REVIEW

Arch. Immunol. Ther. Exp., 2009, **57**, 435–445
PL ISSN 0004-069X
DOI 10.1007/s00005-009-0051-8

Cardiomyocyte death in doxorubicin-induced cardiotoxicity

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Received: 2009.03.16, **Accepted:** 2009.05.20, **Published online:** 2009.10.29

Abstract

Doxorubicin (DOX) is one of the most widely used and successful antitumor drugs, but its cumulative and dose-dependent cardiac toxicity has been a major concern of oncologists in cancer therapeutic practice for decades. With the increasing population of cancer survivors, there is a growing need to develop preventive strategies and effective therapies against DOX--induced cardiotoxicity, in particular late-onset cardiomyopathy. Although intensive investigations on DOX-induced cardiotoxicity have continued for decades, the underlying mechanisms responsible for DOX-induced cardiotoxicity have not been completely elucidated. A rapidly expanding body of evidence supports the notion that cardiomyocyte death by apoptosis and necrosis is a primary mechanism of DOX-induced cardiomyopathy and that other types of cell death, such as autophagy and senescence/aging, may participate in this process. This review focuses on the current understanding of the molecular mechanisms underlying DOX-induced cardiomyocyte death, including the major primary mechanism of excess production of reactive oxygen species (ROS) and other recently discovered ROS-independent mechanisms. The different sensitivities to DOX-induced cell death signals between adult and young cardiomyocytes will also be discussed.

Key words: cardiomyocyte, doxorubicin, apoptosis, necrosis, autophagy.

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INTRODUCTION

The anthracyclines, primarily doxorubicin (DOX) but also including daunomycin, epirubicin, and idarubicin, are among the most widely used and successful antitumor drugs. Cardiotoxicity is a major limiting factor in anticancer therapy (Singal and Iliskovic 1998; Yeh et al. 2004). DOX-induced cardiotoxicity may present as either acute or chronic cardiomyopathy. Acute cardiotoxicity is now rare, occurring after receiving a high dose, and may present as acute tachyarrhythmia and acute heart failure, while chronic DOX-induced cardiac toxicity is dose dependent. In this case, the patient may develop dilated cardiomyopathy many years after receiving the last DOX treatment. Both acute and chronic DOX-induced cardiac toxicity may lead to cardiac dysfunction, cardiomyopathy, and eventually to severe heart failure and death (Wallace 2003; Yeh et al. 2004). Children and adolescents are particularly susceptible to the cardiotoxic effects of anthracycline chemotherapy and there is no safe dose of anthracyclines for this population (Lipshultz et al. 1991; Von Hoff et al. 1977). About half of the young adult survivors of childhood cancer have received anthracyclines at some point in their treatment. Hence the development of novel therapeutic strategies to improve survivor outcome is important, particularly in children, as a large number of pediatric cancer survivors are now expected to live in a cancer-free status for decades after cancer therapy.

Intensive investigations on DOX-induced cardiotoxicity have been continuing for decades. The different lines of evidence have provided putative mechanisms, but the precise mechanism underlying DOX-induced cardiotoxicity is not completely elucidated. Most studies favor free radical-induced oxidative stress playing a piv-

otal role, as it can be interpreted by the chemical structure of DOX, which possesses a tendency to generate reactive oxygen species (ROS) during drug metabolism (Iarussi et al. 2001; Neilan et al. 2007; Wallace 2003). Recent findings indicate that the endothelial nitric oxide synthase reductase domain converts DOX to an unstable semiquinone intermediate that favors ROS generation (Neilan et al. 2007). Also, mitochondrial DNA lesions induced by ROS or directly by DOX further lead to respiratory chain failure and ROS liberation (Lebrecht and Walker 2007). Other contributors to DOX-induced cardiotoxicity include dysregulation of calcium handling, adrenergic dysfunction, and selective inhibition of cardiomyocyte-specific genes expression (Iarussi et al. 2001; Takemura and Fujiwara 2007). Most of these cellular events contribute to cardiomyocyte death, which is a primary mechanism for DOX-induced cardiomyopathy. Cell death is classified by the morphology of the affected cells as apoptosis, necrosis, and autophagy. Although most *in vitro* and *in vivo* studies during the past several decades have suggested that DOX-induced cardiac toxicity is associated with cardiomyocyte apoptosis and necrosis, other forms of cell death can also be related. In this review we focus on recent new findings on the possible mechanisms underlying DOX-induced cardiomyocyte death (Fig. 1). Differences in sensitivity to DOX-induced cell death signals between adult and young cardiomyocytes will also be discussed.

