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Novel Mechanisms of Anthracycline-Induced Cardiovascular Toxicity: A Focus on Thrombosis, Cardiac Atrophy, and Programmed Cell Death

Silvio Antoniak[1,2*†], *Sukanya Phungphong*[3†], *Zhaokang Cheng*[3*] *and Brian C. Jensen*[4,5,6]

[1] Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, United States, [2] Blood Research Center, University of North Carolina School of Medicine, Chapel Hill, NC, United States, [3] Department of Pharmaceutical Sciences, Washington State University, Spokane, WA, United States, [4] Cardiology Division, Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, United States, [5] Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC, United States, [6] McAllister Heart Institute, University of North Carolina School of Medicine, Chapel Hill, NC, United States

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***Correspondence:**
Silvio Antoniak
antoniak@email.unc.edu
Zhaokang Cheng
zhaokang.cheng@wsu.edu

[†]These authors have contributed equally to this work and share first authorship

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Anthracycline antineoplastic agents such as doxorubicin are widely used and highly effective component of adjuvant chemotherapy for breast cancer and curative regimens for lymphomas, leukemias, and sarcomas. The primary dose-limiting adverse effect of anthracyclines is cardiotoxicity that typically manifests as cardiomyopathy and can progress to the potentially fatal clinical syndrome of heart failure. Decades of pre-clinical research have explicated the complex and multifaceted mechanisms of anthracycline-induced cardiotoxicity. It is well-established that oxidative stress contributes to the pathobiology and recent work has elucidated important central roles for direct mitochondrial injury and iron overload. Here we focus instead on emerging aspects of anthracycline-induced cardiotoxicity that may have received less attention in other recent reviews: thrombosis, myocardial atrophy, and non-apoptotic programmed cell death.

Keywords: anthracycline cardiotoxicity, thrombosis, myocardial atrophy, programmed cell death, protease activated receptor, FOXO1 (forkhead box O1)

INTRODUCTION

Considerable research effort has been invested in understanding the complex and multifactorial mechanisms underlying anthracycline-induced cardiotoxicity. Longstanding evidence has established causative roles for oxidative stress in contributing to cardiomyocyte dysfunction and death (1). Mitochondrial dysfunction generates much of this oxidative stress and the central role of multifaceted mitochondrial injury in anthracycline-induced cardiotoxicity has been comprehensively reviewed recently (2). Here, we will focus on emerging, though less-studied, mechanisms underlying the adverse effects of anthracyclines on both the heart and the vasculature.

ANTHRACYCLINES AND THROMBOSIS

Observational data suggest that some anti-cancer therapies are associated with increased risk for thrombotic events in the venous and arterial vasculature including deep vein thrombosis (DVT), pulmonary embolism (PE), and arterial thrombosis (AT) as recently summarized by

Grover et al. (3). Indeed, Weiss et al. reported that 5% of stage II breast cancer patients (22/443) with 2 years of post-mastectomy chemotherapy developed venous thrombosis without signs of metastasis (4). Interestingly, no thrombosis was observed after completion of the chemotherapy (4). In another study of Stage IV breast cancer patients, thrombosis incidence rose to 17.6% in those who received anthracyclines (5). Interestingly, analysis of common risk factors for thrombosis (ambulatory status, obesity, family history, smoking, diabetes mellitus, hypertension, liver dysfunction, thrombocytosis, and previous endocrine therapy) showed no association with the observed thrombotic events (5). With specific regard to anthracyclines, multiple myeloma patients were at an increased risk of DVT (16%) when doxorubicin (DOX) was added to thalidomide and that risk increased with age (6). Importantly, the thrombotic risk for all three of these trials is reported relative to a control group that did not receive an anthracycline. Increased thrombosis incidence (7.5%) was also observed in breast cancer patients undergoing an anthracycline-containing chemotherapy regimen with age-dependent risk increase (27%) in patients over 60 years, though this study did not include a control group that was not exposed to anthracyclines (7).

