Thioredoxin-1 Ameliorates Myosin-Induced Autoimmune Myocarditis by Suppressing Chemokine Expressions and Leukocyte Chemotaxis in Mice

Wenrui Liu, MD; Hajime Nakamura, MD, PhD; Keisuke Shioji, MD, PhD; Masaki Tanito, MD, PhD; Shin-ichi Oka, PhD; M. Kaimul Ahsan, MD; Aoi Son, DDS; Yasuyuki Ishii, PhD; Chiharu Kishimoto, MD, PhD; Junji Yodoi, MD, PhD

Background—Cardiac myosin–induced myocarditis is an experimental autoimmune myocarditis (EAM) model used to investigate autoimmunological mechanisms in inflammatory heart diseases and resembles fulminant myocarditis in humans. We investigated the therapeutic role of thioredoxin-1 (TRX-1), a redox-regulatory protein with antioxidant and antiinflammatory effects, in murine EAM.

Methods and Results—EAM was generated in 5-week-old male BALB/c mice by immunization with porcine cardiac myosin at days 0 and 7. Recombinant human TRX-1 (rhTRX-1), C32S/C35S mutant rhTRX-1, or saline was administered intraperitoneally every second day from day 0 to 20. In addition, rabbit anti-mouse TRX-1 serum or normal rabbit serum was administered intraperitoneally on days 1, 2, and 6. Animals were euthanized on day 21. Histological analysis of the heart showed that TRX-1 significantly reduced the severity of EAM, whereas mutant TRX-1 failed to have such an effect, and anti–TRX-1 antibody enhanced the disease markedly. Immunohistochemical analysis showed that TRX-1 significantly suppressed cardiac macrophage inflammatory protein (MIP)-1α, MIP-2, and 8-hydroxydeoxyguanosine expression and macrophage infiltration into the heart in EAM. Although serum levels of MIP-1α were not suppressed by TRX-1 until day 21, both an in vitro chemotaxis chamber assay and an in vivo air pouch model showed that TRX-1 significantly suppressed MIP-1α– or MIP-2–induced leukocyte chemotaxis. However, real-time reverse transcription–polymerase chain reaction showed that TRX-1 failed to decrease chemokine receptor expression increased in the bone marrow cells of EAM mice.

Conclusions—TRX-1 attenuates EAM by suppressing chemokine expressions and leukocyte chemotaxis in mice. (Circulation. 2004;110:1276-1283.)

Key Words: thioredoxin ■ myosin ■ myocarditis

Thioredoxin-1 (TRX-1), a small (12-kDa) protein with a highly conserved redox-active dithiol/disulfide in the active site sequence Cys32-Gly-Pro-Cys35, plays a variety of redox-related roles in organisms ranging from Escherichia coli to human.1 TRX-1 has a crucial role in the cellular regulation of redox. It catalyzes the reduction of disulfide bonds and quenches reactive oxygen species by coupling with TRX-dependent peroxidase. TRX-1 also has important extracellular functions. It has been reported that TRX-1 is secreted from cells and that extracellular TRX-1 is transported into cells,2 indicating that it shuttles between the cytoplasm and extracellular space. However, to date, any receptor or target molecule for TRX-1 has not yet been identified. Previous reports have shown that plasma TRX-1 is a good mediator of oxidative stress responses and have suggested that it is involved in various inflammatory, immune, and autoimmunne diseases because plasma levels of TRX-1 are elevated in HIV infection,3,4 hepatitis C virus infection,5 and rheumatoid arthritis.6 Recently, we reported that circulating TRX-1 prevents lipopolysaccharide- and chemokine-induced neutrophil chemotaxis, which indicates that TRX-1 has an antiinflammatory effect, especially in pathogen-free disorders initiated by leukocyte infiltration.7 These findings suggest that recombinant TRX-1 ameliorates autoimmune disorders.

See p 1178

Myosin-induced myocarditis is a model of experimental autoimmune myocarditis (EAM) used to investigate autoimmunological mechanisms in inflammatory heart diseases and resembles fulminant myocarditis in humans.8–11 This model
of EAM in susceptible mouse strains is histologically similar to human myocarditis, with the recruitment of macrophages, lymphocytes, and neutrophils accompanying myocardial necrosis. During the development of disease in this model, myosin first elicits a focal inflammation mediated by neutrophil- and bone marrow–derived macrophage infiltration, which then triggers the activation of mononuclear effector cells and initiates the EAM.