APOPTOSIS

Apoptosis is a highly conserved, tightly regulated, and energy-dependent active form of cell death. It is crucial for normal development and cell homeostasis. The typical morphological changes are cell shrinkage, DNA fragmentation, chromatin condensation, and packaging of the cell into a form called an "apoptotic body" that allows its removal by phagocytosis. Apoptosis starts from two canonical signaling pathways, the extrinsic and intrinsic pathways. In the extrinsic pathway, the binding of death ligands (FasL, TNF-α, TRAIL) with their receptors induces recruitment and activation of caspase 8, which subsequently activates downstream effector caspases such as caspase 3. The intrinsic pathway is mediated by mitochondrial cytochrome *c* release. This process is regulated by the members of the Bcl-2 family, which includes three groups: the anti-apoptotic members Bcl-2, Bcl-X_L, and Mcl-1, the pro-apoptotic members Bax and Bak, and the BH3-only proteins such as Bad, Bid, Nix, and BNip3 that enhance apoptosis via inhibition of anti-apoptotic Bcl-2 proteins or activation of pro-apoptotic Bax and Bak (Shi and Wei 2007). Activation of BH3-only proteins by stress stimuli promotes Bax/Bak translocation from the cytosol to the outer membrane of mitochondria, resulting in increased mitochondrial outer membrane permeabilization leading to protein release from the intermembrane space to the cytoplasm, including apoptogenic molecule cytochrome *c*. In the cytosol, cytochrome *c* forms a complex with the adaptor protein apoptosis protease activator protein-1 (Apaf-1), dATP, and caspase 9. The result is the formation of a structure known as the apoptosome, which in turn activates caspase 9. Both the extrinsic and intrinsic apoptotic pathways converge on the activation of the downstream executioners caspases 3, 6, and 7.

These two apoptotic signaling pathways are evolutionally conserved, but the precise molecular events involved in the regulation of caspase enzymatic cascades are often specific to cell type and death stimulus. The

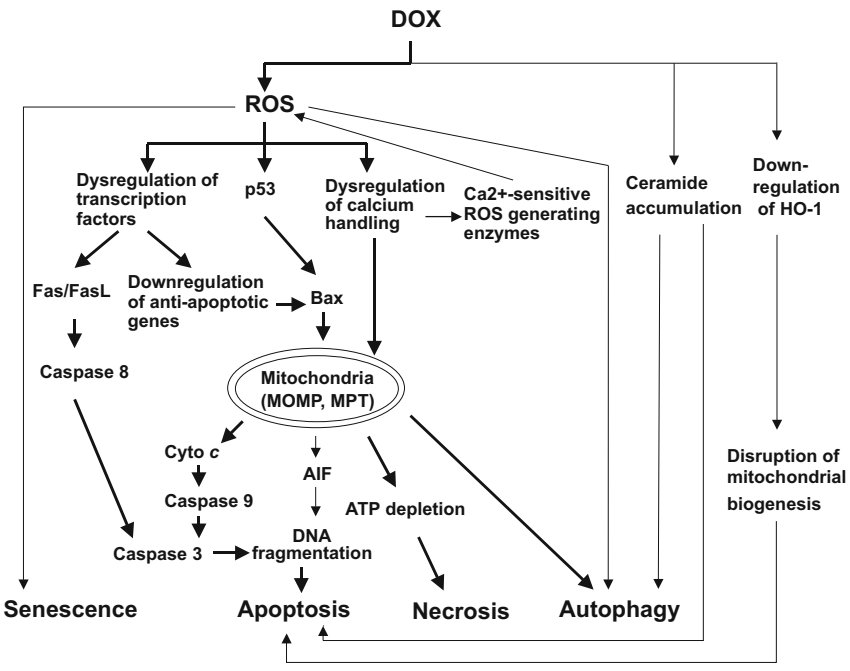

Fig. 1. Potential signaling pathways involved in DOX-induced cardiomyocyte death, as described in the text. Mechanisms of cell death include apoptosis, necrosis, autophagy, and senescence. Crosstalk between these different types of cardiomyocyte death may occur at multiple levels. Thick lines represent major mechanisms and thin lines represent alternative pathways.