Patient-specific factors that enhance risk of anthracycline-induced thrombosis are poorly defined, though one intriguing possibility is the metabolic syndrome. Individuals with the metabolic syndrome are at higher risk of both thrombotic events (8), and anthracycline-induced cardiotoxicity (9), possibly as a result of the chronically proinflammatory systemic milieu. Obesity (10) and insulin resistance (11, 12) components of the metabolic syndrome, also independently enhance risk for anthracycline-induced cardiotoxicity, though a direct link to thrombosis has not been established.

PRO-THROMBOTIC EFFECTS ON VASCULAR CELLS

How do anthracyclines, such as DOX, contribute to a prothrombotic phenotype? Multiple studies have shown that anthracyclines increase phosphatidylserine (PS) exposure on the outer cell surface on vascular cells (13–16). Negatively charged PS-rich membranes enhance the coagulation cascade reaction by increasing the activity of gamma carboxyglutamic acid (GLA)-dependent coagulation factors like factor VIIa (FVIIa), FXa, FIXa, and thrombin (17). Liaw's group showed that DOX induces a procoagulant phenotype in human endothelial cells (ECs) by increasing the PS flip to the cell surface which enhances activity of preexisting tissue factor (TF), without increasing its expression level (16). Interestingly, this effect was not seen for methotrexate nor 5-fluorouracil treated ECs (16). Further, the increase in surface PS on the ECs was associated with DOX-induced EC apoptosis (16). Later, Boles et al. (15) confirmed that the anthracycline daunorubicin also increased cellular TF activity without affecting TF protein levels, but rather by enhancing PS surface exposure on the human monocytic cell line THP-1 (**Figure 1**). DOX had a similar effect on platelets, causing increased PS surface exposure due to apoptotic pathway activation in DOX-exposed human platelets and subsequently resulting in enhanced procoagulant activity (14). The authors linked the increased PS exposure to DOX-induced platelet mitochondrial dysfunction at doses of 2.5–7.5 mg/kg in rats (13). Interestingly, at a cardiotoxic DOX dose of 25 mg/kg apoptosis-dependent thrombocytopenia was observed as early as 4 h after DOX injection in rats (13).

Moreover, daunorubicin was shown to increase the release of TF+ extracellular vesicles (EV) from THP-1 cells *in vitro* (**Figure 1**) (15). Increased anthracycline-induced EV release was confirmed by others (18–20). DOX-induced EVs are enriched for 4-hydroxy-2-nonenal (4-HNE), a marker for oxidative stress (19). 4-HNE can directly induce the release of TF+EVs from perivascular cells which can contribute to a prothrombotic state (21, 22). In line with this observation, TF+EVs were shown to enhance thrombus formation in multiple murine models of cancer-associated thrombosis (23, 24). Aside from its procoagulant effects, DOX is known to negatively affect the anticoagulant properties of ECs by downregulating the expression of the endothelial protein C receptor, leading to decreased protein C pathway activation (25).

EFFECTS ON BLOOD FLOW AND THROMBUS FORMATION *IN VIVO*

Injection of DOX (8 mg/kg) leads to occlusive vasoconstriction of smaller vessels ($<15\,\mu m$) and vascular leakage in the murine femoral microvasculature within 4 min (26). Moreover, the same dose of DOX also reduces the blood flow in testicular arteries in mice within 15 min of injection (27). The authors linked these phenomena to DOX-induced vascular toxicity leading to EC-platelet interactions and the formation of EC-bound platelet microthrombi (27). Blood flow was restored by pre-treatment with low molecular weight heparin or the anti-platelet drug eptifibatide, suggesting that anti-platelet/anti-coagulant agents might be effective in reducing the detrimental vascular effects of DOX (27). DOX doses up to 7.5 mg/kg significantly enhanced thrombus sizes in a modified rat $FeCl_3$ vena cava thrombosis model, without causing thrombocytopenia (14). In addition, in a vena cava stasis model DOX (7.5 mg/kg) caused increased thrombus formation that was reduced by administration of clopidogrel, aspirin or an inhibitor of platelet activated factor (28). These findings strongly suggest that DOX-induced venous thrombosis is dependent upon platelet activation (28).

