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Doxorubicin Cardiotoxicity: Multiple Targets and Translational Perspectives

Antonella De Angelis, Donato Cappetta,
Liberato Berrino and Konrad Urbanek

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Abstract

Anthracycline cardiotoxicity remains a serious problem in pediatric and adult cancer survivors. This chapter discusses the involvement of multiple cardiac cell types in the pathogenesis of the onset and progression of doxorubicin cardiotoxicity. In addition to cardiomyocytes, considered the classical cellular target, the role of cardiac fibroblasts and vascular cells together with progenitor cells of cardiac and extra-cardiac origin is addressed with a focus on oxidative stress, DNA damage, senescence, cell death, and molecular signals involved in cellular injury and response. Current strategies for primary and secondary prevention aiming at contrasting the onset of early and late doxorubicin-induced toxic events do not completely resolve the growing clinical problem. Thus, there is the necessity to understand cellular processes that operate within and beyond cardiomyocyte, to develop more effective tools for the prevention and treatment of progressive cardiomyopathy in otherwise successfully treated oncologic patients.

Keywords: anthracycline cardiomyopathy, cellular targets, molecular mechanisms, progenitor cells, cardioprotective strategies

1. Introduction

Cardiotoxicity is one of the most serious consequences of cancer therapy. Over the last decades, this complication has reached alarming dimensions due to aging of the population, epidemics of chronic comorbidities, and improvement in patient survival through to the increasing access to innovative diagnostic and therapeutic approaches. Cardiovascular death may represent a greater threat of mortality than cancer itself, and its risk, following cancer treatment,

may be higher than the actual risk of tumor recurrence. Indeed, cardiovascular death is the leading cause of death in female breast cancer patients, and exceeds that of cancer recurrence in childhood cancer survivors [1]. The growing awareness of the anticancer therapies-related cardiovascular risk, which can influence the appropriate management of patients, stimulates continuous effort in basic and clinical research.

Anthracyclines, such as doxorubicin (DOX), discovered in Italy, have been widely used in the treatment of various malignancies since the 1960s, although they can cause ventricular dysfunction and cardiomyopathy that were recognized right after their introduction to clinical practice [2, 3]. Contrary to public awareness, the scale of the anthracycline cardiotoxicity issue is by no means minor. A nine-year follow-up of patients treated with anthracyclines showed, respectively, 17.9 and 6.3% of subclinical and overt cardiotoxicity [4]. The cardiotoxic effect may be either reversible or irreversible. The former occurs during or shortly after drug infusion, is dose independent, and manifests as a transient decline in myocardial contractility with various electrocardiogram abnormalities. The latter can be classically divided into early and late onset toxicity. The early onset effects appear several weeks or months after the last dose of anthracycline. This is the most frequent and clinically relevant form of cardiotoxicity, usually presenting as a dilated cardiomyopathy leading to heart failure (HF). The late onset toxicity may appear with the occurrence of HF symptoms, years or even decades after the end of chemotherapy, especially in childhood cancer survivors [5]. The prognosis in anthracycline-related HF is relatively poor. However, the increasing attention to early detection of ventricular dysfunction and appropriate management can significantly improve the outcome.

Although the introduction of new synthetic and biological molecules has improved the course of many cancers, anthracycline chemotherapy regimens still have a prominent role in clinical protocols. Long-term survival creates a growing population of cancer patients who will be, for years to come, at risk of cardiovascular morbidity and mortality due to anthracycline chemotherapy.

2. Molecular and cellular mechanisms

Anthracycline-induced cardiotoxicity has stimulated substantial interest of basic and clinical researchers over the years, although the pathogenetic mechanisms have not been completely clarified.

2.1. Cell loss

It is well known that cardiotoxicity induced by anthracycline involves the activation of molecular pathways triggering the loss of cardiomyocytes, and that these cell death mechanisms, either apoptotic or necrotic, may occur in an acute phase, soon after anthracycline exposure. Experimental studies have shown anthracycline to induce cell death in a concentration-dependent fashion, with apoptosis occurring at lower and necrosis at higher concentrations of the drug [6, 7]. Specifically, DOX activates mitogen-activated protein kinases (MAPK), such as p38 and c-Jun N-terminal kinase (JNK), which leads to apoptosis via oxidative stress, calcium handling impairment, mitochondrial swelling, and caspase activation [8]. Although

autophagy is referred to as a physiologic process, abnormal autophagy may be involved in various diseases, taking part in the pathogenesis of HF including anthracycline-induced cardiomyopathy [9].

