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Research Article

Effect of Doxorubicin on Cardiac Myocytes: Update of the Role of Apoptosis, Autophagy and Other Proteolytic Pathways

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Abstract

Anthracyclines are very effective chemotherapeutic agents. However, their use is limited by serious cardiac side effects, including long-term irreversible and potentially fatal cardiac failure. These effect are related to the cumulative dose but other factors also play a role. Through a systematic search in an electronic database, manuscripts dealing with this matter were included. Exposure of the heart to anthracyclines result in three types of stress: 1) genotoxic, 2) energetic and 3) oxidative stress. These effects are closely related with the dependence of the heart on oxidative phosphorylation, its low defenses against reactive oxygen species and with the peculiarities of mitochondria, which are abundantly present in cardiac tissue. In most manuscripts, the cellular effects of anthracyclines are documented by changes in enzymatic pathways within cardiomyocytes. These pathways have mutual influences, which complicates the interpretation of results. These pathways often result in proteolysis, cellular damage and apoptotic cell death. Necrosis and autophagy are also involved as mechanisms. Most of the included manuscripts deal with in-vitro experiments with anti-oxidant agents, using cardiomyoblasts and H9c2 cell lines as well as in-vivo short-term murine models. However, long-term animal models are lacking. Clinical articles and experiments with cells other than cardiomyocytes are few. The effects of anthracyclines on the extracellular matrix also needs further exploration. These areas need further exploration.

Keywords

Anthracyclines; Apoptosis; Autophagy; Energetic stress; Genotoxic stress; Mitochondria; Reactive oxygen species

Abbreviations

ADP	:	Adenosine Diphosphate
AMP	:	Adenosine Monophosphate
AMPK	:	AMP-Activated Protein Kinase
ATP	:	Adenosine Triphosphate
Bc-L	:	B-cell Lymphoma
BCRAgene	:	Breast Cancer Gene
BNP	:	B-type Natriuretic Peptide
CHF	:	Congestive Heart Failure
CMP	:	Cardio Myopathy
DNA	:	Deoxyribo Nucleic Acid
ECM	:	Extra Cellular Matrix
GSH	:	Glutathione
JNK	:	c-JunN-terminalkinase
LV	:	Left Ventricle
LVEF	:	Left Ventricular Ejection Fraction
MAPK	:	Mitogen-Activated Protein Kinase
MDR1	:	Multi Drug Resistance Protein-1
MMP	:	Matrix Metalloproteinase

mTOR	:	Mammalian Target Ofrapamycin
NADPH	:	Nicotinamide Adenine Dinucleotidephosphate
ROS	:	Reactive Oxygen Species
PI3	:	Phosphatidyl-Inositl-3-kinase
PKB	:	Protein KinaseB
PPAR	:	Peroxisome Proliferator-Activate Receptor
TCL1A	:	T-cell Lymphoma1A
TGF	:	Transforming Growth Factor
TIMP	:	Tissue Inhibitor of MMP
UPP	:	Ubiquitin Proteasome Pathway

Introduction

Anthracyclines have been introduced from 1960's [1] as an effective treatment against several types of cancer. These "cell-cycle non-specific agents" are derived from Streptomyces bacteria [2] and are very effective. Their use, however, is limited by their short and long-term cardio-toxic effect which ranges from asymptomatic cardiac changes such as reduced Left Ventricular (LV) mass, diastolic and systolic dysfunction, abnormal regional wall contractility to cardiomyopathy, overt heart failure and even cardiogenic shock [3-7]. There is a distinction between acute, early onset and late onset anthracycline toxicity [1,8]. The acute toxicity manifests itself as hypotension, tachycardia and arrhythmias [9]. It appears immediately after treatment and disappears after the discontinuation of the treatment. In 1973, a high lethality of Congestive Heart Failure (CHF) was observed. This was refractory to every treatment available at that time. A maximal dose of 550 mg/m² was proposed to avoid such calamities. This event could be reversible [1]. The early-onset cardiotoxicity can appear in the first year after treatment with anthracycline; it presents itself as Chronic Dilated Cardiomyopathy (CMP). The late-onset cardiotoxicity develops after a period of seemingly normal Left Ventricular Function (LVF), with a latency period which can last several years. Echocardiographic abnormalities have been found in 65% of the patients [1]. LV dysfunction evolves to an irreversible CHF, with arrhythmias and fibrosis. This is clinically most relevant [9]. Cumulative dose, irradiation and length of follow-up had a significant effect [10]. These events can are dose dependent, cumulative and irreversible [11]. Its mechanisms are poorly understood. In an old retrospective series, prevalence of clinical heart failure was 2%with a mortality of 71% [12]. LV dysfunction at 10 years was detected in 18% in patients who received less than 500 mg/m² doxorubicin; this was 63% in patients who received more than 500 mg/m² [10]. High age and prior cardiovascular morbidity will increase these events. Nowadays, the medical treatment and the follow-up have improved considerably. One-third of the patients, however, do not recover from a reduction of Left Ventricular Ejection Fraction [LVEF] after treatment with anthracyclines [13]. The reason for this irreversibility in long-term cardiac dysfunction is cell death, either by apoptosis or by necrosis [1]. These events are responsible for the increase in need for heart transplantation as definitive treatment for end-stage heart failure in long-term cancer survivors [14].

