly ameliorated hyperglycemia in KKAy mice, with no significant difference being found between the two treatment groups. Interestingly, WY14643 increased plasma FFA levels on day 0 in the WY14643-early group and on day 4 in the WY14643-late group, but significantly decreased FFA levels on day 4 and day 9 in the WY14643-early group and on day 9 in the WY14643-late group.

Immunohistochemical findings: Cardiac TNF-α was positive in the vehicle group on days 4 and 9 but only weakly positive in the WY14643-early and WY14643-late group (Figure 5A); cardiac expression of adiponectin protein was more strongly positive in the two WY14643 treatment groups than that in the vehicle group (Figure 5B). Cardiac UCP3 was more strongly positive on day 4 in the two WY14643 treatment groups, but more strongly positive on day 9 in the vehicle group (Figure 5C). Cardiac adiponectin and UCP3 were positive on day 0, whereas TNF-α was nearly negative in the vehicle and WY14643 treatment groups (Figures 5A, 5B and 5C).

TNF-α, adiponectin, and UCP3 mRNA expression in heart tissue: The comparative expression levels of TNF-α, adiponectin, and UCP3 mRNA in heart tissue on days 0, 4, and 9 after EMCv inoculation are shown in Figure 6. Significantly higher levels of TNF-α mRNA were observed on days 4 and 9 in EMVc-inoculated mice, with WY14643 treatment reducing this increase markedly, especially on day 9. The expression levels of adiponectin mRNA were significantly enhanced by WY14643 treatment on days 0, 4, and 9. Three days of prior WY14643 treatment increased the UCP3 mRNA expression levels significantly with re-

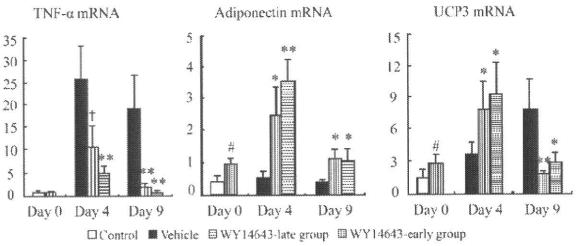

Figure 6. Expression of cardiac TNF-α , adiponectin, and UCP3 mRNA by R-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\dagger}P = 0.059$ with respect to vehicle; $^{\#}P < 0.05$ with respect to control.

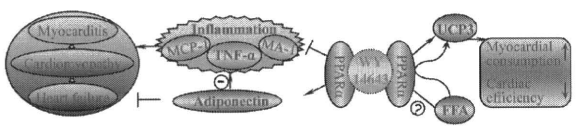

Figure 7. The dual effect of WY14643 on acute viral myocarditis and heart failure in obese diabetic mice. The cardioprotective effect of WY14643 may contribute to reduced inflammatory[3] and increased cardiac adiponectin levels, whereas the reduced cardiac efficiency may be due to up-regulation of UCP3 mRNA expression. ← denotes promotion and ⊣ inhibition. MCP-1 indicates monocyte chemoattractant protein-1; MA-1, macrophage antigen-1; FFA, free fatty acid; and UCP3, uncoupling protein 3.

spect to those in control on day 0. UCP3 mRNA expression levels were significantly higher on day 4, but lower on day 9, in the two treatment groups than those in the vehicle group. No significant difference was found between the two WY14643 treatment groups as regards cardiac TNF-α, adiponectin, and UCP3 mRNA expression on days 4 and 9.

Discussion

The above results demonstrate that the PPARα agonist WY14643 has different effects on the survival of obese diabetic mice with EMCv-induced heart failure. Thus, simultaneous WY14643 treatment increased the survival rate at the endpoint of experiment 1 mainly due to its anti-inflammatory effects and its enhancement of cardiac adiponectin expression, whereas WY14643-treated mice, especially those in the WY14643-early group, had high mortality in the first 5 days after viral inoculation, possibly as a result of the higher UCP3 levels leading to reduced cardiac efficiency (Figure 7).

The survival rate in our study was similar to that in previous clinical and experimental reports. For example, although fenofibrate, another well-known PPARα agonist, reduced the risk of nonfatal myocardial infarctions and coronary revascularisations in patients with type 2 diabetes in the FIELD study, sudden cardiac deaths and deaths from heart failure numbered 54 and 11, respectively, in the placebo group and 70 and 13, respectively, in the fenofibrate group.[5] In an animal experiment, Ichihara, *et al* reported that the survival rate for heart-damaged mice was slightly lower in one of the fenofibrate treatment groups than in the vehicle group at 13 weeks, although the survival rates were significantly higher in the treatment groups than in the vehicle group at 18 weeks.[4] All these results suggest that PPAR ± agonists have a dual effect on the impaired heart.

Inflammation is one of the pivotal factors contributing to the transition from myocarditis and cardiomyopathy to heart failure, therefore, anti-inflammatory treatments play an important role in preventing this transition.[17] Recent studies have revealed that WY14643 is capable of inhibiting inflammation in white adipose tissue by suppressing the expression of TNF-α, monocyte chemoattractant protein-1, and macrophage antigen-1.[3] These inflammatory cytokines are also implicated in myocarditis, dilated cardiomyopathy, and heart failure.[18,19] In our recent study, EMCv inoculation into KKAy mice induced severe inflammation in heart tissue and heart failure, which was strongly associated with increased local TNF-α levels.[12] In this study, high pathological scores, a large number of infiltrating cells, and a higher LC/LW ratio were found in viral-inoculated KKAy mice on day 9. Treatment with WY14643 ameliorated the inflammation in cardiac tissue and reduced the LC/LW ratio by inhibiting TNF-α expression, which might result in a beneficial effect as regards myocarditis, dilated cardiomyopathy, and heart failure, and thereby decrease the mortality.

Adiponectin has recently also been found to be expressed by cardiomyocytes, and the locally produced hormone could be involved in the regulation of cardiac metabolism and function[20] and myocardial hypertrophy.[21] Our previous study showed that adiponectin was expressed in injured myocytes in autopsy cases[22] and that suppressed cardiac adiponectin mRNA expression in obese mice was associated with the development of acute EMCv-induced myocarditis.[12] An elevated local expression of adiponectin in cardiac tissue can decrease the severity of myocardial injury associated with the attenuation of cardiac hypertrophy and inflammation in obese mice with acute viral myocarditis.[13] In agreement with this previous study, we found that adiponectin mRNA expression levels were markedly higher in WY14643-treated mice on days 0, 4, and 9, and that the myocardial diameter decreased and the inflammation improved. These findings imply that the adiponectin-associated antihypertrophic and anti-inflammatory effects might be involved in WY14643 treatment of EMCv-inoculated KKAy mice.

UCP3 is a recently identified member of the mitochondrial transporter superfamily that is expressed predominantly in heart and skeletal muscle to inhibit the synthesis of ATP in these tissues.[23] An elevated expression of local UCP3 has been reported to correlate with increased myocardial oxygen consumption and reduced cardiac efficiency.[8] Murray, *et al* reported that high plasma FFA can increase cardiac UCP3 levels via a PPARα-dependent mechanism.[9] Previous reports concerning the effect of WY14643 on plasma FFA levels are contradictory.[3,9] For example, Tsuchida, *et al* reported that an 8-week treatment with WY14643 decreased serum FFA levels significantly in KKAy mice,[3] whereas Murray, *et al* found that a one-week treatment with WY14643 did not reduce plasma FFA levels, although they found a nonsignificant increase in wild-type mice,[9] We found that the plasma FFA levels in KKAy mice increased significantly in the WY14643-early group on day 0 (after 3 days of treatment) and in the WY14643-late group on day 4, but decreased significantly in the WY14643-early group after 7 and 12 days of treatment (on days 4 and 9) and in the WY14643-late group after 9 days of treatment (on day 9). The cardiac UCP3 levels therefore appear to vary with plasma FFA levels in a PPARα-dependent manner, as reported by Murray, *et al*,[9] who also found that WY14643 treatment increased myocardial UCP3 levels in wild-type mice by 54%. In our study, WY14643 treatment increased the expression of UCP3 mRNA in cardiac tissue on days 0 and 4, but decreased it on day 9, which might be due to the combined effect of WY14643 and plasma FFA on PPARα. The increased UCP3 level on day 4 might further decrease ATP synthesis in the inflamed myocardium in WY14643-treated mice, and may therefore be responsible for the lower contractile movement and ATP-dependent iron transport. This would aggravate the heart failure and might be a predictor for the higher mortalities on day 4 in the two WY14643 treatment groups than in vehicle.[24]

