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Differences in Doxorubicin-Induced Apoptotic Signaling in Adult and Immature Cardiomyocytes

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Abstract

A proposed mechanism for cardiotoxicity of doxorubicin (DOX) involves apoptosis in cardiomyocytes. Here we investigated the molecular basis for the differences in DOX-induced toxicity in adult rat cardiomyocytes (ARCM), neonatal rat cardiomyocytes (NRCM), and rat embryonic H9c2 cardiomyoblasts. Activation of caspase-9 and -3 was considerably lower in DOX-treated ARCM as compared to NRCM and H9c2 cardiomyoblasts. Addition of cytochrome *c* caused the activation of caspase-9 and -3 in permeabilized NRCM and H9c2 cardiomyoblasts but not in permeabilized ARCM. Expression of proapoptotic proteins, apoptotic protease activating factor-1 (Apaf1) and procaspase-9 was significantly lower, and abundance of antiapoptotic X-linked inhibitor of apoptosis protein (XIAP) was higher in ARCM, as compared to immature cardiac cells. Despite the abundance of XIAP in ARCM, its role in the inhibition of apoptosome function was dismissed, as second mitochondria-derived activator of caspases. (Smac)-N7 peptide, had no effect on caspase activation in response to cytochrome *c* in these cells. Adenoviral expression of Apaf1 exacerbated the activation of caspase-9 and -3 in DOX-treated NRCM, but did not increase their activities in DOX-treated ARCM. This finding points to a major difference in the apoptotic signaling between immature and adult cardiomyocytes. Mitochondrial apoptotic pathway is limited in ARCM treated with DOX.

Keywords

cardiomyocytes; apoptosis; doxorubicin; caspases; cytochrome *c*

Introduction

Doxorubicin (DOX) has been used in cancer treatment for nearly three decades (1,2). However, its clinical use became restricted because of the dose-dependent cardiomyopathy (3). Specifically, survivors of childhood cancer represent one of the largest new groups at risk of developing premature cardiovascular diseases (4). DOX causes dilated cardiomyopathy, cardiac muscle wasting, and congestive heart failure in animal models (5). Severe cardiac damage and mortality were detected in patients treated with DOX, suggesting that DOX was selectively more toxic to cardiac muscle cells, as compared to other cell

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types. Many mechanisms for DOX cardiotoxicity were proposed, including impaired Ca^{2+} fluxes, intracellular oxidant formation, suppression of cardiac specific gene expression, and myofibrillar and mitochondrial degeneration in cardiomyocytes (CM) (6-9). Recent *in vitro* and *in vivo* studies implicate DOX-induced myocardial apoptosis as a primary cause of cardiac damage (10-13).

The proapoptotic effects of DOX are well established. DOX causes DNA damage and induces the expression of the tumor suppressor protein, p53 (14). p53 upregulates the transcription of a number of proapoptotic genes, including BH3-only members of Bcl-2 family of proteins (15,16). BH3-only proteins initiate the release of cytochrome *c* and other proapoptotic proteins from mitochondria *via* a mechanism involving a complex interaction with other members of Bcl-2 family of proteins. Cytochrome *c* triggers the formation of apoptosome and activation of initiator caspase-9. Initiator caspases are activated in response to DOX in different cell types, and converge on the induction of the effector caspases responsible for the execution of programmed cell death (17-19). This apoptotic cascade triggered by DOX was identified mostly in tumor cells and in other actively dividing cells. The mechanism of DOX-induced apoptosis in terminally differentiated cardiomyocytes has not been studied in detail. The differences in apoptotic signaling between adult and immature cardiomyocytes are not known. As cardiomyocytes are the target cell type of DOX-induced myocardial toxicity, we assessed the activation of initiator caspases, along with the effector caspase-3-like activity, cytochrome *c* release, and expression of key proteins that regulate the activity of apoptosome in DOX-treated cardiac cells. Results indicate that adult rat cardiomyocytes (ARCM) are significantly more resistant than NRCM and the embryonic cardiomyoblasts to DOX-induced apoptosis. In this study we show that mitochondrial apoptotic pathway is more active in immature cardiac cells than in adult cardiac cells, thus contributing to DOX-induced injury in immature hearts. Furthermore, the contribution of cytochrome *c* -Apaf1-caspase-9 pathway to DOX cardiotoxicity may be limited in the adult myocardium.