The risk for adverse cardiac events increases with the cumulative dose [15], starting from histopathologic changes at 240-250mg/m² [1], and a sharp increase of CHF from 550mg/m² [1], with 3 times increase in cardiac mortality after 20 to 30 years [16]. Remarkably, some patients who received more than 1000 mg/m², however, did not develop CMP [15]. This indicates that unknown factors are involved, which could include differences in metabolism or differences in sensitivity to Reactive Oxygen Species (ROS). It is also possible that events in other cells than cardiomyocytes, present within the heart need to be investigated [16]. Moreover, age below 4 years or over 65 years, hypertension, coronary artery disease, prior mediastinal irradiation, concomitant treatment with trastuzumab or paclitaxel and genetic predisposition increases the risk for cardiotoxicity [1,17]. The early recognized actions of anthracyclines are attributed to inhibition of synthesis of DNA and RNA by intercalation of base pair, inhibition of topo-isomerase II enzyme preventing transcription and replication of genetic information [18] and inhibition of the ligase enzyme to repair DNA, resulting in fragmentation of DNA [19]. Amore recently postulated mechanism is eviction of histones [20]. The aim of this review is to describe the more recently observed cardiac specific events after administration of anthracyclines.

Methods

Search through Web of Science, from 2011 to 2016 resulted for the search terms (autophagy AND anthracyclines AND heart) in 5 articles. The search terms (programmed cell death AND anthracyclines AND heart / cardiac / myocard*) led to one additional article while (apoptosis AND anthracyclines AND heart) resulted in 42 manuscripts. Secondary literature was included if historically relevant. The search was focused on the effect on cardiomyocytes in vitro as well as in vivo studies, not on arrhythmias, ischemia or pericarditis. In vivo as well as in vitro studies were included.

Results

Peculiarities of the heart, of mitochondria and of mitochondrial DNA (Table 1) are of major importance in understanding the effects of anthracyclines. The included studies showed that administration of doxorubicin results in a dose dependent reduction of the weight of the heart, with edema, disorganization of myofibrils, formation of vacuoles, lymphocytic infiltration and increase of fibroblasts and collagen, as well as LV(Left Ventricle) failure, with reduction of LVEF and of fractional shortening [21]. There is an effect on the cytoskeletal apparatus: 1) an impaired actin-myosin interaction [22] 2) a disruption of the formation of desmin which acts as a regulator of sarcomeres [23] resulting in a disorganization of these sarcomeres [24], and 3) a reduction of dystrophin, resulting in an increased permeability of the sarcolemma [21].

The Heart	Mitochondria
High volume % of mitochondria (45%) with heavy reliance on oxidative phosphorylation	High amount of cardiolipine in the inner membrane with high affinity for doxorubicin
Low in catalase and dismutase	Mitochondrial DNA without histone
Small pools of ATP, which can be replenished by phosphocreatine	Close proximity of mitochondrial DNA to respiratory enzymes
	Lack of reparative systems for mitochondrial DNA
	No histones in mitochondrial DNA with consequently accumulation of oxidative damage in comparison to nuclear DNA
	Variable copies of mitochondrial DNA, depending on the cell type and developmental stage

Table 1: Peculiarities of the heart and of mitochondria through which anthracyclines exert their cardiotoxic effects.

From these studies, it becomes also evident that mitochondria are a main target of anthracyclines. Since mitochondria make up to 45% of the volume in myocardial tissues [6,7], the heart is very vulnerable to anthracyclines. Moreover, doxorubicin concentration in mitochondria is 100 times higher compared to plasma [25,26]. Many of these effects can be related to ROS, such as hydrogen peroxide, hydroxyl radicals and superoxide. Mitochondria are the main generators but also the targets of ROS. These organelles are involved in the signaling and buffering of calcium of the cytosol which has an importance of apoptosis. Apoptosis can be the result of mitochondrial damage [6]. Mitochondria have some peculiarities. Cardiolipin is a complex four-tail lipid structure which constitutes 20-45% of the inner mitochondrial membrane. It serves as a proton trap and as cofactor for the mitochondrial respiratory enzymes. It plays a role in the coupling of the respiratory chain and phosphorylation [6,7,9].

