Overexpression of Thioredoxin-1 in Transgenic Mice Attenuates Adriamycin-Induced Cardiotoxicity

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Background—Adriamycin (ADR) is an anticancer drug known to cause severe cardiac toxicity by generating free radicals. We investigated the role of a redox-regulating molecule, thioredoxin-1 (TRX1), in ADR-induced cardiotoxicity.

Methods and Results—The in vitro study showed that TRX1 was dose-dependently increased concomitant with the formation of hydroxyl radicals in ADR-treated neonatal rat cardiomyocytes. Lactate dehydrogenase–releasing assay showed that treatment with recombinant human TRX1 suppressed cardiomyocyte injury in ADR-treated cardiomyocytes. To examine the biological significance of TRX1 in vivo, we used transgenic mice expressing increased levels of human TRX1 (TRX1-TG mice). Electron microscopy revealed that mitochondria, myofibrils, and other cellular details were much better maintained in ADR-treated TRX1-TG mice than in ADR-treated nontransgenic (WT) mice. The increase in the protein carbonyl content, a marker of cellular protein oxidation, was suppressed in ADR-treated TRX1-TG mice compared with ADR-treated WT mice. The formation of hydroxyl radicals in ADR-treated heart homogenates of TRX1-TG mice was decreased compared with WT mice. For the survival study, all WT mice treated with ADR died within 6 weeks, but 5 of 6 TRX1-TG mice treated with ADR survived >8 weeks.

Conclusions—TRX1 is upregulated by intracellular oxidative stress generated by ADR. TRX1 has a protective role against ADR-induced cardiotoxicity by reducing oxidative stress. (Circulation. 2002;106:1403-1409.)

Key Words: antioxidants • free radicals • cardiomyopathy

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driamycin (ADR), a quinone-containing anthracyclin, is an important anticancer drug widely used in the treatment of many human tumors. However, treatment with this agent is limited by a potentially lethal and dose-dependent congestive cardiomyopathy.1 The mechanism for the antitumor activity of ADR has been suggested to be attributable to direct DNA damage or interference with DNA repair.2 Cardiac tissue is not an actively proliferating tissue. Accordingly, the mechanism for ADR-induced cardiotoxicity has been suggested to be attributable, at least in part, to free reactive oxygen species (ROS)-mediated cardiomyocyte damage.3,4 It has been reported that treatment with n-acetylcysteine2 and an antioxidative drug, such as probucol,6 protected against ADR-induced cardiotoxicity and that overexpression of human manganese superoxide dismutase (Mn-SOD) in transgenic mice attenuated ADR-induced cardiotoxicity.7 Accordingly, the modulation of the redox state in cardiomyocytes reduces the severity of the disease in ADR-induced cardiotoxicity.

Thioredoxin (TRX) is a small multifunctional protein that contains a redox-active disulfide/dithiol within the conserved active site sequence -Cys-Gly-Pro-Cys-.8 There is TRX1, a cytosolic and nuclear form, and TRX2, a mitochondrial form. TRX1 is stress-inducible, which protects cells from various types of stresses, eg, viral infection, exposure to ultraviolet light, x-ray irradiation, and hydrogen peroxide.8 Moreover, TRX1 is a scavenger of ROS,9,10 and overexpression of human TRX1 (hTRX1) in transgenic (TRX1-TG) mice showed a protective activity to postischemic reperfusion injury in the brain and autoimmune and streptozotocin-induced diabetes in vivo.11,12 TRX1 expression levels correlated with the resistance to ADR in T-cell leukemia cell lines.13 These findings prompted us to investigate the importance of the redox states controlled by TRX1 in ADR-induced cardiotoxicity. In the present study, we examined the induction mechanism and biological role of TRX1 in ADR-treated neonatal rat cardiomyocytes in vitro. To analyze the biological significance of TRX1 in vivo, we also subjected TRX1-TG and nontransgenic (wild-type [WT]) mice to ADR and investigated the role of TRX1 in ADR-induced cardiotoxicity.

