This information is current as of December 4, 2021.

Matrix Metalloproteinase (MMP)-9, but Not MMP-2, Is Involved in the Development and Progression of C Protein-Induced Myocarditis and Subsequent Dilated Cardiomyopathy

Yoh Matsumoto, Il-Kwon Park and Kuniko Kohyama

*J Immunol* published online 4 September 2009

http://www.jimmunol.org/content/early/2009/09/04/jimmunol.1.0900871

**Why The JI? Submit online.**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Matrix Metalloproteinase (MMP)-9, but Not MMP-2, Is Involved in the Development and Progression of C Protein-Induced Myocarditis and Subsequent Dilated Cardiomyopathy

Yoh Matsumoto, Il-Kwon Park, and Kuniko Kohyama

Repeated or continuous inflammation of the heart is one of the initiation factors for dilated cardiomyopathy (DCM). In previous studies, we established a DCM animal model by immunizing rats with cardiac C protein. In the present study, we analyze the role of matrix metalloproteinases (MMPs) in experimental autoimmune carditis (EAC) and subsequent DCM to elucidate the pathomechanisms of this disease. In this model, inflammation begins ~9 days after immunization. At that time, MMP activities were detected by in situ zymography. Real-time PCR analysis revealed continuous up-regulation of MMP-2 mRNA from 2 wk and thereafter. MMP-9 mRNA, however, had only a transient increase at 2 wk. Double staining with in situ zymography and cell markers demonstrated that gelatinase (MMP-2 and MMP-9)-expressing cells are infiltrating macrophages during the early stage and cardiomyocytes at later stages. Minocycline, which inhibits MMP-9 activities more strongly than MMP-2, significantly suppressed EAC, but an MMP-2-specific inhibitor, TISAM, did not affect the course of the disease. Furthermore, immunohistochemical examination revealed that minocycline treatment suppressed T cell and macrophage infiltration strongly, whereas TISAM did not. These findings indicate that MMP-9, but not MMP-2, is involved in the pathogenesis of the acute phase of EAC, and further suggest that MMP-9 inhibitors, minocycline and its derivatives, may be useful therapies for EAC and DCM. The Journal of Immunology, 2009, 183: 0000 – 0000.

Dilated cardiomyopathy (DCM) is a progressive disease of the myocardium that is characterized by dilatation and impaired contraction of either the left or both ventricles of the heart (1). The causes of DCM are multifactorial, but a viral or immune mechanism is suspected in some cases (2) because examination of heart tissues obtained during surgery has revealed inflammatory lesions in many patients with DCM (3). On the basis of these findings, several immunosuppressive therapies, including steroid and azathioprine administration (4, 5), immunoadsorption (6), and Ig therapy (2), have been attempted. Although the general outcome of these treatments was disappointing, immunoadsorption therapy increased the survival rate for a subset of patients by 5 years after treatment (7). Because the pathogenesis of DCM is still poorly understood, finding effective therapies has been a difficult challenge. The establishment of an animal model that mimics human DCM provides useful information on the pathogenesis of DCM and the development of effective therapies.

In previous studies, we have demonstrated that cardiac C protein localizes in thick filaments of cardiac muscles (8) and has a strong cardiitis-inducing ability (9, 10). Experimental autoimmune cardiitis (EAC) that is induced in Lewis rats by immunization with C protein is a T cell-mediated disease, but various types of autoantibodies are involved for the shift from EAC to DCM (10). In addition, it was shown that several chemokines play an important role in disease progression (9).

Matrix metalloproteinases (MMPs) form a family of enzymes that mediate various functions in tissue destruction, remodeling, and immune responses by hydrolyzing components of the extracellular matrix under physiological and pathological conditions (11). In the heart, MMPs localize in the sarcomere within cardiomyocytes and become activated by acute cardiac injury (reviewed in Ref. 12). Therefore, it is essential to investigate the role of MMPs produced by exogenous infiltrating inflammatory cells and endogenous cardiomyocytes during the development and progression of DCM. Unfortunately, there is little information on MMP production in the heart during autoimmune processes.