Materials and Methods

Cardiomyocyte culture

ARCM were isolated from 36 male Sprague-Dawley rats which had been anesthetized with pentobarbital (100 mg/kg i.p.) as previously described (20), plated on laminin-coated culture dishes, and cultured in Medium 199 containing 10% FBS, 2 mg/ml bovine serum albumin, 10 $\mu\text{mol/L}$ antimetabolic agent cytosine- β -D-arabinofuranoside (Ara-C), 100 IU/ml penicillin, and 100 μg streptomycin. Primary cardiomyocytes were cultured for 48 h before the start of incubation with DOX. NRCM were isolated from 1-day old Sprague-Dawley pups as described (21). Briefly, neonatal hearts were digested at 37°C with collagenase type II and pancreatin (all from Invitrogen, Carlsbad, CA, USA). After preplating, to minimize non-myocyte contamination, cells were plated on tissue culture dishes precoated with fibronectin (Sigma Chemical Company, St. Louis, MO, USA). NRCM were cultured at 37°C and 5% CO_2 in the same culture medium (without Ara-C) as ARCM. Newly isolated NRCM were cultured for 48 h before starting experiments with DOX. H9c2 cardiomyoblasts derived from the heart of rat embryo (ATCC) were cultured in the same culture medium (without Ara-C) and used in experiments upon reaching subconfluency.

Apoptosome function and caspase activity

Experiments were performed on cardiomyocytes and H9c2 cardiomyoblasts permeabilized with saponin, which perforates the sarcolemma while leaving the mitochondria morphologically and functionally intact (22). Cells were permeabilized with 20 $\mu\text{g/ml}$ saponin (23) in the buffer containing 0.25 mol/L sucrose, 20 mmol/L HEPES, 10 mmol/L

MgSO₄, 3 mmol/L KH₂PO₄, 2 mmol/L DTT, 1 mmol/L ATP, and 1 mmol/L EDTA (permeabilizing buffer). We used 1 μmol/L cytochrome *c* and 25 μmol/L second mitochondria-derived activator of caspases (Smac)-N7 peptide to activate apoptosome in separate experimental sets. Smac-N7 peptide (H-AVPIAQK-OH) (Calbiochem, EMD Biochemicals, Gibbstown, NJ, USA) contains the N-terminal seven residues of Smac protein and binds to baculovirus inhibitor of apoptosis protein (IAP) repeat domains of XIAP, thus promoting the catalytic activity of caspase-9 (24). Permeabilized CM and H9c2 cardiomyoblasts were incubated at 37°C for 30 min, and activities of caspase-9 and -3 were measured as described below. In a separate set of experiments, we used cell-permeable Smac-N7 peptide (Smac-N7 peptide where lysine carboxy terminal is linked to the arginine of *Antennapedia* homeodomain peptide via the proline linker to make it cell-permeable) in intact (non-permeabilized) CM treated with DOX. Cell lysates were incubated with fluorimetric substrates Ac-DEVD-AMC (caspase-3 substrate) and Ac-LEHD-AFC (caspase-9 substrate). Fluorophores released from the substrate as a result of specific caspase activities were measured using a fluorescence plate reader (Perkin Elmer, Waltham, MA, USA). Liberated fluorescence was normalized to cell lysate protein measured using BCA protein assay (Pierce, Rockford, IL, USA).

