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Chronic heart damage following doxorubicin treatment is alleviated by lovastatin

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The anticancer efficacy of anthracyclines is limited by cumulative dose-dependent early and delayed cardiotoxicity resulting in congestive heart failure. Mechanisms responsible for anthracycline-induced heart damage are controversially discussed and effective preventive measures are preferable. Here, we analyzed the influence of the lipid lowering drug lovastatin on anthracycline-induced late cardiotoxicity as analyzed three month after treatment of C57BL/6 mice with five low doses of doxorubicin (5×3 mg/kg BW; i.p.). Doxorubicin increased the cardiac mRNA levels of BNP, IL-6 and CTGF, while the expression of ANP remained unchanged. Lovastatin counteracted these persisting cardiac stress responses evoked by the anthracycline. Doxorubicin-induced fibrotic alterations were neither detected by histochemical collagen staining of heart sections nor by analysis of the mRNA expression of collagens. Extensive qRT-PCR-array based analyses revealed a large increase in the mRNA level of heat shock protein Hspa1b in doxorubicin-treated mice, which was mitigated by lovastatin co-treatment. Electron microscopy together with qPCR-based analysis of mitochondrial DNA content indicate that lovastatin attenuates doxorubicin-stimulated hyperproliferation of mitochondria. This was not paralleled by increased expression of oxidative stress responsive genes or senescence-associated proteins. Echocardiographic analyses disclosed that lovastatin protects from the doxorubicin-induced decrease in the left ventricular posterior wall diameter (LVPWD), while constrictions in fractional shortening (FS) and ejection fraction (EF) evoked by doxorubicin were not amended by the statin. Taken together, the data suggest beneficial effects of lovastatin against doxorubicin-induced delayed cardiotoxicity. Clinical studies are preferable to scrutinize the usefulness of statins for the prevention of anthracycline-induced late cardiotoxicity.

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Introduction

The anthracycline derivative doxorubicin (Doxo) is a potent and widely used antineoplastic drug [1]. However, its clinical use is limited by normal tissue damage, in particular cardiotoxicity, which can occur acute or delayed and manifests as congestive heart failure due to cardiomyopathy [2–4]. The molecular mechanisms involved in anthracycline-induced cardiotoxicity remain elusive. The suggested mechanisms comprise generation of cytotoxic peroxynitrite by inducible nitric oxide synthases (iNOS), the induction of reactive oxygen species (ROS), inhibition of membrane-associated ion pumps by anthracycline metabolites (e.g. doxorubicinol) and/or inhibition of topoisomerase II isoforms (topo II) [4–7]. The anticipated relevance of iNOS-mediated increase in peroxynitrite for doxorubicin-induced cardiac damage [7] could not be confirmed

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; Brca, breast cancer associated; BW, body weight; Col, collagenase; Con, control; CTGF, connective tissue growth factor; DAPI, 4′,6-diamidino-2-phenylindole; Doxo, doxorubicin; Gpx1, glutathione peroxidase 1; Gstm1, glutathione S-transferase Mu 1; γH2AX, serine 139 phosphorylated histone 2AX; pH3-S10, serine 10 phosphorylated histone H3; HE, hematoxylin and eosin stain; HO-1, heme oxygenase-1; Hspa1b, heat shock protein A1B; IL-6, interleukin-6, Keap1kelch-like ECH-associated protein 1; Lova, lovastatin; Nfe2l2, nuclear factor (erythroid-derived 2)-like 2; p16INK4a, cyclin-dependent kinase inhibitor 2A; p21(waf1), cyclin-dependent kinase inhibitor 1; Rho, Ras-homologous; ROS, reactive oxygen species; Topo II, topoisomerase type II; Txnrd1, thioredoxin reductase 1.

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in transgenic mouse model. Using iNOS knockouts, mitochondrial damage and cardiotoxicity provoked by doxorubicin were observed to be even enhanced as compared to wildtype controls [8,9]. Moreover, NOS inhibition by N-nitro-L-arginine methyl ester increased the mortality in doxorubicin-treated mice [10]. Therefore (NO independent) alternative cardioprotective strategies for the prevention of anthracycline-induced are preferable and of high clinical importance [11]. Nowadays, the iron chelator dexrazoxane (ICRF-187), which is structurally related to EDTA, is the only clinically approved drug for the prophylaxis of anthracycline-induced cardiac damage [12]. Yet, the molecular effects of dexrazoxane are still unclear [12–14]. For instance, apart form chelating iron, thereby inhibiting ROS formation from Fe-catalyzed redox cycling, dexrazoxane also inhibits topoisomerase II isoforms [15,16], which are major targets of anthracyclines. Moreover, dexrazoxane might have negative effects on the antitumor efficacy of anthracyclines [17,18] and might even increase the risk of secondary tumor formation when used in the therapy of childhood leukemia [19,20]. Therefore, its clinical use was restricted by the FDA in 2011. Bearing this in mind, the development of alternative cardioprotective strategies for anthracycline-based anticancer therapy are inevitable. Off-label use of already approved pharmaceuticals might be preferential for this purpose.