In the present study, we investigated the role of MMPs in the development and progression of EAC. We determined when active MMP-producing cells appear in the heart and whether they are inflammatory cells, cardiomyocytes, or both. The relationship between the MMP expression and inflammatory lesions was also examined. The results suggest that MMP-2 and MMP-9 play important, but different roles in lesion formation of EAC and DCM. Furthermore, treatments with an MMP-9 inhibitor, but not with an MMP-2 inhibitor,ameliorated the pathology of EAC. Thus, immunotherapies to EAC, and subsequent DCM, should be designed on the basis of the diseases mechanism.

Materials and Methods

Unless otherwise indicated, all reagents and apparatuses used in the present study were obtained in Tokyo, Japan.

Animals

Lewis rats were purchased from SLC Japan and were bred in our animal facility. Male and female rats were used at ~12 wk of age. All animal
experiments were approved by the institute ethics committee and performed in accordance with institutional guidelines.

**Preparation of recombinant C protein fragments and synthetic peptides**

The preparation of recombinant C protein was precisely described previously (9). PCR products corresponding to cardiac C protein fragment 2 (CC2) were inserted into a cloning vector, pCR4-Blunt-TOPO, in the Zero Blunt TOPO Kit (Invitrogen), and clones with correct sequences were subcloned into the pQE30 expression vector (Qiagen). Recombinant CC2 produced in transformed Escherichia coli was isolated under denaturing conditions and purified using Ni-NTA agarose (Qiagen).

**EAC induction and tissue sampling**

Lewis rats were immunized once on day 0 with CC2 emulsified in CFA (2.5 mg/ml Mycobacterium tuberculosis) in the hind footpads. In some experiments, rats received an i.p. injection of pertussis toxin (Seikagaku Kogyo) at the time of immunization. Histological and immunohistochemical examinations were performed at the indicated time points using frozen and paraflin-embedded sections of the heart. Although evaluation of EAC and DCM was mainly based on histological examinations (see below), the clinical score was also recorded, as follows: grade 1, dyspnea; grade 2, dyspnea plus ruffling of fur; and grade 3, moribund condition or dead.

**Histological grading of inflammatory lesions and immunohistochemistry**

EAC inflammatory lesions were evaluated using H&E-stained sections according to the following criteria: grade 1, rare focal inflammatory lesions; grade 2, multiple isolated foci of inflammation; grade 3, diffuse inflammation involving the outer layer of the muscle; grade 4, grade 3 plus focal transmural inflammation; and grade 5, diffuse inflammation. When dense fibrosis made it difficult to estimate the grade of inflammation using H&E-stained sections, ED1-stained sections were used for grading. The extent of fibrosis revealed by Azan staining was graded into five categories, as follows: grade 1, rare scattered foci of fibrosis; grade 2, multiple isolated foci of fibrosis; grade 3, fibrosis involving the outer layer of the muscle; grade 4, grade 3 plus patchy transmural fibrosis; and grade 5, diffuse fibrosis. The perivascular connective tissue staining found in the normal heart was not included in this scoring system.

Single immunoperoxidase staining was performed, as described previously, using mAbs against TCR-α/β (R73) and macrophages (ED1), as described previously (13). Briefly, frozen sections were air dried and fixed in ether for 10 min. After incubation with normal sheep serum, sections were reacted with mAb, biotinylated horse anti-mouse IgG (Vector Laboratories), and HRP-labeled VECTSTAIN Elite ABC Kit (Vector Laboratories). Biotin binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide.