2.2. The redox element

Traditionally, the main mechanism accounting for cardiotoxic potential of anthracyclines has been attributed to the excessive production of reactive oxygen and nitrogen species (ROS and RNS) [10]. This overproduction is facilitated by permissive conditions due to low antioxidant capacity, in terms of ROS-scavenging enzyme synthesis, possessed by a cardiomyocyte. Antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, cytochrome P450, and glutathione transferases are less expressed in the heart compared to other organs. In addition, the level of antioxidants is further lowered by DOX that decreases the expression of catalase and Cu/Zn-SOD [11–13].

The reduction of the quinone moiety in ring C transforms DOX to semiquinone, which generates $O_2^{\cdot-}$ (when reacting with oxygen) that in turn is neutralized into low-toxic hydrogen peroxide (H_2O_2) by superoxide dismutase. Alternatively, H_2O_2 and $O_2^{\cdot-}$ react with one another to generate highly reactive OH^{\cdot} according to the iron-catalyzed Haber-Weiss reaction [14].

The subcellular compartment where most of ROS are produced is the mitochondrion, where DOX, being accumulated for its high affinity to cardiolipin, a phospholipid located in the mitochondrial inner membrane, determines deleterious effects by producing ROS and disrupting the electron-transport chain. In addition to mitochondrial dysfunction, high levels of ROS and RNS, triggering cytotoxic signaling, lead to DNA and protein oxidation, and impairment of intracellular calcium homeostasis. Upregulation of Mn-SOD, situated in the mitochondrial matrix and serving as another ROS scavenger, prevents the accumulation of free radicals in mitochondria [15]. DOX impairs the correct synthesis also of this antioxidant enzyme [16].

At the same time, lipid peroxidation is induced by DOX through a non-enzymatic reaction that reduces Fe^{3+} and forms DOX- Fe^{2+} free radical complexes. As a main consequence, lipid peroxidation alters the membrane structure and permeability influencing on cell signaling and function. To control the process of free radical generation, the iron is sequestered by storage and transport proteins. By counterpart, DOX impairs iron homeostasis and determines iron accumulation in mitochondria, leading to cardiac cell damage [17].

Numerous enzyme systems are accountable for ROS production, including reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), xanthine oxidases, uncoupled nitric oxide (NO) synthase (NOS), and enzyme complexes of peroxisomes. NOXs are multi-subunit transmembrane enzymes that utilize NADPH as an electron donor to reduce oxygen to $O_2^{\cdot-}$ and H_2O_2 . NOX2 and NOX4 isoforms are predominantly expressed in the heart where they contribute to enhancement in oxidative stress. Growing evidence has demonstrated an increased and persistent activation of these enzymes in response to DOX exposure [10].

NOSs are another source of DOX-dependent ROS. DOX, by binding to endothelial NOS (eNOS), interferes with NO generation in favor of superoxide formation. ROS, reacting with NO to generate RNS, boost the generation of oxidants that, in turn, force the uncoupling of

eNOS, altering the enzyme function to produce more $O_2^{\cdot-}$ and less NO. The formation of peroxynitrite leads to mitochondrial oxidative damage with a consequent apoptosis and/or necrosis [18].

Nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine zipper protein, is involved in the expression of diverse antioxidant proteins and plays a crucial role in DOX-induced cardiomyopathy. Whereas deficiency of Nrf2 aggravates cardiotoxicity and cardiac function, overexpression of Nrf2 has a protective effect on the myocardium. The exact mechanism remains elusive, but it seems that Nrf2 mediates the balance between oxidative stress and autophagy [19].

2.3. Topoisomerases

DOX-dependent oxidative stress is not the only phenomenon at the base of anthracycline cardiotoxicity. Recent findings have attributed DOX binding to topoisomerase II, a leading mechanism driving cardiac abnormalities. DOX anticancer activity is partly due to the ability to form, in cancer cells, ternary complexes with topoisomerase II α and DNA, causing DNA double-strand breaks and cell cycle arrest, and eventually activating death processes. However, other topoisomerase isoforms are also targeted by DOX. In contrast to α isoform (mainly expressed in rapidly dividing malignant and non-malignant cells), topoisomerase II β is highly expressed by quiescent cells (i.e. adult cardiomyocytes), and its inhibition mediates DOX-driven cardiotoxic effects through DNA damage and defective mitochondrial biogenesis [20, 21]. According to this scenario, the interaction between DOX and topoisomerase II β accounts as the initial event of cardiotoxicity whereas oxidative stress must be considered as a consequential step. Being either originating or downstream event, oxidative stress plays a fundamental role in anthracycline cardiotoxicity and its modulation is still subjected to experimental and clinical investigation.