COAGULATION-DEPENDENT SIGNALING IN ANTHRACYCLINE-INDUCED CARDIOTOXICITY

While coagulation activation leads to fibrin deposition, the coagulation proteases that are generated in the process also lead to cleavage of protease-activated receptors (PARs) (29). PAR1 and PAR4 are activated by thrombin and are expressed on human platelets; their cleavage is the strongest platelet-activating stimulus. PAR3 also is activated by thrombin, but PAR3 mostly acts as co-factor for PAR4 and has only limited

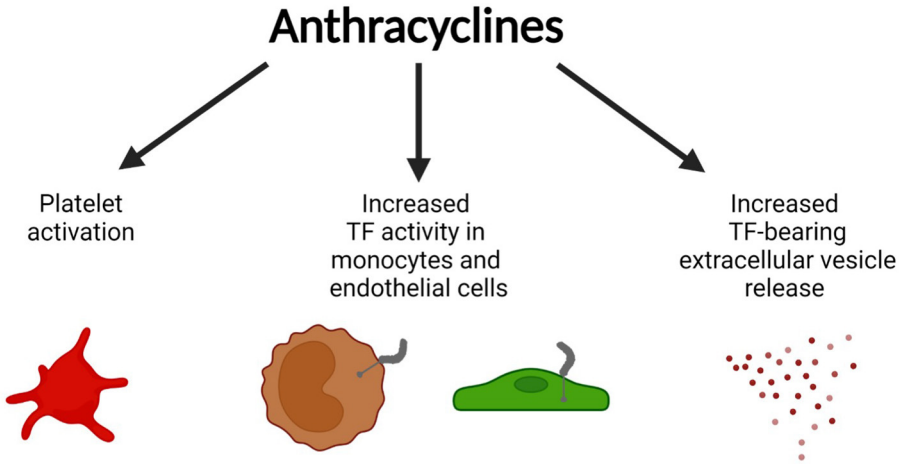

FIGURE 1 | Prothrombotic effects of anthracyclines. Anthracyclines (doxorubicin, daunorubicin) activate vascular cells including platelets, monocytes, and endothelial cells leading to surface phosphatidylserine (PS) exposure, increased activity of pre-existing tissue factor (TF) on monocytes and endothelial cells, and the release of TF-bearing extracellular vesicles (EV). Figure created with BioRender.com.

signaling function in humans (30). PAR2 is rather thrombin-insensitive and is primarily activated by the TF:FVIIa complex or FXa (31). Though PARs frequently are considered for their roles in platelets, they also are expressed on cardiomyocytes, where they contribute to the cardiac response to multiple injury models (29, 31, 32). The absence of PAR1 and PAR2 reduced infarct size and adverse cardiac remodeling in experimental heart failure (29, 31, 32). PAR4 activation can be cardioprotective or detrimental dependent on the chosen injury model and time point analyzed (31, 33–36).

With regard to chemotherapy-induced toxicity, PAR1 deficiency and PAR1 inhibition with the FDA-approved drug vorapaxar protected against DOX cardiotoxicity in mice (37). PAR1 activation exacerbated mitochondrial dysfunction and apoptosis in cardiac cells exposed to DOX *in vitro* (37). PAR1 deficiency was associated with reduced oxidative stress and apoptosis as well as decreased circulating cardiac troponin I and improved cardiac contractile function in the hearts of mice treated with 20 mg/kg DOX (37). PAR1 deficiency was also protective in a chronic DOX cardiotoxicity model (5 mg/kg/week for 5 weeks) (37). In line with these observations, PAR1 inhibition with the PAR1 inhibitor Q94 reduced toxic renal effects of DOX (15 mg/kg) in mice (38). Whether PAR2 or PAR4 contribute to DOX cardiotoxicity is the objective of ongoing investigations. Interestingly, PAR2 inhibition with FSLLRY-NH2 reduced nephropathy in a chronic rat DOX kidney injury model (1 mg/kg/day for 6 weeks) suggesting that PAR2 deficiency/inhibition might also be cardioprotective during DOX chemotherapy (39).