**Film in situ zymography (FIZ), in situ zymography (ISZ), and gelatin zymography**

To localize the activity of gelatinases (MMP-2, -9), zymography with gelatin-coated films (FIZ) was performed, according to the manufacturer’s instructions. Frozen sections (4 μm) were cut and mounted onto MMP in situ Zymo-Films (WAKO). The films were incubated in a moist chamber at 37°C overnight. After being air dried, the films were stained with Biebrich scarlet stain solution (WAKO) for 4 min, followed by a wash with distilled water. Areas possessing gelatinolytic activities were stained white, as shown in Fig. 2, and were quantitated using NIH Image software. The films were first scanned with a Canoscan 8000F scanner (Canon), and the images were saved as 300-kb files. Using inverted images, the areas showing gelatinase activities were calculated as pixels. In 2007, WAKO stopped supplying gelatin-coated films, so additional FIZ experiments were not performed.

ISZ with FITC-labeled gelatin was performed using DQ gelatin (Invitrogen), according to Oh et al. (14). Briefly, 8-μm frozen sections were incubated with reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl2, and 1 mM Na2HPO4, pH 7.6) containing 40 μg/ml DQ gelatin at 37°C overnight. Sections were washed with PBS before observation. The fluorescent intensity of ISZ sections was semiquantitatively analyzed using the Image J software (National Institutes of Health). All sections for analysis were stained, and microphotographs of each were obtained under the same conditions with a confocal microscope (TCS-SF; Leica Microsystems). Bright areas (above threshold, 115) were calculated and evaluated statistically.

To determine the type of cells showing gelatinolytic activities, sections after ISZ were fixed with 4% paraformaldehyde, washed, and incubated with R73 (anti-T cell), ED1 (anti-macrophage), anti-mast cell tryptase (sc-32473, anti-mast cell; Santa Cruz Biotechnology), anti-α-actin (EA-53; anti-cardiacite), or anti-vimentin (SP20; Epitomics) for 1 h, followed by Cy3-labeled anti-mouse IgG (Chemicon International) for 1 h. After being mounted with Vectashield (Vector Laboratories), the sections were observed with a confocal microscope (Leica TCS NT; Leica Microsystems).

Gelatin zymography was performed using the Novex In-gel Zymography System (Invitrogen), according to the manufacturer’s instructions. Heart tissues were homogenized in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. After centrifugation, supernatants were harvested and 100 μg of protein was mixed with Novex Tris-glycine SDS sample buffer and run on Novex 10% Zymogram Gelatin Gel, followed by incubation with Zymogram Renaturing Buffer, and then with Zymogram Developing Buffer. After an overnight reaction, the gel was stained with SimplyBlue Safestain (Invitrogen).

**Identification of mast cells**

To identify mast cells, toluidine blue staining was performed. Frozen sections were fixed with ether, and paraffin-embedded sections were deparaffinized and rehydrated. They were then incubated with 0.05% w/v toluidine blue for 30 min, followed by counterstaining with 0.01% eosin for 1 min. Some sections were immunohistochemically stained with Abs against mast cell tryptase (Santa Cruz Biotechnology) to identify mast cells.

**Real-time PCR**

Total RNA was extracted, as described above, and first-strand cDNA was synthesized from 1 μg of total RNA using random hexamer primers and ReverTra Ace (Toyobo). SYBR Green real-time PCR were performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems) in a total volume of 25 μl using the SYBR Premix Ex Taq (Takara Bio). Each reaction was performed in duplicate using the following thermocycler conditions: 95°C for 10 min for one cycle, followed by 50 cycles of 95°C for 15 s and 58°C for 1 min. All primers were designed over an intron-exon junction to prevent the coamplification of genomic DNA. The relative quantification of mRNA was performed using the standard curve method. MMP and cytokine mRNA were normalized to GAPDH for each sample (15). The absence of nonspecific amplification was confirmed by dissociation curve analysis.

**Treatment of EAC with MMP inhibitors**

Minocycline treatment of EAC was performed using a protocol applied for experimental autoimmune encephalomyelitis, with a few modifications (16, 17). Minocycline hydrochloride (Sigma-Aldrich) was dissolved in PBS and administered daily by i.p. injection at a dose of 50 mg/kg body weight for 3 wk. On day 21 postimmunization (PI), rats were sacrificed under deep anesthesia, and the effects of minocycline were evaluated histologically.

