Glutathione S-transferase overexpression protects against anthracycline-induced H9C2 cell death

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L’Ecuyer, Thomas, Zuhair Allebban, Ronald Thomas, and Richard Vander Heide. Glutathione S-transferase overexpression protects against anthracycline-induced H9C2 cell death. Am J Physiol Heart Circ Physiol 286: H2057–H2064, 2004. First published January 15, 2004; 10.1152/ajpheart.00778.2003.—Anthracyclines (AC) are potent antibiotics used to treat a wide variety of malignancies. The primary antioxidant enzymes that protect cells from oxidative stress are SOD, glutathione S-transferase (GST), catalase, and glutathione peroxidase. Increasing the expression of these enzymes catalase, glutathione peroxidase, and superoxide dismutase (SOD) (41). Despite these encouraging results, no data in large animal or human studies support the use of exogenous antioxidants to reduce this problem.

ANTHRACYCLINES (AC) are potent antibiotics used to treat a wide variety of malignancies. The chief limitation to more widespread use of these highly effective agents has been the development of significant AC-induced cardiotoxicity (ACT). Therefore, reducing oxidant stress should be protective against ACT. To determine whether antioxidant protein overexpression can reduce ACT, we developed a cell culture model system using the H9C2 cardiac cell line exhibiting controlled overexpression of the α6-isofrom of glutathione-S-transferase (GST). Treatment with the AC doxorubicin (DOX) produced both oncosis, manifested by an increase in the number of cells staining positive for Trypan blue, and apoptosis, indicated by the presence of positive terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. In both cases, the loss of cell viability was preceded by an AC-induced increase in fluorescence with carboxy-2,7’-dichlorofluorescein diacetate, demonstrating the presence of high levels of reactive oxygen species (ROS). The DOX-induced increase in ROS was reduced to control levels by maximal GST overexpression. Coincident with this elimination of oxidative stress, there was a reduction in both Trypan blue and TUNEL-positive cells, indicating that GST overexpression reduced both ROS and cell death in this model system. We conclude that GST overexpression may be an important part of a protective strategy against ACT and that this model system will aid in defining steps in the pathway(s) leading to AC-induced cell death that can be therapeutically manipulated.

ACT is generally believed to be due to oxidative injury to cardiac myocytes, a postreplicative cell type with limited defenses against oxidative stress (12, 37). Attempts to improve resistance to oxidative stress and thereby reduce AC cardiomyocyte injury have been successful in some experimental model systems. For example, in cultured cardiac myocytes, administration of the antioxidants amifostine, trolox, 5-aminosalicyclic acid, or α-phenyl-tert-butyl nitrate before AC exposure reduced indexes of oxidative stress and myocyte injury (12, 13). In small animal models, the degree of AC-induced cardiac injury has also been reduced by pretreatment with exogenous antioxidants, including thymoquinone (3), butylated hydroxanisole (48), and the lipiddowering antioxidant protocol (27). Probucol-induced protection was accompanied by an increase in activity of the antioxidant enzymes catalase, glutathione peroxidase, and superoxide dismutase (SOD) (41). Despite these encouraging results, no data in large animal or human studies support the use of exogenous antioxidants to reduce this problem.

The primary antioxidant enzymes that protect cells from oxidative stress are SOD, glutathione S-transferase (GST), catalase, and glutathione peroxidase. Increasing the expression level of SOD and catalase in the hearts of transgenic mice protects against ACT (20, 50), as does overexpression of the redox-sensitive protein thioredoxin (40). However, in each of these reports, the AC was given as a single large bolus rather than mimicking the human intermittent dosage schedule, so it is not clear whether improving oxidant defenses would protect against a more clinically relevant AC administration schedule.