As indicated in Table 1 and Figure 6, 3 days of prior treatment in the WY14643-early group increased plasma FFA levels and cardiac UCP3 mRNA expression, which enhanced myocardial consumption and reduced cardiac ATP production. In the WY14643-late group, however, the plasma FFA levels, cardiac UCP3 mRNA expression, and heart efficiency were the same as those in the control group when attacked by EMCv on day 0, which means that although the heart was protected by the anti-inflammatory effects and increased adiponectin levels equally in the two treatment

groups during the initial few days of treatment, the heart efficiency was lower in WY14643-early mice. Moreover, WY14643 treatment could significantly replace cardiac n-3 polyunsaturated fatty acids (PUFA) by n-6 PUFA, which would be detrimental to the heart since n-3 PUFAs possess cardioprotective and antiarrhythmic properties.[25] In addition, WY14643 treatment might lead to cardiac dysfunction in the diabetic heart by influencing the activity of PPARα.[26] These lines of evidence might be the possible foundation for the higher death rate in the WY14643-early group than in the WY14643-late group.

WY14643 treatment also significantly improved the hyperglycemia and reduced the body weight of KKAy mice. It is well known that cardiovascular disease is the leading cause of death for patients with type 2 diabetes and that maintaining glucose homeostasis is crucial for reducing its mortality.[27] In addition, an increased body-mass index is also associated with an increased risk of heart failure and death.[28] WY14643 therefore appears to improve the survival and heart failure risk for KKAy mice at this point of antihyperglycemia and body weight loss.

The PPARγ signal in mice increased weakly after WY14643 treatment.[29] Likewise, PPARγ ligands have been reported to attenuate AngII-induced cardiac fibrosis.[30] The pathological findings of this study also showed that treated mice had a lower level of fibrosis. Further studies should therefore aim to determine whether this decrease is due to the slight activation of PPARγ or the activation of PPARα.

PPARα agonists such as WY14643 and fenofibrate have very complex biological effects resulting from the activation or suppression of dozens of genes,[1] the biological effects of which remain largely unknown. Despite the fact that this study only provides limited targets for WY14643, its findings suggest that WY14643 has both beneficial and harmful effects on obese diabetic mice with severe myocarditis and heart failure. In addition, both WY14643 and plasma FFA can increase cardiac UCP3 levels by a PPARα-dependent pathway, and WY14643 can also affect the plasma FFA level. Further studies are therefore required to elucidate the competitive binding of WY14643 and plasma FFA to PPARα when WY14643 is used as a therapeutic agent.

Conclusion: This study has shown for the first time the cardioprotective and cardio-suppressive effects of WY14643, a potent PPAR ± agonist, on acute viral myocarditis and heart failure in obese diabetic mice in the initial stages after viral inoculation. The cardioprotective effect of WY14643 may contribute to reduced inflammatory and increased cardiac adiponectin levels, whereas the reduced cardiac efficiency may be due to up-regulation of UCP3 mRNA expression.

Acknowledgments

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REVIEW ARTICLE

Macrolide-resistant *Mycoplasma pneumoniae*: characteristics of isolates and clinical aspects of community-acquired pneumonia

Miyuki Morozumi · Takashi Takahashi · Kimiko Ubukata

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Abstract *Mycoplasma pneumoniae* is one of the main pathogens causing community-acquired respiratory tract infections in children and adults. Macrolide (ML) antibiotics are recognized generally as first-choice agents for *M. pneumoniae* infections, and these antibiotics were thought to have excellent effectiveness against *M. pneumoniae* for many years. In 2000, however, *M. pneumoniae* showing resistance to macrolides was isolated from clinical samples obtained from Japanese pediatric patients with community-acquired pneumonia (CAP). Since then, prevalence of ML-resistant *M. pneumoniae* isolates in pediatric patients has increased rapidly. In 2007, ML-resistant *M. pneumoniae* isolates were obtained from Japanese adults with CAP; numbers of such isolates also have gradually increased in Japan. Recently, similar antimicrobial resistance in *M. pneumoniae* has begun to emerge worldwide. In this review, we focus on changes of ML-resistant *M. pneumoniae* from year to year and consider resistance mechanisms as well as clinical features of patients with resistant *M. pneumoniae* infection.

M. Morozumi (✉) · K. Ubukata
Laboratory of Molecular Epidemiology for Infectious Agents,
Graduate School of Infection Control Sciences,
Kitasato Institute for Life Sciences, Kitasato University,
5-9-1 Shirokane, Minatoku, Tokyo 108-8641, Japan
e-mail: miyuki@lisci.kitasato-u.ac.jp

T. Takahashi
Laboratory of Infectious Diseases,
Graduate School of Infection Control Sciences,
Kitasato Institute for Life Sciences,
Kitasato University, Tokyo, Japan

Keywords *Mycoplasma pneumoniae* · Macrolide resistance · Mechanisms of antimicrobial resistance · Community-acquired pneumonia · Clinical features · Real-time PCR

Introduction

Sixteen *Mycoplasma* species have been isolated from human respiratory and urogenital specimens [1]. One of these species, *M. pneumoniae*, is a main pathogen in respiratory tract infections (RTI) acquired in the community. In school-aged children and young adults with community-acquired pneumonia (CAP), *M. pneumoniae* accounts for as many as 10–30% of cases [2–8]. In distinction, *M. pneumoniae* pneumonia in younger children and in the elderly is infrequent but not rare [9–15].

M. pneumoniae pneumonia is diagnosed based on characteristic chest radiographic abnormalities, patient symptoms, and clinical laboratory data. Diagnosis ultimately is confirmed using serologic tests performed upon paired sera obtained during both acute and convalescent phases. Conventional culture using pleuropneumonia-like organism (PPLO) broth, which requires more than 2 weeks, has not been carried out routinely. As a consequence, antibiotic choice usually is empirical.

Development of molecular methods such as polymerase chain reaction (PCR) assays to be used in combination with conventional diagnostic tests using serology and culture have contributed to improved diagnosis and characterization of *M. pneumoniae* infection in pediatric and adult patients [16–28]. Macrolides (ML) such as clarithromycin (CAM) and azithromycin (AZM) generally are recognized as first-choice agents against *M. pneumoniae* [29–33]. However, in parallel with increased use of oral ML with a

14-membered ring (14-ML) and AZM for RTI, *M. pneumoniae* showing resistance to ML has been isolated increasingly in clinical samples from Japanese pediatric patients with CAP [31, 34, 35]. Prevalence of ML-resistant (ML^r) *M. pneumoniae* in pediatric patients has increased rapidly [35, 36] and ML^r *M. pneumoniae* is beginning to be isolated worldwide [37–41].

In this review, we summarize the emergence and increase of ML^r *M. pneumoniae* isolates, resistance mechanisms, and clinical features of ML^r *M. pneumoniae* infection.

Diagnosis of infection

M. pneumoniae infection tends to show cyclic epidemics every 3–5 years; these outbreaks are particularly likely to occur in summer or early fall [42–46]. Although the incidence of *M. pneumoniae* pneumonia is highest in children 5–9 years old, such pneumonia also occurs frequently in younger children and in the elderly. Prevalence of *M. pneumoniae* infection also may vary according to population and diagnostic methods used.