2.4. Myocardial senescence

Induction of long-term cardiotoxicity is attributable to molecular mechanisms other than those of apoptosis and necrosis. Premature myocardial senescence has been recognized as an essential phenomenon in the development of HF [22]. DOX affects several molecular and cellular events, such as activation of p53, p21^{Cip1}, and p16^{INK4a}, leading to the cells' replicative stress and impaired cellular functions [23]. Importantly, terminally differentiated cells, including adult cardiomyocytes, with a senescent phenotype, are still metabolically active and capable of synthesizing pools of cytokines, growth factors, and regulatory enzymes known as senescence-associated secretory phenotype or senescence messaging secretome that affects the surrounding microenvironment [24].

2.5. Ion homeostasis

DOX cardiotoxicity is associated with dysregulation of sodium and calcium homeostasis that develops after ROS generation. DOX alters the expression of many calcium exchange regulating proteins such as sodium-calcium exchanger, ryanodine receptor, sarco/endoplasmic reticulum adenosine triphosphate (ATP) hydrolase 2a, and phospholamban. From the functional

point of view, changes in the expression of sodium/calcium-regulating genes impair both systolic and diastolic performance. Notably, the activation of calcium/calmodulin-dependent protein kinase II was found to be a key aspect leading to the changes in genes involved in ion signaling in DOX cardiomyopathy [25, 26].

2.6. Ultrastructural changes

An additional effect of anthracyclines on cardiomyocytes implicates the disruption of sarcomeric structure. Studies conducted on patients and rodents exposed to anthracyclines have demonstrated ultrastructural changes, such as loss and disarray of sarcomeric myofibrils, dilation of sarcoplasmic reticulum, swelling of mitochondria, and cytoplasmic vacuolization. The integrity of sarcomere is essential to myocyte dynamics, and deficit in assembly or organization of cardiac sarcomeres ultimately leads to impaired cardiac function [27].

2.7. Cell energetics

Reduced contractility can be also linked to the disturbed myocardial energetics. Negative inotropic effect of anthracyclines as well as the irregular energy-dependent phase of cell relaxation can be linked to intracellular abnormalities. DOX decreases ATP and phosphocreatine levels, thus decreasing cardiac energetic reserve in terms of the availability of high-energy phosphates. Interestingly, the reduced phosphocreatine/ATP ratio was observed in patients without clinical evidence of cardiomyopathy and even years after chemotherapy [28]. Along with the decreased energy production, DOX impairs energy sensing and high-energy phosphate transfer, affecting creatine kinase isoenzymes. Also, in addition to abnormal fatty acids oxidation, DOX limits the compensative response in terms of glucose utilization [29]. Overall, deficit in high-energy phosphate metabolism and poor performance of compensatory and regulatory circuits significantly contribute to the onset and progression of anthracycline cardiomyopathy.

2.8. Non-coding RNAs

Biological importance of regulatory function of RNA is reflected in the fact that more than 98% of the transcriptional product of the genome is a non-coding RNA. In a recent study, global transcriptional profiling has identified DOX-induced changes in the levels of several cardiac RNA-binding proteins (RBPs), including downregulation of Quaking isoform 5 (Qki5). RBPs control the function of coding and non-coding RNAs. Qki5 was shown to regulate the formation of circular RNAs and has protective role against DOX-induced cardiomyocyte death and cardiac dysfunction. Several circular RNAs, controlled by overexpression of Qki5, were also downregulated in response to DOX. Interestingly, inhibition of titin gene-derived circular RNA increased the susceptibility of cardiomyocytes to DOX [30].

3. Cardioprotective strategies

The uncertainties regarding the management of anthracycline cardiotoxicity have evidenced the necessity to develop a multidisciplinary *modus operandi* that intertwines cardiologists'

and oncologists' expertise for the best management of cardiovascular outcomes in patients undergoing anticancer therapy. Prevention of cardiotoxicity begins before starting anticancer therapy, with the evaluation of the cardiovascular risk profile and the adoption of strategies to reduce such a risk (blood pressure and blood glucose control, smoking cessation, and cholesterol reduction).