Iron chelation has appeal as a potential tool against ACT because iron participates in the nonenzymatic generation of free radicals. In isolated myocytes, iron chelation reduced apoptosis induced by exposure to a doxorubicin (DOX) concentration near the peak plasma level seen in human use but did not protect from oncosis induced by exposure to high-dose DOX (38). Small clinical trials, mostly in patients with advanced cancer with poor prognosis, showed that iron chelation reduced short-term symptomat ic ACT, permitting higher cumulative AC dosages to be administered (42, 43). Because there is some relation between the cumulative AC dose and tumor response rate (7), it is possible that iron chelation will improve long-term cancer survival. While effective in small clinical trials, the treatment has myeloid toxicity, has not been shown to influence delayed ACT, and does not influence oxidative stress generated by AC via the enzymatic route (18). It therefore may represent an important, but perhaps not exclusive component of a protection strategy from ACT.
We have recently developed a cell culture model of AC injury using cardiac-derived H9C2 myocytes, which differentiate and can express transcribed foreign genes in a tightly regulated manner (25). We have suggested that this cell line represents a useful model in which to study the relationship between expression level of a putatively protective gene and the degree of protection from AC injury. In this report we describe using this cell line to overexpress the antioxidant protein α4 GST and examine the effect on AC injury. This gene was selected because it is an antioxidant with an important role in repair of oxidized lipid, DNA, and protein, all of which are generated by AC exposure. In addition, the observation that polymorphisms of GST genes have altered susceptibility to disease states (4, 23) raises the possibility that such polymorphisms may explain a portion of variability in susceptibility to ACT that is not explained by cumulative dose received.

MATERIALS AND METHODS

Cell culture. The H9C2 cell line was originally derived from embryonic rat heart tissue using selective serial passage (22) and was purchased from the American Tissue Type Collection (Manassas, VA; catalog no. CRL-1446). Cells were maintained in DMEM with 4.5 mM l-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 1.0 mM sodium pyruvate, with 10% fetal bovine serum. To prevent the loss of differentiation potential, cells were not allowed to become confluent. Cells were grown on tissue culture dishes for transfection, flow cytometry, Trypan blue (TB) staining, or to generate cell lysates, and were grown on Nunc Lab-Tech Permanox plastic chamber two-well slides (Fisher Scientific; Pittsburgh, PA) for the fluorescent oxidative stress and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays. In all experiments, the AC DOX was added to the complete culture medium and was washed off with PBS at the end of the defined incubation period. In some studies, cells were incubated with the general caspase inhibitor benzylcarnobyl-valine-alanine-aspartate fluoromethyl ketone (Z-VAD-fmk) (Calbiochem; La Jolla, CA) before treatment with DOX.

Transfection. H9C2 cells were plated onto 100 mm tissue culture dishes and were permitted to become 50% confluent in complete medium. Cells were then transfected with the regulator plasmid pTet-Off (Clontech; Palo Alto, CA). Transfection utilized 50 µl of a reagent (Lipofectamine, GIBCO; Gaithersburg, MD) complexed with 12 µg of pTet-Off plasmid DNA in serum-free medium for 20 min at room temperature (25°C). After the cells were washed twice with serum-free medium, the transfection cocktail was added for 5 h, after which an equal volume of complete medium with 20% serum was added to bring the final concentration of serum to 10%. The pTet-Off plasmid contains a neomycin-resistance cassette that permits selection of stably transfected clones by the addition of G418 to the culture medium. The plasmid directs the constitutive production of a tetracycline transactivator protein that can activate high-level expression from promoters with a Tet response element as long as Tet is not present in the culture medium (15). Clones surviving selection were expanded and screened by transient transfection with a plasmid (pTReLuc) directing the production of luciferase. The clone with the greatest induction of luciferase on Tet removal was transfected on a 100 mm dish with cells at 50% confluence using the same procedure with a plasmid (pTRe-GST) that expresses the α4 isoform of GST. The pTRE-GST plasmid was constructed by excising the SacII-HindIII fragment from pUbAA4 plasmid (a generous gift of Dr. Philip Board, Australian National University) and ligating it into the SacII-HindIII site of a similarly digested pTRe vector. pTRe contains a promoter that renders expression of cloned genes sensitive to the presence of Tet, permitting the expression level of GST to be controlled by the concentration of Tet in the culture medium. pTRE-GST was cotransfected with a second plasmid (pTK-Hyg) that permitted double stable transfectants to be selected by inclusion of hygromycin in the culture medium. After individual clones were selected and expanded, they were grown either in medium that contained or lacked Tet. Cell extracts were harvested when the cells became subconfluent and were analyzed by immunoblotting using an antibody to α4-GST (a kind gift of Dr. Piotr Zimm尼亚k, University of Arkansas) as described (26). The first antibody was used at a 1:50,000 dilution, and chemiluminescence with a second antibody at a dilution of 1:2,000 was used to detect antibody binding (Amersham; Arlington Heights, IL). To define the relationship between the Tet dosage and the GST expression level, the clone with the highest induction on removal of Tet (GST-45) was plated in varying Tet concentrations, cell lysates were harvested and were subjected to immunoblotting. Autoradiograms were scanned and densitometry was performed to quantify GST expression level.

