DNA damage is an early event in doxorubicin-induced cardiac myocyte death

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L’Ecuyer, Thomas, Sanjeev Sanjeev, Ronald Thomas, Raymond Novak, Lauri Das, Wendy Campbell, and Richard Vander Heide. DNA damage is an early event in doxorubicin-induced cardiac myocyte death. Am J Physiol Heart Circ Physiol 291: H1273–H1280, 2006. First published March 24, 2006; doi:10.1152/ajpheart.00738.2005.—Anthracyclines are antitumor agents the main clinical limitation of which is cardiac toxicity. The mechanism of this cardiotoxicity is thought to be related to generation of oxidative stress, causing lethal injury to cardiac myocytes. Although protein and lipid oxidation have been documented in anthracycline-treated cardiac myocytes, DNA damage has not been directly demonstrated. This study was undertaken to determine whether anthracyclines induce cardiac myocyte DNA damage and whether this damage is linked to a signaling pathway culminating in cell death. H9c2 cardiac myocytes were treated with the anthracycline doxorubicin at clinically relevant concentrations, and DNA damage was assessed using the alkaline comet assay. Doxorubicin-induced DNA damage, as shown by a significant increase in the mean tail moment above control, an effect ameliorated by inclusion of a free radical scavenger. Repair of DNA damage was incomplete after doxorubicin treatment in contrast to the complete repair observed in H2O2-treated myocytes after removal of the agent. Immunoblot analysis revealed that p53 activation occurred subsequent in time to DNA damage. By a fluorescent assay, doxorubicin induced loss of mitochondrial membrane potential after p53 activation. Chemical inhibition of p53 prevented doxorubicin-induced cell death and loss of mitochondrial membrane potential without preventing DNA damage, indicating that DNA damage was proximal in the events leading from doxorubicin treatment to cardiac myocyte death. Specific anthracycline-induced DNA lesions included oxidized pyrimidines and 8-hydroxyguanine. DNA damage therefore appears to play an important role in anthracycline-induced lethal cardiac myocyte injury through a pathway involving p53 and the mitochondria.

Reactive oxygen species, such as generated by AC exposure, induce detrimental modifications to multiple cellular macromolecules, including proteins, lipids, and DNA. Although AC-induced oxidative lesions to cardiac myocyte lipids (26) and proteins (23) have been demonstrated, damage to DNA has been inferred (28) but never directly demonstrated. In contrast, DNA damage is a prominent feature produced by AC and other oxidants in tumor cells (32) and lymphocytes (27). Oxidative DNA base modifications have been demonstrated in hearts and isolated myocytes subjected to oxidant injury, including ischemia-reperfusion (38) and antioxidant depletion (11), indicating that this cell type is susceptible to oxidant-induced DNA damage. In addition to oxidative DNA damage, AC bind avidly to DNA in the nucleus of cancer cells, forming adducts that can interfere with binding of proteins such as transcription factors and DNA polymerase, potentially interfering with the important cellular functions of DNA, including replication and transcription (7).

DNA damage in proliferative cells activates a pathway that arrests cell division to allow either DNA repair or the induction of cell death by apoptosis. p53 is an effector protein in this pathway that plays a critical role in the induction of cell cycle arrest and apoptosis (39). Once activated, p53 translocates to the nucleus where it induces expression of genes that prevent cell division (i.e., p21) and cause apoptosis (e.g., Bax; see Ref. 6). In more differentiated skeletal muscle cells, DNA lesions induced by AC exposure also induce apoptosis in a p53-dependent fashion (17). An alternate response by cells subjected to DNA damage is lesion repair rather than apoptosis. DNA repair utilizes enzymes that excise oxidized bases before DNA replication (3), remove oxidized bases from the nucleotide pool (8), or remove oxidized bases from DNA after replication (33). Because cardiac myocytes are postreplicative, repair of DNA lesions is important to avoid deterioration of cardiac function that would eventually attend myocyte loss. Indeed, nucleotide excision repair, particularly of the transcribed strand, is very active in myocytes after DNA injury induced by irradiation (35). This study was undertaken to define whether AC induce DNA lesions in cardiac myocytes, whether myocytes are able to repair DNA lesions, and to define the pathway transducing the DNA damage into lethal cell injury.