Construction and use of Ad-rApaf1 adenovirus

Rat Apaf1 was cloned from the total RNA isolated from H9c2 cardiomyoblasts. Apaf1 cDNA was amplified using PCR with specific Apaf1 primers and subcloned into pGEMT vector (Supplementary Material). For making the adenovirus, Apaf1 cDNA was released from pGEMT vector by restriction enzymes and subcloned into pShuttle-CMV vector. Adenoviral construct was created and titered by Vector Biolabs, Inc. (Philadelphia, PA, USA). Ad-rApaf1 adenovirus expressed enhanced green fluorescent protein (eGFP), which was used for visualization of the expression of viral proteins in cardiac cells using fluorescent microscopy. NRCM and ARCM were transduced with Ad-rApaf1 adenovirus, and treated with DOX 48 h later. In separate experiments, we used Ad-eGFP adenovirus (supplied by Dr. Andrey Sorokin, Medical College of Wisconsin) for control experiments with NRCM and ARCM expressing eGFP only.

Cell fractionation and immunoblotting

For cytochrome *c* release studies, cells were fractionated using saponin. CM and H9c2 cardiomyoblasts were incubated for 15 min on ice in the permeabilizing buffer containing 20 μg/ml saponin. This procedure releases over 99% of cytosolic enzyme lactate dehydrogenase, yet, unlike mechanical disruption of cells, is gentle enough to maintain mitochondrial integrity (22). Released cytosol was collected after centrifugation (×1000g for 5 min at 4°C), and sedimented cells were used as a mitochondrial cytochrome *c* fraction. A similar protocol for separation of cytosolic and mitochondrial cytochrome *c* fractions in cardiomyocytes has been used before (25). Immunoblotting was performed as described earlier (12). Membranes were incubated with mouse monoclonal anti-XIAP and anti-cytochrome *c* antibodies (Pharmingen, BD Biosciences, Franklin Lakes, NJ, USA), rabbit polyclonal anti-Apaf1 and mouse monoclonal anti-caspase-9 antibodies (Cell Signaling Technology, Danvers, MA, USA), and anti-actin antibody (Chemicon International, Temecula, CA, USA).

Statistical analysis

All data are presented as mean±SEM. Data were subjected to one way ANOVA and Student's *t*-test by using SigmaStat software (version 2.03, SPSS, Inc., Chicago, IL, USA). Significance was accepted at the P<0.05 level. All experiments were performed in triplicate from three to four different primary cell preparations.

Results

Activation of caspases in cardiac cells by DOX

The activation profiles of caspase-9 and -3 was compared in neonatal and adult cardiomyocytes, and in H9c2 cardiomyoblasts incubated with DOX. Activation of caspase-9 was most rapid and pronounced in H9c2 cells (Fig. 1A) and was significantly delayed in ARCM; only a 2-fold activation of caspase-9 was detected after 48 h incubation with DOX in ARCM. Overall, the onset and magnitude of activation of caspase-9 decreased as follows: H9c2>NRCM>ARCM. Caspase-3 activity was significantly increased at 8 h in H9c2 cardiomyoblasts (Fig. 1B), and was delayed and less pronounced in ARCM. In addition, we observed that procaspase-9 expression was dramatically reduced in ARCM, as compared to NRCM and H9c2 cardiomyoblasts (Fig. 1C).

Release of cytochrome *c* and apoptosome activation

Cytochrome *c* released into the cytosol of H9c2 cardiomyoblasts was detectable after 16 to 24 h of incubation with DOX (Fig. 2A). Cytochrome *c* was elevated in cytosolic fractions after 8 h incubation in NRCM, and continued to increase up to 32 h of incubation with DOX. Concomitantly, the amount of cytochrome *c* decreased in the particulate fraction in NRCM (Fig. 2B). Increased release of cytochrome *c* was also detected in ARCM after an 8 h incubation with DOX. Longer incubation times further increased the accumulation of cytosolic cytochrome *c* in these cells. Despite the high levels of cytochrome *c* present in cytosolic fractions of DOX-treated ARCM, caspase-9 was not significantly activated in these cells, as described earlier. We then used a permeabilized cell model to assess the activation of caspase-9. Cytochrome *c* (in the presence of ATP) is known to activate caspase-9. As shown in Fig. 3, incubation of permeabilized H9c2 cardiomyoblasts with exogenous cytochrome *c* activated caspase-9 and -3. A similar response was observed when permeabilized NRCM were incubated with cytochrome *c* (Fig. 3B). In contrast, cytochrome *c* failed to activate caspase-9 and -3 in ARCM (Fig. 3C). Thus, caspase-9 was not responsive to cytochrome *c*, suggesting that the mitochondrial apoptotic pathway was not active in ARCM under these conditions.