ANTHRACYCLINES INDUCE MYOCARDIAL ATROPHY

Anthracycline-based chemotherapies are known to cause abnormalities in heart morphology in cancer patients. Childhood cancer survivors who received anthracycline treatment have reduced ventricular wall thickness and myocardial mass later in life (40, 41). Recent evidence suggests that anthracyclines also cause a reduction in left ventricular mass in adult cancer patients (42–44). Importantly, an early decline in heart mass is associated with worse heart failure outcomes, emphasizing the importance of this phenomenon (42). A decrease in heart mass can be caused by reduced cardiomyocyte size (atrophy) and/or number (i.e., loss of cardiomyocytes due to cell death). Here, we summarize recently identified mechanisms underlying anthracycline-induced atrophy and cell death (**Figure 2**).

Similar to the clinical findings, exposure to the anthracycline DOX also reduces heart weight in mice (44–46). At the molecular level, DOX induces p53 expression, which is necessary for inactivation of mammalian target of rapamycin (mTOR), a serine-threonine kinase essential for protein synthesis (46). Interestingly, DOX-induced reductions in heart weight and myocyte size are abolished by cardiac-specific expression of dominant-interfering p53 or constitutively active mTOR, suggesting that DOX induces cardiac atrophy through p53-dependent inhibition of mTOR (46). Activation of mTOR by vascular endothelial growth factor-B (VEGF-B) gene therapy also prevents DOX-induced cardiac atrophy (47). Conversely, inducible ablation of mTOR in adult heart is sufficient to reduce cardiomyocyte size within 1–2 weeks (48). Taken together, these data indicate that mTOR inhibition is an important mechanism underlying DOX-induced atrophy.

DOX also induces expression of muscle RING finger 1 (MuRF1), a striated muscle-specific ubiquitin ligase and a key mediator of cardiac atrophy (44, 45). Mice lacking MuRF1 are resistant to DOX-induced reduction in heart mass, suggesting that MuRF1 is necessary for DOX-induced atrophy (44). Mechanistically, DOX exposure induces cyclin-dependent kinase 2 (CDK2)-mediated phosphorylation of forkhead box O1 (FOXO1) at Ser 249, resulting in FOXO1 activation and