MATERIALS AND METHODS

Cell culture and treatment. The H9c2 cardiac cell line was derived from embryonic rat heart by selective serial passage (15). Cells were plated on 35- or 100-mm dishes in DMEM with 10% FCS and used

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when 70–90% confluent. The AC doxorubicin (DOX) was added to complete medium and incubated at 37°C for various intervals at a concentration (0.5 μg/ml) similar to plasma concentrations encountered in clinical use (10). In some experiments, the free radical scavenger amifostine was included during DOX treatment at a concentration of 14 mM (4). Alternatively, a chemical inhibitor of p53, pifithrin (16), was added at a concentration of 30 μM during DOX treatment. Pifithrin appears to act downstream of accumulation of p53, reducing apoptosis by inhibiting production of pro-apoptotic proteins (21). Amifostine and pifithrin were added to cultures 60 min before addition of DOX. Cells were alternatively treated with H2O2 at a concentration of 10 μM in PBS on ice for 5 min. At the end of the treatment interval, cells were washed two times with PBS before harvesting. In repair experiments, treated cells were allowed to recover in fresh complete medium at 37°C for varying intervals between harvest.

Single-cell electrophoresis assay. Oxidative stress, radiation, and AC cause DNA damage that can be detected and quantified using the alkaline single-cell electrophoresis assay (comet assay; see Refs. 4 and 31). During electrophoresis, undamaged DNA is largely confined to the nucleus, whereas damaged DNA migrates apart from the nucleus in the shape of a comet. The length and fluorescent intensity of the comet are proportional to the number of DNA strand breaks. The extent of DNA damage can be quantified by the use of this sensitive technique, with the most frequently reported measure being the tail moment, a product of tail length and percent tail DNA (34). We performed the assay essentially as described by Singh et al. (31).

Evaluation of DNA damage. After electrophoresis, DNA was stained with 1% propidium iodide, and slides were coverslipped and analyzed by fluorescent microscopy using an excitation filter of 515–560 nm and a barrier filter of 590 nM. An image analysis system (Kinetic Imaging, Bromborough, Wirral) was used to measure indexes of DNA damage. Digitized images were obtained of 50 randomly selected cells per slide, and the tail moment was determined from two slides per condition. The median tail moment was determined for each slide, and the mean of at least eight of these values per condition (i.e., at least 2 slides/condition from at least 4 separate days) was used as the index of DNA damage. The tail moment was expressed as the degree of increase in elevation above a negative control slide from the index of DNA damage. The tail moment was expressed as the degree of increase in elevation above a negative control slide from the index of DNA damage. The tail moment was expressed as the degree of increase in elevation above a negative control slide from the index of DNA damage.

RESULTS

H2O2 induces myocyte DNA injury. To document that nuclear DNA damage occurred in cardiac myocytes treated with a known oxidant, we applied the alkaline comet assay to measure DNA damage in H9c2 cells treated with H2O2. Representative micrographs of a control cell and a cell treated with 10 μM H2O2 for 5 min on ice (to prevent DNA repair) are shown in Fig. 1. In control cells, the undamaged DNA migrates in an electrophoretic field within the nucleus (Fig. 1A), whereas some DNA from cells treated with H2O2 migrates away from the nucleus, forming a tail, or comet (Fig. 1B). Software analysis allows semiautomated collection of indexes of DNA damage from multiple cells, which can be analyzed statistically. As shown in Fig. 2, DNA damage occurs rapidly in H9c2 cells treated with 10 μM H2O2, with a 5-min exposure producing a mean tail moment significantly above untreated control cells [100 ± 3.1 (SE); P < 0.0001]. If cells were returned to the incubator at 37°C with fresh medium after the 5-min treatment with H2O2, DNA damage was repaired rapidly, as shown in Fig. 2. The mean tail moment after all repair intervals was significantly less than that without repair (P < 0.0001) and after a 90- or 120-min repair interval was not significantly different from untreated control cells (P = 0.145 and 1.0, respectively).
respectively). These experiments indicate that oxidative stress rapidly induces cardiac myocyte DNA damage and that H9c2 myocytes have significant DNA repair capacity from a purely oxidative stress. We found that more extensive DNA damage occurred with higher H2O2 concentrations (data not shown).

DOX induces myocyte DNA injury. In the next series of experiments, H9c2 cells were treated with the anthracycline DOX for varying intervals before performing the alkaline comet assay. DOX was used at a concentration of 0.5 μg/ml, a concentration achieved after bolus use in human pharmacokinetic studies (10). Although DNA damage was detectable after treatment intervals of 2 h, the mean tail moment did not become statistically different from the untreated control cells until 4 h at 23 ± 4.3 (Fig. 3; P < 0.0001). Inclusion of the free radical scavenger amifostine in the culture medium during DOX treatment decreased the mean tail moment to 7.7 ± 1.8 (P < 0.0001 vs. DOX alone), suggesting that the injury induced by DOX was at least partly oxidative.

