Doxorubicin Induces Apoptosis in Normal and Tumor Cells via Distinctly Different Mechanisms

INTERMEDIACY OF H₂O₂- AND p53-DEPENDENT PATHWAYS

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Doxorubicin (DOX), a widely used chemotherapeutic agent, exhibits cardiotoxicity as an adverse side effect in cancer patients. DOX-mediated cardiomyopathy is linked to its ability to induce apoptosis in endothelial cells and cardiomyocytes by activation of p53 protein and reactive oxygen species. We evaluated the potential roles of H₂O₂ and p53 in DOX-induced apoptosis in normal bovine aortic endothelial cells and adult rat cardiomyocytes and in tumor cell lines PA-1 (human ovarian teratocarcinoma) and MCF-7 (human breast adenocarcinoma). Time course measurements indicated that activation of caspase-3 preceded the stimulation of p53 transcriptional activity in endothelial cells. In contrast, DOX caused early activation of p53 in tumor cells that was followed by caspase-3 activation and DNA fragmentation. These findings suggest that the transcriptional activation of p53 in DOX-induced apoptosis in endothelial cells may not be as crucial as it is in tumor cells. Further evidence was obtained using a p53 inhibitor, pifithrin-α. Pifithrin-α completely suppressed DOX-induced activation of p53 in both normal and tumor cell lines and prevented apoptosis in tumor cell lines but not in endothelial cells and cardiomyocytes. In contrast, detoxification of H₂O₂, either by redox-active metalloporphyrin or overexpression of glutathione peroxidase, decreased DOX-induced apoptosis in endothelial cells and cardiomyocytes but not in tumor cells. This newly discovered mechanistic difference in DOX-induced apoptotic cell death in normal versus tumor cells will be useful in developing drugs that selectively mitigate the toxic side effects of DOX without affecting its antitumor action.

The antitumor drug doxorubicin (DOX)1 has been widely used in the clinic for the treatment of a broad spectrum of cancers (1, 2). A major adverse side effect associated with DOX usage in the clinic is the onset of cardiomyopathy and heart failure (2). Several reports suggest that DOX-induced apoptosis plays an important role in its cardiotoxicity that is linked to formation of reactive oxygen species (ROS) derived from redox activation of DOX (3–5). Recent studies have focused on DOX-induced apoptotic signaling mechanisms (6–8).

The transcription factor p53 has been reported to play a very important role in apoptosis (9, 10). As a tumor suppressor, p53 is responsible for protecting cells from tumorigenic alterations (10, 11). Mutational inactivation of p53 is frequently observed in various human cancers (12). Activation of p53, which in turn promotes apoptosis of tumor cells, is considered to be a key mechanism of action of antitumor drugs, including DOX (13, 14). Various mutations of p53 and attenuation of p53 activation by DOX were reported as important mechanisms of drug resistance in tumor cells (15–17). However, relatively fewer studies have focused on the role of p53 in DOX-induced toxicity in endothelial cells, cardiomyocytes, and other non-cancerous cells.

DOX was reported to induce ROS generation in various tumor cells (18–20). However, the exact role of ROS in DOX-induced tumor cell killing still remains uncertain and somewhat controversial. Several groups reported that inhibiting DOX-induced intracellular oxidative stress by the overexpression of antioxidant enzymes prevented apoptosis in tumor cells (20, 21), and that depleting endogenous antioxidants (e.g. glutathione) made tumor cells more susceptible to DOX (22, 23). However, other studies did not support the role of oxidative stress in tumor cell apoptosis induced by DOX and other chemotherapeutic agents (24, 25).

In the present study, we investigated the effect of DOX-induced H₂O₂ and p53 in two non-transformed cell types (bovine aortic endothelial cells (BAECs) and adult rat cardiomyocytes (ARCMs)), and two tumor cell lines (PA-1 and MCF-7). To our knowledge, we provide evidence, for the first time, that p53 and H₂O₂ have distinctly different roles in mediating DOX-induced apoptosis in these normal cell types and tumor cells. Although p53 plays a crucial role in tumor cell apoptosis, H₂O₂ is predominantly responsible for apoptosis in endothelial and myocardial cells. These results suggest that DOX-induced apoptosis is mediated by distinctly different signal transduction pathways in non-transformed and tumor cells, further suggesting that targeted drug development to attenuate DOX-induced side effects without compromising its chemotherapeutic effect is feasible.