Mechanisms and reasons for mitochondrial cardiotoxicity of anthracyclines

The classic mechanisms of doxorubicin have been explored for some time. There are several levels through which doxorubicin acts [16,27]. These include 1) energy stress, 2) oxidative stress and 3) genotoxic stress. It should be noted that these levels show mutual interaction which complicates their representation. Moreover, some other organelles are involved. These types of stress result in loss of cellular components and in cell death. The more important are ubiquitin proteasome proteolysis, apoptosis, autophagy, and some intermediary modes of cellular demise. These are summarized in Table 2.

Energy stress

An increase in adenosine monophosphate/adenosine triphosphate (AMP/ATP) ratio indicates a loss of ATP and is observed in an in vitro model [6,27]. In case of high energy demand, the small pool of ATP in cardiomyocytes can be replenished by phosphocreatine. Creatine kinase act as modulator of the energy reservoir but this can be damaged by ROS, thereby disrupting energy homeostasis [7,28]. As adaptation to decreased ATP output in doxorubicin-toxicity, cardiomyocytes switch over from fatty acid to glucose as substrate [7,29,30]. However, phosphofructokinase and the expression of Peroxisome Proliferator-Activate Receptor (PPAR)-gamma are also affected by doxorubicin. This impairs the uptake of glucose and makes matters worse [31]. The heart is less resistant to additional energy challenges in presence of growth stimulating signals. As compensation for the inability of AMP-activated protein kinase (AMPK) to fulfill its role in energy homeostasis, the creatine kinase energy buffer and transfer system has been upregulated in the acute and subacute setting [27]. In an combined murine in-vivo and in-vitro (i.e. the validated H9c2 cell line of rat cardiomyoblasts) model, the effect of doxorubicin on the pathways of PPAR alpha–PGC-1alpha co-activator were investigated using PPAR inhibitors and activators. Peroxisome proliferator-activated receptor-gamma coactivator (PCG-1alpha) plays a role in the biogenesis of mitochondria making muscles more dependent on oxidative metabolism and less on glycolysis. In the doxorubicin group, PPAR/PGC was lower, with mitochondrial dysfunction and low ATP, and reduced mitochondrial membrane potential. Glucose and free fatty acid consumption had increased. More ROS was present, as was also apoptosis. The PPAR/PGC-1alpha pathways are involved in energy metabolism and in apoptosis in cardiomyocytes [32]. The cardiac function can acutely be impaired by the energy stress [7,28].

Oxidative stress

Doxorubicin has a high affinity to cardiolipin, which is located within the inner mitochondrial membrane, and binds irreversibly to it [1,6,7,33,34]. It places doxorubicin close to the electron-transport chain, permitting formation of ROS, causing damage throughout the cytosol, the mitochondria and other subcellular components [35]. Protein oxidation is mainly expressed as carbonyl formation in the subacute model at 6 weeks [27]. ROS persists for 5 weeks after discontinuation of anthracyclines [16]. The Rac1 component of Nicotinamide Adenine Dinucleotide Phosphate(NADPH) oxidase plays an important damaging [16] role in the formation of ROS: its inhibition mitigated ROS production and hence apoptosis, while overexpression of Rac1 worsened apoptosis. Inhibition of Rac1 preserves the histone HA2X deacetylase activity and prevents activation of p53. Scavenging ROS did not change the histone deacetylase H2AX activity, neither was activation of p53 altered. This indicates that Rac1 mediated cardiotoxicity

Energy stress	Oxidative stress	Genotoxic stress	Other organelles, with increase in the calcium load of the cytosol	Apoptosis
Peroxisome proliferator-activated receptor-alpha (PPAR)	NADPH oxidase (with Rac1 component as essential part)	Inhibition of topo-isomerase II	Interaction with ryanodine receptors of the endoplasmatic	Upregulation of the extracellular signal-regulated kinase (ERK1/2) pathway
Peroxisome proliferator-activated receptor receptor-gamma	NADH dehydrogenase	Inhibition of RNA polymerase	Activation of the non-lysosomial protease calpain	Activation of the pro-apoptotic c-Jun N-terminal kinase (JNK) pathway
AMP-activated protein kinase (AMPK)	Histone HA2X deacetylase	Activation of Akt / Protein kinase B with T-cell lymphoma 1A as cofactor	Stimulation of Ca/calmodulin protein kinase	Inactivation of phosphatidyl-inositl-3-kinase (PI3/Akt or protein kinase B) pathway
Damaging creatine kinase	Cytochrome c reductase	Inhibition of AMPK – related to ATP/AMP ratio		Inactivation of neuregulin
Failure of the respiratory chain	Acetylation of p53	Activation of Mitogen-activated protein kinase (MAPK)		Downregulation of GATA4, which is responsible for the embryonic cardiac development
	Endothelial nitric oxide synthase			BcL-2, p53, Bak/Bax and cytochrome c and activation of caspase-3
	Extraction of iron from ferritin with subsequent complexation			
	Inactivation of glutathione peroxidase			
	Decrease of Cu-Zn superoxide dismutase			
	Clearance of catalase by increased autophagy			
	MDR1 or multi drug resistance protein-1 as efflux pump			