To determine whether DNA damage induced by DOX could be repaired, cells were treated with 0.5 μg/ml DOX for 4 h and allowed to repair DNA damage in fresh medium at 37°C for varying intervals before the comet assay was performed. As shown in Fig. 4, DOX treatment for 4 h induced significant DNA damage, with a mean tail moment of 21 ± 1.7 (P < 0.0001 vs. untreated control). DNA damage progressed in the first 4 h of repair, with the mean tail moment increasing to 29 ± 1.7 (P < 0.0001 vs. negative control, P < 0.006 vs. 4 h treatment without repair). The mean tail moment remained significantly elevated after repair intervals as long as 24 h at 18 ± 1.2 (P < 0.0001 vs. untreated control). Some treated cells began to detach during repair intervals longer than 24 h, making it difficult to assess DNA injury after longer repair intervals. The sum of these results indicate that DOX induces DNA damage in cardiac myocytes that is less extensive and

Fig. 1. Oxidants damage H9c2 cell nuclear DNA. Alkaline comet assay was performed as described in MATERIALS AND METHODS. Representative micrographs of untreated control H9c2 cell (A) with intact DNA migrating exclusively in the nucleus and cell treated with 10 μM H2O2 (B), showing damaged DNA migrating away from the nucleus, forming a tail or comet.

Fig. 2. H2O2 induces DNA damage in H9c2 cells that is rapidly repaired. Quantitative presentation of comet assay data from 5 independent experiments was performed as described in MATERIALS AND METHODS. Cells were untreated (negative control) or treated on ice for 5 min with 10 μM H2O2 and processed for the comet assay immediately or allowed repair intervals from 30 to 120 min in fresh medium at 37°C before the assay. Mean of tail moments as the degree of increase in negative control is shown as index of DNA damage. *Significantly different from negative control at P < 0.0001.

Fig. 3. Time course of H9c2 cell DNA damage induced by doxorubicin (DOX) treatment. Quantitative presentation of comet assay data from 5 independent experiments performed as described in MATERIALS AND METHODS. Cells were untreated or treated for indicated intervals with 0.5 μg/ml DOX with or without 14 mM amifostine and processed for the comet assay. Mean of tail moments as the degree of increase of negative control is shown as an index of DNA damage. *Significantly different from negative control at P < 0.0001.
Fig. 4. DOX-induced DNA damage is incompletely repaired in H9c2 cells. Quantitative presentation of comet assay data from 4 independent experiments performed as described in MATERIALS AND METHODS. Cells were untreated or treated with 0.5 μg/ml DOX for 4 h and processed for the comet assay immediately or allowed various repair intervals in fresh medium at 37°C before the assay. Mean of tail moments as the degree of increase of negative control is shown as index of DNA damage. *Significantly different from negative control at P < 0.0001.

Fig. 5. Specific DOX-induced DNA lesions recognized by DNA repair enzymes. The alkaline comet assay detects single- and double-stranded DNA breaks as well as alkaline-labile sites (34). When the assay is coupled with enzymes that repair specific lesions in DNA, it is possible to define the DNA lesions induced in DOX-treated cells more specifically. An increase in the tail moment is noted in enzymes-treated cells compared with buffer-treated cells if the specific DNA lesion recognized by the enzyme is present in the cell population. In these studies, we first used endonuclease (Endo) III to detect oxidized pyrimidines. As shown in Fig. 5, DOX-treated cells subsequently treated with Endo III had a mean tail moment of 42 ± 5.5, significantly above untreated control cells (P < 0.001) and DOX-treated cells subsequently treated with Endo III buffer alone (19.8 ± 2.1; P = 0.018). This result indicates that DOX induces oxidative damage to pyrimidines in cardiac myocytes, as it does in lymphocytes (5). Similarly, the base excision repair enzyme formamidopyrimidine-DNA glycosylase (Fpg) recognizes and nicks 8-oxoguanine, the most common DNA lesion in cells subjected to oxidative stress (9, 38). As shown in Fig. 5, DOX-treated cells subsequently treated with Fpg had a mean tail moment of 56 ± 7.0, significantly above untreated control cells (P < 0.0001) and DOX-treated cells subsequently treated with Fpg buffer alone (22.8 ± 2.2; P < 0.0001). This result indicates that DOX induces 8-oxoguanine in cardiac myocytes.