Assay of oxidative stress. 2′,7′-Dichlorofluorescein diacetate (DFDA) is a cell-permeable compound, which is transformed into the fluorescent compound dichlorofluorescein on oxidation. DFDA is capable of detecting oxidative stress in primary cardiac myocytes induced by exposure to DOX (37). In the present experiments, control and GST-expressing cells were grown on chamber slides and were exposed for 4–6 h to 5 µg/ml DOX in culture medium before adding DFDA (Molecular Probes; Eugene, OR) for 40 min in the dark at a final concentration of 10 µM. In some experiments, we found that DOX alone generated a weak fluorescent signal in H9C2 cells, although not to the degree as when DFDA was included. Similar results were obtained using dihydrocalcein (Molecular Probes) as an independent indicator of oxidative stress (data not shown). The cells were washed several times with PBS and were viewed immediately with a Nikon TE 300 microscope equipped with a fluorescent attachment and a RT Color Spot Digital Camera. Continuous illumination of cells loaded with DFDA slowly increases dye fluorescence. For this reason, slides were scanned for several seconds to localize cells, and a high-resolution image was obtained with a constant exposure time by a single scan of a new field using ImagePro Plus software (Media Cybernetics; Silver Spring, MD). Fluorescence intensity, which correlates with the degree of oxidant stress (37), was measured in cells using ImagePro Plus software. The mean intensity of all fluorescent signals in a low-power field was compared between DOX-treated and control cells in the presence or absence of high-level GST expression.

Preliminary experiments demonstrated that Tet had no consistent influence on assays of oxidative stress and cytotoxicity (data not shown).

TB exclusion. TB is a vital dye excluded by viable cells with intact cell membranes. Cells dying via oncosis fail to exclude this dye indicating loss of membrane integrity. After membrane rupture, cells release intracellular enzymes such as lactate dehydrogenase, which can be assayed as a parallel measure of cell death. In preliminary studies, we found that lactate dehydrogenase release and TB exclusion yielded similar estimates of cell death in response to treatment with DOX. Therefore, we utilized the simpler, more rapid procedure (TB) to assess the development of oncotic cell death.

Wt-type (WT) and GST-expressing myocytes were plated in the absence of Tet and subjected to treatment with DOX (0.5 or 5 µg/ml for 7 h) or vehicle. After the cells were rinsed, they were incubated for an additional 36–48 h in fresh medium to allow the injury to evolve (25). Cells were collected by gentle trypsinization, followed by neutralizing with serum-containing medium. Subsequently, a small volume of 1% TB was added to cells collected from each dish, and the number of cells excluding or staining with the dye was counted on the Nikon microscope as described (52). A cell was considered positive if the entire cytoplasm was diffusely stained with any shade of blue. Cells were counted immediately after addition of the vital dye to prevent eventual staining of all cells as they became depleted of substrate. Data are presented as the percentage of cells staining with TB, i.e., nonviable.