Table 2: Enzymes and pathways involved in energy, oxidative and genotoxic stress as well as apoptosis after administration of anthracyclines.

involves both ROS dependent and independent pathways [36]. ROS can damage creatine kinase and contribute to the energy stress. Increase in production of ROS [6,37] by doxorubicin occurs by utilizing NADH dehydrogenase/cytochrome c reductase and endothelial nitric oxide synthase [38] and by complexing iron, which is extracted from ferritin [21,39-43]. Additionally, doxorubicin releases iron from its stores [9] and complexes this metal. This catalyzes hydrogen peroxide into hydroxyl radicals, adding to oxidative stress [1]. The reduction of doxorubicin, by adding one electron to the quinone moiety of the C-ring leads to formation of semiquinone which can react with molecular oxygen. By formation of superoxide, radicals can be formed by redox cycling, whereby the quinone structure has regenerated [1,44]. This leads to the formation of ROS [45] but also to the abolishment of anti-oxidant defenses such as inactivation of glutathione (GSH-1) peroxidase with a decrease of Cu-Zn superoxide dismutase [7,40,41]. Overexpression of manganese superoxide dismutase alleviates apoptosis [1]. One molecule of doxorubicin leads to many molecules of free radicals through redox cycling! The respiratory chain starts to fail which contributes to the energy stress [46]. This generation of ROS causes peroxidation of lipids, inflammation and apoptosis [9], which is clinically relevant [16]. The clinical relevance of oxidative stress was documented in women with breast cancer: those who experienced a lowering of LVEF also had elevated levels of plasma byproducts of ROS [17]. The only other clinical article investigated the effect of BRCA 1 and 2 gene mutations on the effects of anthracyclines on the heart in breast cancer patients. Except for higher oophorectomy and early menopause, groups with and without those mutations and without mutations were comparable [47].

Genotoxic stress

Anthracyclines enter cells through passive diffusion and bind to proteasomes [9,48]. This complex is translocated to the nucleus, especially in dividing cells. Anthracyclines dissociate from the proteasomes and binds to DNA by intercalation. This has a DNA adenine methyltransferase effect and results in inhibition of protein synthesis and alteration of many transcriptional factors.Nuclear proteins are displaced and chromatin aggregates. Topoisomerase II and RNA polymerase are inhibited [9]. Lesions in nuclear and mitochondrial DNA are found during the acute in-vitro experiments, resulting in apoptosis. DNA-dependent protein kinase is a crucial component in the DNA repair machinery. It signals to Akt/PKB (protein kinase B) which promotes survival after genotoxic stress. At the same time AMPK is inhibited [27]. DNA damage does occur by breaks of DNA strands. Histone eviction caused by anthracyclines occurs irrespective from these breaks. The H2AX histone variant, which is a key component in the reaction to DNA damage is also evicted. This eviction is responsible for a weakened repair of DNA [20]. As a response to DNA damage, p53 is activated by acetylation and the histone H2AX is phosphorylated [36]. An example of

interrelation between genotoxic, energetic and oxidative stress has been observed in the effect of doxorubicin on AMPK or AMP activated protein kinase [27,36]. AMPK is activated by stress such as lowering of ATP and hence ADP/ATP ratio. ATP generating processes are activated by AMPK in order to restore the energy supply. Doxorubicin can induce "energy stress" in this respect because AMPK is paradoxically suppressed and hence the shortage of ATP remains. There was no evidence for an altered "upstream signaling" by doxorubicin which could account for this effect. Another mechanism by which AMPK is downregulated by doxorubicin might be the cross talk of AMPK with Akt/PKB and with Mitogen-Activated Protein Kinase (MAPK). The activation of Akt pathway is a consequence of genotoxicity, while stimulation of MAPK/ERK (Extracellular Signal-Regulated Kinase) is due to an increase in ROS [1,27]. This contains some cytoprotective elements and could counteract apoptosis caused by genotoxicity [27]. Remarkably, defects in DNA repairing enzymes such as polymerases seem to protect against doxorubicin toxicity. However, defects in breast cancer-2 (BCRA2) gene exaggerates apoptosis and CHF. Cellular DNA reparative mechanisms are of paramount importance but defects in some reparative pathways results in enhanced sensitivity to anthracyclines while other defects have a protective effect [9].