Table 1. Potential mechanisms and therapeutic targets involved in DOX-induced cardiomyocyte apoptosis

Mechanisms	Cell type/animal	References
Increase in intracellular Ca^{2+}, mitochondrial dysfunction	Adult rat cardiomyocytes, mouse, rat	Childs et al. 2002; Kim et al. 2006; Kluza et al. 2004
Dysregulation of apoptosis-related proteins: p53, Akt, ERKs, Bcl-2 family, etc	H9c2, mouse, rat cardiomyocytes	Fan et al. 2008; Kawamura et al. 2004; Liu et al. 2008; Zhu et al. 2009
Dysregulation of transcription factors/coactivators: GATA-4, CARP, NF-κB, NFAT, p300, etc	H9c2, mouse, rat, neonatal rat cardiomyocytes	Aihara et al. 2000; Aries et al. 2004; Jeyaseelan et al. 1997; Kalivendi et al. 2005; Kim et al. 2003; Kim et al. 2007; Li et al. 2007; Wang et al. 2002
Caspase 12 mediated SR-apoptotic pathway	Rat	Jang et al. 2004
Heme oxygenase-1 down-regulation	H9c2, mouse	Bernuzzi et al. 2009; Piantadosi et al. 2008; Suliman et al. 2007
Ceramide accumulation	Neonatal rat cardiomyocytes	Armstrong 2004; Parra et al. 2008
Reduced ARC expression	Neonatal rat cardiomyocytes	An et al. 2009
TLR-2	Mouse	Nozaki et al. 2004
eNOS (NOS3)	Mouse	Neilan et al. 2007

eNOS: endothelial nitric oxide synthase

mechanism of DOX-induced cardiomyocyte apoptosis has been extensively studied in both acute and chronic cardiotoxicity (Arola et al. 2000; Bennink et al. 2004; Fisher et al. 2005; Kawamura et al. 2004; Kotamraju et al. 2000; Wang et al. 2001). These studies have shown that multiple pathways are involved (Table 1). It is worth noting that the studies of DOX-induced cardiotoxicity utilized a wide variety of treatments (e.g. single vs. multiple doses, differences in total dose, differences in timing of assays). The underlying mechanisms reported in these studies may vary in experimental conditions, species differences, *in vitro* versus *in vivo* studies, and so on.

Intrinsic apoptotic pathway

DOX treatment increases oxidative stress and disrupts cytosolic calcium homeostasis. ROS increases intracellular calcium levels by promoting the release of calcium from the sarcoplasmic reticulum (SR) via opening of the ryanodine receptor and by impairing calcium clearance systems in cardiomyocytes (Camello-Almaraz et al. 2006; Gen et al. 2001; Kim et al. 2006; Zeng et al. 2008; Zima and Blatter 2006). The increased intracellular calcium in turn induces ROS production through calcium-sensitive ROS-generating enzymes (Kim et al. 2006). In cardiomyocytes, the mitochondria are located near calcium-release sites on the SR and can capture a large quantity of the released calcium. Due to the significantly raised oxidative stress, mitochondrial calcium level increases beyond a threshold. This mitochondrial calcium overload triggers mitochondrial permeability transition (MPT), resulting in a loss of mitochondrial membrane potential, mitochondrial swelling, outer-membrane rupture, and, consequently, the release of cytochrome *c* and apoptosis inducing factor from mitochondria (Camello-Almaraz et al. 2006; Childs et al. 2002; Deniaud et al. 2008).

Numerous studies have shown that DOX-induced cardiomyocyte apoptosis is associated with increased expression and activation of p53 tumor suppressor protein (L'Ecuyer et al. 2006; Liu et al. 2004; Liu et al. 2008). DNA lesions induced by ROS or directly by DOX activated ERK1/2, followed by increased phosphorylation of p53, the latter further up-regulating p53-downstream genes such as Bax. As a result, the intrinsic apoptosis pathway was activated. Pifithrin-α, an inhibitor of p53, did attenuate the increased protein levels of Bax and effectively inhibited DOX-induced apoptosis in H9c2 cells, neonatal rat cardiomyocytes, and mouse hearts (Liu et al. 2004; Liu et al. 2008). Inhibition of DOX-induced cardiomyocyte apoptosis was also observed in p53-knockout mice (Shizukuda et al. 2005) and in adult mouse hearts expressing cardiomyocyte-restricted dominant-interfering p53 (Zhu et al. 2009). p53 may also mediate DOX-induced cardiotoxicity through other pathways independent of cardiomyocyte apoptosis. For example, p53-mediated inhibition of mammalian target of rapamycin signaling may contribute to the cardiac mass reduction and dysfunction observed in acute DOX cardiotoxicity (Zhu et al. 2009).