TUNEL assay. DNA fragmentation was visualized by use of the ApopTag kit (Intergen; Norcross, GA). This system labels free 3′ OH

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termi of DNA in cells with digoxigenin-tagged nucleotides with the use of the enzyme terminal deoxynucleotidyl transferase. Binding of a fluorescent antibody to digoxigenin in positive cells is visualized using fluorescent microscopy. H9C2 and GST-45 cells were grown on chamber slides in the absence of Tet to induce GST expression. DOX was added at a concentration of 0.5 or 5 μg/ml for 8 h. One thousand attached cells were scored as being TUNEL positive or negative from each condition per experiment. Cells were only scored as being positive under direct visualization if nuclear fluorescent staining was detected at a level significantly above background, as described by Akao et al. (2).

Flow cytometry. Annexin and propidium iodide staining was performed in conjunction with flow cytometry to confirm TUNEL assay data on a larger, unselected population of cells using the Annexin V-FITC Apoptosis Detection Kit (Oncogene Research Products; San Diego, CA). This assay takes advantage of the binding by annexin to phosphatidyl serine residues exposed on the cell surface soon after induction of apoptosis before nuclear breakdown occurs (when propidium iodide staining is present). H9C2 cells or GST-45 cells were plated on 60 mm dishes until 70% confluent, at which time they were treated with vehicle or DOX at doses of 0.5 or 5 μg/ml. Twenty-four hours later, cells were collected by trypsinization and were suspended at a concentration of 10^6 cells/ml in PBS. All subsequent steps were performed with the cells and reagents on ice. Cells were pelleted and resuspended in binding buffer before the addition of 1.25 μl annexin V-FITC and incubating for 15 min in the dark. Cells were pelleted again and resuspended in a second binding buffer before the addition of 10 μl propidium iodide. Cells were assayed by flow cytometry using a Beckman Flow Cytometer, with results positive or negative for both propidium iodide and annexin. Viable cells do not stain with either reagent, early apoptotic cells stain with annexin only, and either late apoptotic or necrotic cells stain with both annexin and propidium iodide. Because we were mainly interested in the number of cells that were clearly apoptotic, cells positive for annexin alone were compared as a function of GST expression and treatment condition.

Data analysis. SPSS software for Windows 98 version was used for all statistical comparisons. The means ± SD of each measure (percentage of cells positive for TB, fluorescent intensity, number of TUNEL positive or annexin-positive cells) was obtained from at least three experiments. Observations are presented as means ± SE, and one-way ANOVA was used to compare means, followed by the post hoc Sidak test (P < 0.05 was considered significant).

RESULTS

Regulated expression of GST by stable transfectants. We previously described the generation of multiple H9C2 cell lines stably transfected with the pTet-Off plasmid (25). Each transfected clone constitutively expresses a tetracycline transactivator protein at high level (15). The H9C2 clone with the greatest luciferase induction on Tet removal was subsequently transfected with the pTre-GST plasmid cotransfected with a plasmid conferring hygromycin resistance, allowing double stable transfectants to be selected. The clone from this second transfection with the highest GST induction by immunoblotting was subsequently grown in varying Tet concentrations and cell lysates were analyzed by immunoblotting to determine the dose-response relationship. Figure 1A shows a representative immunoblot, whereas Fig. 1B shows the average GST expression level as a function of the Tet concentration from five separate experiments. GST was not detectable in untransfected H9C2 cells (WT), was expressed at a very low level in the presence of 1 μg/ml Tet, was induced 2-fold when the Tet concentration was reduced to 0.01 μg/ml, and reached maximal induction of 13-fold when Tet was removed from the medium. Although not shown, higher concentrations of Tet did not further suppress GST expression. Therefore, the GST expression level was reproducibly regulated by the concentration of Tet in the culture medium, particularly between 0.01 and 0.001 μg/ml. This cell line (GST-45) was subsequently treated with DOX and assayed for extent of injury and oxidative stress as a function of GST expression compared with WT H9C2 cells.