Effect on other organelles than mitochondria

Doxorubicin also interferes with ryanodine receptors/ion channels of the sarcoplasmatic reticulum which are responsible for the release of calcium ions during the excitation-contraction coupling and the buffering of cytosolic calcium [49]. Hence, the calcium homeostasis is disturbed. The cytosolic calcium content increases. The protein folding capacity of the endoplasmatic reticulum alters because the combination of increased ROS and disturbed calcium homeostasis. The accumulation of oxidative damaged and misfolded proteins leads to endoplasmatic reticulum stress. Moreover, the removal of such proteins by the ubiquitin proteasome pathway could also be disturbed. Toxic aggregates contribute to myocardial dysfunction and worsen the effect of ROS [33]. Another effect of the increased load of intracellular calcium is the activation of the non-lysosomial protease calpain which might be responsible for the damage to the myofibrillar proteins, including dystrophin [21]. Anthracyclines also stimulate Ca/calmodulin protein kinase, which results in an increased leak of calcium from the sarcoplasmatic reticulum [16], with an impact on contraction and relaxation of cardiomyocytes [9].

Modes of loss of cellular components and of cell death

Doxorubicin modulates p53 activity [9], which causes a series of events, including a decrease in membrane potential [6]. The mitochondrial transition pore opens [6,7] with release of cytochrome C [1,50] as trigger for apoptosis [25,27,51]. Apoptosis is also enhanced by the upregulation of the extracellular signal-regulated kinase (ERK1/2) pathway [52,53]. Activation of the pro-apoptotic c-Jun N-terminal kinase (JNK) pathway with inactivation of the anti-apoptotic phosphatidyl-inositol-3-kinase (PI3/Akt or protein kinase B) pathway by daunorubicin has been observed in an in-vitro rodent model. However, JNK and Akt pathways show "cross-talk" which can complicate their actions. Anyway, activation of PI3/Akt pathway could make cells resistant to chemotherapy, while down regulation of Akt could make tumor cells more sensitive. B-cell lymphoma-2 (BcL-2) and BcL-xL are initially up regulated after exposure to doxorubicin, but this followed by a decrease. However, treatment with anti-oxidants improves the LV function through increase in Akt, Bcl-2 and decrease of caspase activity [1]. Neuregulin, which is a pro-survival factor, plays a role herein and protects against apoptosis [1,16]. For cardiac stem cell survival, a combination of chemotherapeutic agents may be promising [54]. However, in an acute setting – a perfused isolated heart [27], ERK as survival promoting kinase was activated and apoptosis was low, while in the subacute model in living animals with 4 weeks after last injection, the pro-apoptotic JNK was activated. Cardiomyocytes are relatively resistant to apoptosis, but when it occurs, it contributes heavily to heart failure. Upregulation of Akt/PKB preserves viability of the cells and is cardioprotective in doxorubicin treatment [27]. Anthracyclines trigger poly(ADP-ribosyl)ation of p53, which in turn serves as a mechanism for p53 nuclear accumulation. Consistently, daunorubicinpromotes increased nuclear expression of p53, without changing total cellular p53 expression. This anthracycline-induced nuclear presence of p53 is regulated through redox-dependent mechanisms. GATA4 is a cardiomyocyte survival factor and regulates many cardiac genes including the embryonic cardiac development. Its downregulation plays a role herein [16,36,55].If anti-oxidant defenses are overwhelmed, the mitochondrial inner membrane permeability transitional pore opening allows an influx of cytosolic solutes, causing a mitochondrial swelling, degeneration and cardiomyocyte necrosis [9]

Mitochondrial fission produces small rounded mitochondria and is as a sign of stress. This fission has been associated with the production of ROS and with autophagy of mitochondria [56]. An increase in autophagy flux with an increase in accumulation of autophagosomes was observed in cultured rat cardiomyocytes. It seems that autophagy is initially upregulated as compensation for cytotoxic stress [57] by activating Bcl-2 and the autophagy related genes and recruitment of light-chain-3 [46]. But this is followed by apoptosis and necrosis after longer exposure to anthracyclines. The protein degradation machinery is downregulated leading to an accumulation of poly-ubiquinated proteins and autophagosomes. This accelerated aging of cardiomyocytes could make them more susceptible for exposure to anthracyclines [57]. Mitochondrial biogenesis is suppressed as a chronic consequence of exposure to anthracyclines [58].