Effect of Smac-N7 on apoptosome activity

XIAP is a known inhibitor of apoptosome function that binds to and inhibits the activation of caspase-9. We found that XIAP expression was significantly higher in ARCM than in NRCM and H9c2 cells (Fig. 4A). We then determined whether the upregulation of XIAP could blunt the activation of caspase-9 in ARCM. The Smac-N7 peptide was used to test this hypothesis. Smac-N7 mimics the effect of the full length Smac protein, a proapoptotic protein that is released from mitochondria, counteracting the inhibitory action of XIAP on caspase-9. Cytochrome *c* induced the activation of caspase-9 in permeabilized H9c2 cardiomyocytes that increased further upon adding Smac-N7 peptide (Fig. 4B). Caspase-3 activity also increased in these cells in the presence of cytochrome *c* and Smac-N7 peptide. Similarly, both cytochrome *c* and Smac-N7 peptide increased the activity of caspase-9 and -3 in permeabilized NRCM (Fig. 4C). In contrast to NRCM and H9c2 cells, the catalytic activity of caspases in ARCM did not increase in the presence of cytochrome *c* and Smac-N7 peptide. These results suggest that XIAP may not be responsible for inactive caspase-9 in ARCM. In addition, the cell-permeable Smac-N7 peptide neither activated caspase-9 and -3 in intact (non-permeabilized) ARCM, nor exacerbated their activities in DOX-treated ARCM (data not shown).

Apaf1 expression and activation of caspases by DOX

Next we investigated the expression of caspase-9 adaptor protein, Apaf1, which provides the scaffold for the assembly of apoptosome and the activation of caspase-9. Apaf1 protein levels were negligible in intact and DOX-treated ARCM, whereas the expression of this protein in NRCM and H9c2 cells was higher (Fig. 5A). We then examined whether diminished and delayed activity of caspase-9 in ARCM could be attributed to the low levels of Apaf1. Overexpression of Apaf1 protein using Ad-rApaf1 adenovirus caused increased activation of caspase-9 in DOX-treated NRCM (Fig. 5B). Caspase-3 activation was also exacerbated in control and DOX-treated NRCM. Treatment of NRCM with Ad-eGFP did not alter the activities of caspase-9 and -3, as compared to untreated adenovirus cells (data not shown). Overexpression of Apaf1 protein using Ad-rApaf1 adenovirus in DOX-treated ARCM did not increase the activity of caspase-9 (Fig. 5C). Similarly, caspase-3 activity was not affected by Apaf1 overexpression in DOX-treated ARCM. Thus, overexpression of Apaf1 upregulated the activation of caspase-9 in NRCM but not in ARCM. We have presented the results of Apaf1 overexpression in only NRCM and ARCM. These are primary cells with abundant cell surface receptors necessary for the transduction of cells with adenovirus. Therefore, we were able to use relatively low MOI (multiplicities of infection) to induce a robust expression of viral proteins (as evaluated by GFP expression after transduction with adenoviruses). On the other hand, H9c2 cardiomyoblast cells did not exhibit expression of GFP at MOIs similar to the ones used with primary cells. Transduction of H9c2 cells with increased MOIs caused changes in cell morphology and cell damage, making it virtually impossible to complete these experiments with adenoviral transduction of H9c2 cells.