Isolation of *M. pneumoniae* by culture from clinical samples such as throat or nasopharyngeal swabs was considered standard for diagnosis several years ago. However, *M. pneumoniae* is difficult to grow in liquid culture; cultures using PPLO broth require at least 2 weeks for completion. Compared with serologic tests or molecular techniques, including PCR, sensitivity of cultures may be <60% or 70%, even in laboratories with expertise [6, 18, 20]. Therefore, culture methods are only used rarely for routine diagnosing *M. pneumoniae* infection.

Serologic methods are used most frequently to diagnose *M. pneumoniae* infection. Frequently used, widely available serologic tests for *M. pneumoniae* include complement fixation (CF), enzyme immunoassay (EIA), and particle agglutination (PA) assays. However, these ordinarily require paired sera obtained during acute and convalescent phases in order to demonstrate rises in antibody titers; fourfold increase is thought to be significant. The second sample should be obtained 7–14 days after the first, provided that *M. pneumoniae* infection still is suspected.

Although one alternative, the ImmunoCard based on an immunoglobulin M (IgM) assay, is rapid and easy to perform, reliable diagnosis of *M. pneumoniae* infection still cannot be made on the basis of single acute-phase sera; confirmation using convalescent-phase samples still is necessary, as otherwise, both false-positive and false-negative results are frequent [24, 47, 48]. Additionally, sensitivity of IgM assays tends to be low, particularly for adult patients who are known to have weak IgM responses during primary infection or reinfection [49, 50]. Neither culture nor serologic testing can provide timely information for guiding choice of chemotherapeutic agents to use for early intervention. Serologic methods generally have only low sensitivity during the acute phase of disease.

Development of molecular methods (e.g. PCR) has lessened the importance of culture as a way to detect *M. pneumoniae* directly. Two specific targets in the P1 adhesion gene and the 16S ribosomal RNA (rRNA) gene are the main ones used in PCR assays to detect *M. pneumoniae* DNA. Various PCR methods have been developed in several laboratories. *M. pneumoniae* detection by conventional PCR or real-time PCR has been examined as an approach to early diagnosis. Many studies have described application of multiplex PCR, hybridization assays, nucleic acid sequence-based amplification (NASBA), real-time PCR using a molecular beacon probe or TaqMan probe, or cycling probe, to the identification of *M. pneumoniae* DNA [16–28]. Among these methods, real-time PCR has both high sensitivity and high specificity and can detect pathogen DNA even when damaged by empirical administration of antibiotics. Sensitivity (60–100%) and specificity (96.7–100%) of real-time PCR are both higher than those of serologic assays for *M. pneumoniae* [18, 19, 22, 24–26]. Almost all PCR-positive cases (>90%) also were confirmed serologically [22, 24]. In addition, real-time PCR using a fluorescent probe allows continuous monitoring of in vitro DNA amplification, eliminating nonspecific amplification product using fluorescence; no gel electrophoresis is needed. This makes the method suitable for clinical laboratory settings. A molecular approach including real-time PCR is useful for confirming *M. pneumoniae* and providing rapid diagnosis of CAP, thus permitting rational choice of effective antimicrobial agents.

Mechanisms of ML resistance in *M. pneumoniae*

Intrinsically, absence of cell walls in *M. pneumoniae* confers resistance to β-lactams, and indeed to all antibiotics that inhibit synthesis of cell walls. In *M. pneumoniae* infections, 14-ML and 15-membered ring ML (15-ML) usually are considered the first-line agents. *M. pneumoniae* ordinarily are susceptible to all ML, including ketolides.

Main mechanisms of microorganism resistance to ML identified in previous studies include modification of the target sites in 23S rRNA by methylation or mutation, as well as on efflux pump [51–53]. ML act on the 50S ribosomal subunit to inhibit protein synthesis. The site of peptide bond formation on the large 50S ribosomal subunit is associated with the central loop in domain V of 23S rRNA. Nucleotides present in the central loop are necessary constituents of the ML binding site, which implies that all ML bind to the same site (Fig. 1a). A loop of hairpin 35

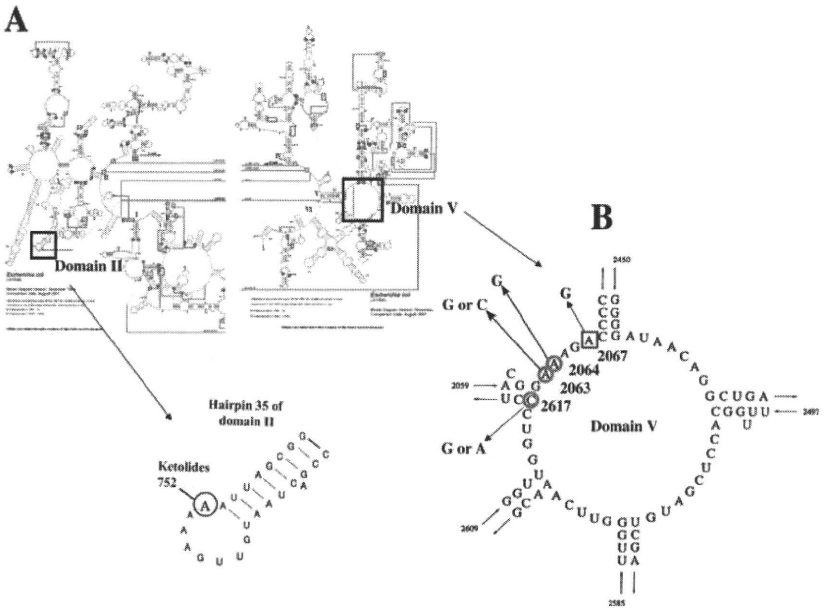

Fig. 1 Binding site of macrolides on the 23S ribosomal RNA. **a** Secondary structure of the large subunit of ribosomal RNA (*Escherichia coli*; http://www.rna.ccbb.utexas.edu/). **b** Central loop in domain V (*Mycoplasma pneumoniae* numbering) and the loop of hairpin 35 in domain II (*E. coli* numbering) of 23S rRNA as modified according to [34, 51]. *Circles* indicate positions of mutations associated with macrolide resistance in clinical isolates. The *square* shows the position of a substitution associated with 16-membered-ring macrolide resistance in a mutant selected in vitro

in domain II also may be part of binding site of ketolides; mutations involving ribosomal proteins L4 and L22 may contribute as well [51–54].

ML inhibit protein synthesis mainly by binding to domain V of 23S rRNA at nucleotide positions 2063 and 2064 (*M. pneumoniae* numbering); these positions appear essential for binding (Fig. 1b) [55]. Mutations at A2063 or A2064 confer the highest resistance to these antimicrobials. A lower level of antibiotic resistance is caused by mutations at positions A2067 and C2617, which are near A2063 and A2064 in the secondary structure, although slightly present outside the central point of ML interaction. The A2067G mutation was reported to cause highest levels of resistance only for 16-membered-ring ML (16-ML), as it interferes with formation of the covalent bond specific to 16-ML [56].

Ketolides are a new class of antimicrobial agent characterized by the substitution at position 3 from L-cladinose to keto group. These antibiotics are active against *Streptococcus pneumoniae* strains resistant to ML because of interaction at both A2058/2059 (*Escherichia coli* numbering) in domain V and A752 in domain II [51, 54, 57]. Substitutions in ribosomal proteins L4 and L22 have been found to confer resistance in *S. pneumoniae* [58, 59], but mutations in domain II of 23S rRNA and in ribosomal proteins L4 and L22 are not involved in ML resistance of clinical *M. pneumoniae* isolates [60]. Furthermore, neither plasmids nor *erm* genes mediating ribosomal modification have been described with respect to *M. pneumoniae*.