EXPERIMENTAL PROCEDURES

Materials—DOX and Pifithrin-α were purchased from Sigma and Tocris Cookson (Ellisville, MO), respectively. Carboxy-H₂DCFDA (5-(and 6)-carboxy-2′,7′-dichloro-dihydrofluorescein diacetate) was pur-
chased from Molecular Probes (Eugene, OR). The glutathione peroxidase-1 (GPx-1) expression plasmid was a generous gift from Dr. L. Oberley (University of Iowa, IA).

**Cell Culture**—Both BAEC and PA-1 cells were obtained from the American Type Culture Collection. BAECs were cultured in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml), incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. All experiments were performed in a similar medium containing 2% FBS. PA-1 cells were cultured in Eagle's Minimal Essential medium containing 10% FBS, L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), sodium bicarbonate (1.5 g/L), and sodium pyruvate (1 mM), incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. MCF-7 cells were a gift from Dr. C. Chitambar (Medical College of Wisconsin). These cells were cultured in MEM/H2O2 medium containing 10% FBS, 2 mM L-glutamine, 25 mM sodium bicarbonate, 10 μg/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. MCF-7 cells were seeded 24 h before experiments and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

**Isolation and Culturing of Cardiomyocytes**—Adult rat ventricular cardiomyocytes were isolated from male Sprague-Dawley rats (175 to 225 g of body weight) as described previously (26). Briefly, hearts were perfused with collagenase type II (200 units/ml) (Invitrogen). After 30 min of perfusion, ventricular tissue was minced and incubated for 30 min in a perfusion buffer with 1% bovine serum albumin and 20 μg/ml deoxyribonuclease (Sigma). Cells were released from chunks of tissue by gentle pipetting. The cell suspension was filtered through an 80-mesh screen, washed twice by gentle centrifugation, and resuspended in a buffer containing 1 mM CaCl2. Ventricular myocytes were allowed to settle and then plated onto 60-mm dishes precoated with laminin. The culture medium was Medium 199 supplemented with 25 mM HEPES, 2 mg/ml of bovine serum albumin, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 nM insulin, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. Intact cardiomyocytes adhered to the culture plates, and damaged cells were washed away during the medium change 2 h after plating. Cardiomyocytes were pretreated with Fe(III) tetrakis (4-benzoyl acid) porphyrin (FeTBAP) (10 μM) or pifithrin-α (PFT-α) (5 μM) for 1 h and incubated with 0.5 μM DOX for 72 h.

**Luciferase Assay**—Cells (1 × 10^5) suspended in 1 ml of complete medium were seeded onto each well of a 12-well plate. After incubating at 37 °C for 24 h, cells were transiently transfected by 1 μg of p53-luciferase reporter plasmid and 1 μg of β-gal plasmid in the medium without FBS and antibiotics using the lipofectamine reagent (Invitrogen). The cells were incubated for 4 h, and then complete medium was

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**Fig. 1.** DOX-induced, time-dependent apoptosis and caspase-3 activation in BAECs and PA-1 cells. **A**, cells were incubated with 0.5 μM DOX for different time periods as indicated, stained for TUNEL-positive cells, and examined by fluorescence microscopy. The percentage of apoptotic nuclei in BAECs (left) and PA-1 cells (right) is shown in the graph. **B**, cells were treated with 0.5 μM DOX for different incubation times as indicated and harvested, and caspase-3 activity was measured in BAECs (left) and PA-1 cells (right). Values are mean ± S.E. of three separate experiments. Asterisks indicate a significant increase as compared with control (p < 0.05).
added. Sixteen h later, the cells were starved for another 16 h in 2% FBS medium, followed by exposure to different treatments. The luciferase activity was determined using the luciferase assay system with reporter lysis buffer from Promega (Madison, WI). Briefly, the cells were harvested by scraping in 200 μl of reporter lysis buffer into a 1.5-ml microcentrifuge tube, vortexed for 15 s, and centrifuged at 12,000 rpm for 30 s, and then the supernatant cell lysates were collected. The luciferase activity was measured with 60 μl of cell lysate and 60 μl of substrate, using a Monolight luminometer.