3.1. Dose reduction

The DOX cumulative dose is the most important risk factor for the development of cardiotoxicity. The estimation of patients with DOX-related HF rises from 5% at a cumulative dose of 400 mg/m², to 16% (500 mg/m²), 26% (550 mg/m²), and 48% (700 mg/m²) [31]. Therefore, clinical protocols recommend not exceeding 400–450 mg/m² cumulative dose. It is worth noting that late onset cardiac abnormalities have been observed in patients treated with DOX cumulative dose well below the “safety threshold,” suggesting that there may be no safe dose of anthracyclines [32]. In children exposed to DOX dose lower than 100 mg/m², cardiac abnormalities were detected in 30% of survivors after several years [33].

3.2. Pharmacokinetic approach and analogs

Alternative or additional strategies of primary intervention are used to reduce or prevent deleterious effects that anthracyclines have on the heart. Standard approaches are based on the differences in pharmacokinetic aspects of antitumor activity and cardiotoxicity. Whereas anthracycline antitumor efficacy corresponds to total exposure, cardiotoxicity correlates with the peak plasma level. Thus, these methods consist in changing administration schedule by replacing bolus with slow infusion and switching from conventional to liposomal formulations. Continuous slow infusion lowers peak concentration, thus reducing cardiotoxicity, but retains anticancer activity. However, cardioprotective benefits have been proved in limited therapeutic protocols, and elevated costs of longer hospitalization and risk of infections represent additional causes that have limited its use [14]. Liposomal encapsulation enables a preferential crossing of irregular vasculature (tumor tissue) instead of less permeable vessels of healthy tissues, thus modifying tissue distribution of DOX. Uncoated or pegylated liposomal anthracyclines have proved to be as effective as conventional formulations but with less toxic effects. Nonetheless, liposomal anthracyclines have been investigated in relatively few randomized trials so that their use is approved for only limited clinical indications, such as metastatic breast cancer, ovarian cancer, multiple myeloma, and acquired immune deficiency syndrome-related Kaposi sarcoma [34]. The costs of such preparation represent also a limiting factor. DOX analogs (i.e. epirubicin and idarubicin) have been introduced into clinical practice in place of DOX with the scope of reducing cardiotoxicity. These molecules are effectively less cardiotoxic than DOX, but being less active as well, it is necessary to augment the dose to maintain the antitumor activity equivalent to DOX, thus increasing the risk of HF, particularly in patients with defined risk factors [35].

3.3. Antioxidants

The role of ROS in the pathogenesis of anthracycline-related cardiotoxicity and HF has provided the basis for testing the coadministration of synthetic drugs or natural compounds with

antioxidant properties to counteract the development of cardiotoxicity. Early studies assessing the efficacy of dietary supplements such as vitamin A, vitamin E, coenzyme Q10, and other compounds known to prevent oxidative damage have produced disappointing results. Although some of these molecules (of which there are data on the tumor response rate) do not disempower the antineoplastic efficacy of the anthracycline, the benefits on myocardial function were modest at most [36].

Dexrazoxane is the only approved drug used in clinical settings as cardioprotective agent in pediatric and adult patients exposed to DOX cumulative dose known to induce HF. Dexrazoxane interferes with DOX-dependent redox reactions, and decreases ROS production and tissue damage by chelating iron before it catalyzes the conversion of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) into highly reactive hydroxyl radicals (OH^{\cdot}) [37]. Recently, it has been demonstrated that dexrazoxane can compete for the ATP-binding site of topoisomerase II β , thus precluding the formation of anthracycline-DNA-topoisomerase II β complex and thus preventing DNA double-strand breaks and cardiomyocyte death. This new mechanism of cardioprotection may explain why it has succeeded while other antioxidants have not [38]. However, the clinical use of dexrazoxane has been limited following few reports of its possible interference with antitumor activity of anthracyclines, and the potential risk of a second malignancy in pediatric patients. Although numerous evidence has denied these concerns, the regulatory agencies have maintained the limitation of the use of dexrazoxane in restricted clinical conditions [39].

