Tumor necrosis factor receptor deficiency exacerbated Adriamycin-induced cardiomyocytes apoptosis: an insight into the Fas connection

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Abstract
Cardiomyopathy is a major dose-limiting factor for applications of Adriamycin, a potent chemotherapeutic agent. The present study tested the hypothesis that increased tumor necrosis factor (TNF)-α signaling via its receptors protects against Adriamycin-induced cardiac injury. We used mice in which both TNF receptor I and II have been selectively inactivated (DKO) with wild-type mice as controls. Morphometric studies of cardiac tissue following Adriamycin treatment revealed greater ultrastructural damage in cardiomyocyte mitochondria from DKO mice. Biochemical studies of cardiac tissues showed cytochrome c release and the increase in proapoptotic protein levels, suggesting that lack of TNF-α receptors I and II exacerbates Adriamycin-induced cardiac injury. The protective role of TNF receptor I and II was directly confirmed in isolated primary cardiomyocytes. Interestingly, following Adriamycin treatment, the levels of Fas decreased in the wild-type mice. In contrast, DKO mice had an increase in Fas levels and its downstream target, mitochondrial truncated Bid. These results suggested that TNF-α receptors play a critical role in cardioprotection by suppression of the mitochondrial-mediated associated cell death pathway. [Mol Cancer Ther 2006;5(2):261–9]

Introduction
Adriamycin (common name doxorubicin) is a prototype of anthracycline antibiotics that has been widely used as a chemotherapeutic agent since the late 1960s for hematologic and solid tumors. However, the clinical application of Adriamycin is limited in that its dose-related cardiomyopathy may lead to fatal congestive heart failure (1, 2). The precise biochemical mechanism of Adriamycin cardiotoxicity remains uncertain. The most prevailing hypothesis is that an increase of oxidative stress (2, 3) may be associated with alterations in calcium homeostasis (3, 4), defects in mitochondrial integrity and deterioration of myocardial bioenergetics (5–7), mutation of mitochondrial DNA (8), altered cardiomyocyte gene expression, and induction of apoptosis (9, 10).

Cytokines, such as tumor necrosis factor (TNF)-α, interleukin-1, interleukin-2, interleukin-6, and interleukin-10, have been shown to be involved in different cardiac diseases and in the complex syndrome of heart failure (11). TNF-α is a potent proinflammatory cytokine produced by many cell types, including cardiomyocytes. It is recognized as a cytokine with pleiotropic biological capacities, and it can elicit broad cellular responses in inflammation, cell survival, proliferation, differentiation, and apoptosis (12). The main biological actions of TNF-α are initiated by binding to two distinct receptors, TNFR-I (p55) and TNFR-II (p75). TNF-α has been shown to have both adverse and beneficial effects on the heart (12–14). TNF-α may serve as a stress response protein. Short-term and low-level expression of TNF-α may provide the heart with an adaptive response to stress, occurs during postischemic remodeling and adaptive growth, but long-term or high-level expression may be maladaptive and result in inflammation and apoptosis.

To investigate whether TNF-α has a beneficial effect on Adriamycin-induced cardiac injury and elucidate its possible mechanisms, we conducted a series of studies using TNF receptor–deficient mice. Our results showed that TNF-α signaling via its receptor I and II pathway may suppress both the extrinsic and intrinsic apoptosis pathways leading to the protection of mitochondria against Adriamycin-induced cardiac injury.

Materials and Methods
Generation of TNF Receptor – Deficient Mice
The p55 (TNFR-I)-deficient and p75 (TNFR-II)-deficient mice were generated by targeted gene disruption (15), and
the homozygote double TNFRI/TNFRII-deficient mice (DKO) were obtained by an appropriate cross of TNFRI- and TNFRII-deficient mice. The DKO mice had no overt phenotype or alterations in myocardial structure or histology (16). DKO mice were maintained on a random C57BL/6 × 129/SvEv hybrid background. Wild-type (WT) mice maintained on the same C57BL/6 × 129/SvEv hybrid background were used as controls. Ten- to 14-week-old male mice were used for experiments. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

**TNF-α Levels**

The TNF-α level of serum was determined using the Quantikine mouse TNF-α immunoassay kit (R&D Systems, Minneapolis, MN). Mice were treated i.p. with 20 mg/kg Adriamycin or the same volume of saline (control) and various samples were collected after injection at the times indicated. The Adriamycin dose used was similar to the maximum single clinical therapeutic dose (60–75 mg/m²; refs. 17, 18) by the calculation according to the conversion factor described by Freireich et al. (19).