**Terminal Deoxynucleotidyl Transferase-mediated Nick-End Labeling (TUNEL) Assay**—The TUNEL assay was used for microscopic detection of apoptosis (28). This assay is based on labeling of 3'-free hydroxyl ends of the fragmented DNA with fluorescein-dUTP catalyzed by terminal deoxynucleotidyl transferase. Procedures were followed according to the ApoAlert DNA fragmentation assay kit (Clontech Laboratories, Palo Alto, CA). Apoptotic cells exhibit a strong nuclear green fluorescence that can be detected using a standard fluorescein filter (520 nm). The areas of apoptotic cells were detected by fluorescence microscopy equipped with rhodamine and fluorescein isothiocyanate filters. The photographs and quantification of apoptosis were obtained using a MetaMorph Imaging System (Universal Imaging Corporation).

**Caspase-3 Activity**—The caspase-3-like activity is increased through a protease cascade during apoptosis in the early stage (29). After treatment with DOX and other agents, cells were washed with ice-cold PBS and lysed with cell lysis buffer (caspase-3 assay kit, Sigma). Samples were incubated on ice for 10 min and centrifuged in a microcentrifuge at 12,000 × g for 5 min at 4 °C to precipitate the cellular debris. The caspase-3 activity in the supernatant was measured in a spectrophotometer, using DEVD-p-nitroanilide as a substrate, according to the manufacturer’s instructions provided with the assay kit. Alternatively, cells were washed twice in cold PBS and lysed in buffer containing 10 mM Tris-HCl, 10 mM NaH2PO4/Na2HPO4 (pH 7.5), 130 mM NaCl, 1% Triton, and 10 mM sodium pyrophosphate. Cell lysate was incubated with caspase-3 fluorogenic substrate N-acetyl-DEVD-7-amino-4-meth-
FIG. 3. Effects of p53 inhibitor on DOX-induced apoptosis. A, the luciferase assay for DOX-induced p53 activation. Transfected BAECs and PA-1 cells were pretreated for 1 h with 2 μM PFT-α, followed by treatment with 0.5 μM DOX for 16 h, and the p53 activity was measured by luciferase activity assay. B, cells were treated with 2 μM PFT-α for 1 h and with DOX (0.5 μM) for 16 h, stained for TUNEL-positive cells, and examined using a fluorescence microscope. Similar treatments were performed with DOX in the absence of PFT-α. The average intensity values were obtained from three different fields of view using MetaMorph software. C, adult rat cardiomyocytes (ARCM) and MCF-7 cells were pretreated with 5 μM PFT-α for 1 h and with 0.5 μM DOX for 48 h (MCF-7) or 72 h (ARCM) and lysed as described in “Experimental Procedures.” Cell lysates were incubated with fluorogenic caspase-3 substrate for 1 h at 37 °C. Caspase-3 activity was normalized to cell lysate protein and expressed as fold activation as compared with control. Values are mean ± S.E. of three separate experiments. Single asterisks indicate a significant increase as compared with control; double asterisks indicate a significant decrease compared with DOX treatment (p < 0.05).
ylcoumarin (BD Pharmingen) at 37°C for 1 h. 7-Amino-4-methylcoumarin liberated from the fluorogenic substrate was measured using a fluorescence plate reader (Perkin Elmer Life Sciences) with λex = 380 nm and λem = 440 nm. Liberated fluorescence was normalized to cell lysate protein measured with the BCA protein assay kit (Pierce).

