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Identification of novel biomarkers for doxorubicin-induced toxicity in human cardiomyocytes derived from pluripotent stem cells

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ABSTRACT

Doxorubicin is a chemotherapeutic agent indicated for the treatment of a variety of cancer types, including leukaemia, lymphomas, and many solid tumours. The use of doxorubicin is, however, associated with severe cardiotoxicity, often resulting in early discontinuation of the treatment. Importantly, the toxic symptoms can occur several years after the termination of the doxorubicin administration. In this study, the toxic effects of doxorubicin exposure have been investigated in cardiomyocytes derived from human embryonic stem cells (hESC). The cells were exposed to different concentrations of doxorubicin for up to 2 days, followed by a 12 day recovery period. Notably, the cell morphology was altered during drug treatment and the cells showed a reduced contractile ability, most prominent at the highest concentration of doxorubicin at the later time points. A general cytotoxic response measured as Lactate dehydrogenase leakage was observed after 2 days' exposure compared to the vehicle control, but this response was absent during the recovery period. A similar dose-dependant pattern was observed for the release of cardiac specific troponin T (cTnT) after 1 day and 2 days of treatment with doxorubicin. Global transcriptional profiles in the cells revealed clusters of genes that were differentially expressed during doxorubicin exposure, a pattern that in some cases was sustained even throughout the recovery period, suggesting that these genes could be used as sensitive biomarkers for doxorubicin-induced toxicity in human cardiomyocytes. The results from this study show that cTnT release can be used as a measurement of acute cardiotoxicity due to doxorubicin. However, for the late onset of doxorubicin-induced cardiomyopathy, cTnT release might not be the most optimal biomarker. As an alternative, some of the genes that we identified as differentially expressed after doxorubicin exposure could serve as more relevant biomarkers, and may also help to explain the cellular mechanisms behind the late onset apoptosis associated with doxorubicin-induced cardiomyopathy.

1. Introduction

Anthracyclines, such as doxorubicin, are amongst the most successful chemotherapy compounds for the treatment of a wide range of cancers, including hematologic malignancies, soft tissue sarcomas, and solid tumours in both children and adults. Doxorubicin binds to DNA associated enzymes such as topoisomerase I and II, responsible for separating the double strands of DNA during replication (Hilmer et al., 2004). The ability of doxorubicin to kill rapidly dividing cells and in turn slowing disease progression has been acknowledged for over 30 years. However, its toxicity on noncancerous cells, with cardiac toxicity being the most

Abbreviations: cTnT, cardiac specific troponin T; hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; hPSC, human pluripotent stem cells; LDH, lactate dehydrogenase; SAM, statistical analysis of microarray.

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prominent, limits its clinical use (Ferreira et al., 2008; Minotti et al., 2004). Anthracycline-induced cardiotoxicity is exponentially dose-dependant (Carvalho et al., 2009) and categorized as acute, early, or late (Zhang et al., 2009). Acute cardiac toxicity occurs during or immediately after initiation of doxorubicin treatment resulting in tachyarrhythmia's, including sinus tachycardia, premature ventricular contractions, and ventricular tachycardia, as well as bradycardia. Early cardiotoxic events develop within one year of exposure and results in dilated cardiomyopathy. The late cardiac toxicity may develop one or several years after initial exposure, leading to a life-threatening form of cardiomyopathy (Wallace 2003; Yeh et al., 2004). Notably, children and adolescents are particularly susceptible to the cardiotoxic effects of anthracycline chemotherapy compared to adult patients (Lipshultz et al., 1991).

Despite intensive research and progress made over the past two decades, the molecular mechanisms responsible for doxorubicin-induced cardiotoxicity remain incompletely understood. Published reports so far have focused mainly on free radical-induced oxidative stress and apoptosis (Childs et al., 2002; Pointon et al., 2010; Zhang et al., 2012). Cardiac mitochondria are the key mediators of anthracycline-induced cardiomyocyte death (Wallace 2007). Additionally to mitochondrial damage, several signalling pathways are triggered by reactive oxygen species and by anthracyclines causing activation of the intrinsic apoptotic pathway. Apart from the intrinsic mitochondrial apoptotic pathway, anthracyclines also activate the extrinsic apoptotic pathway by several mechanisms contributing to cardiomyocyte damage and death (Nakamura et al., 2000; Nitobe et al., 2003; Niu et al., 2009).