Autophagy acts as a double edged sword. On the one hand, it serves as a clearance and renewal mechanism and could reduce cell death, when it inhibits apoptosis. This has been observed in in-vitro as well as in-vivo murine models: production of ROS and apoptosis were mitigated by stimulation of autophagy and the morphology and function of mitochondria improved. On the other hand, inhibition of autophagy by bafilomycin-1 results in mitochondrial fission and cell death [59]. Moreover, autophagy could lead to cell death in chronic doxorubicin toxicity, by allowing or acting synergistically with apoptosis [59-61] with ensuing loss of cardiomyocytes and cardiomyopathy. This could depend on the cell type in use (neonatal versus adult, the concentration) and the duration of treatment with doxorubicin. Inhibition of autophagy or activating protein synthesis through mammalian target of rapamycin (mTOR), Akt/PKB and ERK1/2 pathways seems to restore the expression of contractile proteins [62]. The interplay between autophagy and apoptosis is documented by the fact that in acute doxorubicin toxicity, p53 inhibits mTOR. Ablation of p53, however, is not sufficient to reduce cardiac dysfunction.The functional and structural integrity of the cardiac sarcomere requires a tight balance between protein synthesis and protein degradation by autophagy and UPP. Between both systems, a cross-talk exists [59]. It should be noted that cell death with autophagy is not the same as cell death by autophagy. But over-activation of autophagy can lead to cell death with other characteristics such as an increase in autolysosomes, typical nuclear changes and dilated endoplasmatic reticulum. A new term, "autosis" has been coined [63]. Moreover, other types of cell death such as necroptosis by doxorubicin also has been observed. This is caused by damage to DNA, increase ROS production followed by depletion of ATP and cell death, independent from caspase. Both apoptosis and necrosis have been observed using anthracyclines [54]. Autophagy could remove catalase which make increased autophagy responsible for increase in ROS [46,64]. Autophagy is also sensitive to ROS, with an increase in endogenous superoxide dismutase, catalase and glutathione peroxidase. These enzymes might have an effect on autophagy [46].

One of the few clinical/epidemiologic study investigated the difference in genome wide transcript between women who had a LVEF below versus above 40%. Especially TCL1A or T-cell lymphoma 1A (a co-activator of the Akt pro-survival factor) and an efflux pump of anthracycline the MDR1 or multi drug resistance protein-1 were involved. Inhibition of the latter in an in-vitro experiment made the heart more susceptibleto anthracyclines. TCL1A reduction causes increased sensitivity to apoptosis and leads also to a reduced level of Multi Drug Resistance Protein-1 (MDR1) which enhances cardiac levels of anthracyclines [17]. The Akt pathway as well as the role of MDR1 has been established in a clinical article where breast cancer patients were treated with anthracyclines: women who experienced a reduction of LVEF below 40% were compared with those without such changes according RNA transcripts in plasma: those with a decreased LVEF showed also a lowering for MDR1 and for a co-activator of Akt transcripts [17].

The Ubiquitin Proteasome Pathway (UPP) is activated by doxorubicin [33,65], with an increase in 20S proteasomes, E3 ubiquitin ligases and hence increased degradation of proteins [16]. Ischemia and other cardiac disorders can damage this pathway, adding to the effects of chemotherapy [59]. The in-vivo tumor bearing murine as well as in-vitro rat cardiomyoblasts show that myofibrils become less organized after exposure to doxorubicin because of their targeting and subsequent breakdown by UPP [33]. Actin becomes disorganized and depolymerized. Absence of dystrophin increases the sensitivity of the heart to anthracyclines [16]. In lower dosage, UPP was upregulated [33,46,53]. These seemingly contradictory events depend on the amount of doxorubicin molecules bound on the proteasome [48].

Effect of anthracyclines on other cells and on the extracellular matrix (ECM)

The effect of anthracyclines have mostly been investigated in cardiomyoblasts and cardiomyocytes. Their effect in other cells such as endothelial cells, cardiac stem cells and the cardiac extracellular matrix have been investigated in lesser degree. The undifferentiated cardiac stem cells are supposed to be more vulnerable but results seem to suggest that in differentiated cells, upregulation of oxidative phosphorylation is observed, with a compensatory increase in super oxide dismutase. The higher degree of reliance of mature cells on mitochondrial metabolism could be related to the toxicity induced by doxorubicin [8]. Nevertheless, cardiac stem cells are a target of doxorubicin [54] and a therapeutic goal is their protection. Their elimination by anthracyclines could be held responsible for long-term CHF. Shortening of telomeres and premature expression of p16 could account for senescence. Abnormalities were also observed in endothelial progenitor cells [16]. Anthracyclines activate the nuclear factor kappa-B (NFkB) factor in endothelial cells, which results in apoptosis. Decreased plasma levels of endothelin-1 can be found after exposure which correlates with a decreased cardiac function. In cancer survivors, there is an increase in vascular inflammation, vascular wall stiffness and of atheromatosis. These are long-term effects. Erythropoietin derivatives restore the cardiac microvasculature and endothelial differentiation. One needs to take into account that acute effects in the laboratory differs from the mid and long-term effects on the heart, which are clinically more relevant [16].