In cardiomyocytes, transcriptional factor GATA-4, a critical regulator in heart development, has been shown to be a pivotal survival factor for the postnatal period. GATA-4 transcriptionally regulates the apoptotic pathway via activating the anti-apoptotic gene Bcl-X_L, thus preserving mitochondrial function and integrity. An early event observed in DOX cardiotoxicity was GATA-4 depletion, which sequentially caused cardiomyocyte apoptosis (Aries et al. 2004; Kim et al.

2003). DOX-induced inhibition of Akt phosphorylation was suggested to be involved in the underlying mechanism, which increased active GSK3β, a negative regulator of GATA-4 in the nucleus (Suliman et al. 2007). Moreover, cardiac p300 mRNA, a transcriptional coactivator required for the maintenance of the differentiated phenotype of cardiac myocytes, was depleted in mouse hearts after DOX treatment, but the overexpression of p300 protein in cardiomyocytes could prevent DOX-induced apoptosis and cardiac dysfunction. It was believed to be due to the up-regulation of Bcl-2 and Mdm2 (Kawamura et al. 2004). Other studies reported that DOX caused p38 MAPK activation and p300 degradation via hyperphosphorylation (Lou et al. 2005; Poizat et al. 2005).

The apoptosis repressor with a caspase recruitment domain (ARC) is an endogenous inhibitor of apoptosis and is restricted primarily to terminally differentiated cells such as skeletal myocytes, neurons, and cardiomyocytes. It disables apoptotic pathways through preventing Bax translocation to the mitochondrion or binding to components of the extrinsic pathway such as Fas, FADD, and caspase 8 to prevent the formation of death-inducing signaling complex (Mercier et al. 2005). Down-regulation of ARC mRNA and protein expression levels was observed in neonatal rat cardiomyocytes and mouse hearts upon DOX exposure. p53-dependent transcriptional down-regulation or p53-induced ubiquitin E3 ligase Mdm2 may be linked to the ARC decrease. In contrast, enforced ARC expression markedly attenuated DOX-induced cardiomyocyte apoptosis and prevented the activation of the mitochondrial death pathway and subsequent cardiomyocyte death (An et al. 2009).

In addition, a recent study has shown that DOX--induced up-regulation of Ser/Thr phosphatase PP1 may be involved in the dephosphorylation of Akt and Bad, resulting in caspase 3 activation (Fan et al. 2008). DOX--induced ceramide generation may also contribute to cardiomyocyte apoptosis through mitochondrial fragmentation, mitochondrial outer-membrane permeabilization, and cytochrome *c* release (Armstrong 2004; Parra et al. 2008).

Extrinsic apoptotic pathway

Although cardiomyocytes are usually resistant to Fas-induced apoptosis, studies indicate that cardiomyocyte apoptosis in DOX-induced cardiomyopathy can be executed through a Fas-mediated pathway (Nakamura et al. 2000). Cardiac-targeted expression of soluble Fas, a competitive inhibitor of FasL, could attenuate DOX-induced cardiotoxicity partly by inhibiting cardiomyocyte apoptosis and reducing ROS and peroxynitrite formation in mice (Niu et al. 2009). Other studies showed that DOX treatment of rat cardiomyocytes increased mitochondrial ROS production, activated the calcium/calcineurin signaling pathway, and further activated nuclear factor-activated T cell 4 (NFAT4), leading to up-regulation of Fas/FasL (Kalivendi et al. 2005). Interestingly, NFAT5, a novel member of NFAT family, was degraded by proteolysis in cultured rat neonatal cardiomyocytes after DOX exposure. As the result, the neonatal cardiomyocytes became more susceptible to cell damage (Ito et al. 2007). Transcription factor NF-κB was activated by ROS in DOX-treated neonatal rat cardiomyocytes and myocardium and exerted a pro-apoptotic effect via direct activation of apoptotic genes, including FasL, Fas, c-Myc, and p53 (Kim et al. 2007; Li et al. 2007; Wang et al. 2002). ROS down-regulated the expression of FLIP, a FLICE/caspase 8 inhibitory protein, and thereby, at least in part, sensitized Fas-mediated apoptosis (Nitobe et al. 2003). In addition, an innate immune system member has been implicated in the regulation of apoptotic pathway. Studies reported that Toll-like receptor-2 (TLR-2) functions as a novel "death receptor" that employs the apoptotic apparatus such as FADD and caspase 8 without a conventional cytoplasmic death domain (Aliprantis et al. 2000). In one study, fewer TUNEL-positive nuclei and less caspase 3 activity in myocardium were observed in TLR-2-knockout mice than that in wild-type mice after DOX treatment. This could partly involve the inhibition of NF-κB activation and reduction of proinflammatory cytokines (e.g. TNF-α) in TLR-2-knockout mice (Nozaki et al. 2004).