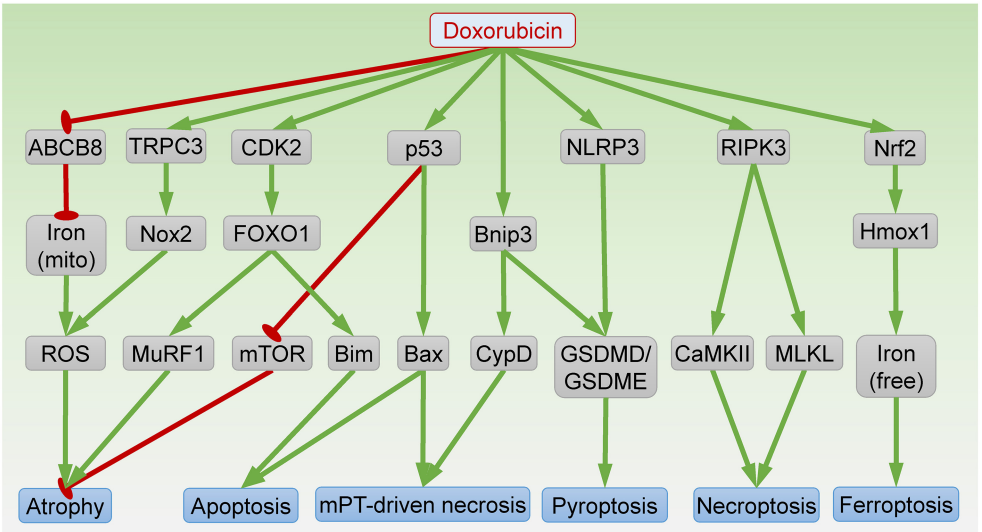

FIGURE 2 | Signaling pathways in DOX-induced cardiomyocyte atrophy and death. ABCB8, ATP-binding cassette protein-B8; CaMKII, Ca^{2+}-calmodulin–dependent protein kinase; CDK2, cyclin-dependent kinase 2; CypD, cyclophilin D; FOXO1, forkhead box O1; GSDMD/GSDME, gasdermin D/E; Hmox1, heme oxygenase-1; mito, mitochondria; MLKL, mixed lineage kinase domain like pseudokinase; mPT, mitochondrial permeability transition; mTOR, mammalian target of rapamycin; MuRF1, muscle RING finger 1; Nox2, NADPH oxidase 2; RIPK3, receptor-interacting protein kinase 3; ROS, reactive oxygen species; NLRP3, NLR family pyrin domain containing 3; TRPC3, transient receptor potential canonical 3. Arrows indicate activation; bar-headed lines indicate inhibition.

transcription of MuRF1 (45). Treatment with a FOXO1 inhibitor prevents DOX-induced cardiac atrophy and dysfunction (45). Collectively, FOXO1-dependent MuRF1 expression mediates DOX-induced atrophy.

Cardiac atrophy can occur as a result of oxidative stress. DOX exposure induces reactive oxygen species (ROS) generation through mitochondrial iron accumulation, owing to repression of ATP-binding cassette protein-B8 (ABCB8)-mediated mitochondrial iron export (49). Cardiac-specific ABCB8 transgenic mice are protected from DOX-induced ROS generation and atrophy (49). In addition, DOX exposure induces transient receptor potential canonical 3 (TRPC3)-dependent upregulation of NADPH oxidase 2 (Nox2) (50). Formation of the TRPC3-Nox2 complex amplifies ROS production and results in cardiac atrophy. Knockdown of TRPC3 or pharmacologic inhibition of TRPC3-Nox2 interaction attenuates DOX-induced atrophy in neonatal rat cardiomyocytes (NRCMs) (50). Moreover, mice lacking Nox2 are also resistant to DOX-induced cardiac atrophy (51). These findings suggest that enhanced ROS production resulting from mitochondrial iron accumulation or TRPC3-Nox2 complex formation also contributes to DOX-induced atrophy.

CONTRIBUTIONS OF PROGRAMMED CELL DEATH TO ANTHRACYCLINE CARDIOTOXICITY

Exposure to anthracyclines triggers a variety of cell death modalities in the heart, resulting in cardiac cell loss. Anthracycline-induced cell death pathways have been reviewed in detail quite recently (52). A brief summary of the novel mechanisms of anthracycline-induced cardiomyocyte death is provided below.

Apoptosis

Apoptosis is undoubtedly the most intensively studied form of cell death in anthracycline cardiotoxicity. DOX targets topoisomerase-IIβ to cause DNA double-strand breaks and initiate the intrinsic apoptosis pathway (53). DNA damage induces p53-dependent oligomerization of the Bcl2 family members Bak and Bax, which forms a pore in the outer mitochondrial membrane, resulting in cytochrome *c* release, caspase activation, and apoptosis. Accordingly, pharmacological inhibition of p53 or Bax blocks apoptosis and prevents DOX-induced cardiomyopathy (54, 55). It is noteworthy that p53 plays complicated roles in DOX-induced cardiotoxicity by modulating apoptosis-independent processes including mitochondrial biogenesis (56) and clonal hematopoiesis (57), as well as atrophy (46). In addition to the pore-forming effectors Bak and Bax, the pro-apoptotic Bcl2 family proteins also include activators (Bim, Bid, and Puma) that directly interact with the effectors to trigger apoptosis (58). DOX induces expression of Bim through CDK2-dependent FOXO1 activation (45, 59). Inhibition of either CDK2 or FOXO1 attenuates DOX-induced apoptosis and cardiac dysfunction (45, 59). Young age, a major risk factor for anthracycline cardiotoxicity in humans, is associated with higher sensitivity to apoptosis, further supporting an important role of apoptosis in anthracycline-related cardiotoxicity (60).