Figure 2 schematically illustrates experiments subsequently performed on GST overexpressing and control H9C2 cells. DOX-induced oxidative stress is prevented by GST overexpression. Oxidative stress precedes the development of irreversible cell injury after DOX exposure in H9C2 cells (25). To determine whether GST overexpression mitigated DOX-induced early oxidative stress, control cells were compared with GST-45 cells induced to maximally express GST. Cells were loaded with DFDA after exposure to 5 μg/ml DOX or vehicle for 4 h. A representative set of fluorescent micrographs from such an experiment is shown in Fig. 3. Control cells had a low baseline level of fluorescence (Fig. 3A). Exposure to DOX without GST induction (Fig. 3B) significantly increased fluorescence, indicating that DOX generated oxidative stress. DFDA fluorescence in DOX-treated GST overexpressing cells (Fig. 3C) was reduced compared with treated cells without GST expression (Fig. 3B). Figure 4 shows the mean fluorescent intensity from 8 independent experiments as a percentage of fluorescent intensity in control (vehicle treated) cells. DOX treatment in H9C2 cells without high-level GST expression increased the level of oxidative stress >100-fold above vehicle treatment (P < 0.001). GST induction reduced DOX-induced oxidative stress to the level seen in cells exposed to vehicle alone.

Fig. 1. Glutathione S-transferase (GST) expression is regulated by the concentration of tetracycline (Tet) in the culture medium of stably transfected H9C2 cells. A: representative immunoblot showing GST expression from lysates of a single stably transfected clone grown for at least 1 wk in the indicated concentrations of Tet (in μg/ml). A lysate from an untransfected [wild type (WT)] H9C2 culture is included. B: bar graph summarizing densitometry averaged from five immunoblots of GST expression as a function of Tet concentration, expressed as fold elevation above the GST level in 1 μg/ml Tet.

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Cell membrane injury is reduced by GST overexpression. In a separate series of experiments, H9C2 and GST-45 cells were treated for 7 h with 0.5 or 5 μg/ml DOX, followed by a medium change and incubation for an additional 36–48 h to allow for the development of injury. The percentage of nonviable cells in each condition (i.e., that stained with TB) is shown in Fig. 5 as a function of DOX dose and cell type. High-dose treatment of control H9C2 cells with 5 μg/ml DOX increased the proportion of TB-positive cells, from 5 to 50%, indicative of oncotic cell death (P < 0.001 compared with untreated control cells). When GST-45 cells were treated with the same DOX dose, 39% of cells became TB positive, indicating that increased GST expression provided protection against cell death (P < 0.006), albeit not to control levels. Low-dose treatment of control H9C2 cells with 0.5 μg/ml DOX induced a slight increase in TB-positive cells above control, from 5 to 15%, a significant increase (P < 0.001). Treatment of GST-45 cells with the same DOX dose resulted in 8% TB-positive cells; significantly less than treated H9C2 cells (15%, P < 0.001) and not significantly different from untreated control cells (5%, P = 1.0), indicating that GST expression eliminates cell death at this DOX dose.