Methods

Culture of Neonatal Rat Cardiomyocytes In Vitro
Cardiac ventricles from 1- to 4-day-old Lewis rats were minced and dissociated with 0.125% trypsin (Sigma). The dispersed cells were
incubated for 60 minutes at 37°C. Nonattached myocytes were collected and cultured in DMEM supplemented with 10% FCS in a humidified atmosphere of 5% CO₂ at 37°C. Bromodeoxyuridine (BrdU, 100 µmol/L) was added during the first 48 hours to prevent proliferation of nonmyocytes. Spontaneously beating myocyte-rich cultures (final cell density, 10⁶ cells/cm²) were then incubated for 48 hours without BrdU, followed by a final incubation in 10 mL fresh DMEM containing 10% FCS. During this final incubation, myocytes were treated with ADR (0.1 to 0.5 µmol/L or without) for 24 hours, and the medium or lysate was collected. Recombinant human TRX1 (rhTRX1, 10 to 1000 nmol/L or absence) was added to the medium 1 hour before the treatment of ADR. ADR was provided by Kyowa Hakko Kogyo Company, Ltd (Tokyo, Japan).

Western Blot Analyses
The expressions of TRX1, Mn-SOD, and copper/zinc (CuZn)-SOD proteins were estimated by Western blot analysis using rabbit anti-mouse TRX1 polyclonal antibody, mouse anti-human monoclonal antibody, rabbit anti-Mn-SOD antibody (StressGen), or rabbit anti–CuZn-SOD (StressGen) and semiquantitatively analyzed using the NIH Image system, as described previously.

Detection of Oxidized Proteins
Oxidative inactivation of enzymes and oxidative modification of proteins by metal-catalyzed oxidation reactions are accompanied by the generation of protein carbonyl derivatives. Oxidized protein was measured by detecting protein carbonyl derivatives, which is a hallmark of the oxidation status of all of the proteins (OxyBlot, Oncor), as described previously.

Electron Spin Resonance Spectroscopy
The formation of hydroxyl radical (·OH) was detected with 5,5′-dimethyl-1-pyrroline-1-oxide (DMPO) (Labotec) as a spin trap. In vitro, 1×10⁶ cells were incubated with ADR (50 to 200 µmol/L or without) in 0.15 mL PBS for 10 minutes and then added to 0.05 mL of 9.0 mol/L DMPO. Ex vivo, the hearts were homogenized in cold PBS (0.2 g hearts/mL), incubated with 200 µmol/L ADR (WT mice, n=5; TRX1-TG mice, n=6) or without (WT mice and TRX1-TG mice, n=3 each) for 10 minutes, and then added to 0.05 mL of 9.0 mol/L DMPO. The generation of hydroxyl radicals was observed as DMPO·OH adduct on a JEL-FR30 spectrometer. Scan conditions were as follows: microwave frequency, 9.42 GHz; power, 4.0 mW; modulation frequency, 100 kHz; time constant, 0.1 seconds; and scan time, 120 seconds.

Cytotoxicity Assay
Cell viability was assayed by lactate dehydrogenase (LDH)-releasing assay. LDH released from damaged cells in the medium was measured using an LDH assay kit (Kyoruto, Japan), according to the manufacturer’s procedure. The percent cell lysis was determined as the ratio of LDH in the medium/total LDH (medium plus cell lysates) per well.

Transgenic Mice
The generation and maintenance of TRX1-TG mice was described previously. hTRX1 cDNA was inserted between the β-actin promoter and the β-actin terminator. The transgene was cut out of the plasmid by XbaI and 3′-SpI digestion, purified, and used to generate transgenic mice. The pronuclei of fertilized eggs from superovulated C57BL/6j were microinjected with this DNA construct. Animals were screened by Southern blot analysis of their tail DNA. Among 3 established lines (βAc-ADF-2, βAc-ADF-5, and βAc-ADF-10) of transgenic mice, 1 line (βAc-ADF-2) was used for additional experiments. There were no differences in the expressions of Mn-SOD, CuZn-SOD, and glutathione peroxidase between WT and TRX1-TG mice, analyzed by immunohistochemistry and Western blotting. The presence of the TRX1 transgene was confirmed by reverse transcription–polymerase chain reaction analysis before the experiments.

Treatment Protocol In Vivo
ADR dissolved with saline (cumulative dose, 6 [n=7], 15 [n=7], or 24 [n=8] mg/kg body weight) was administered intraperitoneally to 8-week-old WT mice in 3 equal injections for 2 weeks. Littermate controls (n=6) were injected with saline. For the experiment using TRX1-TG mice, ADR (cumulative dose, 15 mg/kg body weight) was administered to 8-week-old WT mice (n=10) and TRX1-TG mice (n=6) in 3 equal injections for 2 weeks. Littermate controls (WT mice, n=4; TRX1-TG mice, n=4) were injected with saline. In each protocol, animals were killed under ether anesthesia at the end of the 2-week posttreatment periods. The hearts were removed and additionally processed for pathological studies. For the survival study, WT mice (n=5) and TRX1-TG mice (n=6) were treated with 24 mg/kg ADR in 3 equal injections for 2 weeks and were followed for 8 weeks.