Freund’s complete adjuvant alone as a control. BALB/c mice were injected subcutaneously on days 0 and 7 in an equal volume of Freund’s back with 0.1 mL of porcine cardiac myosin (1 mg/mL) emulsified with Mycobacterium tuberculosis H37Ra (DIFCO) or with Freund’s complete adjuvant alone as a control.

Administration of Recombinant Human TRX-1
Five-week-old BALB/c mice were randomly grouped as follows: unimmunized (n = 5), immunized with intraperitoneal administration of either 0.1 mL of saline (n = 11), 40 μg per mouse of recombinant human TRX-1 (rhTRX-1) (n = 11), or C32S/C35S mutant rhTRX-1 (n = 11) in 0.1 mL of saline every second day from day 0 to day 20. Animals were euthanized under ether anesthesia 21 days after the first immunization.

Administration of Anti–TRX-1 Antibody
Five-week-old immunized BALB/c mice with myosin immunization were randomly grouped as follows: intraperitoneal administration of 0.1 mL rabbit anti-mouse TRX-1 serum (n = 11), normal rabbit serum (DAKO) (n = 10), or saline (n = 11) on days 1, 2, and 6. Animals were euthanized in the same manner as described above.

Histopathology
Under euthanasia, the heart was removed, fixed in formalin for 24 hours, embedded in paraffin, and stained with hematoxylin and eosin. Myocarditis was determined by identifying both infiltrating mononuclear cells and myocyte necrosis. The percentage of myocardial inflammation was determined by semiquantitative image analysis.

Immunohistochemistry
Immunohistochemical detection of macrophages, MIP-1α, MIP-2, and 8-hydroxydeoxyguanosine (8-OHdG) was performed with the use of rat anti-mouse macrophage F4/80 antigen (Serotec), rabbit anti-mouse MIP-1α or rabbit anti-mouse MIP-2 polyclonal antibody (HyCult Biotechnology), and anti–8-OHdG monoclonal antibody (NOF Corporation) as primary antibodies, respectively. Briefly, mice were euthanized 21 days after the first immunization, and the hearts were removed and made into paraffin sections. After a dewaxing procedure, antigen retrieval was performed in 10 mmol/L citric acid buffer (pH 6.0) with a 10-minute microwave. To detect murine macrophages, the slides were incubated with Alexa Fluor 488 goat anti-rat IgG (Molecular Probes), and counterstaining was performed with propidium iodide. Murine macrophages were visualized by merge. To detect MIP-1α or MIP-2, endogenous peroxidase activity was blocked with 0.6% H2O2 at room temperature for 30 minutes. MIP-1α and MIP-2 were recognized with the HistoScan HRP Universal Rabbit Kit (BIOMEDA). For 8-OHdG, the slides were incubated with a biotinylated rabbit anti-mouse immunoglobulin (Bayer), and the reaction was visualized with the ABC complex (Vector Laboratories) followed by diaminobenzidine (DAKO).

ELISA for Serum MIP-1α and MIP-2 and rhTRX-1
ELISA assay was performed to determine serum levels of MIP-1α and MIP-2 in EAM. Blood was obtained from the abdominal aorta of myosin-immunized mice 17 and 21 days after the first immunization and from normal mice on day 0. Serum MIP-1α and MIP-2 levels were quantified with the use of Murine Quantikine M kits (R&D System).

To examine the pharmacological kinetics of intraperitoneally administered rhTRX-1, serum levels of rhTRX-1 were measured by sandwich ELISA as described previously, with the use of a TRX ELISA kit (Redox Bioscience), which does not cross-react with murine TRX-1. The reported value is the mean±SD of duplicate samples.

Chemotaxis Chamber Assay
Bone marrow cells were collected from 6-week-old male BALB/c mice under sterilized conditions. MIP-1α– and MIP-2–induced chemotaxis was evaluated with a 48-well microchemotaxis chamber. Saline or chemoattractant solution (30 μL) was added to the lower compartment, and 50 μL of cell suspension (5×107/mL), preincubated at 37°C with or without 100 ng/mL of rhTRX for 20 minutes, was added to the upper compartment. The chamber was incubated at
Chemotaxis In Vivo (Air Pouch Model)
A dorsal air pouch was created as described previously. Briefly, 6-week-old male BALB/c mice were injected subcutaneously with 4 mL of air on days −7 and −3. On day 0, 1 mL of saline alone or with 100 ng of MIP-1α or 10 ng of MIP-2 was injected into the air pouch, immediately followed by intraperitoneal administration of 0.1 mL of saline alone or saline with 40 μg of rhTRX. Four hours later, the air pouch was flushed quantitatively with saline, the volume recovered was measured, and the number of cells recovered was determined with a hemocytometer.