TISAM, an N-sulfonfylamino acid derivative (18), was provided by Discovery Research Laboratories of Shionogi. Rats were orally fed with TISAM suspended in 0.5% w/v methylcellulose 400 solution (WAKO) at a dosage of 5 mg/kg/day, according to a previous protocol (19). Rats were sacrificed on day 21 under deep anesthesia, and hearts were removed and analyzed histologically.

**Statistical analysis**

Data were analyzed by Student’s t test or Mann-Whitney’s U test. Values of p less than 0.05 were considered significant.

**Results**

**Early immunopathological events and the switch from inflammation to fibrosis in the heart with C protein-induced EAC**

One of the advantages of using C protein-induced EAC for analysis is that the onset and progression of the disease are rather uniform (9, 10). Therefore, this model is suitable for examining early immunopathological events occurring in the heart. We performed immunohistochemical staining for T cells with R73 mAb and macrophages with ED1 mAb using frozen sections of hearts taken from unimmunized and immunized rats at 0, 7, 9, 11, and 14 days after immunization. Consistent with our previous work, very few T cells might be trapped in blood vessels and macrophages residing in the hearts of control animals (10). On day 7, T cells and
macrophages increased in number, but they did not form inflammatory foci (data not shown). Small inflammatory foci appeared on day 9. As shown in Fig. 1, A and B, the foci were composed of T cells and macrophages (indicated by arrows). On day 14, the number and size of inflammatory foci increased considerably (Fig. 1, C and D). When the disease peaked (2–4 wk), diffuse T cell and dense macrophage infiltration was noted, as shown in Fig. 1, C and D. However, in some lesions, T cells were sparsely distributed in the cell cluster (inset of Fig. 1C). These findings indicated that definite lesion formation starts on day 9 and suggested that subtle inflammatory processes began on day 7.

Using H&E- and Azan-stained sections, inflammation and fibrosis were scored according to the grading system described under Materials and Methods. Representative features at early (2 wk; Fig. 2, A and B) and late (12 wk; Fig. 2, C and D) stages are shown. During the early stage, there was extensive inflammatory cell infiltration in the parenchyma of some rats (Fig. 2A), whereas fibrosis shown in Azan staining was minimal (Fig. 2B). In sharp contrast, very small number of inflammatory cells was present at the later stage (Fig. 2C). Instead, dense fibrosis was recognized in Azan staining (Fig. 2D). Consistent with these findings, inflammation was not obvious on days 7 and 9 (Fig. 2E). However, inflammation was

**FIGURE 1.** A–D. Immunohistochemical staining for T cells (A and C) and macrophages (B and D) of hearts taken on days 9 (A and B) and 14 (C and D). T cells and macrophages are colocalized in a small inflammatory focus in serial sections (A and B). At the peak of inflammation, T cells are distributed diffusely, and a much larger number of macrophages infiltrate the parenchyma. The same field of serial sections was photographed. An inset of C demonstrates that in some areas of lesions, T cells are almost absent (below the dotted line). A and C, R73 staining; B and D, ED1 staining. A–D, Original magnification ×75; insets ×150.

**FIGURE 2.** Estimation of inflammation and fibrosis using H&E- and Azan-stained sections. Representative feature at early (2 wk; A and B) and late (12 wk; C and D) stages is shown. During the early stage, there was extensive inflammatory cell infiltration in the parenchyma of some rats (A), whereas fibrosis shown in Azan staining was minimal (B). In sharp contrast, very small number of inflammatory cells was present at the later stage (C). Instead, dense fibrosis was recognized in Azan staining (D). A–D, Original magnification ×180. E. Longitudinal study of inflammation and fibrosis in the hearts during autoimmune carditis and subsequent dilated cardiomyopathy. Inflammation and fibrosis were scored according to the grading system provided under Materials and Methods. There was severe inflammation between 2 and 6 wk, and fibrosis was significantly beginning at 4 wk. Between 4 and 6 wk, inflammation and fibrosis were colocalized in diseased hearts. Asterisks indicate score 0 for inflammation and fibrosis.
prominent after 2 wk (the early inflammation phase) and lasted until week 6. In contrast, marked fibrosis appeared after 4 wk and became predominant after 8 wk (the late fibrosis phase). Between weeks 4 and 6, inflammation and fibrosis were colocalized in diseased hearts (Fig. 2).