Immunohistochemistry
We used the immunoperoxidase technique to perform immunohistochemistry for TRX1, as described previously.

Ultrastructural Studies
Ultrastructural studies were performed, as described previously. For the ultrastructural studies, 3 hearts in each group were processed. Six electron micrographs in each mouse were observed in a Hitachi H-7000 transmission-electron microscope.

Statistical Analysis
Values were expressed as the mean±SD. Statistical analysis of the data was determined by unpaired Student’s t test or one-way ANOVA with Fisher’s protected least-significant difference test. Survival data was analyzed by the Kaplan and Meier method.

Results
Upregulation of TRX1 Expression Associated With Increased Oxidized Protein in WT Mice Treated With ADR In Vivo
No mice died throughout the entire study period in each group. Massive pleural effusion was observed in mice treated with 24 mg/kg ADR but not in mice injected with saline or mice treated with 6 or 15 mg/kg ADR. In the H&E sections, the changes of cardiomyocytes were not apparent in mice treated with 6 or 15 mg/kg ADR, but vacuolization and edema of cardiomyocytes were observed in mice treated with 24 mg/kg ADR.

We first determined whether treatment with ADR increased the expression of TRX1 protein in the heart of WT mice. Western blot analysis demonstrated that the expression of TRX1 protein was dose-dependently enhanced in the hearts of ADR-treated mice (Figures 1A and 1C). However, the expressions of CuZn-SOD and Mn-SOD proteins were not changed by ADR treatment (Figures 1A and 1C).

Immunohistochemistry was performed to determine the histological localization of TRX1 in ADR-induced cardiomyopathy. Control specimens injected with saline showed the trivial immunoreactivity for TRX1. TRX1 was strongly stained in damaged cardiomyocytes in mice treated with 24 mg/kg ADR (Figure 1D).

We measured oxidized protein by detecting protein carbonyl derivatives, because ADR was suggested to increase intracellular protein oxidation by ROS via quinone-semiquinone recycling. Oxidized protein was dose-dependently increased in the heart of ADR-treated mice.
Densitometric analysis showed that the levels of oxidized protein in mice treated with 6, 15, or 24 mg/kg ADR were enhanced by 1.08 to 1.10-fold (n=4, P<0.05), 1.59 to 1.60-fold (n=4, P<0.01), or 2.24 to 2.25-fold (n=4, P<0.01), respectively, relative to mice treated with 0 mg/kg ADR (n=4).

ROS Produced by ADR Increased Expression of TRX1 Protein in Neonatal Rat Cardiomyocytes In Vitro

We next examined the mechanism and role of TRX1 increased by ADR treatment in primary cultures of neonatal rat cardiomyocytes. TRX1 expression (Figure 2A) and oxidized protein (data not shown) were dose-dependently increased by ADR treatment.

Figure 2B showed the representative electron spin resonance (ESR) spin adduct of ADR-treated samples, which showed a dose-dependent increase in the quartet signal patterns specific for the hydroxyl radical up to 100 μmol/L ADR. Hyperfine coupling constants for the spin adduct were analyzed with values for N of 1.49 mT and H of 1.49 mT. This component of the spectrum was assigned to DMPO·OH, as described previously. Ethanol is a scavenger of hydroxyl radical and forms DMPO-carbon-centered radicals. Incubation with 20 μL of 20% ethanol decreased the signal, suggesting that the hydroxyl radical formed from ADR were free hydroxyl radicals trapped by DMPO (data not shown). Although DMPO was also used to screen for the superoxide radical, none of the samples showed any evidence of superoxide-related signals. Accordingly, it is suggested that hydroxyl radicals have an important role in ADR-induced cytotoxicity.
In addition, preincubation with rhTRX1 suppressed the cytotoxicity induced by ADR in a dose-dependent manner (rhTRX1 0 nmol/L, 19.2±9.3%; 10 nmol/L, 16.3±6.6%; 100 nmol/L, 12.8±7.3%; 1000 nmol/L, 5.1±4.9%, P<0.01 versus 0 nmol/L rhTRX1, n=4 each) (Figure 3). Accordingly, TRX1 might confer cellular defense against ADR by scavenging intracellular toxic oxidants generated by ADR.