GST overexpression reduces apoptosis in response to DOX treatment. When complete protection from high-dose DOX injury was not observed by GST expression, we considered the possibility that GST may protect against apoptotic rather than oncotic cell death. In support of this concept, it has been shown that in primary cardiac myocytes that apoptosis is induced by exposure to low concentrations of DOX (e.g., 1 μM or less), whereas oncotic death is induced by exposure to higher concentrations (e.g., 10 μM or higher) (38). Consistent with this observation, we observed extensive TB staining on exposure to DOX concentrations near 10 μM (~5 μg/ml), but less so at 1 μM (~0.5 μg/ml, Fig. 5). To examine whether apoptosis occurs in response to DOX exposure in our system, H9C2 or GST-45 cells were subjected to a TUNEL assay immediately after exposure to 0.5 or 5 μg/ml DOX for 8 h, and the number of TUNEL-positive cells per 1,000 cells was counted. (After longer DOX treatment, cells begin to lift off the slides, precluding evaluation by TUNEL staining.) A representative TUNEL micrograph of H9C2 cells exposed to 0.5 μg/ml DOX is shown in Fig. 6. Cells containing brightly fluorescing nuclei were scored as positive (arrows). When H9C2 or GST-45 cells were exposed to vehicle alone, no TUNEL-positive cells were detected. After exposure of H9C2 cells to 0.5 μg/ml DOX, TUNEL-positive cells developed at a rate of 2.9% (29 cells per 1,000). Exposure to a DOX concentration of 5 μg/ml produced only 1.4% positive cells (14 per 1,000) suggesting that apoptotic cell death predominates at the lower DOX dose and that an additional mechanism is operative when cells are treated with high-dose DOX. The number of TUNEL-positive cells was significantly higher at both doses compared with untreated control cells (P < 0.001). When GST-45 cells were similarly treated, the prevalence of TUNEL-positive cells in response to treatment with 0.5 or 5 μg/ml DOX was significantly below that in H9C2 cells treated with the same doses (1.5% and 0.8%, respectively; P < 0.002 for both groups).

Fig. 2. Diagram of experimental design with WT and GST-overexpressing H9C2 cells. Z-VAD, benzylcarbonyl valine-alanine-aspartate-fluoromethyl ketone (Z-VAD-fmk); DOX, doxorubicin; DFDA, 2',7'-dichlorofluorescein diacetate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Fig. 3. DOX exposure produces oxidative stress, which is reduced by GST overexpression. Micrographs from DFDA staining of control (A), untreated cells, control cells treated with DOX for 4 h (B), and GST-overexpressing cells treated with DOX for 4 h (C).

Fig. 4. DFDA intensity in control, untreated cells, DOX-treated control cells, and DOX-treated GST-expressing cells, expressed on logarithmic scale as a percentage of control. Signal intensity from eight independent experiments was averaged for each condition. *P < 0.001, significantly different from control untreated cells.
Flow cytometry studies. Annexin staining, indicative of exposure of phosphoserine residues, is an additional hallmark of early apoptotic cell death. Flow cytometry confirmed that exposure to DOX caused cells to stain positive for annexin, consistent with apoptosis (data not shown). The extent of apoptosis was abrogated by GST expression at both DOX doses, although the differences did not reach statistical significance. Preincubation of H9C2 cells with a general caspase inhibitor (Z-VAD-fmk) significantly reduced the number of TB-positive cells after treatment with 5 μg/ml DOX from 45 to 31% and after treatment with 0.5 μg/ml DOX from 21 to 14% (both P < 0.01) (Fig. 7A) albeit not to untreated control levels. Caspase inhibition also reduced the number of TUNEL-positive cells after 5 μg/ml DOX treatment from 1.5 to 0.5%, and after 0.5 μg/ml DOX treatment from 3.6 to 1.5% (Fig. 7B, P < 0.01). The sum of these results indicate that apoptosis and oncosis occur in response to DOX treatment at high and low doses and that high-level GST expression reduces both types of cell death.

DISCUSSION

This report demonstrates for the first time that overexpression of the α4 isoform of GST markedly reduces fluorescent indexes of oxidative stress induced by DOX exposure and significantly reduces apoptotic and oncocytic cell death caused by this agent. GST may conjugate glutathione directly to oxidized derivatives of DOX or alternatively may exert its protective effect by sequestration of DOX from cellular elements necessary to generate reactive oxygen species. Indeed, a sequestration role has been demonstrated for members of the α-class of GST enzymes and may be especially prominent at high cellular GST levels (29). Apoptosis induced by low concentrations of DOX is almost completely eliminated by GST overexpression, whereas oncosis induced by high concentrations of DOX is less extensively reduced by GST overexpression. Because GST overexpression eliminates oxidative stress generated by DOX, this suggests that other factors in addition to oxidative stress play a role in DOX-induced cell death at high doses. Oxidative stress, likely, however, plays an etiologic role at serum concentrations of DOX encountered clinically.