Fluoroquinolone (FQ)-resistant *M. pneumoniae* mutants may be obtained in vitro by exposure to subinhibitory concentrations of FQ [61]. These mutants possess mutations in *gyrA* and *gyrB* genes, which express topoisomerase II, and in *parC* and *parE* genes, which express topoisomerase IV; both of these enzymes are essential for bacterial DNA replication. FQ often are chosen as empiric treatment of respiratory tract infection in adults, as they are active against the main pathogens, including *S. pneumoniae*, *Haemophilus influenzae*, and *M. pneumoniae*. In contrast to in vitro findings, no FQ-resistant strains have been observed in clinical settings.

Tetracyclines inhibit protein synthesis by preventing association of aminoacyl-tRNA to the acceptor (A) site of the ribosome [62, 63]. These antibiotics bind to the ribosomal 30S subunit involving 16S rRNA and several ribosomal proteins. Only the *tetM* determinant associated with conjugative transposon Tn*916*, which confers resistance to the tetracyclines in *M. hominis*, has been documented so far [64]. These agents are administered for treatment of *M. pneumoniae* infection in adults and in pediatric patients >8 years, but development of resistance to tetracyclines has not yet been reported.

Association of ML resistance and mutation site in 23S rRNA from ML^r *M. pneumoniae*

Specific sites of the 23S rRNA mutation contribute to ML resistance in *M. pneumoniae*. In actuality, based on our results, clinical isolates with mutations at positions A2063 and A2064 show high resistance to 14-ML and 15-ML, with minimal inhibitory concentration (MIC) $\geq$ 32 μg/ml (Table 1). Especially, the A2063G mutation conveys the highest level of resistance to 14-ML, 15-ML, and telithromycin (TEL) but intermediate resistance to josamycin and rokitamycin belong to 16-ML. The A2063C and

Table 1 Antimicrobial activity of macrolides, fluoroquinolones, and minocycline against *Mycoplasma pneumoniae* strains isolated from clinical samples

ML^r class		MIC range (μg/ml)				
Antimicrobial agent	Susceptible strains (n = 423)	A2063G (n = 96)	A2064G (n = 7)	A2063C (n = 1)	C2617A (n = 1)	C2617G (n = 1)
Erythromycin	0.00195–0.0313	32 to >64	64 to >64	>256	1	8
Clarithromycin	0.00049–0.0313	32 to >64	16 to >64	>256	0.5	1
Azithromycin	0.00012–0.00195	16 to >64	16–64	16	0.0313	0.0313
Telithromycin	0.00024–0.0039	16 to >64	1–16	ND	0.0625	ND
Josamycin	0.0156–0.0625	0.0625–64	64 to >64	64	0.0625	0.25
Midecamycin	0.0625–0.25	2 to >64	>64	64	ND	0.25
Rokitamycin	0.0039–0.0313	0.0156–16	8–16	4	0.0313	0.0625
Levofloxacin	0.125–1	0.5–1	0.5–1	ND	1	ND
Moxifloxacin	0.0625–0.125	0.0625–0.125	0.0625–0.125	ND	0.125	ND
Sitafloxacin	0.0313–0.0625	0.0313–0.0625	0.0313–0.0625	ND	0.0625	ND
Garenoxacin	0.0313–0.0625	0.0313–0.0625	0.0313–0.0625	ND	0.0313	ND
Minocycline	0.0313–2	0.0625–1	0.0313–1	ND	1	ND

MICs of susceptible strains and resistant strains with A2063G, A2064G, and C2617A mutations were determined in our laboratory [31, 36]

MIC data for strains with A2063C and C2617G mutations were adapted from [35, 65]

MIC minimal inhibitory concentration, *ML^r* macrolide resistance, *ND* not determined

A2064G mutations result in a high level of resistance to all ML [36, 65]. The ketolide TEL is affected by both mutations, although A2063G was associated with MICs higher than those associated with A2064G.

MICs for minocycline (MINO) and FQ in ML^r strains were equivalent to those in susceptible strains. No strains having resistance to MINO and FQ have been observed among clinical isolates.

Several strains with the C2617 to G or A mutations show slight increases in MIC of ML compared with those with mutations at positions 2063 and 2064. A2063G in domain V of 23S rRNA is the most frequent mutation association with ML resistance, followed by A2064G; others (A2063C, C2617G, and C2617A) are rare.

Ribosomal protein L4 and L22 mutants with a few amino acids deleted or inserted have been described, but roles of these mutations in resistance to ML are uncertain [60]. No mutation has been detected in domain II of 23S rRNA.

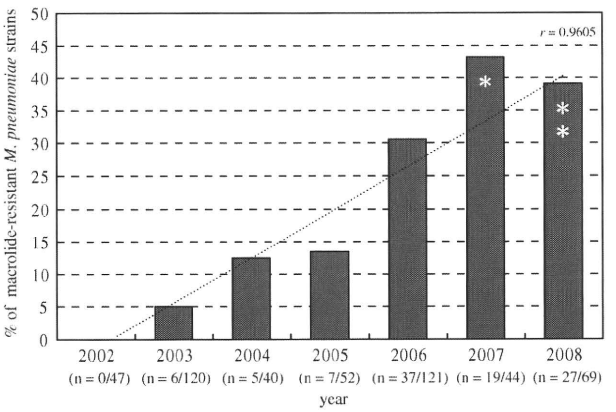

Fig. 2 Increases in numbers of macrolide-resistant *Mycoplasma pneumoniae* strains from 2002 to 2008 in Japan according to our analyses. *Asterisks* indicate macrolide-resistant strains isolated from adult patients. The *dotted line* can be expressed by the equation $y = 7x - 8.62$ ($r = 0.9605$)

Emergence and increase of ML^r strains

ML^r *M. pneumoniae* isolates possessing a nucleotide mutation in 23S rRNA first were isolated from pediatric patients with CAP, as reported by Okazaki and colleagues in 2001 [34].

Together with growing use of ML in Japan, resistant strains increased rapidly year by year (Fig. 2) [36]. Along with greater overall prevalence of *M. pneumoniae* infection in 2006, ML^r strains increasingly were isolated and identified in diverse regions across Japan.

In Japanese adult CAP patients, ML^r strains first were isolated in 2007; since then, occasional isolations have continued [66]. An increase in ML^r strains among adult patients, las in pediatric patients, may occur in the future. However, FQ are recommended for CAP treatment in adults, and no FQ-resistant strains have been observed among isolates from adult patients with CAP.

Emergence of ML^r isolates having either an A2063G or an A2064G mutation has been reported not only in Japan but also in other countries, including France, the USA, Denmark, and China [37–41]. A notably high isolation rate (92%) of ML^r strains was reported in China [38, 40].

The emergence and increase of ML^r *M. pneumoniae* has attracted the attention of microbiologists worldwide. Several recent studies demonstrated direct detection of ML^r isolates using real-time PCR [37, 41, 67].

DNA profiles of ML^r- and ML-susceptible (ML^s) strains obtained by pulsed-field gel electrophoresis (PFGE) have suggested classification into two groups [36, 68]. Significant differences in these DNA patterns were not recognized between strains, geographic locations, or years.

PCR-based restriction fragment length polymorphism (RFLP) types of the P1 adhesin gene also were used to divide strains into two types. ML^r isolates were present in both types [38, 39, 69, 70], showing no predominance.

Clinical aspects of ML^r *M. pneumoniae* infection

The clinical course of patients with ML^r *M. pneumoniae* infection appears to be prolonged [36, 66, 71, 72]. Patients infected with ML^r strains mostly had been treated with ML previously [36, 40]. Among CAP patients infected with ML^r, treatment frequently was changed from ML to MINO or levofloxacin (LVFX) because of either persistent symptoms (i.e., fever and cough) or unresolved or worsening chest radiographic abnormalities. In our observations, treatment was changed from ML to MINO or LVFX in 8.2% of patients with ML^s strains and in 39.7% of patients with ML^r strains, representing a significant difference between the two patient groups ($P < 0.01$). ML also was more frequently discontinued in favor of MINO among ML^r patients than ML^s patients in a report by Suzuki et al. [71]. Neither LVFX nor MINO are recommended for pediatric patients. However, when ML are ineffective against *M. pneumoniae* infection, pediatricians have little choice of antimicrobials except for MINO. In adult inpatients infected with ML^r strains, antibiotic agents were changed from CAM to intravenous pazufloxacin when symptoms did not improve [66]. Orally administered respiratory FQ such as moxifloxacin, sitafloxacin, and garenoxacin may be selected for adult outpatients with ML^r strains.