3.4. Cardiovascular drugs

Additional options include the use cardiovascular prophylaxis with β -blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor antagonists (ARBs), and statins. Two β -blockers, carvedilol and nebivolol, have shown to be protective against DOX-induced cardiotoxicity. The mechanisms have not been fully elucidated, but it seems clear that the protective effects lie beyond β -adrenergic antagonism, as not all β -blockers are effective. According to several studies, the antioxidant activity of carvedilol prevents lipid peroxidation and endogenous scavenger breakdown, while the inhibition of peroxynitrite generation and NOS uncoupling may be underlying protective mechanisms of nebivolol [40, 41]. Numerous evidence suggests that a key role in the development and progression of anthracycline-induced cardiotoxicity is played by the renin-angiotensin-aldosterone system, highlighting a potential benefit from the use of ACE inhibitors and ARBs. Preclinical and clinical studies have demonstrated that the inhibition of the renin-angiotensin-aldosterone system can mitigate cardiac dysfunction induced by anthracyclines [1]. Of note, the association of a β -blocker (carvedilol) with the ACE-inhibitor (enalapril) has provided the most effective response toward the amelioration of anthracycline-caused myocardial functional deficit, when either administered preventively, without any signs of systolic dysfunction, or promptly given after the detection of ejection fraction fall [5]. Also, statins have shown to be effective in the prevention of cardiotoxicity by reducing the risk of HF and cardiac-related mortality, due to their pleiotropic effects. The antioxidative property and the capacity of reducing cardiac inflammation may be the main mechanisms that support the role of statins as cardioprotective agents in this scenario [42].

3.5. Non-pharmacological approach

Lifestyle changes and exercise represent non-pharmacological measures to counter anthracycline cardiotoxicity. Clearly, exercise positively modulates several cardiovascular risk factors that need to be considered when assessing the risk of cardiotoxic event. But there is also a possible mechanistic link as exercise diminishes pro-apoptotic signals, improves calcium handling and myocardial energetics, and reduces ROS production. However, more data are needed to assign a role of exercise in the prevention and treatment of anthracycline cardiotoxicity [43, 44].

Despite all the progress made, more and more researches and prospective studies are needed to develop clinical practice guidelines that may direct the specialists to choose an interventional strategy (prophylactic and/or therapeutic) tailored to the characteristics of cancer patients.

4. Beyond a cardiomyocyte

Decades of research to elucidate anthracycline cardiotoxicity have exclusively focused on cardiomyocytes. However, they account for less than one-third of the total number of cells within the heart, whose proper function depends on a complex cellular network. Therefore, attention may be warranted to other cardiac and non-cardiac cell populations such as progenitor cells, vascular cells, and fibroblasts that have been suggested as additional targets in the development as well as management of anthracycline toxicity [45–48].

The following sections will expose the research on representative populations of cardiac cells (non-cardiomyocytes), whose depressed function induced by the anthracycline provides additional insights to further elucidate the complexity of cardiotoxicity. This expanding knowledge may also serve as the basis for innovative preventive and therapeutic interventions.

4.1. Cardiac progenitor cells

It has been repeatedly demonstrated that the myocardium contains an endogenous reservoir of cells with the ability to repopulate the damaged tissue. One of the best characterized populations of primitive cells is represented by cardiac progenitor cells (CPCs). They are cells expressing the stem cell marker (c-kit), residing in the myocardium, where they contribute to tissue homeostasis/repair [49–57].

4.1.1. CPC senescence and functional deficit

The increasing number of researches showing that DOX has CPCs as a cell target, with negative effects on their biological function, suggests a novel mechanism of cardiotoxicity [58–64]. In a rodent model of anthracycline cardiomyopathy, DOX increased ROS-dependent DNA damage, cellular senescence, cell cycle arrest, and apoptosis, affecting CPC viability, growth, and functional activities. In the failing heart, the loss of CPC pool interfered with mechanisms that account for the restoration of structural integrity and functional performance [58].

Supporting evidence has come from another study analyzing the heart of oncologic patients, who died of HF after being treated with anthracyclines. In comparison to age-matched patients who died of non-cardiovascular causes, the myocardium of DOX-treated patients showed a higher number of CPCs positive for the phosphorylated form of histone H₂AX and p16^{INK4a}, indicating increased levels of DNA damage and cellular senescence, respectively. In addition, *in vitro* exposure to DOX of human CPCs displayed the activation of senescent and apoptotic pathways, corroborating the hypothesis that a CPC dysfunction may be responsible for a higher susceptibility of the myocardium to the increased workload and injury [59]. Indeed, DOX-exposed human CPCs were no longer able to promote structural and functional recovery when injected in the heart of animals with DOX cardiomyopathy, confirming the ineffectiveness of DOX-treated CPCs in fulfilling their functional role in the diseased myocardium [60]. A recent study conducted on human CPCs confirmed the role played by senescence and apoptosis as main mechanisms activated by DOX. The rate of senescent CPCs, increased after DOX, was significantly reduced by the pretreatment with a human amniotic fluid stem cell secretome [62]. Similarly, the *in vitro* treatment with resveratrol, a sirtuin 1 activator with intrinsic antioxidant properties, was able to prevent DOX-induced senescence and growth arrest of CPCs by decreasing accumulation of intracellular ROS and enhancing antioxidant enzymatic defense. The uselessness of DOX-exposed CPCs to guarantee supportive role in the diseased myocardium has been demonstrated with the lack of any structural and functional recovery after the administration of *in vitro* DOX-treated CPCs in the heart of animals with anthracycline cardiomyopathy. On the other hand, priming CPCs with resveratrol partly restored their capacity to counteract the progressive ventricular dysfunction induced by DOX [60].