Other mechanisms

The endoplasmic/SR (ER/SR)-mediated apoptotic pathway was reported to mediate cardiac apoptosis induced by DOX (Jang et al. 2004). Caspase 12, an essential caspase to initiate SR-mediated apoptosis and located in the SR, was activated by calpain in DOX-treated rat hearts. As shown by recent studies, heme oxygenase-1 (HO-1) expression was down-modulated in H9c2 cells exposed to DOX (Bernuzzi et al. 2009), and other *in vivo* studies indicate that the HO-1/Akt/Nrf2 pathway mediates cardiac mitochondrial biogenesis and that down-regulation of HO-1 by DOX disrupts cardiac mitochondrial biogenesis, which promotes intrinsic apoptosis (Piantadosi et al. 2008; Suliman et al. 2007). Other potential mechanisms involved in DOX-induced cardiomyocyte apoptosis include: dysregulaiton of a phosphodiesterase 3A/inducible cAMP early repressor feedback loop (Yan et al. 2007), activation of the endocannabinoid system (Mukhopadhyay et al. 2007), activation of volume-sensitive chloride channels and subsequent apoptotic volume decrease (D'Anglemont de Tassigny et al. 2004), and oxidative stress-induced up-regulation of lectin-like oxidized LDL receptor-1 (Spallarossa et al. 2005).

NECROSIS

Necrosis is typically described as early rupture of the plasma membrane and swelling of cytoplasmic

organelles, in particular the mitochondria, and is often described as an uncontrolled, energy-independent process. However, recent studies have shown that necrotic cell death can be well controlled and programmed (Diwan et al. 2009; Dorn 2009). Numerous studies showed that cardiac expression of proinflammatory cytokine, inflammatory cell infiltration, and necrosis are increased in DOX-treated mouse hearts (Ikegami et al. 2007; Li et al. 2006; Riad et al. 2009). Oxidative stress is implicated in necrotic cardiomyocyte death. The use of free radical scavengers protected cardiomyocytes from anthracycline-induced necrosis (Ikegami et al. 2007). The rationale is that increased ROS leads to mitochondrial calcium overloading, promotes MPT pore opening, causes mitochondrial swelling and ATP depletion, and hence triggers necrotic cell death (Dorn 2009; Gustafsson and Gottlieb 2008). For that reason, disturbance of mitochondrial calcium homeostasis may exert a critical factor in the accumulative and irreversible cardiomyopathy associated with long-term DOX treatment. DOX also induces mitochondrial DNA damage, mitochondrial respiration mutilation, mitochondrial dysfunction, and ATP depletion. All these events contribute to necrosis (Lebrecht and Walker 2007; Solem et al. 1996; Wallace 2003; Wallace 2007; Zhou et al. 2001). In addition, ROS--induced lipid peroxidation may also contribute to cardiomyocyte necrosis (Casey et al. 2007). Furthermore, degradation of titin, the largest myofilament protein, was enhanced in the early stage of DOX treatment by activation of the calcium-dependent proteases calpains, which may represent an important proximal step that leads to accelerated myofilament degradation and necrosis (Lim et al. 2004).

AUTOPHAGY

Autophagy, first described in the 1960s in mammalian cells, is a highly regulated dynamic process involving cytosolic proteins and organelle degradation through engulfment into double-membraned vesicles called autophagosomes, which then fuse with lysosomes and subsequently degrade the contents. Autophagy plays important roles in cell growth and development, organelle biogenesis and turnover, and controlling the precise balance between protein synthesis and degradation. Many excellent reviews have discussed some aspects of the molecular mechanism of autophagy (Rubinsztein et al. 2005; Schmid and Munz 2007; Tsujimoto and Shimizu 2005; Yorimitsu and Klionsky 2005). Autophagy normally occurs in the myocardium, represents the most prevalent renewal mechanism of cellular constituents, and is substantially enhanced under pathological conditions, including cardiac hypertrophy, cardiomyopathy, and heart failure. Studies indicate that autophagy serves as a double-edged sword in the heart under stress; on one hand, it functions by removing protein aggregates and damaged organelles as a pro-survival pathway maintaining energy homeostasis, while on the other, intense enhancement of autophagy can lead to cell death (De Meyer and Martinet 2008; Gustafsson and Gottlieb 2009; Matsui et al. 2008; Rothermel and Hill 2008; Shimomura et al. 2001; Terman and Brunk 2005).