Discussion

We report here that adult cardiomyocytes are more resistant to DOX-induced apoptosis than cardiomyoblasts and neonatal cardiomyocytes. Activation of initiator caspase-9, effector caspase-3, and cell death was delayed and significantly diminished in ARCM. Although cytochrome *c* was released into the cytosol of ARCM, the levels of expression of mitochondrial proapoptotic proteins caspase-9 and Apaf1 were significantly lower as compared to immature cardiac cells, thereby limiting the role of cytochrome *c* – Apaf-1 – caspase-9 pathway in DOX cardiotoxicity in the adult hearts. In contrast, the expression of XIAP (antiapoptotic protein) was much more abundant in adult cardiomyocytes, as compared to NRCM and cardiomyoblasts. Results described in this study have ramifications in DOX-mediated chronic cardiotoxicity in pediatric patients.

The activation of caspase-9 was detected even in Apaf1-null animals, as a result of the cleavage of caspase-9 by the effector caspase-3 (26). We also observed that the activation of caspase-3 in ARCM preceded the activation of caspase-9 (Suppl. Fig. 2). Failure to activate caspase-9 increased the resistance of tumor cells to cisplatin (27). On the other hand, the overexpression of Apaf1 and caspase-9 augmented etoposide-induced apoptosis in glioma cells (28). Regulation of apoptosome assembly and activation is a complex phenomenon (29). Other intracellular proteins such as XIAP, and Smac also contribute to this process (24,29). It is likely that adult cardiomyocytes, like other primary postmitotic cells, adult neurons and skeletal myotubes that lack Apaf1 expression, are more resistant to mitochondrially-driven apoptosis (30,31). In contrast, a recent study showed that Apaf1 present in abundance in neonatal cardiomyocytes interacts with caspase-9 (32). However, another study reported low Apaf1 expression in neonatal cardiomyocytes that were resistant to cytochrome *c*-induced apoptosis (33). In the present study, we have shown that levels of expression of Apaf1 in NRCM and H9c2 cardiomyoblasts are similar. In addition, caspase-9 was activated both by supplying cytochrome *c* to the permeabilized NRCM, and by overexpression of Apaf1 in DOX-treated NRCM. The enhanced expression of Apaf1

increased the sensitivity of NRCM to mitochondrially-induced apoptosis, as reported by others (33).

The H9c2 cardiomyoblasts derived from rat heart embryo can proliferate and differentiate into postmitotic myotubes (34,35). Progenitor cells are also present in adult hearts, as previously reported (36,37). They are able to divide and commit to myogenic lineage. In contrast to the adult postmitotic cardiomyocytes, cardiomyoblasts contain a large amount of the proapoptotic proteins, caspase-9 and Apaf1, and low levels of the antiapoptotic factor XIAP. As a result, the H9c2 cardiomyocytes are sensitive to DOX, displaying a significant activation of caspase-3 and -9. These data suggest that the myocardial progenitor cells could be a primary target of DOX toxicity in the heart. Because of enhanced sensitivity of dividing cardiomyoblasts to its proapoptotic action, DOX may also inhibit the regenerative capacity of the heart. By this mechanism, it is likely that the cardiotoxicity of DOX persists years after its initial use as a chemotherapeutic agent in pediatric patients. These patients are clearly at risk of developing premature cardiomyopathy.

Postmitotic adult cardiomyocytes are the most functionally significant cell type in the heart, and numerous studies have shown various defects induced by DOX in these cells. However, these cells appear to be resistant to DOX-induced apoptosis. Although adult cardiomyocytes showed abundant expression of antiapoptotic XIAP, functional inhibition of this protein using Smac-N7 peptide did not restore the activation of caspase-9 in permeabilized ARCM. In addition, adult cardiomyocytes have a negligible level of expression of Apaf1 protein, but overexpression of this protein in ARCM did not restore the activation of the mitochondrial apoptotic pathway. The present results point to a concerted downregulation of mitochondrial apoptotic pathway proteins in adult cardiomyocytes that makes them more resistant to proapoptotic influences as compared to neonatal rat cardiomyocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Apaf1	apoptotic protease activating factor-1
ARCM	adult rat cardiomyocytes
CM	cardiomyocytes
DOX	doxorubicin
IAP	inhibitor of apoptosis protein
NRCM	neonatal rat cardiomyocytes
Smac	second mitochondria-derived activator of caspases
XIAP	X-linked inhibitor of apoptosis protein

