Upregulation of thioredoxin (TRX) expression in giant cell myocarditis in rats

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Abstract To examine the possible involvement of a redox regulating mechanism in the pathogenesis of immune-mediated myocarditis, myocarditis was induced by immunization of porcine cardiac myosin in rats and immunohistochemistry and Western blot for thioredoxin (TRX) were performed. Immunohistochemistry for 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nuclear factor kappa-B (NF-κB) was also performed. TRX was upregulated in the acute stage, but not in the chronic stage, and the expression was correlated with the severity of the disease. Damaged myocytes were strongly immunostained for 8-OHdG and NF-κB. Thus, TRX may be specifically induced by acute inflammatory stimuli, and the development of acute immune-mediated myocarditis may be regulated by the cellular redox state via TRX.

Key words: Myocarditis; Thioredoxin; 8-Hydroxy-2′-deoxyguanosine; Nuclear factor kappa-B

1. Introduction

Myocarditis is an important cause of heart failure among adolescents and young adults. Viral myocarditis frequently precedes the development of dilated cardiomyopathy (DCM) and is considered as a cause of DCM. Some cases of DCM may be mediated by autoimmune responses to cardiac antigens. At present, we have two animal models of myocarditis to analyze the pathogenetic mechanisms responsible for immune or autoimmune mechanisms. One is elicited by cardiotropic virus infection in mice and the other by immunization of cardiac myosin in rodents [1,2]. The myosin immunization model in rats mimics human fulminant myocarditis in the acute phase and human DCM in the chronic phase [2,3]. Accordingly, this model is particularly suitable for studying the immunopathology of inflammatory heart diseases [2,3].

Adult T cell leukemia-derived factor was originally defined as an interleukin-2 (IL-2) receptor α-chain inducer in human lymphotropic virus-1-transformed cells and is identical to thioredoxin (TRX) [4,5]. TRX is a small multifunctional protein which contains a redox-active disulfide/dithiol within the conserved active site sequence: -Cys-Gly-Pro-Cys [6]. TRX has various important biological activities for both intra- and extracellular components. For example, TRX is stress-inducible which protects cells from various types of stresses, e.g. viral infection, exposure to ultraviolet (UV) light, X-ray irradiation and hydrogen peroxide [7,8]. Moreover, TRX is a scavenger of reactive oxygen intermediates (ROIs) and overexpression of TRX in transgenic mice showed a protective function against postischemic reperfusion injury in brain in vivo [9]. In myocarditis, ROIs are able to induce severe cardiovascular dysfunction and it was reported that oxygen free radicals may be involved in the pathogenesis and development of coxsackievirus B3 myocarditis and that appropriate dosages of superoxide dismutase have a therapeutic potential for myocarditis [10,11]. However, it has not been reported whether myocarditis is controlled by the redox regulatory protein, TRX. To examine the cellular redox regulation in immune-mediated myocarditis, giant cell myocarditis was induced by the immunization of porcine cardiac myosin in rats. The role of the cellular redox state in the development of immune-mediated myocarditis was discussed.

2. Materials and methods

2.1. Antigen preparation and immunization

Autoimmune myocarditis was induced as previously described [2]. Porcine cardiac myosin (Sigma, M0531) was dissolved in phosphate buffered saline (PBS) at a concentration of 2 mg/ml. Six- to 7-week-old Lewis rats (Shimizu Laboratory Supplies Co., Japan) were injected subcutaneously in their foot pads with 0.1 ml of myosin (1 mg/ml) mixed with an equal volume of Freund's complete adjuvant (FCA) supplemented with *Mycobacterium tuberculosis* H37Ra (Difco, 3113-60) on days 1 and 7. Rats were killed serially on days 17 ($n=8$), 21 ($n=14$), 24 ($n=10$) and 54 ($n=6$) under ether anesthesia and were processed for the immunohistochemical study and Western blotting analysis. Littermate controls ($n=5$) were injected with FCA alone and were killed on day 21. After macroscopic examination, the ventricles were fixed in 10% formalin, transversely sliced, embedded in paraffin and stained with hematoxylin and eosin. Tissue preparations were examined with a microscope for the degree of myocardial damage and the infiltration of inflammatory cells.