Kinetics of MMP-2, MMP-9, TIMP-1, and TGF-β1 mRNA in the heart

We quantitated the mRNA and protein concentration of inflammation- and fibrosis-related molecules in the hearts of normal and immunized animals by real-time PCR and gelatin zymography. As shown in Fig. 3, A and B, MMP-2 and MMP-9 mRNA had different kinetics. MMP-2 mRNA increased gradually until the chronic phase, with significant increase at 2 and 4 wk compared with normal hearts (Fig. 3A). In sharp contrast, MMP-9 mRNA increased abruptly at 2 wk and decreased thereafter (Fig. 3B). Interestingly, TIMP-1 mRNA had essentially the same kinetics as MMP-9 mRNA (Fig. 3C). TGF-β1 mRNA increased at 2 wk, and expression remained high during the chronic phase (Fig. 3D).

Gelatin zymography (Fig. 3E) demonstrated that the MMP activity correlated well with mRNA expression revealed by real-time PCR analysis. MMP-9 increased abruptly at 2 wk PI (Fig. 3G), whereas both active (Fig. 3F) and latent (data not shown) MMP-2 showed continuous up-regulation during the inflammation and fibrosis phases.

Kinetics of gelatinase activity in the heart during EAC

To quantitatively estimate gelatinase activity, we performed FIZ. Representative FIZ figures at different time points (Fig. 4, A–D) and a summary of the results (Fig. 4E) are shown in Fig. 4. Under normal conditions, gelatinase activities were not detected in the heart (Fig. 4A). Gelatinase activity was seen in the hearts as early as day 7. Second, gelatinase activity as detected by FIZ analysis dropped down quickly at 6 wk as fibrosis became prominent. The latter finding, along with previous reports (20), suggests that an imbalance of the MMP/TIMP ratio may facilitate cardiac fibrosis.
vessel walls and the perivascular region were positive for gelatinase (Fig. 4, H and I). At the peak of inflammation (2 wk), vascular and parenchymal staining was strong and diffuse (Fig. 4, J and K). Thereafter, inflammation was gradually replaced by fibrosis (Fig. 2E), but gelatinase activity, as detected by ISZ, was maintained throughout the observation period (Fig. 4, L and M). Considering that the level of MMP-9, but not MMP-2, mRNA dropped very rapidly at 4 wk (Fig. 3), we concluded that the gelatinase activity at 6 wk is mainly due to MMP-2.

Identifying cells with gelatinase activities in the heart

Using ISZ and immunohistochemistry, we identified which cells expressed gelatinase in the heart by using markers for T cells (R73), macrophages (ED1), mast cells (anti-mast cell tryptase), cardiomyocytes (anti-α-actinin), and myofibroblasts (anti-vimentin). The results are illustrated in Fig. 5. At 2 wk (Fig. 5, A–L), T cells (Fig. 5, A–C), and mast cells (Fig. 5, G–I) did not express gelatinase activity (arrows in Fig. 5, C and I). The majority of gelatinase-expressing cells were infiltrating macrophages (Fig. 5, D and E, and arrowheads in F). Cardiomyocytes showed diffuse and faint staining (Fig. 5, J–L). As clearly seen in Fig. 5L, cells showing strong gelatinase activity were negative for α-actinin (arrowheads in Fig. 5L), suggesting that they are infiltrating inflammatory cells. At 6 wk (Fig. 5, P–X), T and mast cells were also negative for gelatinase (data not shown). Macrophages and cardiomyocytes had different staining patterns. At 6 wk, macrophages were negative for gelatinase (Fig. 5, P and Q, and arrows in R), and cardiomyocytes stained strongly for gelatinase (Fig. 5, S–U). Vimentin, one of myofibroblast markers, was positive for a few cells at 2 wk (Fig. 5N), and vimentin-positive cells increased in number considerably at 6 wk (Fig. 5W). At both time points, it was clearly demonstrated that vimentin-positive cells were negative for gelatinase (arrows in Fig. 5, O and X).