Understanding the mechanisms by which doxorubicin induces cardiac injury is crucial not only to inhibit its cardiotoxic action but also to improve the therapeutic use of doxorubicin. To this end, a number of preclinical models, both long-term and short-term, have been developed in order to predict and understand the cardiac toxicity of doxorubicin and other anthracycline analogues (Herman et al., 1985; Jaenke 1974; Maral et al., 1967; Platel et al., 1999; Pouna et al., 1996). Common for these models is that they all are of non-human origin. However, due to species-related variations in general physiology and drug metabolism, studies in laboratory animals are in many cases of limited value for prediction of the potential toxic effects in humans. To address this issue, Licata et al. developed an *in vitro* human heart system in which cytosolic fractions from myocardial samples disposed during coronary artery bypass surgery were used to study doxorubicin metabolism (Licata et al., 2000). However, human heart tissue samples are usually difficult to source and the material that can be made available is usually derived from non-healthy donors. Thus, the establishment of new human myocardium models is essential in order to develop *in vitro* assays that more accurately can predict cardiac toxicity in patients.

Human pluripotent stem cells (hPSC), of either embryonic origin (hESC) or induced by genetic modification (hiPSC), offer a new approach for generating a variety of cells for *in vitro* models. The ability of unlimited propagation and the potential to differentiate into all cell types in the human body makes hPSC very attractive as a source for human specialized cells (Takahashi et al., 2007; Thomson et al., 1998). The protocols for differentiation of hPSCs into cardiomyocytes have improved substantially in recent years, and today cardiomyocytes with high purity can be derived in large scale and in a reproducible fashion (Burridge et al., 2014; Lian et al., 2012). The fact that these cells can be derived robustly from well-characterized hPSCs makes them well suited to use as a toxicity assessment model, especially since the genetic background of the hPSC can be selected to address a specific disease phenotype (Liang et al., 2013).

In the present study, we evaluate the use of hESC-derived cardiomyocytes as a model to study doxorubicin-induced cardiotoxicity. Human cardiomyocyte cultures were exposed to doxorubicin at various concentrations and the toxic responses in the cells were assessed during the acute exposure (up to 2 days) as well as after an additional 12-day recovery period. The release of lactate dehydrogenase (LDH) and cardiac specific troponin T (cTnT) was measured to assess general cytotoxicity and cardiotoxicity, respectively. In addition, global gene expression analysis was performed to investigate the mechanisms and cellular pathways activated in the cells during and after doxorubicin treatment. The results from this analysis identified several novel potential biomarkers, which can be used with high sensitivity to predict doxorubicin-induced cardiotoxicity.

2. Materials and methods

2.1. Cell culture

Human cardiomyocytes, Cellartis® Pure hES-CM, were obtained from Takara Bio Europe AB (former Cellectis AB, Gothenburg, Sweden) and handled according to the instructions from the manufacturer. The cells were thawed and seeded at 200 000 cells/cm^2 in 24- or 96-well plates and medium change was performed every second day.

2.2. Compound exposure and toxicity evaluation

Four days post-thawing, the cells were incubated with or without doxorubicin (D-1515, Sigma–Aldrich, Sweden) at various concentrations (50 nM, 150 nM, and 450 nM) for up to 2 days, followed by a 12-day wash-out period without drug exposure. The experiments were performed in triplicates. The cell morphology as well as the contractile ability was monitored during the entire experiment. Cells and conditioned cell culture medium were harvested at four different time points (1, 2, 7, and 14 days counted from the start of compound treatment) during the exposure and recovery period (see Fig. 1 for an overview of the study outline). LDH was measured in the cell culture medium using Lactate Dehydrogenase Activity Assay Kit (MAK066, Sigma–Aldrich Sweden) according to the providerís instructions. The cTnT levels in the cell culture medium were measured using an automated

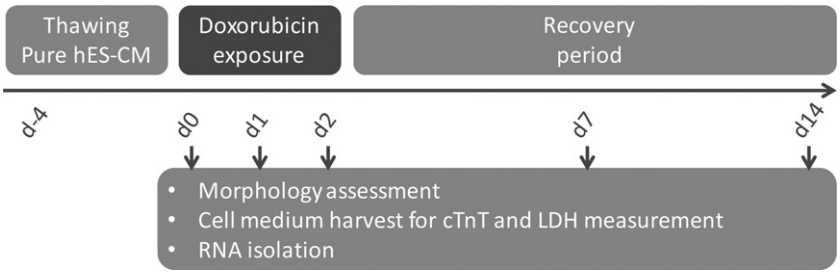

Fig. 1. Study outline. The figure displays a schematic overview of the study outline.