The GST family of proteins is important in detoxification of lipid peroxidation products generated by exposure to oxidizing agents by conjugating glutathione to these products, repairing oxidative damage (54). We found a tight correlation between extent of oxidative stress and cell death after DOX treatment that was reduced by GST overexpression, suggesting that oxidative stress is important in both apoptotic and oncocytic cell death. This observation is consistent with a recent report treating neonatal rat cardiomyocytes with DOX, where oxidative stress was clearly documented after exposure to a dose of DOX producing primarily apoptosis and where elimination of oxidant stress protected against apoptosis (49). Our results differ, however, from another recent study (38) showing that addition of exogenous antioxidants (i.e., ascorbic acid, tocopherol, and N-acetylcysteine) reduced oxidant stress but failed to...
reduce apoptosis induced by low-dose DOX exposure. This discrepancy may reflect the ability of GST to repair oxidative damage, a property not seen with exogenous antioxidants. Whether AC directly cause cardiotoxicity or whether the toxicity is mediated by an intermediate compound has not been conclusively demonstrated. The alcohol metabolite of the AC daunorubicin, for example, is concentrated in heart tissue after bolus administration, persists for at least 3 days after the parent compound has disappeared, and can directly reduce cardiac function (10). This metabolite may produce irreversible cardiac myocyte injury by generation of reactive oxygen species by delocalizing iron from aconitase, a protein critical in normal function (10). This metabolite may produce irreversible cardiac myocyte injury and provides a possible mechanistic explanation for the observed clinical benefit of iron chelation as a prophylactic measure against ACT.

**Dosage of DOX and mechanism of cell death.** Our results confirm previous reports that the mechanism of DOX toxicity is dependent on the concentration of the compound experienced by the target cell, with apoptosis predominating at low doses and oncosis predominating at high doses. Although not the dominant mechanism of cell death, at higher doses apoptosis does occur, as supported by positive TUNEL staining and by a decrease in extent of cell death when a caspase inhibitor was added before DOX exposure. A previous report using primary cardiac myocytes also demonstrated that low-dose DOX exposure induced apoptosis whereas high-dose exposure primarily induced oncosis (38). With the use of a targeting dosage similar to that used in humans, the peak plasma concentration of DOX after bolus infusion in experimental animals (i.e., 1 to 2 μg/ml) was observed to be closer to the level producing apoptosis than oncosis (11). Typical indexes of cardiomyocyte necrosis (i.e., creatine kinase or troponin release) are not detected in patients after treatment with AC, further suggesting that apoptosis may be the more relevant cell death mechanism associated with clinical ACT (14). In leukemic cells, the concentration of DOX also determines the mechanism of cell death, with apoptosis occurring over a concentration range of 1–3 μM, and necrosis at higher doses, possibly because the apoptotic program requires ongoing RNA synthesis, which is inhibited at high concentrations of DOX. Thus the apoptotic pathway may be initiated but not executed at higher DOX concentrations (31). It seems plausible to suggest similar events occur in cardiomyocytes, but this will require further experimental verification.