2.2. Immunohistochemistry

For the analysis of TRX and nuclear factor kappa-B (NF-κB), we used the immunoperoxidase technique [12]. Briefly, after paraffin sections of the heart were deparaffinized and autoclaved for 10 min at 121°C in 10 mmol/l citrate buffer (pH 6.0), endogenous peroxidase activity was inactivated with 3% H_2O_2 for 10 min. The primary antibody (rabbit anti-mouse TRX antibody [12]; rabbit anti-human NF-κB p65 antibody, Santa Cruz, C-20) or normal rabbit serum was added and incubated overnight. It was reported that the rabbit anti-mouse TRX antibody crossreacts on rats [12]. The rabbit anti-human NF-κB p65 antibody crossreacts on humans and rats [13]. Biotinyl-

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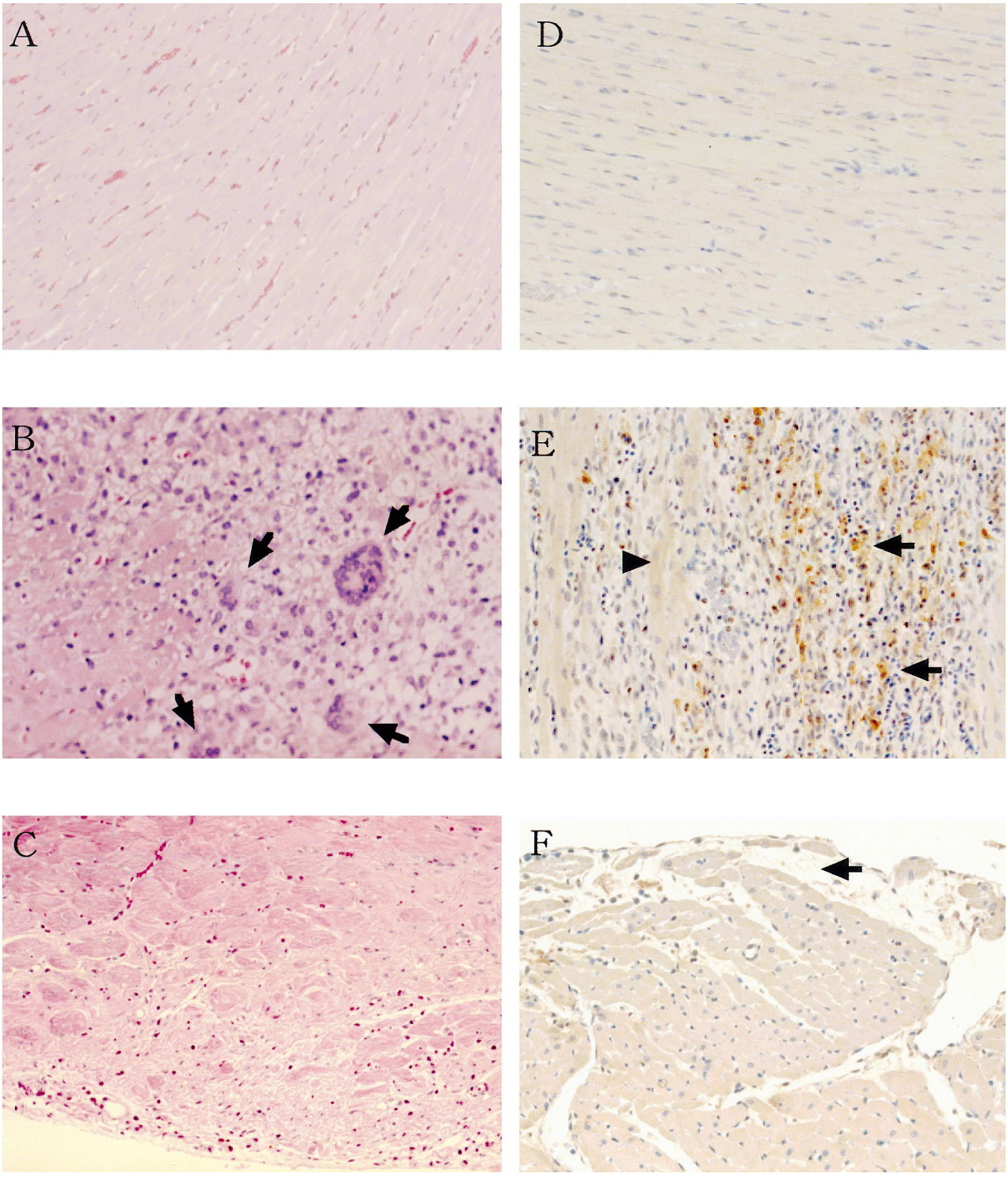