Effect of the Overexpression of TRX1 in Transgenic Mice on ADR-Induced Cardiomyopathy In Vivo

General Observation
To examine the biological significance of TRX1 in vivo, we finally performed experiments using transgenic mice expressing increased levels of hTRX1. The expression of hTRX1 protein in heart tissue was confirmed (Figure 4). No mice treated with 15 mg/kg ADR died during the entire study period in any group. After completion of the ADR treatment, WT mice appeared to be more seriously ill than TRX1-TG mice. In control groups injected with saline, both WT and TRX1-TG mice were healthy. There was no significant difference in the hearts of any group either macroscopically or microscopically.

Attenuation of Morphological Changes in TRX1-TG Mice
Electron microscopic analysis of the left ventricular wall was conducted. In WT mice treated with 15 mg/kg ADR (Figure 5).
5A), morphological changes, such as swelling of mitochondria, sarcoplasmic reticulum, extensive loss of cristae, and myofilament disarray, which were consistent with lesions of ADR-induced cardiotoxicity, were observed. However, in TRX1-TG mice treated with 15 mg/kg ADR (Figure 5B), mitochondria, myofibrils, sarcoplasmic reticulum, and other cellular details were better maintained than in WT mice treated with 15 mg/kg ADR. The sections of both saline-injected WT mice and saline-injected TRX1-TG mice showed a normal appearance.

**Suppression of Oxidized Proteins in TRX1-TG Mice**

There was no significant difference in protein carbonyl contents between saline-injected WT mice (n=4) and saline-injected TRX1-TG mice (0.80±0.15-fold relative to saline-injected WT mice, n=4, P=NS), analyzed by Western blotting for protein carbonyl derivatives in the heart tissues (Figure 6). However, protein carbonyl contents in WT mice treated with 15 mg/kg ADR were increased compared with TRX1-TG mice treated with 15 mg/kg ADR (ADR-treated WT mice, 1.66±0.43-fold relative to saline-injected WT mice; ADR-treated TRX1-TG, 1.05±0.25-fold relative to saline-injected WT mice, n=4 each, P<0.01).

**Suppression of the Formation of Hydroxyl Radical in TRX1-TG Mice**

ESR spectroscopic analyses showed that the formation of hydroxyl radical in ADR-treated heart homogenates of TRX1-TG mice was decreased compared with WT mice (Figure 7A). The hydroxyl radical signals relative to the internal standard of manganese ion were 0.94±0.07 in WT mice (n=5) and 0.74±0.15 in TRX1-TG mice (n=6, P<0.05, Figure 7B). No signal was present in homogenates without ADR heart tissues in both WT and TRX1-TG mice (Figure 7A).
modulated by TRX1 have an important role in the cellular pathways in the modulation of the redox state and the genomic responses to oxidative stress.

Findings suggest that TRX1 per se and the redox system modulated by increased TRX1 have a protective role in the pathogenesis and development of ADR-induced cardiotoxicity, which was shown by ESR spectroscopy in the present study, it is possible that TRX1 is induced by oxidative stress, including hydroxyl radicals. It is suggested that the upregulated expression of TRX1 by ADR is not enough to protect the heart against ADR-induced cardiotoxicity. We also showed that treatment with a high dose, but not low dose, of rhTRX1 reduced ADR-induced injury in neonatal rat cardiomyocytes in vitro. Moreover, TRX1-TG mice, whose TRX1 expressions in the hearts were 50-fold greater than those of WT mice, attenuated ADR-induced cardiotoxicity in vivo, and TRX1-TG mice survived longer than WT mice. The formation of hydroxyl radical in adriamycin-treated heart homogenates of TRX1-TG mice was decreased compared with WT mice. Furthermore, flow cytometric analysis with 2',7'-dichlorofluorescin diacetate demonstrated that exposure to ADR caused a significant increase in the intracellular accumulation of peroxides in the parental hepG2 hepatoma cells, but not in the hTRX1-transfected hepG2 cells (data not shown). Accordingly, TRX1 is an important molecule in the protection against ADR-induced cardiotoxicity via scavenging activities of hydroxyl radicals.

It was reported that mRNA of Mn-SOD was induced by high concentration of TRX1 and that overexpression of Mn-SOD in transgenic mice attenuated ADR-induced cardiotoxicity. However, the present study suggested that overexpression of TRX1 attenuated ADR-induced cardiotoxicity without interfering with the expression of Mn-SOD, because no significant difference was observed in the expression of Mn-SOD protein in heart lysates between WT mice and TRX1-TG mice (data not shown). As previously described, antioxidative enzymes, such as probucol, reduced long-term mortality in animals treated with ADR without reducing the chemotherapeutic activity of ADR against tumor. TRX1 has been shown to play a role in the drug resistance of adult T-cell leukemia cells to ADR. Additional studies are needed to investigate the differential effects of ADR-induced ROS on cardiomyocytes and human tumors. Organ-specific delivery of TRX1 to the heart or organ-specific overexpression of TRX1 would be useful for clinical applications to ADR-induced cardiotoxicity.