Among clinical features of ML^r *M. pneumoniae* infection, fever duration has been significantly longer than for ML^s-strain infection. In our data, fever persisted after ML initiation for 1.6 ± 0.8 days in ML^s *M. pneumoniae* infection and for 4.1 ± 2.3 days in ML^r-strain infection, showing a significant difference ($P < 0.01$). In other studies, febrile days during ML administration also were significantly greater in ML^r-infected patients than in ML^s-infected patients (3.5–4.0 days vs. 1.0–1.5 days) [71, 72]. Furthermore, the mean duration of persistent cough after ML administration was 7.0 days in ML^s-infected patients and 11.4 days in ML^r-infected patients [72]. Finally, the efficacy rate of ML therapy was 91.5% and 22.7% in ML^s- and ML^r-strain infections, respectively [72].

Fever may have resolved spontaneously in some patients with continuing ML treatment for ML^r infections; *M. pneumoniae* infection is associated with occasional spontaneous symptomatic recovery. According to previous reports, fever duration was 1 week in symptomatically treated *M. pneumoniae* infection [73, 74]. In another study, mean fever duration in hospitalized patients receiving AZM or EM was 2.1 days, whereas fever persisted for about 1 week in patients not receiving ML [75]. Similarly, mean cough duration was reported as 8.5 days [76]. Although treatment failure or serious illness has not yet been attributed to ML^r-strain infection, the clinical course of patients infected with ML^r strains may be prolonged.

A brief consideration of the host response is helpful here. *M. pneumoniae* resides on the surface of ciliated human respiratory epithelial cell. Several membrane proteins in *M. pneumoniae* have high affinity for various surface receptors on host cells [77]. Through interaction with recognition receptors, including toll-like receptors 2 and 6, mycoplasma membrane lipoproteins are able to induce host immune responses [77, 78]. Adherence of *M. pneumoniae* to host cells in the respiratory tract is mediated by the P1 adhesin protein and accessory proteins [79–82]. Following adherence, macrophages become activated and release cytokines, and a mononuclear cell inflammatory response is established. Human lung epithelial cells infected with *M. pneumoniae* induce expression of interleukin (IL)-8, which is both a potent chemoattractant and an activator of neutrophils, monocytes, and T lymphocytes [83–85]. In particular, significant production of IL-8 and IL-18, a cytokine that induces interferon gamma (IFN-γ) production and promotes a type 1 cytokine response, is associated with the severity of *M. pneumoniae* pneumonia in children [86–88] and adults [89].

As previously described, the inflammatory response to *M. pneumoniae* infection is considered to play a crucial role in the pathogenesis of the ensuing clinical disease. ML possess antimicrobial activity against *M. pneumoniae*, and also have anti-inflammatory effects. For example, CAM is able to suppress IL-8 induction, even in ML^r *M. pneumoniae* infection [78, 86, 90]. Thus, clinical efficacy of ML in treating *M. pneumoniae* infection may reflect not only direct antimicrobial activity but also anti-inflammatory effects of inhibition of production of cytokines, including IL-8.

Symptoms and severity of illness due to *M. pneumoniae* were similar in younger and older patients, and the mortality rate was low, even in the elderly [13, 91, 92]. This disease ordinarily is mild, and reinfection may occur in adults who already experienced *M. pneumoniae* infection, as protective immunity usually is not established following initial infection [93]. However, symptoms appear to be

prolonged in certain clinical situations regardless of antimicrobial resistance [15, 94]. Takahashi and colleagues reported *M. pneumoniae* DNA in sputum after 2 weeks of MINO therapy. Elderly persons with *M. pneumoniae* pneumonia may have a longer clinical course than younger persons. Further observations from various patients infected with *M. pneumoniae* are necessary.

In the future, alternative treatment strategies, such as promptly initiated steroid therapy, might be considered for patients with ML^r strains as a symptomatic measure [95–97].

Conclusions

In pediatric patients with CAP, ML^r *M. pneumoniae* possessing a 23S-rRNA mutation was first isolated in 2000; numbers of these isolates have increased rapidly year by year in Japan. In other countries, ML^r *M. pneumoniae* also is emerging. Symptoms often are relatively prolonged in patients with ML^r *M. pneumoniae* infection. ML^r *M. pneumoniae* should be considered an emerging issue for not only pediatric patients but also adult patients with CAP.

No FQ-resistant *M. pneumoniae* strain has been observed thus far among specimens from adult patients with CAP. However, emergence of infections with FQ-resistant *M. pneumoniae* might occur, considering the increasing FQ prescription rate among adult patients. Further studies are needed to clarify the prevalence of ML^r *M. pneumoniae* in pediatric and adult patients with RTI and to establish clinical guidelines regarding the most appropriate antimicrobial agents to use against these strains. Interventions to prevent emergence and increases of novel antibiotic-resistant strains are needed, including rapid identification of pathogens by methods such as real-time PCR, as well as antimicrobial treatments capable of eliminating the organisms.

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Original Article

Mao-to Prolongs the Survival of and Reduces TNF-α Expression in Mice with Viral Myocarditis

Zhu Shijie[1], Junji Moriya[1], Jun'ichi Yamakawa[1], Rui Chen[1], Takashi Takahashi[1], Hiroyuki Sumino[2], Takeshi Nakahashi[3], Kunimitsu Iwai[3], Shigeto Morimoto[3], Nobuo Yamaguchi[4] and Tsugiyasu Kanda[1]

[1]Department of General Medicine, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Kahoku-gun, Ishikawa 920-0293, [2]Department of Medicine and Biological Science, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, [3]Department of Geriatric Medicine and [4]Fundamental Research for Complementary and Alternative Medicine, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Kahoku-gun, Ishikawa 920-0293, Japan

Goal of this study was to evaluate effects of Mao-to on development of myocarditis induced by encephalomyocarditis (EMC) virus in mice. Mice were randomly divided into five groups. Group N included uninfected controls ($n = 18$), while group A, B and C underwent intraperitoneal injection of EMC virus. Group A was administered oral saline from day 0 to day 4. Group B was administered oral Mao-to ($500\,mg^{-1}\,kg^{-1}\,day^{-1}$) from day 0 to day 4. Group C was administered Mao-to from day 2 to day 6. Group D was administered Mao-to from day 5 to day 10. Treated mice were followed for survival rates during 2 weeks after infection. Body weight (BW) and organ weights including heart (HW), lungs, thymus and spleen were examined on days 4, 6 and 14. Survival rate of group C (36.4%) was significantly improved compared with group A, B or D (0% of each, $P < 0.05$). HW and HW/BW ratio in group C was significantly ($P < 0.05$) lower than those in group A, B or D. Viral titers of hearts were significantly different among groups A, B and C. Cardiac expression in tumor necrosis factor-α (TNF-α) was significantly reduced in group C in comparison with group A, B or D on day 6 by immunohistochemical study. Administration of Mao-to starting on day 2 improves mortality resulting from viral myocarditis in mice with reduced expression of cardiac TNF-α. These findings suggest that timing of Mao-to is crucial for preventing cardiac damage in mice with viral myocarditis.

Keywords: Mao-to – viral myocarditis – tumor necrosis factor-α

Introduction

Myocarditis is an important cause of cardiomyopathy in young patients (1). Therapeutics for myocarditis have been restricted to supportive care including basic medications (2). Randomized trials of immunosuppressive agents have failed to show a benefit (3). Immunomodulative agents have been tried with limited effects (4). In severe cases, heart transplantation presents the only therapeutic option (1). Anti-inflammatory and anti-viral agents are needed to improve outcomes in these patients.