Treatment of CC2-immunized rats with MMP inhibitors

Finally, we treated CC2-immunized rats with two types of MMP inhibitors to examine whether the suppression of MMPs could modulate EAC development (Figs. 6 and 7). Minocycline, which was reported to be an inhibitor of MMP-2 and MMP-9, was administered at a dose of 50 mg/kg for 3 wk from the day of immunization. Hearts were examined both macroscopically and microscopically on day 21 (Fig. 6). Minocycline treatment significantly ameliorated the histological severity of EAC (Fig. 6A). Although minocycline is considered as an inhibitor of both MMP-2 and MMP-9, real-time PCR analysis of heart tissues revealed that MMP-9, but not MMP-2, mRNA was almost completely suppressed by minocycline (Fig. 6, C and D). Gelatin zymography demonstrated that the treatment resulted in complete inhibition of MMP-9 and moderate suppression of active MMP-2 (Fig. 6B). The findings obtained by in vivo treatments were consistent with those obtained by in vitro studies, showing that, especially at low concentrations, minocycline severely inhibits MMP-9 activity, but has less effects on MMP-2 (16, 21). Collectively, these results indicate that minocycline treatment predominantly suppressed MMP-9 production to ameliorate the severity of C protein-induced EAC. Minocycline treatment also affected the survival rate. Approximately 10% of untreated immunized rats died at this time point (9), whereas there were no dead rats in the treated group. These findings suggest that MMP-9 plays an important role in the development and progression of a certain type of EAC and DCM.

We next treated immunized animals with the MMP-2-specific inhibitor, TISAM (an N-sulfonylamino acid derivative) (22), for 3...
ROLE OF MMPs IN C PROTEIN-INDUCED DCM

We also performed immunohistochemical examination of hearts of rats treated with minocycline (Fig. 8, A and B) and TISAM (Fig. 8, C and D), and untreated controls at 2 wk (Fig. 8, A and F) and 6 wk (Fig. 8, G and H). Compared with untreated controls (Fig. 8E), infiltrating T cells decreased in number drastically in the minocycline-treated groups (Fig. 8A). TISAM treatment did not suppress T cell infiltration (Fig. 8C). At 6 wk, T cells were sparsely distributed in the heart parenchyma in the untreated controls (Fig. 8G). Macrophage staining showed a similar finding, but the number of macrophages was higher than that of T cells (Fig. 8, B, D, F, and H).

Discussion

DCM is a serious problem for patients with heart failure because the disease progresses irreversibly and often causes death. To develop effective therapies, it is essential to elucidate the pathomechanism of DCM development. Unfortunately, there are few sufficient experimental models for DCM. In previous studies, we succeeded in inducing severe EAC by immunizing Lewis rats with cardiac C protein (9, 10). The animals presented characteristics of EAC with a high rate of fatality, and the survivors developed DCM. This animal model is useful not only to understand the mechanism of DCM development from viral and immune induction, but also to develop effective immunotherapies (9). Furthermore, as shown in the present study, the disease course consistently exhibits early inflammatory and late fibrotic phases with a transitional phase between them. This uniform lesion formation enables the precise examinations of disease development and progression. In the present study, we demonstrate the important role of MMPs in the development of EAC and DCM to identify a successful, mechanism-based therapy.