RESULTS

DOX-induced Apoptosis and Caspase-3 Activation in BAECs and PA-1 Cells—Apoptosis was observed using the TUNEL technique in BAECs and PA-1 cells exposed to relatively low concentrations (0.1 to 0.5 μM) of DOX. As shown in Fig. 1A, after incubation with 0.5 μM DOX for 4 h, a significant increase in TUNEL-positive (apoptotic) cells was detected in BAECs. More apoptotic cells were observed with longer incubation times. Although a similar temporal pattern of apoptosis was observed in DOX-treated PA-1 tumor cells, the apoptotic response in PA-1 tumor cells was much lower than that of BAECs.

Next we investigated the effect of DOX on caspase-3 activation, because caspase-3 is an important upstream factor leading to apoptosis (30). Consistent with the TUNEL assay, we found that 0.5 μM DOX caused a significant increase in caspase-3 activation in BAECs and PA-1 cells after incubating for 4 and 8 h, respectively. The activation of caspase-3 increased with increasing incubation time, and the peak activation of caspase-3 was less in PA-1 cells than in BAECs (Fig. 1B).

DOX-induced Apoptosis Is p53-independent in BAECs and ARCMs and p53-dependent in PA-1 and MCF-7 Cells—Fig. 2 shows the results of the luciferase assay for p53 activity measuring the ability of p53 as a transcription factor to regulate the expression of its target genes. BAECs and PA-1 cells, transiently transfected with the p53-luciferase plasmid, were exposed to different concentrations of DOX for 16 h. As shown in Fig. 2A, DOX induced a dose-dependent activation of p53 in BAECs and PA-1 cells, but the peak response was higher in PA-1 cells than in BAECs.

Although similar dose-dependent patterns were found in endothelial cells and tumor cells, the time course of p53 activation induced by DOX was different (Fig. 2B). In BAECs, p53 was not activated until after an 8-h incubation with DOX, which is later than the onset of DOX-induced apoptosis described in Fig. 1A. In contrast, p53 was activated in PA-1 tumor cells starting at 2 h incubation with DOX. There was little or no apoptosis under these conditions (Fig. 1). The distinct differences in the activation profiles of p53 and apoptosis in these cells suggest that DOX-induced apoptosis is p53-independent in endothelial cells but p53-dependent in tumor cells. To further confirm the different roles of p53 (Fig. 2B), a p53 inhibitor, PPT-α, was used. PPT-α blocks p53-dependent transcriptional activation (31). As shown in Fig. 3A, the addition of PPT-α abolished p53 activation induced by DOX in both BAECs and PA-1. The inhibition of p53 in BAECs did not decrease the apoptosis; in contrast, it attenuated DOX-induced apoptosis by ∼50% in PA-1 tumor cells (Fig. 3B).

We also assessed DOX-induced apoptosis in primary cardiomyocytes (ARCMs) and human breast adenocarcinoma (MCF-7) cells. Pretreatment with p53 inhibitor significantly decreased caspase-3 activity in DOX-treated MCF-7 cells (Fig. 3C). However, the antiapoptotic effect of PPT-α was only marginal in ARCMs treated with DOX. These results indicate that although p53 became activated after DOX treatment in endothelial cells, it does not regulate the apoptotic pathway in BAECs and ARCMs. On the other hand, p53 inhibitor significantly diminished DOX-induced apoptosis in tumor PA-1 and MCF-7 cells.

H2O2 Generation in DOX-treated BAECs and PA-1 Cells—DOX has been reported to generate ROS in various cells (3–5, 18–20). To investigate the role of oxidative stress in DOX-induced apoptosis, we used carboxy-H2DCFDA, a cell-permeable fluorescent dye, to examine the ROS generation in both types of cells in response to DOX stimulation. Incubation with DOX for 4 h showed a considerable increase in oxidant-induced 2′,7′-dichlorofluorescein fluorescence in BAECs but not in PA-1 cells (Fig. 4). H2O2-mediated DCF fluorescence occurred after 2 h incubation with DOX in BAECs and increased in intensity over time; however, in PA-1 cells there was no significant change in DCF fluorescence with DOX treatment (Fig. 4). This suggests that DOX, under these conditions, does not induce intracellular oxidative stress in tumor cells.