The definition of the role of growth factors as major determinants of CPC function has been addressed by studying the impact of insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF) signaling. The activation of IGF-1 receptor promotes proliferative and anti-apoptotic effects, while the stimulation of HGF receptor (c-Met) supports cell migration toward injury areas [65–68]. DOX showed reduced expression of IGF-1 receptor and c-Met by CPCs, revealing the impairment of pro-survival signaling and deficiency of migratory capability, thus aggravating the inadequate response of cardiac repair signaling in the injured heart [59].

4.1.2. CPCs and late cardiotoxicity

Despite decades of studying the molecular mechanisms at the base of late onset DOX cardiotoxicity, no general agreement has yet been reached. It is likely that even the exposure to DOX at a dose that does not determine symptomatic manifestations of cardiotoxicity, makes the heart more susceptible to successive injuries, with progenitor cell dysfunction and/or impaired angiogenesis. To address this issue, experiments were done in juvenile mice that were exposed to a cumulative dose of DOX that did not induce acute cardiotoxicity and, once the animals reached an adult age, they were subjected to myocardial infarction. DOX-exposed mice were more sensitive to myocardial infarction, with a greater extent of infarct size, a lower blood vessel formation in the infarct border zone, and more fibrotic tissue accumulation, compared to infarcted mice that were not exposed to DOX as pups. Moreover, DOX reduced the number of CPCs in the myocardium, and significantly overexpressed the cell cycle inhibitor p16^{INK4a}, suggesting the involvement of cellular senescence of cardiac progenitors as one of

the mechanisms responsible for the higher vulnerability of the heart. DOX impaired CPC functional competence by inhibiting cell growth and differentiation capacities *in vitro* [63]. According to this evidence, CPCs “poisoned” by DOX fail to migrate toward the site of injury with a consequent defect in myocardial repair.

The key impact of senescence and time-dependent evolution of molecular effects on CPCs was confirmed in studies on human cells. Human CPCs were briefly exposed to DOX and then cultured in a DOX-free medium. Early after exposure, DOX significantly increased apoptosis along with the expression of proteins involved in DNA damage response, such as ataxia telangiectasia mutated (ATM) kinase and p53. Both cell death and expression of p53 and ATM proteins returned to baseline after DOX washout, while at the same time, the increasing fraction of p16^{INK4a}-positive senescent cells indicated that CPCs entered the irreversible phase of growth arrest [59]. These data support the hypothesis that an early toxic event can justify a delayed response that transforms a latent and asymptomatic myopathy into overt HF.

4.1.3. CPCs and non-coding RNAs

MicroRNAs (miRs) are emerging as regulatory factors in cardiovascular physiology and pathology by contributing in the modulation of biological processes such as response to oxidative stress and cellular damage [69]. miRs have been associated with the regulation of myocardial cell proliferation and differentiation [70], and among others, the miR-34 family, particularly miR-34a, is expressed in the heart and is associated with DNA damage, and pro-senescent and pro-apoptotic mechanisms [71, 72]. Recent study has demonstrated the increased expression of miR-34a in rat CPCs after exposure with DOX. miR-34a increased senescent and apoptotic signaling by activating p16^{INK4a} and p53, and when released by DOX-treated CPCs, affected viability and function of other cardiac cells (cardiomyocytes, fibroblasts, and endothelial cells) [61]. This result indicates a paracrine mechanism, already known in other cardiovascular diseases [73]. However, the implications of miR-34a modulation in oncologic patients, as well as the hypothetical role as biomarker of myocardial damage need to be further assessed.