MMPs form a family of enzymes that mediate various functions in tissue destruction and immune responses in autoimmune diseases. In the heart, it was demonstrated that MMPs play a pivotal role in acute cardiac injury and chronic remodeling (19, 23, 24). However, virtually no reports have shown longitudinal examinations of MMPs at either the RNA or protein level. In the present study, we correlate MMP expression and enzymatic activity with the degree and extent of inflammation and fibrosis in the heart over a 12-wk period. In our model, inflammation peaked after 2 wk, and then fibrosis developed. MMP-2, MMP-9, TIMP-1, and TGF-β1 mRNA all showed peak levels at 2 wk, and gelatinase activity was seen in both the parenchyma and the perivascular space. At 6 wk, and evaluated the histological severity of TISAM-treated and vehicle-treated rats on day 21. TISAM treatment caused no significant difference in pathology between the treated and control groups (Fig. 7A). Then, we determined the mRNA levels of MMP-2 and MMP-9. Real-time PCR analysis revealed that in vivo administration of TISAM did not suppress the level of MMP-2 mRNA (Fig. 7B). Similarly, gelatin zymography revealed no difference in the band density of MMP-2 between the TISAM-treated and vehicle-treated groups (Fig. 7C). Because MMP-2 activities could reappear if the TISAM-MMP-2 complex dissociated during electrophoresis, we performed ISZ to evaluate in situ enzymatic activity. Gelatinolytic activity, mainly by MMP-2 produced by cardiomyocytes (Fig. 5), was diminished in both the parenchyma and the blood vessels compared with controls (Fig. 7D). Semi-quantitative analysis revealed that gelatinolytic activity was significantly suppressed in the TISAM-treated groups compared with the control group. These findings are consistent with previous studies (19), demonstrating that TISAM specifically inhibited MMP-2 activities in a cell-free assay. These findings indicate that, although TISAM treatment inhibited MMP-2 activities in a contact-dependent manner, it does not modulate the development and progression of EAC.
every parameter shifted to the fibrosis phase. Whereas MMP-2 and TGF-β1 mRNA were maintained at a high level, MMP-9 and TIMP-1 mRNA levels dropped after week 2. As shown in the present study, FIZ and ISZ had distinct patterns during the chronic phase. Whereas FIZ activity dropped down rapidly after week 6, ISZ activity remained at a high level during this period. The results obtained by gelatin zymography clearly demonstrated that at 2 wk PI, MMP-9 and, to a lesser degree, MMP-2 represented the gelatinase activity. However, at the 4- and 6-wk time point, MMP-2 was the major gelatinase present in the heart. These findings strongly suggested that FIZ and ISZ mainly represent MMP-9 and MMP-2 activities, respectively.

FIGURE 6. Minocycline treatment ameliorates EAC and down-regulates MMP-9, but not MMP-2, activity. Minocycline hydrochloride was dissolved in PBS and administered daily by i.p. injection at a dose of 50 mg/kg body weight for 3 wk. On day 21, rats were sacrificed under deep anesthesia, and the effects of minocycline were evaluated histologically. A, Inflammation score; B, gelatin zymography; C and D, real-time PCR.

FIGURE 7. TISAM treatment suppresses MMP-2 activities, but does not modulate the severities of EAC. TISAM, suspended in 0.5% w/v methylcellulose 400 solutions, was orally fed at a dosage of 5 mg/kg/day. Rats received TISAM daily for 3 wk after immunization with CC2. Control rats received the vehicle. Rats were sacrificed on day 21 under deep anesthesia, and hearts were removed and analyzed histologically (A). In parallel, real-time PCR (B) and gelatin zymography (C) were performed using heart tissues. Cryostat sections were stained with ISZ (D) and evaluated semiquantitatively (E).
Examinations of ISZ and immunohistochemistry clearly demonstrated that gelatinase (MMP-2 and MMP-9)-expressing cells in the early stage consist of infiltrating macrophages, whereas those in the later stage are cardiomyocytes. Gelatin zymography and real-time PCR analysis suggest that infiltrating macrophages produce both MMP-2 and MMP-9 during the inflammation phase, whereas cardiomyocytes produce mainly MMP-2 at later stages. Although it is well known that MMPs, especially MMP-2, are expressed in cardiomyocytes under various pathological conditions (25–28), the present study clearly demonstrates that MMP-2 expression by cardiomyocytes takes place during later stages, but not during early inflammation. It should be noted that T cells and mast cells, which are reported to secrete MMPs (29, 30), were negative for gelatinase activity throughout the observation period. These findings suggest that, although MMPs can be expressed in a variety of cells, they are limited to certain cell types under individual pathological conditions.