Crosstalk among the autophagic, apoptotic, and necrotic pathways has been frequently reported. Bcl-2 family has been implicated in the crosstalk between apoptosis and autophagy (Hoyer-Hansen et al. 2007; Levine et al. 2008; Maiuri et al. 2007; Nishida et al. 2008; Shimizu et al. 2004; Tsujimoto and Shimizu 2005). ROS-induced increase in intracellular calcium not only triggers apoptosis and necrosis, but also induces autophagy by activation of calmodulin-dependent kinase kinase and AMP-activated protein kinase (Hoyer-Hansen et al. 2007). Other apoptosis-related proteins such as p53 have also been shown to play a role in autophagy (Maiuri et al. 2007). Another recent study suggests that activation of poly(ADP-ribose) polymerase 1, one of the major nuclear targets of caspases, is involved in autophagy which might be cytoprotective during the response to DNA damage (Munoz-Gamez et al. 2009).

Mitochondria function as the crossroads for the autophagic, apoptotic, and necrotic pathways. Under conditions with mild stress, autophagy is induced to degrade and recycle cytoplasmic components. With increasing stresses, apoptosis begins to occur because of cytochrome *c* release from mitochondria. Under extreme stress, the MPT occurs in all mitochondria, the intracellular supply of ATP is exhausted, and necrosis occurs because of ATP depletion. Excessive autophagy induced by severe stimuli can also damage cytosol and organelles, especially mitochondria and ER, and release lysosomal enzymes or other cell death-inducing factors, thereby leading to apoptotic and necrotic cell death (Nishida et al. 2008; Nishida et al. 2009).

The three types of cell death may converge in dying cells on many levels of different pathways, such as oxidative stress, dysregulation of calcium homeostasis, mitochondrial damage, DNA damage, and induction of pro--apoptotic proteins. All of these occur in DOX-induced cardiotoxicity. Although no evidence of autophagy in DOX-induced cardiotoxocity has been reported so far, it is possible that DOX is able to induce cardiomyocyte autophagy, which might be protective or detrimental depending on the stress levels, in particular the dosage of DOX.

SENESCENCE (AGING)

Senescence, which is characterized by progressive accumulation of macromolecular damage, growth arrest of normal somatic cells, and reduction in function, mainly affects long-lived postmitotic cells such as neurons and cardiac myocytes. It is a risk factor for cardiac dysfunction and heart diseases (Terman et al. 2006).

The molecular and cellular pathways controlling senescence include telomere shortening, accumulation of DNA and chromosomal damage as well as the expression of the cell cycle inhibitors p16INK4a and p53 (Bergmann et al. 2008; Kajstura et al. 2006). The known factors involved in the senescence of cardiomyocytes include oxidative stress, altered gene expression/mutations, inflammation, reduced cellular protection and repair, altered cellular metabolism, altered protein degradation machinery and autophagy machinery, and others (Bernhard and Laufer 2008). Cardiomyocyte senescence may play a role in DOX-induced latent myocardial toxicity many years after the last treatment. A recent study showed that cultured neonatal rat cardiomyocytes treated with DOX exhibited characteristic changes similar to the cardiomyocytes of aged rats. These changes included increased positive staining for senescence-associated β-galactosidase and cell cycle inhibitor expression, and decreased cardiac troponin I phosphorylation and telomerase activity. Oxidative stress and p53 acetylation might be involved in this process (Maejima et al. 2008).

DIFFERENT SENSITIVITY TO DOX-INDUCED CARDIOTOXICITY IN YOUNG, ADULT, AND OLD HEARTS

As suggested by clinical studies, children and adolescents are particularly susceptible to the cardiotoxic effects of anthracycline chemotherapy (Von Hoff et al. 1977). The possible rationale was due to the loss of myocytes and impaired cardiac growth resulting in inadequate left ventricular mass and cardiomyopathy a year or more after cessation of chemotherapy (Lipshultz et al. 1991). Cardiomyocyte atrophy and myofiber disarray may also contribute to the cardiac dysfunction observed in DOX-treated juvenile mice (Zhu et al. 2008). Another effect of age is the alteration of DOX pharmacokinetics in the old age group, and the effect was particularly evident in the heart (Cusack et al. 2003). A study showed that age was highly correlated with drug distribution clearance. A reduction of the distribution clearance in heart tissue contributed to DOX-induced cardiotoxicity and was attributed to the decline in regional blood flow with age (Li and Gwilt 2003).