**Ultrastructural Examination of Cardiac Tissues**

Ultrastructural injury in cardiac tissues of mice treated with saline or 20 mg/kg Adriamycin for 5 days was evaluated by electron microscopy using methods described by Yen et al. (20) with minor modifications. Heart tissue was cut into 1 mm³ pieces and fixed in 4% paraformaldehyde and 5% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) for 6 to 8 hours, and then postfixed in Caulfield’s osmium tetroxide with sucrose for 30 to 60 minutes at 4°C. Tissue was dehydrated in graded ethanol series and 100% propylene oxide and embedded in Embed 812. Thin sections were cut with an LKB ultramicrotome (Ultramome NOVA, LKB 2188, Bromma, Sweden) and transferred to copper grids. The grids were stained with lead citrate and uranyl acetate, and observed with a Hitachi H-600 electron microscope. Systematic random sampling was achieved by scanning the grid at low magnification so that cell injury was not apparent. Grids were scanned continuously in equal spaces from top to bottom and left to right. Cardiomyocytes, 30 micrographs per mouse, were taken and analyzed from DKO (n = 7) or WT (n = 6) mice. Quantitation of mitochondrial damage was done using strict criteria for mitochondrial injury and image analysis techniques as previously described (21). Investigators performing morphometry were blinded as to expected results if the hypothesis was correct in treatment groups.

**Isolation and Culture of Cardiomyocytes from Adult Mice**

The isolation of cardiomyocytes from adult mice followed the method used by Wolska and Solaro (22) with minor modifications. The hearts were removed and mounted on a Langendorff perfusion system. Blood was washed out by perfusion with Ca²⁺-free buffer (126 mmol/L NaCl, 4.4 mmol/L KCl, 1 mmol/L MgCl₂, 18 mmol/L NaHCO₃, 30 mmol/L butanedionemonoxime, 4 mmol/L HEPES, 18 mmol/L glucose, and 0.13 units/mL insulin). The perfusion process was done at 37°C by using a heating coil connected to a peristaltic pump at a speed of 1 mL/min. The perfusion buffer was then replaced by enzyme buffer I [100 units/mL collagenase II (Worthington Biochemical Co., Lakewood, NJ) and 60 units/mL hyaluronidase type II (Sigma, St. Louis, MO) in perfusion buffer]. The tissue was digested by perfusion with enzyme buffer I for 14 minutes. The crude digested ventricle was cut into small pieces, transferred to enzyme buffer II [2 mmol/L CaCl₂, 100 units/mL collagenase II, 60 units/mL hyaluronidase type II, 300 units/mL trypsin type IV (Sigma, St. Louis, MO), and 0.02 mg/mL DNAase I (Worthington Biochemical)], and incubated at 37°C in a water bath with shaking (60 rpm) for 10 minutes. The cell suspension was filtered through 500 µm nylon mesh and collected with 10 mL wash medium [perfusion buffer/DMEM (Life Technologies, Inc., Grand Island, NY) 1:1]. The cells were collected by centrifugation at 20 × g, 4°C, for 3 minutes. The cell pellet was resuspended in wash medium and filtered through 6% bovine serum albumin to collect viable cardiomyocytes. Cells were plated onto an eight-well chamber slide (Nalge Nunc International, Lab Tek, Naperville, IL), precoated with 20 µg/mL laminin, and incubated at 37°C for 1 hour before the experiment was done.

**Terminal Deoxynucleotide Transferase – Mediated Nick-End Labeling**

The apoptotic nuclei of cultured cardiomyocytes were detected by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay using Cardio-TACS In situ Apoptosis Detection kit (R&D Systems) following the instructions of the manufacturer with minor modifications. Cardiomyocytes at a density of 2.5 × 10⁴ cells/mL were grown on a laminin-coated eight-well chamber slide (5,000 cells per well) and treated with or without 5 µmol/L Adriamycin for 10 hours. Methanol/acetone (7:3) was used for cell fixation. After permeabilization with cytonin, cells were further permeabilized with 0.2% Triton X-100 in 1× PBS.

**Caspase Activity Assay**

Caspase activity in cultured cardiomyocytes was detected by using CaspaTag fluorescence caspase activity kit (Seralogicals Corporation, Norcross, GA). Cultured cardiomyocytes were treated with or without 5 µmol/L Adriamycin for 3 hours.