It is interesting to note that MMP-2 mRNA was at a high level from 2 wk onward throughout the observation period, whereas MMP-9 mRNA was up-regulated at week 2 and then dropped at week 4. These time points corresponded with the switch from inflammation to fibrosis in the diseased hearts. Although both MMP-2 and MMP-9 belong to the gelatinase family, their role in autoimmune inflammation is substantially different. Morphologic and enzymatic analyses suggested that MMP-9 promotes disease progression during the early inflammatory phase and that MMP-2 is active during the chronic fibrosis phase. To test these hypotheses, we treated rats with MMP inhibitors. Administration of minocycline significantly reduced inflammation (Fig. 6) (16, 21). The effective use of minocycline in brain diseases such as encephalomyelitis (16, 31) and stroke (21) has been demonstrated, but this is the first report to demonstrate the effectiveness of minocycline in heart inflammation. In addition to down-regulation of MMPs, minocycline has several functions, including antiapoptotic and antioxidant actions (32, 33). Therefore, it is possible that cardioprotection may be also exerted by these mechanisms other than MMP suppression. These findings suggest that minocycline and its derivatives could be potential drugs for EAC and DCM therapy.

The role of MMP-2 in various disease conditions remains to be controversial. MMP-2-deficient mice exhibited more severe forms of autoimmune inflammation, such as arthritis (34), encephalomyelitis (35), myocarditis (36), and colitis (37), suggesting that MMP-2 plays a suppressive role in the development of autoimmune diseases. However, targeted disruption of MMP-2 ameliorated myocardial remodeling after myocardial infarction (19, 38) and chronic pressure overload (24). The precise mechanism of MMP-2's differential effects under various disease conditions remains unclear; there are several explanations. Esparza et al. (35) demonstrated a compensatory increase in MMP-9 in MMP-2-deficient mice with encephalomyelitis, which may be an aggravation factor of the disease. In addition, MMP-2 may suppress inflammation by cleaving MCP-3 (39) and IL-1 (40). In contrast, in situation of cardiac remodeling without inflammation, disruption or inhibition of MMP-2 could improve disease conditions. In the present study, we treated immunized animals with a specific MMP-2 inhibitor, TISAM, to evaluate whether MMP-2 inhibition modulates the disease course. TISAM treatment during the early inflammatory phase neither ameliorated nor aggravated the disease, although the treatment did suppress MMP-2 activity (Fig. 7). These findings suggest that MMP-2 has a small role in early lesion formation.

The involvement of MMP-2 and MMP-9 in fibrosis formation during the late stage is controversial. Although some groups insisted that MMP-2 is necessary and sufficient for fibrosis formation (41, 42), other groups reported that both MMP-2 and MMP-9 are involved (43, 44). In contrast, there are several reports suggesting that MMPs play no or little role in fibrosis formation (45–48). Because there are so many differences in the types of diseases, experimental condition, and clinical observations, it is difficult to draw conclusions at the moment. It should be elucidated in the near future.

In summary, we have demonstrated that during the course of EAC and subsequent DCM, different types of cells produced MMPs in a manner that corresponds to the disease phase. During the early inflammation phase, infiltrating macrophages mainly produced MMP-9, and cardiomyocytes produced MMP-2 in later stages. Inhibition of MMP-9, but not of MMP-2, ameliorated the disease status. Thus, immunotherapy against EAC should be performed based on an understanding of the disease development mechanism.

Acknowledgments
We thank Jun Lu for tissue preparation.

Disclosures
The authors have no financial conflict of interest.