**Survival Study for WT and TRX1-TG Mice**

Comparison of survival curves by the Kaplan and Meier method showed a significant difference between the 2 groups (log-rank 6.513, P = 0.01) (Figure 8). Five of six TRX1-TG mice treated with 24 mg/kg ADR survived over 8 weeks, whereas all of WT mice treated with 24 mg/kg ADR died within 6 weeks.

**Discussion**

ADR remains one of the most effective chemotherapeutic agents for cancer therapy. However, the development of severe cardiomyopathy reduces its clinical effectiveness in humans. Thus, it has been particularly important to clarify the precise mechanisms by which treatment with ADR induces cardiotoxicity. The present study provides the first evidence that the redox regulatory protein, TRX1, is upregulated in ADR-induced cardiotoxicity because of oxidative stress, including hydroxyl radicals. Moreover, increased TRX1 and the cellular redox state modulated by increased TRX1 have a protective role in the pathogenesis and development of ADR-induced cardiotoxicity, which was clearly shown in the TRX1-TG mice study.

Increasing evidence has suggested that the modulation of intracellular redox states has important aspects to cellular events, such as proliferation and apoptosis via the regulation of intracellular signal transduction and gene expression. TRX1 has not only a scavenging activity of ROS, but a regulating activity of various intracellular molecules, including transcription factors such as nuclear factor-kB, activator protein 1, myb, redox factor 1, and mitogen-activated kinase. We previously reported the enhanced expression of TRX1 in damaged myocytes in rats with acute myocarditis. We documented that the elevation of serum TRX1 levels correlated with the severity of New York Heart Association functional class and negatively correlated with left ventricular ejection fractions in patients with heart diseases. These findings suggest that TRX1 per se and the redox system modulated by TRX1 have a important role in the cellular defense against oxidative stress in cardiomyocytes as well as in other cell types, such as neurons and hepatocytes.

Most studies support the view that both an increase in oxidative stress and a decrease in antioxidants play an important role in the pathogenesis of ADR cardiomyopathy. ADR has been shown to be a potential source of free radicals. In vitro studies, quinone-containing anticancer agents, including ADR, have been shown to form semiquinone free radical intermediates in the presence of certain flavin enzymes. The aerobic metabolism of ADR in rat heart sarcosomes has been shown to produce hydrogen peroxide, which reacts with ADR semiquinone to produce hydroxyl radicals, as confirmed by the ESR method.

In the present study, we demonstrated that ADR treatment increased the expression of TRX1 accompanied with increased oxidized protein, one of the oxidative stress markers in vivo. As mentioned above, TRX1 is a scavenger of hydroxyl radical. Taken together with the results that hydroxyl radicals were produced by ADR in neonatal rat cardiomyocytes, which was shown by ESR spectroscopy in the present study, it is possible that TRX1 is induced by oxidative stress, including hydroxyl radicals. It is suggested that the upregulated expression of TRX1 by ADR is not enough to protect the heart against ADR-induced cardiotoxicity. We also showed that treatment with a high dose, but not low dose, of rhTRX1 reduced ADR-induced injury in neonatal rat cardiomyocytes in vitro. Moreover, TRX1-TG mice, whose TRX1 expressions in the hearts were 50-fold greater than those of WT mice, attenuated ADR-induced cardiotoxicity in vivo, and TRX1-TG mice survived longer than WT mice. The formation of hydroxyl radical in adriamycin-treated heart homogenates of TRX1-TG mice was decreased compared with WT mice. Furthermore, flow cytometric analysis with 2',7'-dichlorofluorescin diacetate demonstrated that exposure to ADR caused a significant increase in the intracellular accumulation of peroxides in the parental hepG2 hepatoma cells, but not in the hTRX1-transfected hepG2 cells (data not shown). Accordingly, TRX1 is an important molecule in the protection against ADR-induced cardiotoxicity via scavenging activities of hydroxyl radicals.

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In conclusion, the findings of the present study may yield an important insight into both the mechanism of ADR-induced cardiotoxicity in relation to TRX1 and the future clinical application of TRX1.

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