**DOX activates p53 in H9c2 cells.** The transcription factor p53 has a short half-life and is present at a low level in normal cells. Exposure to DNA damaging agents leads to stabilization and accumulation of p53, translocation to the nucleus, and expression of its target genes (6). As shown in Fig. 6A, p53 is not detectable by immunoblot analysis in untreated cells but becomes detectable within 4–8 h of treatment with DOX, and demonstrates a time-dependent accumulation over the first 16 h before returning toward control levels by 48 h. As shown in Fig. 6B, densitometry of p53 immunoblots confirmed significant induction by DOX treatment within 8 h (P < 0.006). Equal loading was confirmed by probing representative immunoblots for β-actin. Activation of p53 correlates with accumulation of the protein and with expression of proapoptotic proteins, including Bax and Bad (17). We were unable to consistently document induction of p53-responsive proapoptotic proteins after treatment with DOX, possibly reflecting inadequate sensitivity of immunoblotting for detection of these proteins in cardiac cells. To confirm the importance of p53 in DOX-induced cell death, H9c2 cells were treated with DOX in the presence or absence of the p53 inhibitor pifithrin, and cell viability was assessed using trypan blue. As shown in Fig. 6C, untreated H9c2 control cells have a rate of spontaneous cell death of 11 ± 0.4% after 48 h in culture. Treatment of cells with DOX significantly increased the percentage of trypan blue-positive cells at 48 h to 19 ± 0.3% (P < 0.0001 vs. untreated control). Inclusion of 30 μM pifithrin during DOX treatment reduced the cell death rate to the untreated control rate of 11 ± 1%. These experiments indicate that p53 activation occurs after DOX treatment and suggest that it plays an important role in mediating DOX-induced H9c2 cell death. To rule out the possibility that pifithrin protects against cell death by preventing DOX-induced DNA damage, a comet assay was performed on H9c2 cells treated for 4 h with DOX in the presence or absence of pifithrin. As shown in Fig. 7, DOX significantly increased the mean tail moment above untreated control to 18 ± 2.5 (P < 0.001), and the inclusion of pifithrin had no significant effect on this DOX-induced DNA damage, indicating that DNA damage induced by DOX is proximal to p53 activation and eventual cell death.

**DOX induces ΔΨ loss in cardiac myocytes.** Oxidative stress induces p53 accumulation in cardiac myocytes, with eventual apoptosis mediated by release of proapoptotic proteins from the mitochondria, including cytochrome c (36). Activation of p53 is followed by loss of ΔΨ and downstream caspase
activation in HeLa cells independent of Bax translocation or Bid activity (20). H9c2 cells were treated with DOX in the presence or absence of pifithrin. After cells were loaded with JC-1, the percentage of cells expressing red fluorescent aggregates (indicating intact ΔΨ) was obtained from each condition. Merged images of the fluorescein and rhodamine channels of a representative culture from each condition are shown in Fig. 8. Nearly all cells are well spread and exhibit red or orange fluorescence in an untreated culture, as shown in Fig. 8A. An image from a culture treated with DOX for 4 h is shown in Fig. 8B, which does not look significantly different from the untreated condition, indicating that ΔΨ is intact. In contrast, a culture treated with DOX for 16 h is shown in Fig. 8C, demonstrating multiple rounded cells, a majority of which fluoresce green exclusively, indicating loss of ΔΨ. A culture treated with DOX for 16 h in the presence of pifithrin appears similar to untreated control, with spread cells exhibiting red or orange fluorescence, as shown in Fig. 8D. The percentage of cells with intact ΔΨ is displayed in Fig. 9. In untreated control cells, 90 ± 1.1% demonstrated intact ΔΨ, with the percentage similar for cells treated with DOX for 4 h. The percentage of cells with intact ΔΨ decreased significantly after 16 h treatment with DOX to 58 ± 4.0% (P < 0.0001 vs. untreated control or 4 h DOX treatment), and this decrease was prevented by inclusion of pifithrin in the medium, indicating that loss of ΔΨ after DOX treatment is mediated by p53.

DISCUSSION

In summary, our results demonstrate for the first time that the anthracycline DOX induces specific DNA lesions in cardiac myocytes. These lesions occur at clinically relevant concentrations of DOX within several hours of exposure and are not completely repairable. This pattern of DNA damage/repair is distinctly different from that seen in purely oxidative lesions, where DNA damage occurs more rapidly and is rapidly and completely repaired. The data also show that DOX causes activation of p53 after DNA lesion development, with subsequent loss of ΔΨ and loss of cell viability. Furthermore, inhibiting p53 eliminated DOX-induced cell death and loss of ΔΨ without reducing the extent of DNA lesions. Taken together, our results demonstrate that DNA damage is an early event in DOX-induced lethal cardiac myocyte injury and that cell death is mediated through p53 and the mitochondria.