There is a role of elevated Transforming Growth Factor- Beta (TGF-beta) in patients with heart failure: this factor remodels the myocardium by fibrosis. Inhibition of the TGF-β pathway alleviates the detrimental effects of doxorubicin on endothelial cells in vivo. The inhibition of (TGF-β) pathway seems a valid strategy for preserving and even enhancing of myocardial capillary networksas well asprevention of microvascular remodeling [66]. Cardiomyocytes and coronary

capillaries are tethered in a network consisting of collagen, proteoglycans, glycoproteins and glycosaminoglycans. This network is maintained and balanced by fibroblasts. This network has several functions. It provides a scaffold for myofiber alignment, prevents overstretching of sarcomeres, transmits and coordinates forces in cardiac tissue. This network is also involved in vasomotor reactivity of the myocardial microcirculation, and controls the release of cytokines. When the synthesis of the ECM exceeds degradation, fibrosis is the result. In the case of the opposite, this scaffold will disrupt. Anthracyclines have acute and chronic effect on the regulation of the ECM [66,67]. The acute effects include upregulation of matrix metalloproteinase MMP-9 (through p38/MAPK), of matrix metalloproteinase MMP-2 (through c-Jun N-terminal Kinase / NADPH oxidase) and of MMP-1 and MT1-MMP.The ECM components such as fibronectin, tubulin, myosin-light chain kinase decrease, which results in fibrosis, vacuolization and loss of cardiomyocytes. The actin cytoskeleton reorganizes whereby cells shrink and detach because the interface between these cells and the ECM is negatively affected. Through the loss of extracellular superoxide dismutase, endothelial cells become damaged and fibroblasts proliferate [68]. The chronic effects are consequences of intracellular changes of activity of NADPH oxidase and of nitrox oxide synthase 2 which leads to apoptosis and fibrosis. The increase in MMP-2 persists and tissue inhibitor of MMP (TIMP-3) reduces. The ECM-preservingcomponentthrombospondin-2 is absent. This is associated to impaired Akt signaling pathway, because the feedback loop between thrombospondin and Akt is disturbed. Cathepsins and cystatins are in close balance in healthy tissues. An increase of Cystatin C correlates with inhibition of cathepsin Bwhich is followed by an accumulation of collagen. Chemotaxis of inflammatory cells results in a wound healing response: necrotic cells and collagen debris are removed. This degradative phase ends after one week, when TIMP are upregulated. Inflammatory cells and myofibroblasts generate TGF-beta1 which deposits collagen and inhibits matrix degeneration. This results in interstitial fibrosis, scarring and extensive cardiac ECM remodeling [69].

There is one interesting acute animal study with pregnant rats. Study of fetal and maternal cardiac tissue showed, after administration of a high dose doxorubicin, unaltered fetal hearts. In contrast, in maternal hearts there was obvious loss of body weight anda reduction of LVF. Apoptosisand decreased DNA turnover was also documented. The fetal plasma concentration of doxorubicin reached about 6% of that in the maternal circulation. Plasma BNP was lowered by doxorubicin, while in fetal circulation, BNP was higher which could be considered as counter-regulatory and protective. It seems that fetal hearts are protected. Fetal growth restriction has been observed in clinical conditions, but this could be explained by indirect mechanisms such as maternal malnutrition and placental dysfunction [70]. These data might be useful for young women who develop a malignancy during pregnancy. The pool of fetal cardiac stem cells, which are potential targets of anthracyclines is protected by the placenta. Heart failure of the newborn, after delivery is not to be expected.

Testosterone had a protective effect against doxorubicin in rat cardiomyoblasts by counteracting senescence, which might be important for gender differences in cardiotoxicity of cancer treatment in young patients. The mechanisms involved were the androgen receptor, phosphatityl-inositol-3 kinase, p53, phosphorylation of Akt and phosphorylation of nitric oxide synthase 3 [71]. These results were not confirmed by an in vivo adult rat model studying the effect of gender on doxorubicin toxicity: signs of cardiomyopathy and mortality were higher in male rats compared to female animals. Although neither the oxidation levels nor the apoptosis signaling pathways were altered by doxorubicin, the level of total AMPK was decreased in male rats, together with the markers of mitochondrial biogenesis and cardiolipin content [72].

Cardio-protective measures

Several attempts to reduce the cardiotoxicity of anthracyclines have been developed. Only few of them proved to be clinically significant. Dexrazoxane has been used as chelator of iron [1,4,16,73,74]. Liposomal and hyaluronic acid preparations of anthracyclines lower the delivery of the agent to the heart [1,9,42]. Polyethylene glycol (PEG)ylated liposomal anthracycline showed a reduced cardiotoxic effect with less ROS and apoptosis and improved cardiac function [16,75]. Endurance exercise also has a protective effect by inhibiting autophagy. Mitochondrial adaptations could make cells more resilient to ROS, proteolysis and apoptosis [27,37].