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Figure 1. Caspase-9 and caspase-3-like activities in cardiac cells treated with doxorubicin

Activities of caspases were assayed using specific fluorimetric substrates, as described in *Methods*. H9c2 cardiomyoblasts, NRCM, and ARCM cultures were incubated with 0.5 μ M DOX for 24 to 48 h. (a) Activity of caspase-9. (b) Caspase-3-like activity. (c) Expression of procaspase-9 in control and DOX-treated (0.5 μ M) cardiac cells for 24 h. Cell lysates were prepared and immunoblotted with anti-procaspase-9 and anti-actin antibodies. Densitometry graphs are shown for the original blot images.

Figure 2. Release of cytochrome *c* into cytosol in doxorubicin-treated cardiac cells

Cells treated with 0.5 μ M DOX for indicated periods of time were fractionated into cytosolic (cyto) and particulate mitochondrial (mito) fractions, as described in *Methods*. Cellular fractions were immunoblotted using anti-cytochrome *c* antibody. (a) H9c2 cardiomyoblasts. (b) NRCM and (c) ARCM. (d) The results of densitometric analysis of the release of cytochrome *c* after treatment of the cardiomyocytes with DOX (based on the data shown in Figure 2). For the cytosolic fractions the band intensities of the samples treated for 40 h (H9c2), 32 h (NRCM) or 48 h (ARCM) have been taken as 100%. For the mitochondrial fractions the band intensities of samples not treated with DOX (0 h) have been taken as 100%.

Figure 3. Caspase-9 and caspase-3-like activities in permeabilized cardiac cells in the presence of cytochrome *c*

Cardiac cells were permeabilized with saponin in permeabilizing buffer containing 1 mM ATP, and treated with 1 μ M cytochrome *c*. Activities of caspases were measured after the incubation of permeabilized cells for 30 min at 37°C using specific fluorimetric substrates. (a) H9c2 cardiomyoblasts, (b) NRCM, and (c) ARCM.

Figure 4. XIAP and caspase activation in permeabilized cardiac cells

(a) Expression of XIAP in control and DOX-treated (0.5 μ M) H9c2 cardiomyoblasts, NRCM, and ARCM. Equal amounts of cellular lysates were loaded onto the gel, and immunoblotted using anti-XIAP and anti-actin antibodies. H9c2 cells (b), NRCM (c), and ARCM (d) were permeabilized using saponin in permeabilizing buffer containing 1 mM ATP, and treated with 1 μ M cytochrome *c* and/or 25 μ M Smac-N7 peptide. Activities of caspase-9 and caspase-3 were assayed after the incubation of permeabilized cells for 30 min at 37°C using specific fluorimetric substrates, as described in *Methods*.

Figure 5. Expression of Apaf1 protein and caspase activation in cardiac cells

(a) Expression of Apaf1 in control and DOX-treated (0.5 μ M) H9c2 cardiomyoblasts, NRCM, and ARCM. Equal amounts of cellular lysate protein were loaded onto the gel and immunoblotted using anti-Apaf1 and anti-actin antibodies. NRCM (b) and ARCM (c) were transduced using Ad-rApaf1 adenovirus. Cells were treated with 0.5 μ M DOX 48 h after adenoviral transduction. Caspase-9 and caspase-3-like activities were assayed using specific fluorescent substrates as described in Methods. *, $p < 0.05$ as compared to untreated control; #, $p < 0.05$ as compared to DOX-treated cells.