*p53 activation in cardiomyocytes by AC. p53 is activated in the hearts of DOX-treated animals. Inhibition of p53 reduced morphological, biochemical, and functional consequences of DOX treatment (21). The function of p53 is best characterized in proliferative cells, where it maintains genomic stability in response to DNA damage, thereby preventing malignant transformation. Highly differentiated cells such as myocytes are also subjected to DNA damage, where p53 activation may maintain the integrity of the transcribed genome to avoid loss of cell function (17). Presumably, death of differentiated cells occurs when DNA lesions exceed the capacity for repair. The
earliest proteins activated in proliferating cells in the DNA damage pathway are ataxia telangiectasia-mutated and ataxia telangiectasia Rad3-related kinase. Subsequent events in this pathway include activation of transducers, including BrcA, Chk-1 and Chk-2, and p53 (39). Apoptosis is one of the responses effected by these transducers. The DNA damage pathway has been demonstrated subsequent to DNA damage in skeletal muscle cells (17), but to our knowledge it has not been demonstrated in cardiac myocytes. Our data demonstrating myocyte DNA damage and p53 activation suggest that the DNA damage pathway is active in this cell type.

Anthracycline-induced DNA injury. Oxidative stress occurs rapidly after DOX treatment of cardiac myocytes (19), and oxidative stress is a known activator of p53 (36), possibly because of oxidative DNA damage. We show in this report that DNA damage is an early event after DOX treatment in cardiac myocytes that is reduced by inclusion of a free radical scavenger. This extends mechanistic observations in cancer cells showing that DOX causes site-specific DNA damage and subsequent generation of H₂O₂ mediated by NAD(P)H oxidase activation (25). However, it appears that DNA lesions in cardiac myocytes may not be exclusively oxidative, since we observed that purely oxidative DNA lesions induced by H₂O₂ were rapidly and completely repaired when the oxidant was removed, whereas the less extensive DOX-induced lesions were not. The alkaline comet assay detects multiple DNA lesions, including single- and double-stranded breaks and alkaline-labile sites. It is possible that the type of DNA lesion(s) induced by H₂O₂ is different and more readily repaired than lesions induced by DOX, a possibility that can be explored by performing the comet assay under neutral conditions, which only detects double-stranded DNA breaks (11). In a previous report, we demonstrated that overexpression of the α₄-isoform of the antioxidant glutathione transferase in cardiac myocytes eliminated DOX-induced oxidative stress and reduced, but did
not eliminate, DOX-induced total and apoptotic cell death (19).
Our previous data therefore are consistent with the current report that oxidative stress may not be the exclusive mediator of cardiac myocyte death after DOX treatment. However, DOX has been shown to influence DNA independent of generating oxidative lesions. DOX rapidly localizes to the nucleus in tumor cells, related to its high affinity for DNA. The drug intercalates rapidly in the DNA strand at specific sequences. Specific DNA-binding proteins, including transcription factors, may thereby be denied access to their usual binding sites by these adducts, potentially inhibiting transcription of specific genes (7).
Specific DNA lesions induced by AC include oxidized pyrimidines and 8-hydroxyguanine. These lesions occur in malignant cells after AC treatment (24) but have not been previously demonstrated in cardiac myocytes. AC treatment of lymphocytes induces DNA damage more rapidly, and repair is more rapid and complete (5) than we observed in cardiac myocytes, suggesting that the DNA damage response is cell type specific.

Therapeutic implications. Although oxidative stress is generally accepted as the mechanism by which AC cause toxicity to the heart, antioxidant therapy has not significantly reduced the magnitude of the clinical problem. For example, the iron chelator dexrazoxane reduces oxidative stress by reducing iron availability for the Fenton reaction, a generator of toxic oxygen-centered free radicals. This agent in clinical trials has reduced, but not eliminated, the DOX-induced decline in cardiac function (22). Moreover, antioxidants are not cardiac specific but rather reduce oxidative stress nonspecifically. This potentially reduces the desired effect of AC, since oxidative stress plays a role in the tumor-killing effect of these agents (14). Knowing that DNA injury occurs in myocytes treated with DOX and that elements of the DNA damage pathway are subsequently activated identifies additional steps that can be inhibited to reduce AC-induced cardiac myocyte death. Inhibition of p53 specifically in the heart, for example, may be a treatment to pursue, and this approach has been validated in animal studies (21). The observation that ΔΨ is lost after p53 activation in cardiac myocytes provides an additional target, since cyclosporine, diazoxide, and pinacidil prevent ΔΨ loss in response to cardiac myocyte oxidative stress (1). Agents that specifically act on cardiac myocyte mitochondria to preserve ΔΨ offer the potential to block the detrimental effects of AC while preserving antitumor activity, and such agents are currently under investigation (13).

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REFERENCES


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