**Isolation of Mitochondria and Cytosol from Mouse Heart**

Mitochondria and cytosol were isolated from fresh pooled hearts (three hearts for each sample) after saline or Adriamycin treatment using the method previously described by Yen et al. (5), but without adding Nargarse in the isolation medium.

**Western Blotting**

For protein analysis, total heart tissue homogenates, cytosol fractions, or mitochondrial fractions were separated by SDS-PAGE. The following antibodies were used: anti–cytochrome c (BD Biosciences/PharMingen, San Jose, California) or anti–actin (Sigma, St. Louis, MO). Blots were visualized using ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

**In situ TACS Apoptosis Detection**

Cultured cardiomyocytes were plated onto an eight-well chamber slide (Nalge Nunc International, Lab Tek, Naperville, IL), precoated with 20 µg/mL laminin, and incubated at 37°C for 1 hour before the experiment was done.

**3′UTR Regulation by Tumors**

The 3′UTR region of the tumor-specific genes was cloned into the pGL3 vector (Promega). The luciferase reporter constructs were transiently transfected into different cell types using Lipofectamine (Life Technologies, Inc., Grand Island, NY). The luciferase activities were normalized to the cotransfected Renilla luciferase activity.
CA), anti-MrSOD (Upstate Biotechnology, Lake Placid, NY), anti-G3PDH (Trevigen, Gaithersburg, MD), anti-caspase-3, anti-Bcl-xs/xI, anti-Fas, anti-truncated Bid (anti-t-Bid; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-CuZnSOD (Calbiochem, EMD Biosciences, Inc., La Jolla, CA).

**Statistical Analysis**

Data were evaluated using either ANOVA with a post hoc multiple comparison Student-Newman-Keuls test, bootstrap analysis, or independent Student’s t test. A difference of $P < 0.05$ was considered significant.

**Results**

**Adriamycin Treatment Increased Serum TNF-α Levels**

To examine the effect of Adriamycin in TNF-α level, serum TNF-α levels from mice treated with 20 mg/kg Adriamycin over a time course was determined by ELISA assay. The data showed that serum TNF-α levels in WT mice increased from 6 hours and reached the peak level at 24 hours after Adriamycin injection (Fig. 1A). The basal level of serum TNF-α was 13.22 ± 1.93 pg/mL. After Adriamycin treatment, serum TNF-α increased ~2.5-fold at 24 hours. Similar changes of serum TNF-α levels in response to Adriamycin treatment were also observed in DKO mice (Fig. 1B).

**TNF-α Mediated Cardioprotection against Adriamycin Toxicity**

To investigate the role of TNF-α in Adriamycin-induced cardiac injury, mice deficient in both TNFRI and TNFRII (DKO) and WT mice were treated with 20 mg/kg Adriamycin for 5 days for ultrastructural pathologic examination. WT mice and DKO mice treated with saline (Fig. 2A-a, c) showed normal ultrastructure of heart muscle with cardiomyocytes showing numerous mitochondria and prominent myofilaments, indicating that TNFRI and TNFRII deficiency does not result in ultrastructural cardiac tissue damage. Mice treated with Adriamycin (Fig. 2A-b, d) showed mitochondrial injury with disruption of mitochondrial membranes, mitochondria with the presence of myelin figures, mitochondrial vacuolization, perimitochondrial swelling, lysosomal degradation of mitochondria, and degeneration of mitochondria. The magnitude of cardiac mitochondrial tissue injury was evaluated by quantification of mitochondrial damage area and total mitochondrial area, and data were expressed as a ratio (Adriamycin/saline; Fig. 2B). The data showed that lack of TNF receptor I and receptor II exacerbated mitochondrial ultrastructural damage in DKO mice after Adriamycin injection with an approximate 40% increase in mitochondrial damage observed in DKO in comparison with WT mice (Fig. 2B). These results suggested that TNF receptor I and II mediated cardioprotection against Adriamycin toxicity.