Mao-to (Ma-Huang-tang in Chinese) is traditionally used in Japan and China for treatment of influenza-like illness (high fever, headache, pain and cough) since ancient times. Component herb names (botanical names) of Mao-to are as follows: *Ephedra Herba* (stem of *Ephedra Sinica* Stapf), *Cinnamomi Cortex* (bark of

For reprints and all correspondence: Tsugiyasu Kanda, MD, Department of General Medicine, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Kahoku-gun, Ishikawa, 920-0293, Japan. Tel: +81-76-286-2211 (ext. 3841); Fax: +81-76-286-2702; E-mail: kandat@kanazawa-med.ac.jp

Cinnamomum cassia Blume), *Armenicae Semen* (semen of *Prunus Armeniacae* Linne) and *Glycyrrhizae Radix* (root of *Glycyrrhizae uralensis* Fisher) (5). *Ephedra Herba* was reported to show the *in vitro* anti-influenza viral effects and augments the production of inflammatory cytokines including interleukin-6 and interleukin-1 (6,7). Moreover, *Cinnamomi Cortex*, which contains Mao-to, suppress IL-1α production in the influenza virus-infected mice (8). Moreover, Mao-to has been studied for its anti-viral and anti-autoimmune effects (9,10). Recently, anti-pyrulent effect of Mao-to was reported in patients with influenza infection (6). However, the effects of Mao-to in viral myocarditis have not been studied. Inflammatory cytokines are involved in the pathogenesis of myocardial injury in viral myocarditis.

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine (11) that plays a crucial role in the initiation and continuation of inflammation and immunity (12). This cytokine has been implicated in the pathogenesis of cardiovascular diseases, especially in viral myocarditis. Our previous work showed that cardiac expression of TNF-α mRNA is increased in a mouse model of viral myocarditis (13). Over production of TNF-α is generally considered to be harmful to the cardiovascular system, because systemic administration of TNF-α results in myocardial depression (14) and cardiomyopathy (15). Cardiac-specific over-expression of TNF-α has been reported to cause severe myocarditis in mice (16,17).

Therefore, we hypothesized that Mao-to would modify viral myocarditis through anti-viral and anti-inflammatory effects and that cardiac expression of TNF-α was studied as an indicator of myocardial damages.

Methods

Mice

Eight-week-old C3H female mice (Charles River, Japan) were used in the experiments.

Virus

A myocarditic variant of encephalomyocarditis (EMC) virus was obtained from Y. Seto, Keio University, Tokyo, Japan. Encephalomyocarditis virus was cultured and purified followed by previous report (18). Animals were inoculated intraperitoneally with 500 plaque-forming units of EMC virus in 0.1 ml of saline.

Chemicals

Mao-to which was supplied by Tsumura Co. (Tokyo, Japan) was dissolved in distilled water, and diluted with distilled water to the appropriate concentration. HPLC finger print pattern of Mao-to is shown in Fig. 1. Mao-to solution at the dose of $500\,mg^{-1}\,kg^{-1}$ was administered orally once daily to the mice. The dose of Mao-to was based on the findings in previous reports (19,20). The control mice were given saline.

Treatment Protocol

A total of 135 mice were assigned randomly to five groups. Mice in groups B and C received Mao-to 10 mg per mouse in 0.1 ml saline ($500\,mg^{-1}\,kg^{-1}\,day^{-1}$) once daily for 5 days. Group A was administered with 0.1 ml saline from day 0 to day 4. Group B was administered with 0.1 ml Mao-to from day 0 to day 4. Group C was administered with 0.1 ml Mao-to from day 2 to day 6. Group D was administered 0.1 ml Mao-to from day 5 to day 10. Group N was the uninfected control group. The survival rate of each group was monitored during the observation period. Body weight and organ weight, and histopathologic changes in the heart were examined on day 4, 6 and 14 after infection.

Pathologic Examination

The heart and other organs were weighed. Body weight (BW) also was recorded. One half of each organ was fixed in 10% buffered formalin and stained with hematoxylin-eosin; the other half was frozen in embedding compound at −120°C for immunohistochemical studies. Transverse sections of ventricular myocardium were graded for severity of necrosis and mononuclear cell infiltration on a scale from 1 to 4 as follows: grade 1, lesions involving <25% of the ventricular myocardium; grade 2, lesions involving 25-50% of the myocardium; grade 3, lesions involving 50-75% of the myocardium and grade 4, lesions involving 75-100% of the myocardium. Tissues were evaluated blindly by an experienced pathologist who was familiar with grading murine viral myocarditis and had no knowledge of the study design.

Measurements of Myofiber Diameter

In the lateral wall of the left ventricle, myocardial fiber diameter was determined by measuring the shortest diameter at the level of the nucleus of 50 myocardial fibers from each group with an ocular micrometer in the stained cross-sectional areas.

Immunohistochemical Examination

To visualize the presence and anatomic localization of TNF-α within the myocardium, immunohistochemical studies were performed using an avidin biotin complex method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) as previously described (21). To minimize the background staining, all sections were first blocked with normal goat serum for 20 min at room temperature.

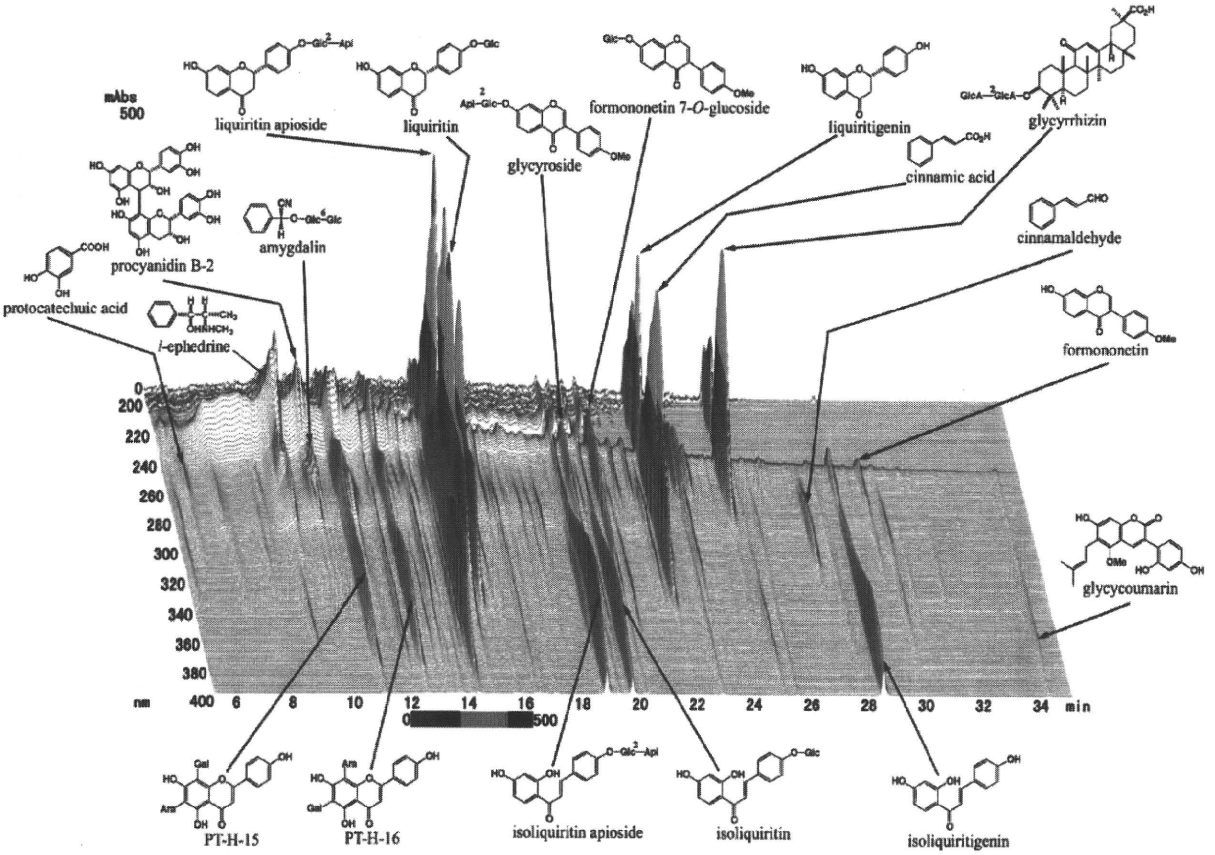

Figure 1. HPLC finger print pattern of Mao-to. HPLC conditions: Pulp: LC-10AD vp (Shimadzu, Japan); column: TSK-GEL ODS-80TS column (250 × 4.6 mm; Tosoh, Japan); mobile phase: 0.05 m AcONH4 (pH 3.6) (5).