HMG-CoA reductase inhibitors (statins) have pleiotropic biological effects beyond their cholesterol lowering activity [21], which likely rest on the inhibition of the prenylation of Ras-homologous (=Rho) low-molecular weight GTPases [21–23]. Rho GTPases play a key role in the regulation of manifold cellular processes including proliferation, cell shape, adhesion and cell death [24–26]. Correspondingly, statins were reported to increase cell killing induced by anticancer drugs [27,28], impair G1-S transition [29] and trigger apoptosis in tumor cells [23,30–33]. Opposite, i.e. cytoprotective, effects were reported for non-malignant cells. For instance, statins reduce cell death of primary human endothelial cells (HUVECs), rat cardiomyoblasts (H9c2) and smooth muscle cells following treatment with doxorubicin and ionizing radiation *in vitro* [34–36]. *In vivo*, statins mitigate ionizing radiation-induced acute and subacute pro-inflammatory and pro-fibrotic stress responses and protect the intestine from fibrotic tissue remodeling following radiotherapy by interfering with Rho-signaling [37,38]. Moreover, statins protect normal tissue of the heart and liver from anthracycline-induced acute and subacute stress responses and fibrosis while enhancing the anti-neoplastic potential of doxorubicin in a xenograft model [39–42]. The small Rho GTPase Rac1 is the most relevant target for the well known cardioprotective statin effects [43] and appears to be of particular relevance for acute doxorubicin-induced cardiotoxicity, too [39,44].

So far, beneficial effects of statins have only been reported with respect to acute (one to three days) and subacute (up to four weeks) cardiotoxicity following anthracycline exposure [39–41,45]. In general, rather high doses of doxorubicin have been used in these studies, which puts the clinical relevance of the data into question. The dose is of particular relevance, because the molecular mechanisms underlying anthracycline-induced cytotoxicity depend on it [46,47]. Since the pathophysiological mechanisms underlying early and late cardiotoxicity following anthracycline treatment likely differ from each other [48,49], it remains unclear whether statins also affect late cardiotoxicity evoked by anthracyclines.

In our previous study we described beneficial effects of lovastatin on doxorubicin-induced acute and subacute cardiotoxic effects, as analyzed on the level of expression of pro-inflammatory and pro-fibrotic markers [39]. In the present study we now addressed the question whether statins are also favorable for the prophylaxis of late cardiotoxicity occuring three month after administration of multiple clinically relevant low doses (5×3 mg/kg BW) of doxorubicin and, if so, whether the mechanisms involved are similar. Apart from the analysis of molecular surrogate markers of doxorubicin-induced heart damage, which we have identified to be mitigated by co-treatment with lovastatin in our previous study [39], we additionally performed functional analyses and electron microscopic studies now. The data obtained are suggestive that lovastatin has beneficial effects also on late cardiotoxicity evoked by doxorubicin treatment.

Materials and methods

Animal experiments

Mice were bred in the local animal facility of the University Medical Center Mainz (Germany) and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory animals". Euthanasia of mice was performed by exposure to 100% carbon dioxide (CO_2) for 5 min in an appropriate chamber. To investigate chronic (late) cardiotoxicity (i.e. 3 month after first dose of doxorubicin) female C57BL/6 mice (8 weeks old; 20–25 g BW) were used. A total of 30 animals were randomly divided into groups of 6–9 animals per group. To determine the effect of lovastatin on late doxorubicin toxicity, mice were treated three times a week with lovastatin (10 mg/kg BW; alternate i.p. and p.o.). Since it is hardly possible to lower the naturally low blood cholesterol levels of wild-type mice any further, even if very high statins doses are administered (i.e. 50 mg/kg BW, daily) [50,51], we did not monitor blood cholesterol levels in this study. Doxorubicin (5×3 mg/kg BW; i.p) was injected in week 1, 2, 3, 5 and 6 of the experiment. 6 weeks after the last injection (i.e. 3 month after injection of the first dose of doxorubicin) analyses were performed. CO_2 euthanasia was used to sacrifice the mice for tissue isolation and subsequent gene expression and histopathological analyses. Body, heart, liver and kidney weight were determined for the calculation of organ weight/body weight ratios. Organs were immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation or were fixed in formalin and embedded in paraffin for histological analysis. Pieces of the upper middle part of the heart were fixed for electron microscopy (performed at the Institute of Pathology, Mainz) as described below. Blood samples were collected at the end of the experiment from tail vein and via puncture of the heart.

Gene expression analyses (real-time RT-PCR)

Total RNA was purified from 20 to 30 mg of tissue using the RNeasy Mini Kit (Qiagen). The RT reaction was performed with the OmniScript Kit (Qiagen) using 500–2000 ng of RNA. In general, real-time RT-PCR analyses were performed in duplicates with pooled RNA samples isolated from $n = 6–9$ mice per group using a MyIQ Thermal Cycler (BioRad) or CFX96 cycler (BioRad) and the SensiMix SYBR Kit (Bioline, London, UK). Denaturation of cDNA/TAQ-polymerase activation was done at 95 °C for 10 min. 45 amplification cycles were performed (each cycle: 95 °C, 15 s–55 °C, 17 s–72 °C, 17 s). The primers used are listed in supplementary Table I. Apart from the analysis of relative mRNA amounts of factors reflecting inflammation, fibrosis, oxidative stress and heart function, a semi-customized PCR-array was used (established in collaboration with Sigma–Aldrich Chemie GmbH, Steinheim, Germany), which contains 94 selected genes involved in metabolism, DNA repair, stress signaling, cell cycle regulation, cell death and metastasis [52]. At the end of each run, melting curves were analyzed to ensure product specificity. PCR products with threshold cycles (Cq) of ≥ 35 were omitted. mRNA levels were normalized to that of the housekeeping genes GAPDH and β-actin. Gene expression in vehicle-treated control animals was set to 1.0. Only changes in gene expression of ≤ 0.5 and

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