**TNF-α Receptors Protected against Adriamycin-Induced Mitochondrial Cytochrome c Release and Caspase-3 Activation**

We have shown TNF receptors protected against Adriamycin-induced mitochondrial injury by ultrastructural examination in Fig. 2. The release of cytochrome c is an indicator of mitochondrial-mediated apoptosis, which is one of the major apoptotic pathways in cardiac cells. As shown in Fig. 3A, 3 days after a single injection of Adriamycin, the cytosolic cytochrome c levels were significantly increased in DKO mice compared with Adriamycin-treated WT mice. Consistent with the increases in cytosolic cytochrome c levels, mitochondrial cytochrome c levels were decreased significantly in DKO mice (Fig. 3B). Caspase-3 is the executive protease for apoptosis. After Adriamycin treatment for 3 days, cleaved caspase-3 (active) protein levels were increased significantly in DKO mice compared with the WT mice (Fig. 3C). These results suggested that TNF receptors protected against Adriamycin-induced apoptosis in cardiac tissues.

**TNF Receptors Suppressed Adriamycin-Induced Apoptosis Directly in Adult Primary Cardiomyocytes**

To determine the direct role of TNF on cardiomyocytes, primary cardiomyocytes were isolated from WT and DKO mice (10–14 weeks of age). Adriamycin was added to cultured primary adult cardiomyocytes and cells undergoing apoptosis were detected by TUNEL assay (Fig. 4A). The results showed that Adriamycin treatment for 10 hours significantly increased apoptotic cell death in cardiomyocytes and the rate of apoptosis was significantly higher in DKO mice compared with WT mice.

Caspases are the principle effectors of apoptosis in both extrinsic and intrinsic pathways. To confirm the effect of Adriamycin in cardiomyocyte apoptosis, studies were done to determine if the activated forms of pan-caspases were present (Fig. 4C). Healthy cardiomyocytes were elongated...
(rod-shaped) with regular oval-shaped nuclei. Apoptotic cells were identified by bright green staining, indicating the caspase-positive cells. Caspase-positive cells analyzed by double labeling with propidium iodide showed nuclei with a condensed, irregular shape. The percentage of caspase-positive cells was significantly increased in DKO mice after Adriamycin treatment for 3 hours compared with WT mice.

To further investigate which TNF receptor mediates the suppression of Adriamycin-induced apoptosis in cardiomyocytes, the primary adult cardiomyocytes isolated from TNFRI-deficient mice (p55+/C0/C0/p75+/+) and TNFRII-deficient mice (p55+/++p75/C0/C0) were used for both TUNEL assay (Fig. 4B) and caspase activity assay (Fig. 4D). The results showed that either TNF receptor I or TNF receptor II was sufficient to protect against Adriamycin-induced apoptosis and the increase of caspase in cardiomyocytes. These results suggested that it is unlikely that an increased level of Bcl-xl in the DKO mice will play a significant role in the protection of cardiac tissue against Adriamycin-induced injury. Consistent with this finding, the basal protein levels of Bcl-xs (a proapoptotic protein) were also significantly higher in the saline or Adriamycin treated DKO mice (Fig. 5C). Consequently, the Bcl-xs/Bcl-xl ratio increased significantly in DKO mice after Adriamycin treatment compared with the WT mice similarly treated (Fig. 5D). These results suggested that the increased mitochondrial dysfunction in DKO mice caused by Adriamycin treatment was not due to a lack of mitochondrial-associated cytoprotective adaptive responses to the oxidative stress, but rather may be due to a change in the relative levels of Bcl-xs and Bcl-xl.

**Lack of Protective Adaptive Response to Oxidative Stress in Mitochondria**

A prevailing hypothesis for Adriamycin-induced cardiotoxicity is increased production of reactive oxygen species. MnSOD, a reactive oxygen species response gene, is the first line of antioxidant defense in mitochondria. After Adriamycin treatment, there was no change in MnSOD immunoreactive protein levels (Fig. 5A) or MnSOD activity. The basal levels of MnSOD in WT or DKO mice were not different. The proteins of the Bcl-2 family are other key regulators of mitochondrial integrity. In contrast to MnSOD levels, the protein levels of mitochondrial Bcl-xl (an antiapoptotic protein) in the saline or Adriamycin-treated DKO mice were significantly higher than the WT mice (Fig. 5B). These results suggested that it is unlikely that an increased level of Bcl-xl in the DKO mice will play a significant role in the protection of cardiac tissue against Adriamycin-induced injury. Consistent with this finding, the basal protein levels of Bcl-xs (a proapoptotic protein) were also significantly higher in the saline or Adriamycin treated DKO mice (Fig. 5C). Consequently, the Bcl-xs/Bcl-xl ratio increased significantly in DKO mice after Adriamycin treatment compared with the WT mice similarly treated (Fig. 5D). These results suggested that the increased mitochondrial dysfunction in DKO mice caused by Adriamycin treatment was not due to a lack of mitochondrial-associated cytoprotective adaptive responses to the oxidative stress, but rather may be due to a change in the relative levels of Bcl-xs and Bcl-xl.