Fig. 1. Histopathology and immunohistochemistry for TRX in immune-mediated myocarditis. A: Histology of the heart immunized with FCA on day 21. The myocardium was intact. B: Histology of the heart immunized with myosin on day 21. Marked cellular infiltrations in the inflammatory focus were observed. Moreover, multinuclear giant cells were observed (arrows). C: Histology of the heart immunized with myosin on day 54. Myocarditis was almost healed and the area of myocardial necrosis was replaced by prominent fibrous tissue. D: Immunohistochemistry for TRX in the heart immunized with FCA on day 21. Marginal or trivial TRX immunoreactivity was observed. E: Immunohistochemistry for TRX in the heart immunized with myosin on day 21. Strong TRX immunoreactivity was observed in both infiltrating inflammatory cells in the inflammatory focus (arrows) and damaged myocytes in the perinecrotic lesion (arrow head). F: Immunohistochemistry for TRX in the heart immunized with myosin on day 54. TRX was slightly stained in myoctyes and was not stained in fibrous tissue (arrow). A–C: Hematoxylin and eosin staining. D–F: Immunohistochemical staining for TRX and counterstaining with hematoxylin. Magnification, ×180.

ated and affinity purified goat anti-rabbit immunoglobulin G (IgG) (Dako) was used as the secondary antibody and incubated for 30 min. Normal goat serum was added for the inhibition of non-specific binding of a secondary antibody. An avidin–biotin complex was sequentially added for 5 min incubation with the substrate 0.1% 3′,3′-diaminobenzidine at room temperature, followed by hematoxylin nuclear counterstaining.

For the analysis of 8-hydroxy-2′-deoxyguanosine (8-OHdG), we used the alkaline-phosphatase technique [14]. 8-OHdG was an established marker for oxidative stress [14]. After deparaffinization, the primary antibody (Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan) or normal mouse serum was added and incubated overnight. Biotin-labelled rabbit anti-mouse IgG (Dako) was used as the second antibody for 40 min, followed by an avidin–biotin–alkaline phosphatase complex (Vector). Counterstaining was not performed.

2.3. Western blot

The hearts were homogenized and then lysed for 30 min with 5 ml of a lysis buffer (0.5% Nonidet P-40, 10 mmol/l Tris–HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l phenylmethylsulfonyl fluoride and 5 mmol/l aprotinin) on ice. The extracts were cleared by centrifugation. Equal amounts of protein of the supernatant (8 μg protein/lane), estimated by the Bradford method using a protein assay (Bio-Rad), were electrophoresed on a 15% sodium dodecyl sulfate–polyacrylamide gel and sequentially electrophoretically transferred to a polyvinylidene difluoride microporous membrane (Millipore). After blocking with 5% bovine serum albumin in PBS containing 0.05% Tween 20 at 4°C overnight, the membrane was incubated with the primary antibody (rabbit anti-mouse TRX antibody) and then with the peroxidase-linked secondary antibody (Amersham Life Science). Chemiluminescence was detected with an ECL Western blot detection kit (Amersham Pharmacia Biotech) and semiquantitatively analyzed using the NIH Image system.

3. Results

3.1. Histopathology

Immune-mediated giant cell myocarditis was induced in all the rats immunized with porcine cardiac myosin. No evidence of myocarditis was shown in the rats immunized with FCA alone (Fig. 1A). In rats immunized with myosin, the hearts showed mild and focal discolored myocarditis and a few infiltrating inflammatory cells were observed on day 17. On days 21 and 24, some of the hearts showed severe and diffuse discolored myocarditis with massive pericardial effusion. In inflammatory foci, extensive injury of myocytes with various kinds of inflammatory changes, such as fragments of necrotic myocardial fibers, mononuclear cells, polymorphonuclear neutrophils, eosinophils and multinucleated giant cells, was observed (Fig. 1B). On day 54, active inflammatory lesions were healed and some cardiomyocytes showed degeneration. The area of myocardial necrosis was replaced by prominent fibrous tissue (Fig. 1C).