Cardio-protective agents such as antioxidants, scavengers of radicals, with anti-apoptotic or anti-inflammatory properties are less convincing and probably clinically less effective. These agents include co-enzyme Q, vitamins C and E, riboflavin, the flavonoid quercetin [76], carvacrol [34], tanshinone 2A [11,77], arjunolic acid [78], and nutrients such as carnitine, thiamine, vitamin D, folic acid and omega-3 fatty acids [6,38]. Melatonin has also been investigated thoroughly [7]. Vitamin E is clinically ineffective as anti-oxidant [1]. Some substances such as safranal with corcins activated mTOR, thereby inhibiting autophagy but also apoptosis with consequently preserving contractile proteins [79]. Some substances such as carnitine improved also the anti-apoptotic status, throughthe generation of prostacyclin,the enhancement ofAkt phosphorylation and signaling [11], as well as through catalase and superoxide dismutase [38]. Docosahexaenoic acid, combined with epirubicin, on the other hand resulted in more oxidized lipids in tumor cells, but not in healthy tissues [80]. Moreover, curcumin, which is also an anti-oxidant and anti-inflammatory agent increased ROS and resulted in a depletion of glutathione during treatment with doxorubicin. This agent did not protect against apoptosis [81]. It seems that not all anti-oxidant agents have a protective effect during administration of anthracyclines.

Tadalafilis a potent long-acting selective inhibitor of cyclic guanosine monophosphate or cGMP-specific phosphodiesterase-5 and has known beneficial effects on acute myocardial infarction. cGMP takes part in regulating apoptosis. Simultaneous administration of tadalafil and doxorubicin in an in-vivo murine model resulted functional, biochemical, and histological improvement on the heart [82]. The same observation could be made with nicorandil, which is a stimulator of cGMP formation and decreases the calcium sensitivity of smooth muscle. It activates the sarcolemma pump to remove calcium and promotes K afflux. This induceshyperpolarization and hence vasodilation. Nicorandilinhibitsoxidative stress-induced myocyte apoptosis through the opening of mitochondrial K-ATP channels which can preserve mitochondrial energy production. The production of ROS has also been reduced and the ultrastructural changes have been mitigated. The ATP/ADP ratio has been preserved.The anti-tumor activity of doxorubicin remained unaltered [35]. Calcium antagonists such as amlodipine and felodipine also have beneficial effects. The latter has anti-oxidant properties which mitigates ultrastructural changes and cardiac damage [44]. Verapamil, another calcium antagonist, however, increases the susceptibility by inhibiting the multidrug resistance protein-I MDR1 [17]. Other agents such as angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, iron chelators, statins, and metformin are also subjected to research efforts [1,9,13].

These tests were mostly performed in short-term animal models or in in-vitro H9c2 rat cardiomyocytes. Although this cell line has been validated, their results and those of in vivo animal models are not always reproducible with humans. This might be due to the fact that this toxicity not only acts through ROS [59] but has a chronic course. In general, all anti-oxidant treatments need further research since no effect has been observed with certainty. This is also true for anti-inflammatory and anti-apoptotic substances [16]. Micro-RNA have a major role as modulators of gene expression. These small molecules have a great potential as biomarkers in this area. Overexpression of miRNA-30 protects cardiomyocytes from doxorubicin-related damage [83]. The only clinically tested and useful measures are use of dexrazoxane, as chelator of iron, statins, ACE inhibitors, Carvedilol, and exercise [16].

Conclusion

The unravelling of the effects of anthracyclines on the heart is very complex and far from complete. Even the study of gender effects did not show unequivocal results. Most affected pathways show mutual interaction, hence there changes are difficult to interpret. Most in-vivo experiments were acute or short term. Their results do not necessarily predict events in human cardiomyocytes and are not necessarily relevant for long-term outcomes in cancer patients. These long-term events can only be examined by cardiac biopsies, which are hard to come by. Genetic variations in susceptibility need to be taken into account. Examples are the presence or absence of HER-2 mediated receptors and single-nucleotide polymorphisms for NADPH oxidase and efflux transporters. Other cells than cardiomyocytes as well as the extracellular matrix have hardly been investigated. Nevertheless, changes induced by anthracyclines can have a major effect on cardiac function, since there is a close structural relationship between all components of the heart which affects cardiac function. Long-term animal studies are warranted. There are some important limitations: the effects of combined chemotherapeutic regimens and of prior heart disease were not included. Follow-up protocols of patients with anthracycline-induced cardiotoxicity was also considered outside the scope of this manuscript.

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