**TNF Receptors Suppressed the Fas-Mediated Cell Death Pathway**

The Fas/Fas ligand–mediated extrinsic pathway is another major pathway for Adriamycin-induced apoptosis. It has been shown that Fas signaling via the type I death-inducing signal complex is independent of Bcl-2 family proteins. The proteins of the Bcl-2 family are other key regulators of mitochondrial integrity. In contrast to MnSOD levels, the protein levels of mitochondrial Bcl-xl (an antiapoptotic protein) in the saline or Adriamycin-treated DKO mice were significantly higher than the WT mice (Fig. 5B). These results suggested that it is unlikely that an increased level of Bcl-xl in the DKO mice will play a significant role in the protection of cardiac tissue against Adriamycin-induced injury. Consistent with this finding, the basal protein levels of Bcl-xs (a proapoptotic protein) were also significantly higher in the saline or Adriamycin treated DKO mice (Fig. 5C). Consequently, the Bcl-xs/Bcl-xl ratio increased significantly in DKO mice after Adriamycin treatment compared with the WT mice similarly treated (Fig. 5D). These results suggested that the increased mitochondrial dysfunction in DKO mice caused by Adriamycin treatment was not due to a lack of mitochondrial-associated cytoprotective adaptive responses to the oxidative stress, but rather may be due to a change in the relative levels of Bcl-xs and Bcl-xl.

* Unpublished data.
protein (23). Figure 6A shows that after Adriamycin treatment, Fas protein levels in the cardiac tissues of WT mice were significantly decreased in a time-dependent manner. In contrast, Fas levels in DKO mice did not change after 1 or 2 days of Adriamycin treatment but significantly increased in the 3-day treated mice (Fig. 6B). The protein levels of Fas ligand were undetectable, which may be due to the sensitivity limitation of Western analysis. Ligation

Figure 3. TNF receptors suppressed Adriamycin-induced mitochondrial cytochrome c release and caspase-3 activation in cardiac tissues. Cytochrome c protein levels were analyzed by Western blotting. Expression of cytochrome c in cytosol fractions (A) and mitochondrial fractions (B) after Adriamycin treatment for 3 d (n > 5). Active caspase-3 protein levels (C) after Adriamycin treatment for 3 d (n = 5) were analyzed by Western blotting. Columns, mean; bars, SD. Independent Student’s t test. *, P < 0.05 compared with the corresponding saline groups.

Figure 4. TNF receptors suppressed Adriamycin-induced apoptosis in primary adult cardiomyocytes. Apoptotic cells in cultured cardiomyocytes were determined by TUNEL assay (A) after Adriamycin treatment for 10 h (n = 4). Arrows, apoptotic cardiomyocytes with blue staining in the nuclei. The rate of apoptosis was expressed as percentage of apoptotic cells over the total cell number (A and B). Columns, mean; bars, SE. Two-way ANOVA with a post hoc multiple comparison Student-Newman-Keuls test. #, P < 0.001 compared with the corresponding control groups. *, P < 0.001 compared with the WT, p55−/−p75+/+, and p55−/−p75−/− Adriamycin groups. Caspase activity in cultured cardiomyocytes were determined by CaspaTag staining (C) after Adriamycin treatment for 3 h (n = 5). Propidium iodide stained the nuclei in cardiomyocytes. Arrows, caspase-positive cells. The rate of caspase-positive cells was expressed as percentage of caspase-positive cells over the total cell number (C and D). Columns, mean; bars, SE. Independent Student’s t test or one-way ANOVA. *, P < 0.001 compared with the WT, p55−/−p75−/−, and p55−/−p75−/− mice.
of Fas and Fas ligand has been shown to result in the activation of caspase-8, with resultant cleavage of Bid, a proapoptotic protein. t-Bid has been shown to migrate to mitochondria and cause mitochondrial dysfunction. After Adriamycin treatment for 3 days, mitochondrial t-Bid protein levels increased significantly in DKO mice (1.4-fold), but there was no change in the WT mice (Fig. 6C). These results suggested that the presence of TNF receptor I and II suppressed Adriamycin-induced activation of the Fas-mediated pathway.