The alteration of cardiac transcriptional activities in response to DOX may contribute to more severe DOX cardiotoxicity in neonatal hearts, as some cardiac transcriptional factors may present at a higher level or a higher degree of sensitivity to DOX in neonatal hearts than in adult hearts (Aihara et al. 2000; Jeyaseelan et al. 1997). Cardiac ankyrin repeat protein (CARP) is present at the earliest stages of cardiac morphogenesis and gradually decreases from neonatal to adult hearts. It may function as a transcriptional regulator of cardiac muscle genes which are important for the growth and/or morphogenesis of myocardium (Aihara et al. 2000; Jeyaseelan et al. 1997; Zou et al. 1997). The early, rapid, and high repression of CARP gene transcription by DOX was observed in neonatal hearts, and the underlying mechanism may involve oxidative stress and subsequent activation of H7-sensitive serine/threonine kinase (Aihara et al. 2000).

The different susceptibilities to DOX may also be due to differences in expression levels of apoptotic signaling molecules between children and adult hearts. Some studies have suggested decreases in apoptotic potential in postmitotic cells such as skeletal muscle cells (Burgess et al. 1999), neuronal cells (Yakovlev et al. 2001), as well as cardiomyocytes (Sanchis et al. 2003). Reduced expression levels of Apaf-1, caspases, and some pro-apoptotic members of the Bcl-2 family may contribute to the reduced apoptotic potential in postmitotic cells (Bahi et al. 2006; Burgess et al. 1999; Sanchis et al. 2003; Yakovlev et al. 2001). Recent *in vitro* studies further support the idea that different pathways may be involved in DOX-induced cell death in adult and immature cardiomyocytes (Bahi et al. 2006; Konorev et al. 2008). The intrinsic apoptotic pathway was more active in immature cardiac cells compared with adult cardiac cells, which may explain why the higher sensitivity to DOX-induced injury is seen in immature hearts. Another *in vivo* study also described a down-regulation of intrinsic apoptotic pathway-related proteins in mouse brain, skeletal muscle, and heart from neonate to adult. The expression levels of Bim, Apaf-1, and caspase 3 were dramatically decreased during postnatal development in the brain, skeletal muscle, and heart, which is consistent with the observation that the TUNEL-positive cells presented a significant reduction in adult brain, skeletal muscle, and heart compared with the neonate (Madden et al. 2007).

It is worth noting that the majority of *in vivo* DOX studies with animals heavily rely on acute or chronic drug administration in young adults (including mouse and rat between the ages of 5–22 weeks) but not in neonates. Given the unique properties of cardiomyocytes during postnatal development, it is therefore important to understand the molecular events involved in cardiomyocyte apoptosis in this age group.

POTENTIAL STRATEGIES TO REDUCE DOX-INDUCED CARDIOMYOCYTE DEATH

As the generation of ROS has been considered a primary mechanism of DOX-induced cardiotoxicity, clinical approaches designed to attenuate DOX-induced cardiotoxicity consist of antioxidants, iron chelators, and free radical scavengers (Table 2). Carvedilol, an adrenergic blocking agent with potent antioxidant activity, has been found to be protective against DOX-induced ROS generation and apoptosis (Armstrong 2004; Machado et al. 2008; Spallarossa et al. 2004). Dexrazoxane, the only cardioprotective drug currently available clinically, is an intracellular iron chelator which has been proved to protect myocardial mitochondria from genetic and func-