**Potential signaling pathways.** Oxidative stress, such as exposure to hydrogen peroxide, causes apoptosis in a number of cell types, including cardiac myocytes. The signal transduction pathway connecting oxidative stress to apoptosis may involve p53 (47), p53 and p66Shc (44), c-Jun NH2-terminal kinase (6, 45, 51), or the mitochondrial death pathway (1). It seems reasonable to assume that because DOX causes oxidative stress in cells and oxidative stress leads to apoptosis, a pathway would connect the two events. In addition to the involvement of members of the MAP kinase family (35, 53), other pathways connecting DOX exposure to apoptosis include p53 (28), erb receptors 2 and 4 (39), phosphatidyl inositol 3-kinase/Akt (34), and the Fas/Fas ligand system (33). Our model system provides a valuable environment in which to define signaling pathways involved in DOX-induced apoptosis. Furthermore, the GST-overexpressing cells described in this report should prove a useful reagent in the study of the specific role of oxidant stress in triggering apoptotic pathways as well as other oxidative disease states that affect cardiac myocytes, including ischemia-reperfusion and septic shock.

**Mechanism of DOX-induced cardiomyocyte death.** Other mechanisms have been put forth to explain the molecular basis of AC toxicity to cardiomyocytes. One explanation is that AC disrupt expression and activity of the transcription factor GATA-4 (21). Because GATA-4 activity induces expression of genes such as glutathione peroxidase and Bcl-x that protect cardiomyocytes from the apoptotic program, interference with its activity might reduce cardiac gene transcription, impair antioxidant defenses, and thereby induce apoptosis. In the current report, we observed apoptosis after a shorter exposure to DOX than was described as necessary to reduce GATA-4 protein levels in the cited study. The steps upstream of inhibition of GATA-4 activity need to be elucidated to further define the contribution of this mechanism to ACT. Alternatively, disruption of myocyte expression of genes important in energy production has also been suggested to be important in the
mechanism of ACT (19), but this disrupted expression could still be a consequence of AC-induced oxidative stress.

On the basis of our results, we propose a potential sequence of events that may serve as a framework within which to define the precise steps linking DOX exposure to myocyte cell death. The initial event occurring after DOX exposure may be lipid peroxidation, which does not require that DOX enter the target cell. This event could generate oxidative stress, which in turn could initiate a signaling pathway ultimately producing apoptotic cell death. Because of the importance of the MAP kinases p38 and JNK in responses to cellular stresses (46), one or both of these pathways are good candidates to become activated after DOX exposure. Because activity of JNK and p38 influences transcription through specific transcription factors, such a scheme can also explain the observation of altered gene transcription after DOX exposure. A link between oxidative stress, JNK activation, and eventual apoptotic cell death involving the mitochondrial death pathway has been demonstrated in cardiomyocytes (1, 6) and may be important.

Future directions and possible clinical relevance. This study shows that the myocyte GST level influences susceptibility to cell death on exposure to clinically relevant concentrations of DOX. The optimal extent of GST overexpression that is necessary and sufficient to provide protection against DOX treatment with clinically important doses is currently being investigated. This observation is consistent with previous descriptions of protection from DOX provided by antioxidant gene overexpression in transgenic mice, including SOD (50), thioredoxin (40), and catalase (20). GST genes are polymorphic in humans, and polymorphisms of specific isoforms are associated with disease susceptibility, including leukemia (4, 23) and liver disease (17). Other antioxidant gene polymorphisms disease susceptibility relationships have been defined for heme oxygenase with coronary disease in diabetics (9), glutamate-cysteine ligase with myocardial infarction (32), and SOD with breast cancer (5). It is possible that polymorphisms of GST or other antioxidant genes may explain a portion of ACT in humans that is independent of cumulative dose received. We are currently exploring this avenue of investigation.

In conclusion, exposure to DOX induces oxidative stress that leads to apoptotic and/or oncocytic cell death, with the predominant mechanism of cell death determined by the DOX dosage. Overexpression of the α4 isoform of GST significantly reduces oxidative stress and subsequent cell death by both mechanisms but particularly apoptotic cell death. Because the concentration of DOX in human plasma after infusion of the drug approximates the concentration that primarily induces apoptosis, we believe that this mechanism of cell death is most important in the induction of clinical ACT. This model system will be useful for dissecting the important signaling pathway(s) leading from DOX exposure to cell death and may reveal therapeutic targets that may reduce the impact of this important clinical problem.

GRANTS

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