Discussion
TNF-α is a multifunctional cytokine that has been shown to mediate different physiologic and pathophysiologic events. Although increased circulating TNF-α has been associated with many forms of cardiac injury, including acute viral myocarditis, myocardial infarction, atherosclerosis, chronic heart failure, cardiac allograft rejection, and sepsis-associated cardiac dysfunction (24), it has also been shown that TNF-α plays an adaptive role in mediating cardiac protection, adaptive growth, and postischemic remodeling in response to stress (12, 13). However, whether and how TNF plays a role in cardiac injury and cardiomyopathy resulting from cancer treatment are unknown. In the present study, we showed that serum TNF-α levels were significantly increased by 24 hours after one Adriamycin injection in both WT and DKO mice, suggesting the involvement of TNF in Adriamycin-induced cardiac injury. However, this low level of TNF-α (~33 pg/mL at 24 hours after Adriamycin injection, compared with TNF-α level in mice after 2.5 mg/kg lipopolysaccharide injection for 1.5 hours, ~13,000 pg/mL; ref. 25) seemed to be beneficial to hearts after Adriamycin treatment when the receptors are present as shown by ultrastructural analyses in TNF receptor I and receptor II deficient mice. Our results showed mitochondrial ultrastructural injury 5 days after Adriamycin treatment, including the disruption of mitochondrial membranes, mitochondria with myelin figures, perimitochondrial swelling, and degeneration of mitochondria, and the levels were greater in Adriamycin-treated DKO mice. The lesions were similar to the subacute cardiomyopathy shown by Rosenoff et al. (26) in 4 days Adriamycin-treated mice, in which they showed that these lesions were similar to the delayed Adriamycin-induced cardiomyopathy noted in human. The protective effect of TNF receptors against Adriamycin-induced apoptosis in cardiac tissues is further shown by the increased levels of mitochondrial cytochrome c release and caspase-3 activation in Adriamycin-treated DKO mice. The heart is an organ composed of multiple cell types, including myocytes and nonmyocytes, such as connective tissue cells, vascular...
smooth muscle cells, and endothelial cells. Cardiomyocytes are the major cell type of cardiac tissue and adult cardiomyocytes are terminally differentiated and non-proliferated cells. The structural and functional integrity of cardiomyocytes is essential for the normal cardiac function, and the injury of cardiomyocytes may lead to permanent loss of cell number and contribute to the pathology of cardiac failure. Our data from the primary adult cardiomyocytes showed the direct apoptotic effect of Adriamycin in cardiomyocytes, which may contribute to the cell loss and Adriamycin-induced congestive heart failure. Our data in isolated primary adult cardiomyocytes also directly confirmed the TNF receptor–mediated cardioprotection, which is consistent with the observations in cardiac tissues. Furthermore, either TNF receptor I or TNF receptor II was sufficient to protect against Adriamycin-induced apoptosis in cardiomyocytes, which may be mediated by the common signaling pathways through TNF receptor I and II or/and by the disparate cytoprotective pathways. Although the levels of mitochondrial cytochrome c release and caspase-3 activation were only slightly increased in the cardiac tissues of WT mice after Adriamycin treatment, the apoptosis levels did increase significantly after Adriamycin treatment in the primary adult cardiomyocytes isolated from WT mice. These results suggested that Adriamycin may have more direct effects on cardiomyocytes, and the levels of apoptosis markers (cytochrome c release and caspase-3 activation) observed in the whole cardiac tissues may be compensated by the other cell types that are not Adriamycin targets or cell types containing less mitochondria. It is also possible that cardiomyocytes were exposed to a higher concentration of oxygen. Our primary cardiomyocyte culture experiments were done at 21% oxygen concentration. Knudson et al. (27) have shown that the basal level of oxygen tension in the myocardium of the left ventricle is only ~6.4%. Adriamycin-induced generation of superoxide anion in the cardiac tissues/cardiomyocytes requires oxygen. Thus, the isolated primary cardiomyocytes were exposed to a higher concentration of oxygen compared with the in vivo oxygen concentration, which may result in the generation of more superoxide anion through Adriamycin redox cycling and greater severity of the cell injury.