Effects of Supplementation with Antioxidants, FeTBAP and GPx-1, on DOX-induced Apoptosis and Caspase-3 Activation—To further investigate the role of ROS in DOX-induced apoptosis, we used a redox-active metalloporphyrin FeTBAP that was reported to detoxify superoxide, hydrogen peroxide, and peroxynitrite (32). Pre-incubation of endothelial cells with 10 μM FeTBAP significantly decreased the DOX-induced p53 activation by 60% but had no appreciable effect on p53 activation in PA-1 cells (Fig. 5A). Treatment with ROS scavenger, FeTBAP, resulted in decreased apoptosis and caspase-3 activation in BAECs and ARCMs (Fig. 5, B and C). In contrast, FeTBAP was not effective in preventing DOX-induced apoptosis in tumor cells PA-1 and MCF-7 cells.

Alternatively, we increased the level of GPx-1, an intracellular enzymatic scavenger of H2O2, by introducing the GPx-1 expression plasmid into the cells (33). In GPx-1-transfected BAECs treated with DOX, a reduction in p53 activation was observed (Fig. 6A). Overexpression of GPx-1 almost completely inhibited the apoptosis induced by DOX (Fig. 6B), and the caspase-3 activity was dramatically decreased with GPx-1 overexpression coincidently (Fig. 6C). On the contrary, the overexpression of GPx-1 did not have any effect on DOX-induced p53 activation, apoptosis, and caspase-3 activity in PA-1 cells (Fig. 6).

These results indicate that both chemical and enzymatic detoxification of H2O2 in BAECs inhibit DOX-induced apoptosis and caspase-3 activation. However, H2O2 does not play an important role in regulating DOX-induced apoptosis in tumor cells.

DISCUSSION

In this study, we demonstrate that DOX induces apoptosis in normal cell types and tumor cells via different mechanisms. In endothelial cells and cardiomyocytes, DOX induced apoptosis...
by a H$_2$O$_2$-mediated mechanism and is largely independent of p53 activation. In contrast, p53 tumor suppressor, and not H$_2$O$_2$, plays a crucial role in inducing apoptosis by DOX in tumor cells.

Numerous reports indicate that p53 tumor suppressor protein is important in regulating the apoptosis pathway, but relatively little is known about the role of p53 in apoptosis induced by DOX in endothelial cells and cardiomyocytes. It has been reported that DOX-induced activation of p53 lead to apoptosis in human umbilical vein endothelial cells and neonatal cardiomyocytes (34, 35). Although the present findings indicate that DOX treatment activates p53, this activation did not play any significant role in the apoptosis process in BAECs and adult cardiomyocytes. This conclusion, albeit contrary to previous reports, is based on the following observations: i) apoptosis in endothelial cells was observed at 4 h after DOX incubation, but significant p53 activation occurred only at 8 h after DOX treatment, and ii) p53 inhibition by PFT-α did not decrease DOX-induced apoptosis in endothelial cells and primary cardiomyocytes.