3.2. Immunohistochemistry and Western blot for TRX

Immunohistochemistry was performed to determine the histological localization of TRX in the heart of immune-mediated myocarditis. A control specimen with FCA alone (intact heart) showed the trivial immunoreactivity for TRX (Fig. 1D). TRX was strongly stained in both infiltrating inflammatory cells in the necrotic areas and damaged myocytes in the perinecrotic lesions on day 21 (Fig. 1E). TRX was slightly stained in myoctyes but not in fibrous tissue on day 56 (Fig. 1F). Immunohistochemistry for TRX was observed in neither the control intact heart nor the myocarditis specimen when the primary antibody was omitted (data not shown). To determine the level and stage of TRX induction in myocarditis, the Western blot and semiquantitative analyses using the NIH Image system for TRX was performed. The Western blot with anti-mouse TRX antibody (Fig. 2) showed that levels of TRX were higher in the rats with myosin immunization than in the rats with FCA alone on day 21. Moreover, the expressions of TRX were more increased in the rats with severe myocarditis than in the rats with mild myocarditis. The expression of TRX was decreased in the rats immunized with cardiac myosin on day 54, compared with that on day 21. The data suggest that the expression was correlated with the severity of the disease and that TRX was upregulated not in the chronic stage but in the acute immune-mediated stage. The rabbit anti-mouse TRX antibody reacted on both smooth muscle cells (Fig. 2A, lane i) and myocytes (Fig. 2A, lanes b–h) in rats.

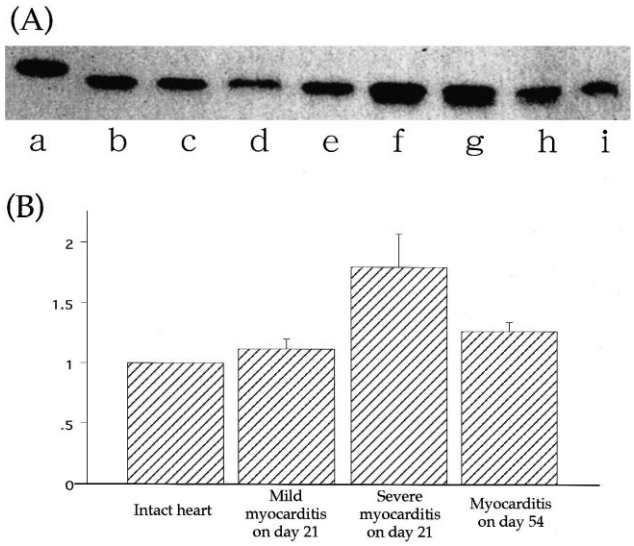

Fig. 2. Western blot analysis for TRX. A: Representative Western blot is shown. Lane a, recombinant TRX; lanes b and c, lysates from two different hearts immunized with FCA on day 21; lanes d and e, lysates from two different hearts immunized with myosin on day 21 (mild myocarditis); lanes f and g, lysates from two different hearts immunized with myosin on day 21 (severe myocarditis); lane h, lysate from the hearts immunized with myosin on day 54; lane i, lysate from rat smooth muscle cells. Protein of 8 μg per lane was loaded except for lane a (recombinant TRX, 100 ng). B: Densitometric band intensities were shown. The level of TRX was higher in the rats with myocarditis induced by myosin immunization than in normal rats immunized with FCA alone. Moreover, the expression of TRX was more intense in the rats with severe myocarditis than in the rats with mild myocarditis. The level of TRX was decreased in the rats immunized with cardiac myosin on day 54, compared with on day 21. Levels of TRX in normal rats immunized with FCA alone were normalized to 100% in each experiment and relative levels of TRX were expressed. Data are mean ± S.E.M. of four independent experiments.

3.3. Immunohistochemistry of 8-OHdG and NF-κB

Immunohistochemistry for 8-OHdG was carried out to investigate whether ROIs were produced in immune-mediated myocarditis. Myocytes of the control specimen with FCA immunization (intact heart) showed trivial nuclear immunostaining (Fig. 3A). On day 21, 8-OHdG was strongly stained in the nuclei of both infiltrating cells and myocytes in the perinecrotic lesions (Fig. 3B). On day 56, weak nuclear staining was observed in the nuclei of fibroblasts and myocytes around fibrous tissue (Fig. 3C). No nuclear staining was seen in the specimen of myocarditis when the primary antibody was omitted (data not shown).