Apoptosis has been shown to be one mechanism of Adriamycin-induced cardiac injury (10, 28–32). Two major cellular apoptosis pathways, extrinsic and intrinsic, have been well established. The extrinsic cell death pathway is mediated by death receptors, such as Fas and TNF receptors. The intrinsic pathway is mitochondria dependent. Both of these pathways have been shown to be involved in cardiac myocyte apoptosis (33). Adriamycin treatment can induce structural and functional injury in mitochondria (5–7). We have previously shown that a single injection of Adriamycin resulted in the decrease of mitochondrial respiratory function and inactivation of mitochondrial complex I, complex II, and creatine kinase in cardiac tissue 5 days after treatment that was consistent with the effect on mitochondrial ultrastructural injury (5). Our present study showed that DKO mice had greater mitochondrial ultrastructural damage, suggesting that TNF receptor I and II are involved in the maintenance of mitochondrial function and integrity following Adriamycin treatment. We have also shown that overexpression of MnSOD in transgenic mice protected against Adriamycin-induced acute cardiac injury (5), suggesting the involvement of reactive oxygen species in Adriamycin-induced cardiac injury. In the present study, our results showed that MnSOD levels did not change, but the Bcl-xs/Bcl-xl ratio was increased after Adriamycin treatment. Because TNF-α can stimulate multiple nuclear factor-κB target genes, the lack of an increase in MnSOD but increases in Bcl-xs/Bcl-xl ratio suggests that reactive oxygen species generated by Adriamycin may also resulted from Adriamycin-enhanced mitochondrial injury. It is well shown that TNF-α can induce MnSOD expression in various cells and tissues. The lack of MnSOD induction by TNF-α in response to Adriamycin treatment in this model may be due to the low level of TNF-α (~33 pg/mL after Adriamycin treatment).

**Figure 6.** TNF receptors suppressed the Fas-mediated pathway in response to Adriamycin treatment. The extrinsic apoptosis pathway proteins, Fas and t-Bid, were analyzed by Western blotting. The levels of Fas in cardiac tissue in WT mice (A) and DKO mice (B) over a time course of 1 to 3 d (n = 5 for WT, n = 6 for DKO). CuZnSOD was used to confirm equal protein loading. Columns, mean; bars, SD. One-way ANOVA with a post hoc multiple comparison Student-Newman-Keuls test. *, P < 0.01 compared with control groups. The expression of t-Bid in isolated cardiac mitochondria (C) after Adriamycin treatment for 3 d (n > 5). Columns, mean; bars, SD. Independent Student’s t test. *, P < 0.05 compared with the corresponding saline groups.
Previous studies in our laboratory showed that the minimum level of TNF-α to induce MnSOD expression varied between 0.5 and 4 ng/mL depending on cell lines used. Thus, it is not surprising that TNF-α level is increased, but MnSOD gene induction did not occur. These results also suggested that MnSOD induction is not a major mechanism for the Adriamycin-induced TNF-mediated protection. Although the possibility of the involvement of other antioxidant pathways could not be completely ruled out, we did not find any difference in the levels of reduced glutathione, oxidized glutathione, and oxidized glutathione/reduced glutathione ratio in the cardiac tissues from WT and DKO mice in an unpublished study using mice of different background. Also, there is no significant difference of these three variables between WT and DKO mice with saline or Adriamycin treatment, suggesting that reduced glutathione/oxidized glutathione pathway may not play an important role in our model.