4.2. Vascular cells

In cancer biology, angiogenesis has a central role, given the necessity to form new blood vessels to support the growth of tumor mass. For this reason, continuous researches have pointed the attention on the effects exerted by cytotoxic drugs upon the vascular system [74]. Vascular damage can be directed on non-tumor tissues as well, supporting the concept that endothelial toxicity may be an additional aspect of antineoplastic therapies, including anthracycline-induced cardiovascular alterations [75, 76]. Studies on endothelial cells demonstrated increases in oxidative stress and DNA damage after anthracycline exposure, with impaired endothelial function and disruption of nitric oxide/superoxide balance [77–79]. In endothelial cells, DOX-induced increase in apoptotic rate was associated to elevated intracellular calcium levels and enhanced transcription of eNOS, suggesting a role for eNOS in DOX-mediated endothelial cell death [77]. Moreover, it has been shown that DOX directly binds to eNOS. This determines the reduction of DOX to the semiquinone radical and a consequent increase in superoxide formation and decrease in NO formation [80]. In this way,

a new concept of cardiac microvascular injury as a potential primary event contributing to anthracycline cardiotoxicity has emerged. DOX may affect the function of cardiac endothelial cell barrier by inhibiting the formation of tight junctions and determining an augmented vascular permeability [81].

Besides a direct effect on skeletal muscle microcirculation [82], the treatment with DOX has also proven to directly affect the biology of vascular smooth muscle cells (SMCs). Exposure to DOX produced DNA damage, generation of ROS, and increased senescence-associated β -galactosidase (SA- β -gal) activity in SMCs, which underwent premature senescence and cell cycle arrest [83]. Toxicity of DOX on vascular system was confirmed in organ culture by the detection of a lower capacity of vessels to relax. The involvement of oxidative stress was evidenced by a partial restoration of contractility in the presence of superoxide dismutase [84, 85]. Moreover, DOX induced an upregulation of endothelin-1, the potent vasoconstrictor and pro-inflammatory peptide, whose effects are noticeable during development of cardiovascular disease. Experimentally, endothelin receptor antagonism ameliorated DOX-induced cardiac dysfunction [86]. These data further support the view of anthracycline cardiotoxicity as a multicellular effects-driven process. This can stimulate future studies aiming at characterizing mechanisms of vascular toxicity and helping to design strategies to prevent or minimize the negative impact of DOX on vascular cell function.

4.3. Cardiac fibroblasts

Cardiac fibroblasts have been underrated for a long time. These cells, however, are essential for maintaining cardiac function and take a vital part in cardiac remodeling during pathological conditions. Initiation and maintenance of fibrogenic response is regulated by a complex interaction of growth factors and cytokines. Transforming growth factor- β (TGF- β) signaling is considered the most potent activator of fibroblasts that differentiate into myofibroblasts, cells capable to release great amounts of extracellular matrix components, such as collagen and fibronectin [87–91]. Myocardial fibrosis is a common feature of a broad variety of cardiovascular pathologies including anthracycline cardiomyopathy [87]. Indeed, treatment with DOX promoted phenotypic transformation of cardiac fibroblasts into myofibroblasts both *in vivo* and *in vitro* [92]. As signaling molecules, ROS, through NADPH oxidases, are implicated in the amplification of TGF- β -related pathways that promote fibroblast differentiation, and ultimately cardiac fibrosis [93]. In a rat model of DOX cardiomyopathy, increase in oxidative stress was associated with the upregulation of TGF- β , connective tissue growth factor, and SMAD3 determining adverse matrix remodeling with accumulation of collagen type I.

Pro-fibrotic phenotype and premature myocardial aging coalesce in anthracycline cardiotoxicity. In cardiovascular diseases, senescence is a well-recognized process that contributes to inflammation and myocardial fibrosis and stimulates the production of several factors including IL-6, IL-8, TGF- β , and tumor necrosis factor α (TNF α) [24, 94, 95]. TNF α may have a relevant role as cardiotoxic molecule, since the upregulation of its receptor was detected after DOX exposure in apoptotic myocardial cells [96, 97]. In a recent study, cardiac fibroblasts exposed to DOX prematurely acquired a senescent phenotype, as shown by the increases in SA- β -gal activity and the expression of senescence markers p16^{INK4a} and p21^{Cip1} [98]. As in other cell types, DOX induces DNA damage-response also in fibroblasts. The activation of the stress

sensor ATM kinase catalyzes the phosphorylation of p53, and determines increased expression levels of p53 and p21^{Cip1}. Cardiac fibroblasts have been even proposed as the principal cells that mediate cardiotoxic effects of DOX. It has been shown that ATM, activated mainly in cardiac fibroblasts, stimulates the release of Fas ligand, thus promoting DOX-induced cardiomyocyte apoptosis [99]. In addition to the activation of DNA damage-response cascade, in pulmonary fibroblasts, DOX produces a prompt reduction in acetyl-CoA carboxylase 1 expression, the enzyme that catalyzes the rate-limiting step in fatty-acid synthesis [100]. Overall, these processes can regulate the equilibrium of the myocardium and contribute to the switch to a pro-fibrotic profile. Further studies will need to determine the relative contribution of cardiac fibroblasts in the pathophysiology of anthracycline cardiomyopathy and establish the significance of fibroblast-cardiomyocyte cross talk in drug-induced cardiotoxicity.