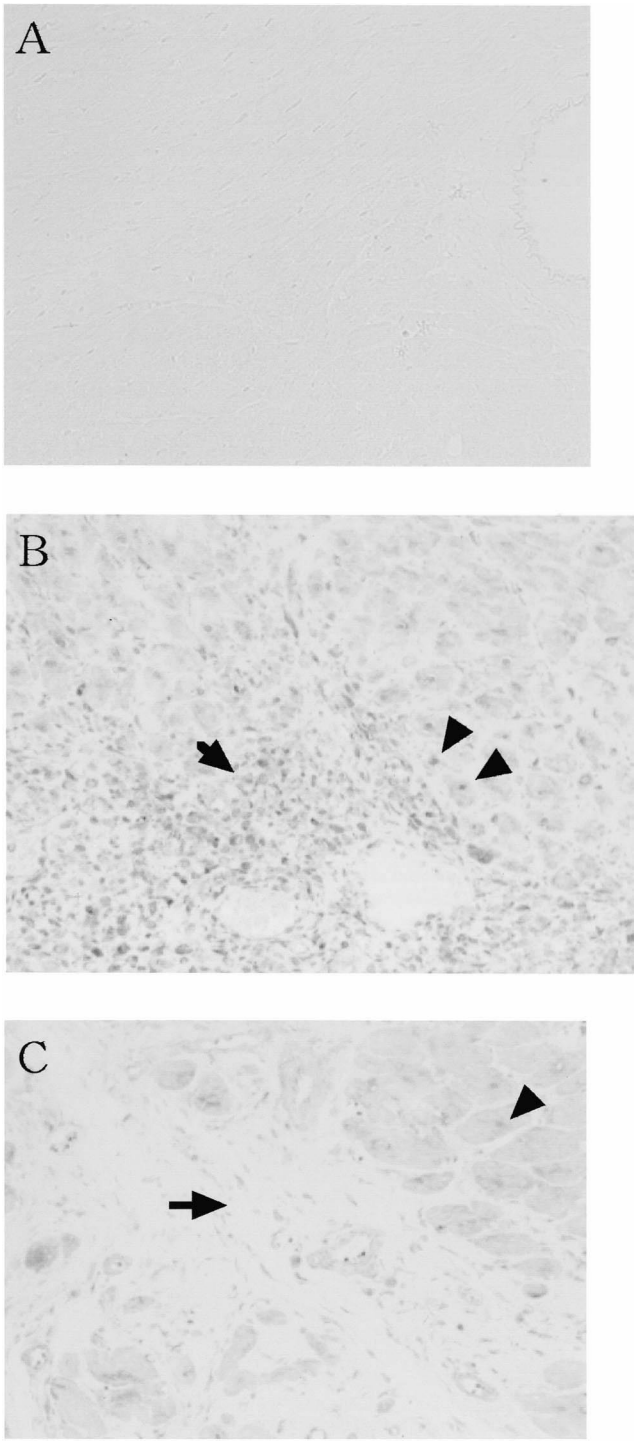

Fig. 3. Immunohistochemistry for 8-OHdG in immune-mediated myocarditis. A: Immunohistochemistry for 8-OHdG in the intact heart immunized with FCA alone on day 21. In control intact heart, slight or no nuclear staining was observed. B: Immunohistochemistry for 8-OHdG in the heart immunized with myosin on day 21. Strong nuclear staining was observed in both infiltrating inflammatory cells in the inflammatory focus (arrow) and myocytes in the perinecrotic lesions (arrow heads). C: Immunohistochemistry for 8-OHdG in the heart immunized with myosin on day 54. Weak nuclear staining was observed in nuclei of fibroblast (arrow) and myocytes around fibrous tissue (arrow head). Counterstaining with hematoxylin was not done. Magnification, ×188.