Interestingly, although the basal level of Bcl-xl, an antiapoptotic member of Bcl-2 family, is increased in DKO mice, apoptosis is higher in cardiomyocytes isolated from these animals. These results suggest that in the absence of TNF receptors, apoptosis in DKO mice may also be mediated via a Bcl-2-independent Fas signaling. The Fas/Fas ligand system is one of the key regulators of the extrinsic apoptotic pathway. Fas is a cell surface receptor expressed in many cell types, including cardiomyocytes (34, 35). Ligation of Fas and Fas ligand results in the activation of caspase-8, an apoptosis initiator. Fas/Fas ligand–mediated cell death has been shown to be important in Adriamycin-induced cardiac myocyte apoptosis (28, 29, 31). Our study showed that after an acute dose of Adriamycin, the myocardial Fas protein level decreased in WT mice in a time-dependent manner, but significantly increased at 3 days in Adriamycin-treated DKO mice. Nakamura et al. (29) showed that Fas-mediated apoptosis was induced in chronic Adriamycin treatment in rats. The difference between our results and Nakamura’s results may be due, in part, to the difference between mice and rats because the heart metabolic rate of mice is significantly higher than rats. Furthermore, after single injection of high dose of Adriamycin (20 mg/kg), we showed that Fas levels increased after Adriamycin treatment in DKO mice, which had a higher level of cardiac injury comparing with the WT mice, suggesting that Fas level increase is associated with greater levels of cardiac injury. Thus, it is possible that Fas levels may increase in WT mice if the animals were subjected to a higher level of cardiac injury. Our studies using a single injection provide a unique opportunity to discover that TNF receptors can suppress Fas-mediated signaling. Thus, although there seem to be a difference in cellular responses between the single-dose Adriamycin injection and the repeated chronic Adriamycin treatment, this acute model provides a unique opportunity to identify the previously unrecognized role of TNF receptors in Adriamycin-induced cardiac injury. The acute effect of Adriamycin in TNF receptor–deficient mice may imitate enhanced mitochondrial injury in the chronic model. Our data showed that lack of TNF receptors resulted in an increase of Fas levels and mitochondrial t-Bid levels after Adriamycin treatment. Bid, a proapoptotic Bcl-2 family protein and a caspase-8 substrate, is a key factor linking an extrinsic apoptotic pathway to an intrinsic apoptotic pathway (36). Activation of Fas results in the cleavage of Bid by caspase-8 and t-Bid translocates to mitochondria and oligomerizes with Bax and Bak to form channels for cytochrome c release (37, 38). Using primary mouse hepatocytes as a model, Ding et al. (39) have shown that Bid was responsible for the majority of reactive oxygen species production following death receptor activation and that reactive oxygen species were important for caspase activation and cell death by promoting degradation of FLICE-inhibitory proteins (FLIP, a caspase-8 inhibitor), mitochondrial cristae reorganization, membrane lipid peroxidation, and cytochrome c release. Liu et al. (40) have shown that t-Bid may affect mitochondrial respiration and enhance cytochrome c release by directly interfering with cardiolipin, a unique phospholipid exclusively found in bacteria and mitochondrial membranes (41, 42). Taken together, our results and the above cited evidence suggested that TNF-α receptor I and II may protect against Adriamycin cardiotoxicity by, in part, prevention of Fas-mediated extrinsic apoptosis and t-Bid mitochondrial translocation, thereby preventing increased generation of reactive oxygen species and interference of cardiolipin and mitochondrial respiratory function that may result in the mitochondria-dependent apoptosis. The results of our studies showed that the presence of both TNF receptor I and II suppresses Adriamycin-induced Fas levels. However, whether Adriamycin-induced Fas expression and TNF-mediated Fas suppression is controlled at the transcriptional, posttranscriptional, or translational levels is unclear and will be a subject of further investigation.

The binding of TNF-α to its receptors, TNFR-I (p55) and TNFR-II (p75), results in the activation of several divergent cytoprotective signaling pathways involved in cell growth, survival, and proliferation. These include the downstream events of nuclear factor-kB, c-Jun NH2-terminal kinase, stress activated protein kinase, and protein kinase C (12). The cytoprotective pathways downstream of the TNF receptors may provide adaptive responses to stress to maintain normal cardiac contractility and homeostasis, including increased regional myocardial blood flow, protection against hypoxic- and ischemia-induced injury, and increase of cytoprotective proteins, such as heat shock protein 72, MnSOD, and Bcl-xl (12–14).

Our results are the first to show that in the absence of TNF receptor I and II, Fas-mediated pathway is quickly activated. Our results also suggest that signaling via TNF receptor I or TNF receptor II can prevent activation of Fas-mediated signaling. Thus, TNF receptors have a novel protective role against Adriamycin cardiotoxicity, in part by suppressing Fas-mediated apoptosis. These results also show a link between TNF-α-mediated cardioprotection and Adriamycin-induced intrinsic and extrinsic apoptosis pathways in cardiac tissues. Scaffidi et al. (23) have shown
that the mechanisms of Fas signaling vary between cell types, each using almost exclusively one of two distinct Fas signaling. In type I cells, death-inducing signaling complex involving caspase-8 activity is not blocked by Bcl-2 or Bcl-xL overexpression. Our results, which indicated that the basal levels of Bcl-xL are higher in DKO mice but apoptosis is increased, are consistent with this finding. Thus, future studies on the specific inhibition of type I components of Fas signaling via TNF receptor-mediated mechanisms may be a strategy for prevention of Adriamycin-induced cardiac injury and may have a significant effect on the quality of life of cancer patients.

References

Molecular Cancer Therapeutics

Tumor necrosis factor receptor deficiency exacerbated Adriamycin-induced cardiomyocytes apoptosis: an insight into the Fas connection

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