Mitochondrial Permeability Transition Pore (mPTP)-Driven Necrosis

Necrosis driven by opening of the mPTP is characterized by rapid loss of the inner mitochondrial membrane potential and is dependent on cyclophilin D (CypD) (61). Recent evidence suggests that DOX treatment provokes mPTP-driven necrosis in cardiomyocytes (62). Mechanistically, DOX induces expression of Bnip3, which binds CypD to trigger mPTP opening and resultant necrosis (62). Bnip3 null mice are protected from DOX-induced mitochondrial damage, necrosis, and cardiac dysfunction (63). In addition, Bax and Bak are necessary for mPTP-driven necrosis (64, 65). Indeed, a small-molecule Bax inhibitor protects against DOX-induced necrosis *in vivo* (55).

Necroptosis

Necroptosis is programmed cell necrosis that is initiated by binding of a death ligand (typically from the TNF superfamily) to a death receptor (such as Fas, TNFR1, or TRAIL) and culminates in plasma membrane permeabilization mediated by mixed lineage kinase domain like pseudokinase (MLKL) (61). MLKL activation and plasma membrane translocation requires phosphorylation by receptor-interacting protein kinase 3 (RIPK3) (66). DOX exposure upregulates cardiac RIPK3 and MLKL *in vivo* and *in vitro* to induce necroptosis (67). RIPK3 knockout mice are resistant to DOX-induced myocardial necrosis, cardiomyopathy and death (68). In this context, RIPK3 induces activation of Ca^{2+}-calmodulin–dependent protein kinase (CaMKII) to trigger necroptosis (68). Moreover, DOX-induced cardiomyocyte death is blocked by the necroptosis inhibitor necrostatin-1, suggesting that necroptosis contributes to DOX-induced cardiomyocyte injury (67).

Ferroptosis

Ferroptosis is a form of programmed cell death associated with mitochondrial damage owing to iron accumulation and lipid peroxidation (61). DOX induces nuclear factor erythroid 2–related factor 2 (Nrf2)-dependent transcription of heme oxygenase-1 (Hmox1) to trigger heme degradation, resulting in free iron accumulation, and ferroptosis (69). Treatment with the Hmox1 antagonist zinc protoporphyrin IX, the iron chelator dexrazoxane, or the ferroptosis inhibitor ferrostatin-1 protects against DOX-induced cardiomyopathy (69). Interestingly, loss of the E3 ubiquitin ligase tripartite motif containing-21 (TRIM21) enhances Nrf2 antioxidant activity but downregulates Hmox1, resulting in reduced ferroptosis and cardiotoxicity following DOX exposure (70). In addition, DOX reduces the levels of glutathione peroxidase 4 (GPx4), acyl-CoA thioesterase 1 (Acot1), and mitochondrial ubiquitin ligase MITOL, all of which augment lipid peroxidation and ferroptosis, in mouse heart (71–73).

Pyroptosis

The major characteristic of pyroptosis is plasma membrane permeabilization mediated by gasdermin proteins such as gasdermin D (GSDMD) and gasdermin E (GSDME) (61). Cleavage of GSDMD by caspases 1, 3, 4, 5 or 11 results in GSDMD pore formation at the plasma membrane and subsequent pyroptosis. Pyroptosis is often pro-inflammatory owing to secretion of interleukin-1β and interleukin-18. DOX exposure induces cardiomyocyte pyroptosis *in vivo* and *in vitro* through NLR family pyrin domain containing 3 (NLRP3)-dependent activation of caspases 1, 3, and 11 (74, 75). In addition, Bnip3-dependent activation of caspase 3 also contributes to DOX-induced pyroptosis in cardiomyocytes (76).

CONCLUSIONS

Here, we have reviewed our emerging understanding of the contributions of thrombosis, myocardial atrophy, and programmed cell death to the complex and multifaceted pathobiology of anthracycline-induced cardiovascular toxicity. Future work in our labs and others will further explicate the importance of these processes to anthracycline-induced cardiovascular toxicity and define whether they could represent novel therapeutic targets for prevention or treatment of these dose-limiting and potentially life-threatening adverse effects.

AUTHOR CONTRIBUTIONS

All authors drafted, edited, and approved the final version of the manuscript.

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