Real-Time Reverse Transcription–Polymerase Chain Reaction
To examine gene expressions of chemokine receptors in bone marrow cells of myosin-immunized mice, real-time reverse transcription–polymerase chain reaction (RT-PCR) for chemokine (C–C) receptor 1 (CCR1)/MIP-1α receptor and chemokine (C–X–C) receptor 2 (CXCR2) was performed. EAM was generated in 5-week-old BALB/c male mice with rhTRX treatment (n=4) or saline treatment (n=4). Seventeen days after the first immunization, total RNA was extracted from the bone marrow cells from normal (n=4) or EAM-induced mice after red cell lysis with the use of TRizol reagent (Invitrogen), and first-strand cDNA was synthesized with SuperScript II (Invitrogen) following the manufacturer’s instructions. Primers of murine CCR1 and CXCR2 were purchased from Applied Biosystem. The reaction was performed with ABI PRISM 7000 Sequence Detection System (Applied Biosystems) under conditions of 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Statistical Analysis
Results are expressed as mean±SD. The Student t test was used for comparisons between 2 groups. The statistical significance of differences among the 3 groups was assessed by the Scheffé multiple-comparison test. Findings of P<0.05 were considered statistically significant.

Results
TRX-1 Attenuates Autoimmune Myocarditis
To investigate the effect of rhTRX-1 on EAM, rhTRX-1 was injected intraperitoneally into inbred mice with myosin immunization every second day. Myocarditis, characterized by inflammatory cellular infiltration and myocardial necrosis, was detected 21 days after the first immunization by hema-toxylin and eosin staining. Postmortem histopathology of cardiac tissue showed that >75% of myosin-immunized mice developed myocarditis. Among different animal groups, the ratio of disease development showed no statistical difference, whereas the severity of disease in myocarditis-infected mice showed a significant difference. EAM was significantly attenuated by both 40 μg (Figure 1A, 1B) and 80 μg of rhTRX-1 administration but not by 10- or 20-μg treatment (data not shown). Therefore, we used 40 μg of rhTRX-1 per mouse in this study. Meanwhile, C32S/C35S mutant rhTRX-1 was used as a negative control peptide, which showed no effect on EAM (Figure 1A, 1B). We reported previously that myocardial expression and/or serum levels of TRX-1 were increased in patients, rats, and susceptible mouse strains with myocarditis. To examine whether endogenous TRX-1 is involved in the attenuation of EAM, we used anti-mouse TRX-1 antibody to neutralize the endogenous TRX-1. The neutralization of TRX-1 resulted in a marked enhancement of EAM, whereas control serum had no such effect (Figure 1A, 1B). Because murine TRX-1 activity is undetectable in vivo, in this study the insulin disulfide reduction assay was performed, which proved that our anti–TRX-1 antibody effectively neutralized TRX-1 reducing activity (Figure 1C). These results suggested that endogenous TRX-1 plays an inhibitory role in the development of EAM and that the inhibition of EAM development by administration of rhTRX-1 occurs through promotion of the intrinsic action of the protein.

TRX-1 Suppresses Cardiac Oxidative Damage in EAM
Oxidative damage was identified in tissue immunopositive for 8-OHdG. Strong 8-OHdG immunoreactivity was evident in the hearts of mice with EAM, particularly in the area with inflammatory cellular infiltration and myocardial necrosis. In contrast, the area immunopositive for 8-OHdG was much smaller in mice treated with TRX-1 (Figure 1D).

Pharmacological Kinetics of Intraperitoneal Injection of rhTRX-1
ELISA assay was used to examine blood levels of intraperitoneally injected rhTRX-1. Within 4 hours of injection, the concentration of rhTRX-1 was >100 ng/mL, which was high enough to suppress the chemotaxis of leukocytes, as shown in Figure 2. Twelve hours later, serum concentrations of rhTRX-1 were below detectable levels (data not shown). The administration of rhTRX-1 was performed every second day in this study, in which EAM was successfully attenuated. These results suggested that continuously high levels of TRX-1 were not necessary for the suppression of EAM.

TRX-1 Suppresses Macrophage Infiltration in EAM
Macrophage infiltration is crucial to the onset of EAM. In the present study, immunohistochemistry studies with the use of anti-F4/80 antibody, a specific marker of macrophages, were performed to detect macrophage infiltration in EAM. Macrophage infiltration into the hearts was confirmed in mice with EAM 21 days after the first immunization, particularly at the peripheral area of necrosis, which was strongly inhibited by rhTRX-1 administration (Figure 3). These results indicated that TRX-1 suppresses the event(s) upstream of macrophage infiltration.