Next, the slides were incubated with an antibody directed against murine TNF-α (Alpha-Diagnostic International Inc., San Antonio, USA). Sections were counterstained with hematoxylin and eosin. TNF-α immunostaining was graded as follows: both nuclear and cytoplasmic staining 4; strong cytoplasm staining 3; moderate cytoplasmic staining 2 and slight cytoplasmic staining 1.

Viral Titer in Heart

The EMC viral titer in the individual hearts was determined in terms of the viral cytopathic effects, and is expressed as the tissue culture mean infectious dose (TCID50). The hearts on day 4 after the inoculation ($n = 3$ of each group) were homogenized in 2 ml of MEM. After the centrifugation, the supernatants were added into 96-well microtiter plates containing human amnion cells in the MEM supplemented with 10% fetal calf serum as described previously (21). The microtiter plates were daily observed for 5 days to find the appearance of any cytopathic effects.

Statistics

Data are reported as means ± SD. The Kaplan–Meier curves were generated to analyse differences in survival. The differences in scores of myocardial damages were examined by two-way analysis of variance to reveal the combined effects of two different agents. Scheffes' *F*-test and Bonferroni/Dunn analysis were used for confirmation. A level of $P < 0.05$ was considered statistically significant.

Results

Prolonged Survival

Survival rates on day 7 were 18.2% in group A, 18.2% in group B, 63.6% in group C and 23.3% in group D. The survival rates on day 14 were 0% in group A, 0% in group B, 36.4% in group C and 0% in group D. The survival rate in group C was significantly higher than those in groups A, B or D ($P < 0.05$, Fig. 2). Thus, treatment with Mao-to starting on the day 2 after EMC

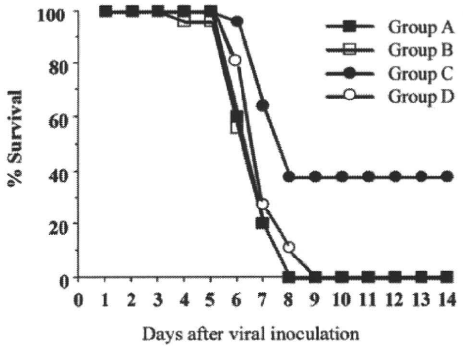

Figure 2. Survival in mice after viral inoculation. survival was significantly ($P < 0.01$) improved in the group C as compared with mice in groups A, B or D. Group A: administered with 0.1 ml saline starting on day 0 to day 6, group B: administered with 0.1 ml Mao-to starting on day 0 to day 4, group C: administered with 0.1 ml Mao-to starting on day 2 to day 6, group D: administered 0.1 ml Mao-to from day 5 to day 10.

virus improved the survival, although Mao-to given earlier or later was not effective in this regard.

Reduced Heart Weight

The results are shown in Fig. 3. The BW in group A and B on day 6 after virus inoculation was significantly ($P < 0.05$) lower than that in group C. The HW/BW ratio in group A on day 4 was elevated compared with that of groups B and C ($P < 0.05$). The HW and HW/BW ratio in group C on day 6 after virus inoculation was significantly ($P < 0.01$) lower than that in group A. Additionally, the HW/BW in group C on days 4 and 6 were significantly ($P < 0.01$) lower than those in group B. However, the HW and HW/BW ratio in group C on days 4, 6 and 14 did not differ significantly from those of the group N. In group D, HW and HW/BW ratio on day 6 were significantly higher than those in group N.

Decrease of Lung Congestion

The lung weight (LuW)/BW ratio on days 4 and 6 was increased in groups A and B compared with group N. The LuW/BW ratio on days 4 and 6 were significantly reduced in group B and C vs. group A. In group C, the LuW/BW ratio on day 4 was significantly lower than that in group B, but that on day 6 was not significantly different than group B. In group D, LuW/BW ratio on day 6 was significantly higher than that in group N.

Reduction of Thymus Weight and Enlarged Spleen

The thymus weight (ThW)/BW ratio in groups A, B, C and D on day 6 was significantly ($P < 0.05$) lower than in the group N and the spleen weight (SpW)/BW ratios in groups A, B and C on days 4 and 6 were significantly ($P < 0.05$) higher than in group N (Fig. 4). Although the ThW/BW ratio on day 6 in group C did not differ from that in group A, the SpW in group C on day 6 was significantly ($P < 0.05$) higher than in groups N and A. In group D, ThW/BW ratio on day 6 was significantly lower than that in group N and SpW/BW was significantly higher than that in group N.

Decreased Myocardial Diameter and Myocardial Damage

The myocardial diameter was significantly smaller in group C than that in group A and B on day 6, as shown in Fig. 5. Myofiber diameter in group A was significantly larger than that in group N. The scores of myocardial necrosis and mononuclear cell infiltration were significantly reduced in group C compared with groups A, B and D on day 6 (Fig. 6A).

Suppression of Expression of TNF-α in Heart

Localized expression of TNF-α in the heart is shown in Fig. 6B. Endothelial cells and myofiber were positive for TNF-α in groups A and B on days 4 and 6. Myofibers from group C were less positive than those from groups A and B on day 6. The grading of TNF-α immunostaining on day 6 was relatively lower in group C compared with those in group A, B or D ($P < 0.05$, $n = 3$ of each, Fig. 4). In addition, comparative expression of cardiac TNF-α mRNA in the group C were significantly less than in the groups A, B or D (Fig. 7).

Viral Titer in Heart

On day 4, viral titer in the hearts was not significantly elevated in the Mao-to groups compared with that in the group A (group B; 3.3 ± 0.8 TCID/mg, group C; 3.0 ± 0.7 TCID/mg vs. group A; 3.5 ± 1.0 TCID/mg), although the titer in group C was slightly lower than that in group A.

Discussion

At present, we have shown that Mao-to, administered 2 days after viral inoculation in C3H/HeJ mice, improves survival rates, and reduces both myocardial necrosis and mononuclear cell infiltration in mice with viral myocarditis. Accordingly, SpW/BW ratios were elevated in these mice. However, the administration of Mao-to at the same time as virus inoculation did not influence survival or myocardial destruction. We conclude that Mao-to has an anti-viral effect on EMC viral myocarditis in this mouse model *in vivo* and involves the modulation of early immune responses with the reduction of cardiac TNF-α