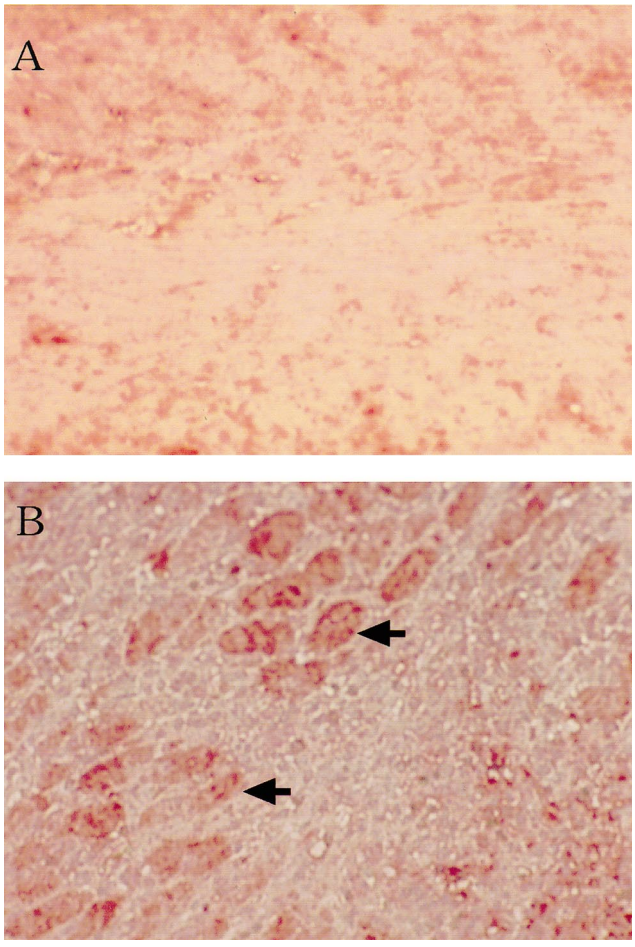

Fig. 4. Immunohistochemistry for NF-κB in immune-mediated myocarditis. A: Immunohistochemistry for NF-κB p65 in the intact heart immunized with FCA alone on day 21. Myocytes were slightly stained. B: Immunohistochemistry for NF-κB p65 in the diseased heart immunized with myosin on day 21. Myocytes in the perinecrotic lesions were strongly stained (arrows). Counterstaining with hematoxylin was performed. Magnification, ×200.

Immunohistochemistry for NF-κB showed that the myocytes in the perinecrotic lesions were strongly stained (Fig. 4B), compared with those of the control specimens with FCA alone (intact heart) (Fig. 4A). No staining was seen in the specimen of myocarditis when the primary antibody was omitted (data not shown). The results suggest that ROIs may be involved in the development of myocardial damage in immune-mediated myocarditis. Taken together, the upregulation of TRX may be a reaction to oxidative stress possibly produced by infiltrating cells in the myocardium.

4. Discussion

Our study provides the first in vivo evidence that the redox regulatory protein, TRX, was upregulated in immune-mediated giant cell myocarditis and that it is upregulated in the acute immune-mediated stage, but not in the chronic stage. TRX per se and the cellular redox state controlled by TRX may play an important role in the pathogenesis and development of the disease.

8-OHdG, one of the major DNA base-modified products, is induced either by hydroxyl radical, singlet oxygen or photo-

dynamic action and is known to be mutagenic by pairing with adenine as well as cytosine, leading to G:C to T:A transversion at DNA replication [15,16]. Accordingly, 8-OHdG is an established marker for oxidative stress. In the present study, 8-OHdG was expressed in the necrotic areas and perinecrotic lesions of myocarditis. It was already reported that TRX was induced by ROIs in the mouse kidney [17]. Thus, taken together, the results of the present study suggest that enhanced TRX expression may be induced by ROIs produced by infiltrating inflammatory cells in acute immune-mediated myocarditis.

It was reported that TRX has the function of redox regulation of the transcription factors such as NF-κB or activator protein-1 [18]. In the present study, the expression of NF-κB was enhanced in the perinecrotic lesions of immune-mediated myocarditis. As TRX is known to regulate NF-κB activation via its thiol redox control [19], enhanced TRX expression may respond to the corresponding overexpression of NF-κB in the cytoplasm. It was reported that ROIs induce translocation of TRX from the cytoplasm into the nucleus and that TRX in the nucleus enhances NF-κB transcriptional activities in its ability to bind to DNA [19]. There is accumulating evidence that the activation of NF-κB mediates cytoprotective signal transductions [20]. Thus, TRX per se may have a protective role against the progressive myocardial damage in acute immune-mediated myocarditis through the activation of NF-κB. An appropriate dosage of TRX may have therapeutic potential for the treatment of the acute stage of myocarditis.

We analyzed TRX expression in spontaneously hypertensive rats (SHR) in another set of experiments. SHR is a genetic model of chronic overload with a lifetime elevated systolic blood pressure and established left ventricular hypertrophy and myocardial fibrosis [21,22]. In 4-week-old SHR, hypertension begins to develop and in 14-week-old SHR, with early hypertensive heart disease, left ventricular hypertrophy and myocardial fibrosis is observed [21,22]. We found that the immunoreactivity of TRX in 14-week-old SHR was equal to that in their normotensive genetic control age-matched Wistar–Kyoto rats. That is, TRX was not induced by the chronic hypertensive stimuli (data not shown). Taking this together with the result that TRX was reduced in the chronic or healed stage of myocarditis, TRX seemed to be specifically induced by the acute inflammatory stimuli.

In summary, TRX was upregulated in acute immune-mediated giant cell myocarditis. Our findings suggest that the development of acute immune-mediated myocarditis may be regulated by the cellular redox state via TRX.

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