TRX-1 Suppresses Cardiac MIP-1α and MIP-2 Expression in EAM
Recent studies have indicated that MIP-1α and MIP-2 are deeply involved in the pathogenesis of EAM, with increased cardiac expression inducing macrophage and T-lymphocyte migration into the heart, leading to myocardial necrosis and resulting in the development of EAM. In the present study, we investigated the effect of TRX-1 on cardiac MIP-1α and MIP-2 expression in EAM. Immunohistochemical analysis revealed that cardiac MIP-1α and MIP-2 levels were increased in EAM and were decreased by rhTRX-1 (Figure

37°C with 5% CO2 for 2 hours, then the chamber was turned upside down for 15 minutes. The filter was stained with Diff-Quik (International Reagents Co). The number of infiltrate cells was taken as the mean of counts in 5 immersion fields.
4A). It has also been reported that serum levels of MIP-2 were significantly elevated in CVB3 myocarditis–infected mice. Next, we analyzed the serum concentrations of MIP-1α/H9251 and MIP-2 in EAM.

TRX-1 Decreases Serum Levels of MIP-1α in EAM

ELISA showed that serum levels of MIP-1α were significantly increased on days 17 and 21 in myosin-immunized mice compared with those of control mice. Administration of rhTRX-1 markedly lowered the serum levels of MIP-1α in myosin-immunized mice on day 21 (Figure 4B). In contrast, there was no significant increase of serum MIP-2 levels in either myosin-immunized mice or TRX-1–treated myosin-immunized mice compared with control mice (data not shown), suggesting that MIP-2 is specifically utilized in the cardiac region in this model of EAM.

TRX-1 Suppresses MIP-1α– and MIP-2–Induced Chemotaxis of Neutrophils, Macrophages, and Lymphocytes

Although TRX-1 suppressed myosin-induced MIP-1α and MIP-2 expression in myocarditic hearts (Figure 4A), serum levels of MIP-1α were not decreased until day 21 (Figure 4B), suggesting that TRX-1 should have an inhibitory effect downstream of MIP-1α and MIP-2 expression. Because previous studies showed that TRX-1 suppresses lipopolysaccharide-induced neutrophil chemotaxis, we investigated...
whether TRX-1 can also suppress MIP-1α and/or MIP-2–induced leukocyte chemotaxis. In the present study both the in vitro chemotaxis chamber assay and the in vivo air pouch model showed that, at a concentration of 100 ng/mL, TRX-1 significantly suppresses MIP-1α– and MIP-2–induced chemotaxis of neutrophils, macrophages, and lymphocytes of murine bone marrow cells (Figure 5A, 5B). Furthermore, ELISA showed that circulating TRX-1 concentrations were >100 ng/mL until 4 hours after intraperitoneal administration.

TRX-1 Does Not Suppress Chemokine Receptor Expression of Bone Marrow Cells in EAM

Real-time RT-PCR showed that gene expression of chemokine receptors CCR1 and CXCR2 increased in bone marrow cells 17 days after the first immunization in EAM, and the administration of TRX-1 did not suppress them (Figure 6).

Discussion

The present study demonstrated that rhTRX-1 attenuates myosin-induced EAM in mice. Administration of rhTRX-1 significantly reduced the severity of the disease, whereas the neutralization of TRX-1 by anti–TRX-1 antibody markedly promoted the disease. It seems unlikely that anti–TRX-1 antibody enters into the tissues and suppresses intracellular TRX-1 actions. It is more likely that anti–TRX-1 antibody suppresses the action of TRX-1 in plasma. Although the plasma levels of murine TRX-1 were not detectable, it has been reported that plasma levels of human TRX-1 are greatly elevated in patients with myocarditis or heart failure. These results suggest that the main action site of TRX-1 is outside...
The cell in this disease. It has been reported that, in the early stage of EAM, myosin induces cardiac chemokine expression, which results in macrophage and T-lymphocyte infiltration into the heart and initiates the disease. The present study showed that TRX-1 did not suppress chemokine receptor expression in bone marrow cells and suppressed the migration of effector cells from the bloodstream into the heart by its direct antimicrobial effect.