Table 2. Potential strategies to reduce DOX-induced cardiomyopathy

Category	Molecules	References
Antioxidants and free radical scavengers	Dexrazoxane (only cardioprotective drug currently available clinically)	Hensley et al. 2009; Lebrecht et al. 2007b
	Carvedilol (clinically approved drug)	Armstrong 2004; Machado et al. 2008; Spallarossa et al. 2004
	Melatonin	Liu et al. 2002
	C-phycocyanin	Khan et al. 2006
	Rosmarinic acid	Kim et al. 2005
	Flavonoid	Bast et al. 2007; Bruynzeel et al. 2007
	Resveratrol (RVT)	Tatlidede et al. 2009
	Statin	Riad et al. 2009
Inhibition of increased $[Ca^{2+}]$	Carnitine	Mijares and Lopez 2001
Inhibitors of ceramide production	Carnitine	Armstrong 2004
Inhibitors of NF-κB	Plantainoside D	Kim et al. 2007
	Pyrrolidine dithiocarbamate	Li et al. 2007
Inhibitors of p53	Pifithrin-α	Chua et al. 2006; Liu et al. 2004
Preservation of p-Akt	Heat shock protein 20	Fan et al. 2008
Modified DOX	Liposomal doxorubicin (DaunoXome, etc)	Rigacci et al. 2007; Yildirim et al. 2008
Prodrugs and derivatives of DOX	Doxazolidine Carbamates, DOXO-EMCH, etc	Burkhart et al. 2006; Kratz et al. 2007; Lebrecht et al. 2007a
Others	Thrombopoietin	Li et al. 2006
	SNAP (exogenous NO donor)	Maejima et al. 2005

tional lesions induced by DOX via removing iron from its complex with DOX and thereby reducing the formation of hydroxyl radicals and superoxide (Armstrong 2004; Lebrecht et al. 2007b). However, the effect of oxidative stress in clinical cardiotoxicity is increasingly questioned. Application with antioxidants, such as vitamin E and N-acetylcysteine, did not provide visible protection in long-term experimental and clinical trials (Gianni et al. 2008).

Other potential approaches to increasing tumor response and decreasing cardiotoxicity include application of liposomal anthracyclines, prodrugs, and derivatives of DOX (Burkhart et al. 2006; Kratz et al. 2007; Lebrecht et al. 2007a; Rigacci et al. 2007; Yildirim et al. 2008). According to a recent study, another promising method is to find candidates with the ability to form diffusible metabolites that eliminate excess anthracycline and prevent accumulation in the heart (Salvatorelli et al. 2009). Results from basic research have provided an increasing number of potential therapeutic targets for the development of new strategies of cardioprotection against DOX-induced cardiotoxicity (Tables 1 and 2). The research in our laboratory has indicated that ROCK1 (Rho-associated coiled-coil containing protein kinase-1) is a key mediator of cardiomyocyte apoptosis (Chang et al. 2006; Shi and Wei 2007). ROCK1 may also mediate DOX-induced cardiotoxicity in adult mouse hearts (Shi et al., unpublished observations). Continuous efforts in elucidating the pathogenic mechanisms, as well as identifying new therapeutic targets, will certainly be helpful for the development of more effective therapies.

CONCLUSIONS AND FUTURE DIRECTIONS

Cardiac dysfunction is the most severe side effect of DOX treatment. Considerable data indicate that cardiomyocyte death through apoptosis, necrosis, and other forms is a primary contributor to the progression of DOX-induced cardiomyopathy. Excessive oxidative stress, damage to nuclear DNA, changes in calcium handling and cellular contractility, suppression of transcription factors that regulate cell survival and sarcomere protein synthesis, and disruption of sarcomere stability are identified as contributors to the mechanisms of cardiomyocyte death. Numerous studies evaluating DOX--induced cardiomyocyte death were performed *in vitro* or *in vivo* with a time window of hours or days after exposure to DOX at high concentrations. Future studies using long-term animal models should be performed to evaluate the contribution of different types of cardiomyocyte death to the chronic and delayed DOX-induced cardiotoxicity associated with clinically relevant doses of the drugs. In addition, in order to draw a comprehensive picture for DOX-induced cardiotoxicity, more information is needed to compare the relative importance of each cell death type and other mechanisms of car-

diomyocyte injury, particularly how these different mechanisms interact during the development of cardiomyopathy.

As mentioned above, the mechanisms of late-onset anthracycline cardiac toxicity in children remain underexplored. Postnatal hearts contain pluripotent stem cells which are capable of giving rise to functional cardiomyocytes (Davani et al. 2005). Like other undifferentiated cells, cardiac stem cells could be more sensitive to DOX and their death will limit the regenerative capacity of heart. It is possible that DOX-induced loss of cardiomyocytes together with early damage to cardiac stem cells in pediatric patients can cause permanent cardiotoxicity among those long-term cancer survivors. Future research will continually validate the essential mechanisms and develop therapeutic strategies to prevent premature cardiomyocyte death in pediatric patients who need anthracycline treatment.

Acknowledgment: This work was supported by National Institutes of Health grants (HL072897 and HL085098 (project 2) to L. Wei) and the Showalter Trust (89829 to J. Shi).

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