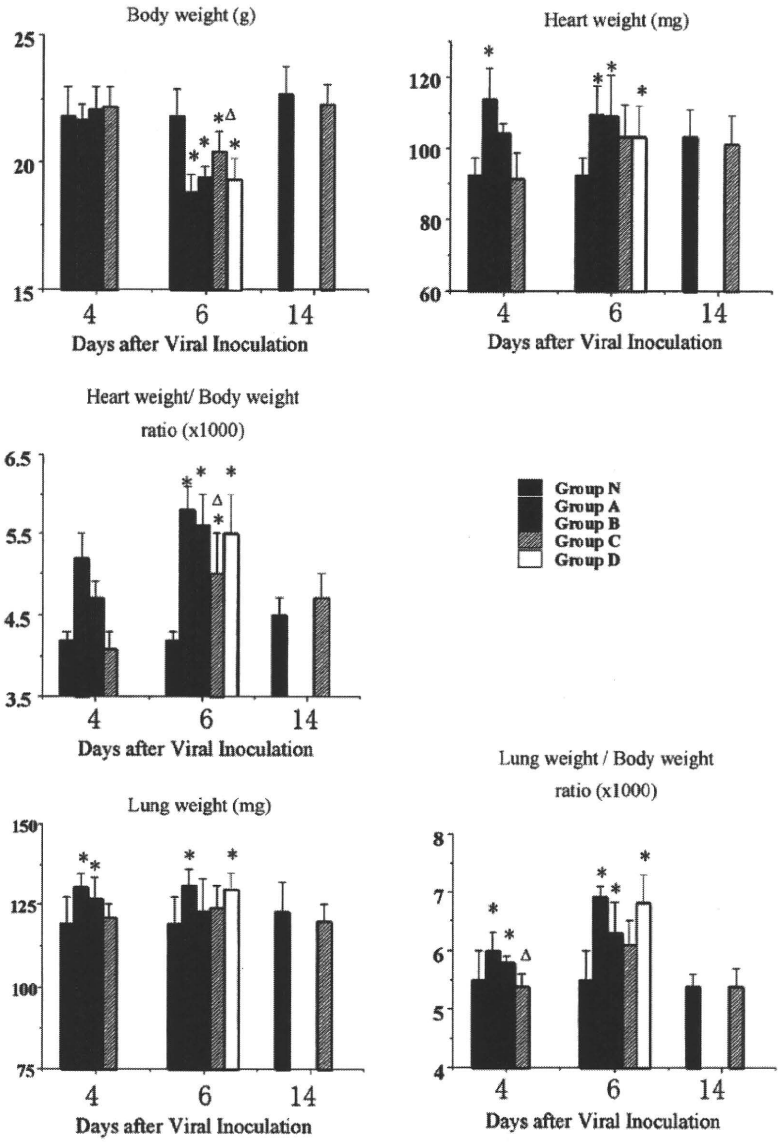

Figure 3. Heart and lung weight in murine viral myocarditis. Heart weight, heart weight/bodyweight ratio and lung weight/body weight ratio on day 6 was significantly reduced in group C compared with group N. Abbreviations; N: uninfected control mice, A: administered with 0.1 ml saline starting on day 0 to day 6, B: administered with 0.1 ml Mao-to starting on day 0 to day 4, C: administered with 0.1 ml Mao-to starting on day 2 to day 6. $*P < 0.05$ vs. group N, $\Delta P < 0.05$ vs. group A.

mRNA, although the viral titer of hearts were not significantly changed.

The LuW/BW ratio reflects the degree of lung congestion. The two days later administration of Mao-to and virus as group C leads to a significant reduction in LuW/BW on day 6. The reduction of LuW/BW ratios in group B are considered to reflect an improvement in congestive heart failure due to viral myocarditis. The effect of Mao-to on histopathological changes depends on the timing of administration. As noted, when administered two days following viral inoculation, Mao-to led to significant reduction of myocardial necrosis and mononuclear cell infiltration.

The reasons why oral administration of Mao-to was started on 2 days after virus inoculation, should be discussed. Our previous data showed that the both peaks of serum TNF-α and interferon were identified on day 2 after viral inoculation in this murine model (21). Neutralizing antibody titer was confirmed on day 4. Oral administration of Mao-to was effective in patients

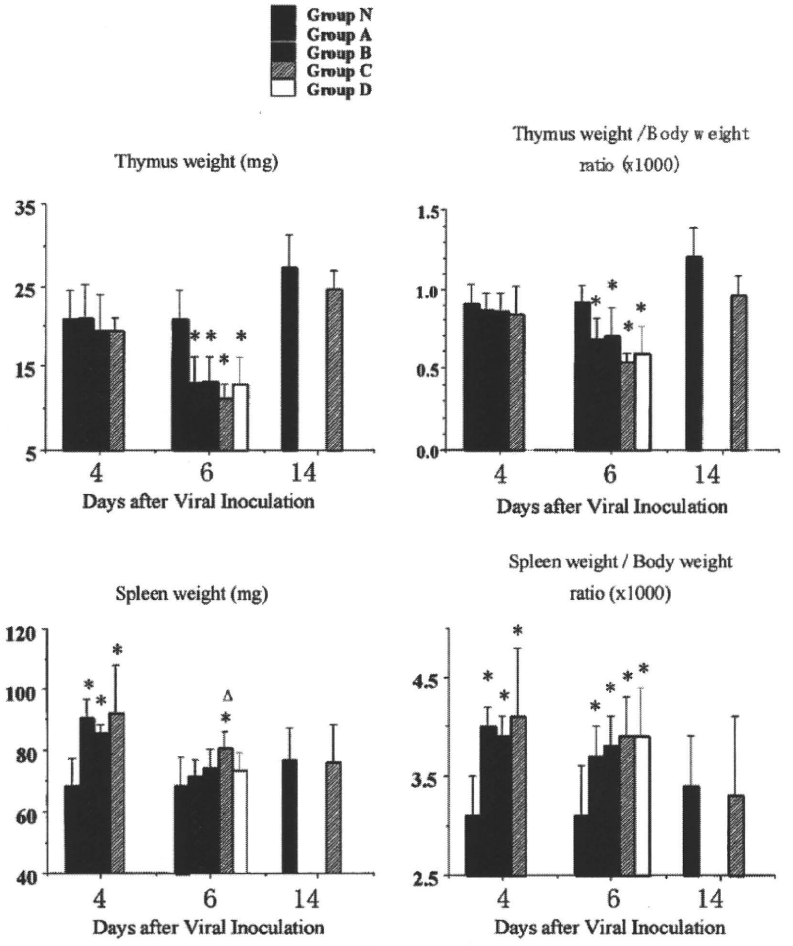

Figure 4. Thymus and spleen weight in murine viral myocarditis. Thymus weight in Mao-to-treated groups A, B, C and D were significantly reduced compared with group N on day 6. Spleen weight/body weight ratio were significantly increased in group C compared with group N. Groups as are described in Fig. 2. $*P < 0.05$ vs. group N, $\Delta P < 0.05$ vs. group A.

already infected with influenza virus (10). Moreover, Mao-to has been reported to show an *in vitro* anti-viral effect (6). These reports suggest that the starting time of administration, 2 days after virus inoculation, could suppress these reactions instead of other days after virus inoculation.

The reasons why Mao-to administration starting on day 0 (group B) did not prolong the survival should be discussed. It is known that Mao-to increase the blood pressure, heart rate and cardiac output and decrease the total peripheral resistance (22). From day 0 to 4, the main course of viral myocarditis is the infection of viral genome to myocytes, not the secondary immune cell infiltration in the heart. Mao-to administration on day 0 to 4 is possible to make myocyte damages worse by inducing excessive contraction of infected myocytes.

TNF-α is secreted primarily by myocytes and macrophages after injury (23). Elevation of TNF-α contributes to the extent of ventricular dysfunction as shown in TNF-α knockout mice (24). Previous studies have demonstrated that cardiac-specific expression of TNF-α results in myocardial inflammation, cardiac hypertrophy, progressive dilatation and increased apoptosis, which leads to heart failure and death (25). TNF-α may play an important role in modulating left ventricular dysfunction (26).

The present study also suggests that Mao-to may play a role in cytokine regulation of host defense mechanisms against viral myocarditis in mice *in vivo*. Comparative studies on anti-immune effects of Mao-to were reported. Mao-to prevents passive cutaneous anaphylaxis and also inhibits histamine and leukotriene C4 release from the mast cells (27). These suppressive effects may be due to the interaction of its components such as *Ephedra Herba* and *Cassia Twig*. The former was showed to suppress interleukin-1 and 6 and the latter suppresses interleukin-1α