The in vitro chemotaxis chamber assay showed that the serum levels of TRX-1 at 4 hours after intraperitoneal injection were high enough to suppress the chemotaxis of leukocytes, which is consistent with previous studies showing that intraperitoneal injection of rhTRX-1 every second day suppressed leukocyte infiltration–associated interstitial pneumonia in the lung; rhTRX-1 was administered every other day in the present study in an animal model of EAM. Although rhTRX-1 was no longer detectable 12 hours after the intraperitoneal injection, it proved to have a suppressive effect on EAM. The initial suppression of leukocyte chemotaxis by TRX-1 may be important for the attenuation of EAM. In addition, our preliminary study showed that administration of rhTRX-1 did not change the subpopulation and proliferation of the splenetic lymphocytes in mice with myocarditis (data not shown), suggesting that TRX-1 does not induce an additional host immune response in this model.

TRX-1 is well known as a scavenger of reactive oxygen species. In the present study immunohistochemistry studies with the use of 8-OHdG, a marker for tissue oxidative damage, were performed, which showed that 8-OHdG was strongly expressed in the myocardial heart, whereas the area immunopositive for 8-OHdG was much smaller in the heart with TRX-1 treatment. It also has been reported that, in the initial stage of EAM, cardiac dendritic cells and infiltrating macrophages and neutrophils attack the cardiomyocytes, forming rosette figures as a sign of active cardiomyocytolysis; subsequently, the infiltration by macrophages and T lymphocytes plays a crucial role in the generation of myocarditis. A recent study showed that reactive oxygen species are involved in the antigen-presenting function of dendritic cells and that antioxidants suppress the activation of dendritic cells. TRX-1 has radical scavenging functions. Therefore, administration of TRX-1 may also attenuate the activation of dendritic cells in this model. Shioji et al also reported that the suppression of dendritic cell functions by immunoglobulin therapy in the early phase of disease attenuates giant cell autoimmune myocarditis. The aforementioned evidence strongly suggests that the reducing activity of TRX-1 plays, at least in part, an important therapeutic role in EAM.

In addition, MIP-1α and MIP-2 have recently been reported to be involved in the recruitment of inflammatory cells in EAM. In the present study the cardiac expression of MIP-1α and MIP-2 was markedly suppressed by rhTRX-1. Our study showed that serum MIP-2 levels in mice with EAM were not significantly different from those of controls, which was inconsistent with previous reports that plasma MIP-2 levels were significantly elevated in CVB3 myocarditis–infected mice on days 7, 10, and 14. This may be due to the different mechanisms involved in antigen mimicry and viral infection–induced autoimmune myocarditis. In the present study both the in vivo air pouch model and in vitro chemotaxis chamber assay showed that TRX-1 suppresses the MIP-1α– and MIP-2–induced chemotaxis of macrophages, lymphocytes, and neutrophils. Taken together, our results suggested that the attenuation of EAM by rhTRX-1 is due to its suppression of MIP-1α– and MIP-2–induced chemotaxis of inflammatory cells in the initial phase of EAM. In fact, later administration of rhTRX-1 from day 14 did not ameliorate EAM (data not shown). It has been reported that, in the early stage of EAM, myosin induces cardiac chemokine expression, which results in macrophage and T-lymphocyte infiltration into the heart and initiates myocarditis. In the
The present study demonstrated that the administration of rhTRX-1 ameliorates autoimmune myocarditis by suppressing chemokine expression and leukocyte chemotaxis, providing evidence obtained in vivo that the use of recombinant TRX-1 offers a novel approach to the treatment of inflammatory diseases initiated by leukocyte infiltration. Therefore, the therapeutic use of TRX-1 for the treatment of inflammatory and autoimmune diseases is promising.

Acknowledgments
This work was supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Wenrui Liu); Japan Society for the Promotion of Science (Masaki Tanito); New Energy and Industrial Technology Development Organization Fellowship Program (Shin-ichi Oka); grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and grant-in-aid for the Research and Development Program for New Bio-Industry Initiatives, Bio-oriented Technology Research Advancement Institution. We thank Yoko Kanekiyo for secretarial support and Akie Teratani for technical assistance.

References
Thioredoxin-1 Ameliorates Myosin-Induced Autoimmune Myocarditis by Suppressing Chemokine Expressions and Leukocyte Chemotaxis in Mice

Wenrui Liu, Hajime Nakamura, Keisuke Shioji, Masaki Tanito, Shin-ichi Oka, M. Kaimul Ahsan, Aoi Son, Yasuyuki Ishii, Chiharu Kishimoto and Junji Yodoi

_Circulation._ 2004;110:1276-1283; originally published online August 30, 2004;
doi: 10.1161/01.CIR.0000141803.41217.B6
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/10/1276