In contrast to the endothelial cell results, we found that p53 did play an important role in tumor cell apoptosis, as reported previously (13, 14). The activation of p53 in human ovarian teratocarcinoma PA-1 cells occurred before the onset of apoptosis, i.e. 2 h after DOX treatment. p53 inhibitor PFT-α effectively blocked DOX-induced apoptosis in human ovarian teratocarcinoma (PA-1) and in human breast adenocarcinoma (MCF-7) cells. It has been reported that caspase-3 essentially acts as a downstream regulator of p53 in stress-induced apoptosis (34, 36, 37). p53 inhibitor PFT-α significantly inhibited caspase-3 activity in MCF-7 cells treated with DOX. These results indicate that disabling p53 transcriptional activity with PFT-α (38, 39) in two tumor cell lines effectively blocks caspase-3 activity and DNA fragmentation.
Besides p53, NF-κB, another transcription factor, is also involved in DOX-induced cellular responses. Previously, we reported that NF-κB is pro-apoptotic in DOX-treated endothelial cells and cardiomyocytes, which is contrary to its usual anti-apoptotic role in various cancer cells (40). The cellular modulatory functions of p53 and NF-κB overlap in many respects, but results have been controversial. NF-κB has been reported to up-regulate the gene expression of p53 (41, 42). However, other groups reported that NF-κB and p53 inhibit each other's function (43, 44). In the present study, we found that inhibiting NF-κB activation did not have significant effects on p53 activation by DOX in endothelial cells, whereas p53 inhibitor did abolish NF-κB activation by DOX (data not shown). These results suggest that p53 does not act as the downstream regulator for NF-κB-mediated apoptosis but does play a tangible role that is upstream of NF-κB activation.

The role of ROS in inducing apoptosis in myocytes and endothelial cells is well established (3, 5, 40, 45). The present study provides additional evidence in support of intracellular H₂O₂ as a mediator in DOX-induced endothelial apoptosis. Pretreatment with FeTBAP, a cell-permeable metalloporphyrin antioxidant enzyme mimetic, dramatically decreased DOX-induced caspase-3 activation and apoptosis in endothelial cells. FeTBAP, a redox-active metalloporphyrin, consists of a redox-active iron (III) located at the center of a porphyrin ring. Recent reports indicate that FeTBAP is an efficient scavenger of both superoxide and H₂O₂ and protects cardiomyocytes against DOX-induced apoptosis (32, 45). Transient transfection of BAECs with GPx-1 expression plasmid also resulted in a dramatic reduction of DOX-induced caspase-3 activation and apoptosis, suggesting that H₂O₂ plays a crucial role in DOX-induced apoptosis in endothelial cells.

The intermediacy of ROS in apoptotic signal transduction in cancer cells still remains questionable (20–25). The present study shows that DOX did not induce H₂O₂ generation to any significant extent in PA-1 cells. Addition of FeTBAP and overexpression of GPx-1 did not cause any significant effect on p53 activation, caspase-3 activation, and apoptosis by DOX in PA-1 tumor cells. The differential effects of free radical scavengers in DOX-mediated apoptosis in normal and tumor cells are noteworthy. For example, DOX-induced transferrin receptor-mediated uptake of iron mediated through enhanced activation of iron regulatory protein-iron response element interaction was inhibited by antioxidant treatment in endothelial cells but not in tumor cells (46). Results obtained in the present study implicate that in human tumor MCF-7 and PA-1 cells, H₂O₂ is not involved in p53-dependent apoptosis induced by DOX.

In summary, results from the present study strongly suggest that DOX-induced apoptosis occurs through a different signal transduction mechanism in non-transformed cells, such as endothelial cells and cardiomyocytes (H₂O₂-dependent), as compared with tumor cells (p53-dependent). This difference in
FIG. 6. Effects of GPx-1 overexpression on DOX-induced p53 activation and apoptosis. A, BAECs and PA-1 cells were transiently co-transfected with the GPx-1 plasmid, p53-luciferase reporter plasmid, and β-galactosidase plasmid. The cells were exposed to various concentrations of DOX for 16 h as indicated, and the p53 activity was measured by the luciferase activity assay. B, BAECs and PA-1 cells were transfected with the GPx-1 plasmid. After the transfection, cells were treated for 16 h with different concentrations of DOX as indicated, and apoptosis was measured using the TUNEL assay. C, the caspase-3 activity was measured under conditions described in B. Values are mean ± S.E. of three separate experiments. Single asterisks indicate a significant increase as compared with control; double asterisks indicate a significant decrease compared with DOX treatment (p < 0.05).
mechanism provides a unique strategy for minimizing DOX cardiotoxicity without mitigating its antitumor potential. It is conceivable that transfection of the wild-type p53 into the target tumor cells will sensitize the tumors to DOX treatment.

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