4.4. Mesenchymal stem cells

To broaden the perspective of the pathophysiology of anthracycline cardiotoxicity, in addition to intracardiac cells (both mature and primitive), extracardiac undifferentiated cells can also be taken into consideration. Mesenchymal stem cells (MSCs) are identified as a fibroblast-like population, originally isolated from bone marrow but present in other tissues as skeletal muscle, adipose tissue, cord blood, dental pulp, lung, and liver. Indeed, bone marrow is among tissues severely injured by DOX, which has detrimental effect on local stem cell compartment including bone marrow-derived MSCs. Although MSCs are equipped with efficient enzymatic and non-enzymatic antioxidant mechanisms [101], accumulation of ROS can influence the growth, self-renewal, and differentiation of MSCs [102, 103]. MSCs may respond to excessive oxidative stress undergoing premature senescence with a decreased ability to secrete trophic factors [104, 105], thus having a deep impact on their anti-inflammatory and immunomodulatory properties [106]. MSCs isolated from animals subjected to DOX administration exhibited a lower proliferation rate, had a limitative capacity to respond to cardiomyogenic stimuli and when treated *in vitro* with DOX, experienced premature senescence and reduced clonogenicity [107, 108].

In the diseased heart, the participation of MSCs in the activation of the local repair machinery has been reported, but their ability to differentiate into cardiomyocytes and contribute, in a direct way, to functional recovery has not been conclusively proven [109–112]. The use of MSCs isolated from sources other than bone marrow, (e.g. adipose tissue) is relatively easy and reproducible, making this cell population a valuable tool in regenerative medicine. The transplantation of adipose tissue-derived MSCs was associated with beneficial effects on heart function after experimental myocardial infarction [113, 114], and on vascular system by promoting revascularization and tissue repair in a murine model of hindlimb ischemia [115, 116]. It is evident that a paracrine mode of action represents the main mechanism of action through which MSCs stimulate tissue repair. In fact, MSCs can produce and release a broad variety of cytokines, chemokines, and growth factors serving as supportive signaling for other cells directly involved in the repair of the injured myocardium.

4.5. Endothelial progenitor cells

The discovery that endothelial progenitor cells (EPCs) can home to the site of injury and regulate vascular repair and local angiogenesis has increased the interest in their potential

use for therapeutic application [117]. The concept that cardiovascular homeostasis requires an adequate number of functional EPCs is supported by the correlation between the number of circulating EPCs and cardiovascular events [118]. The capacity of EPCs to promote angiogenesis after a vascular insult is hampered by stress-induced cellular aging processes. DOX has been shown to affect EPC function by increasing oxidative stress and activating senescence pathways via the activation of NADPH oxidase [119]. In addition, subapoptotic dose of DOX accelerated senescent processes of EPCs by regulating p38 and JNK MAPKs and enhancing p16^{INK4a}-dependent signaling [120]. Therefore, ROS accumulation and induction of senescence seem to be key mechanisms implicated in the effects that DOX exerts on EPCs, thus hindering their functional capacity and leading to the failure of EPC-mediated regenerative processes.

5. Conclusion

The growing cardio-oncology discipline detects and examines cardiovascular signals that emerge from cancer therapies powering reverse-translational research. Not surprisingly, the number of molecular mechanisms and cellular phenomena is growing. This new knowledge, resulting from always more in-depth studying of anthracycline cardiotoxicity, creates an opportunity to gain new insights into myocardial biology and to identify new targets that can be valid also in cardiovascular pathologies unrelated to oncologic treatment.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Antonella De Angelis, Donato Cappetta*, Liberato Berrino and Konrad Urbanek

*Address all correspondence to: donato_cappetta@yahoo.it

Department of Experimental Medicine, Section of Pharmacology, University of Campania "Luigi Vanvitelli", Naples, Italy

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