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Review

Patient-specific pluripotent stem cells in doxorubicin cardiotoxicity: A new window into personalized medicine

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ABSTRACT

In the past ten years, there has been a revolution in our ability to generate human pluripotent stem cells (hiPSCs) from adult somatic cells. hiPSCs can be differentiated into many cell types, including cardiomyocytes (hiPSC-CMs), providing cardiovascular scientists for the first time with a human heart muscle cell line. hiPSC-CMs have several potential uses: to study mechanisms of disease, as a platform for screening drugs for efficacy and toxicity, and as cell therapy for diseases such as cardiomyopathy. In this review, we discuss the potential of using hiPSC-CMs for drug toxicity testing, and in particular to screen genetic variants found to be predictive of which patients develop cardiotoxicity after receiving the chemotherapeutic agent doxorubicin.

1. Introduction

The anthracycline doxorubicin is one of the oldest, yet most effective, anti-cancer agents known. Doxorubicin is used in treating a wide range of malignancies and is currently utilized in 50–60% of breast cancer and 70% of childhood cancer treatment protocols. In children, anthracyclines have contributed to the dramatically increased 5-year survival rates for childhood cancer, now greater than 80% [1]. Almost immediately after the use of doxorubicin began, the presence of dose-dependent cardiotoxicity was recognized [2,3]. A review of three anthracycline trials [4] found that the incidence of significant left ventricular dysfunction (reduction in ejection fraction [EF] of >10% below normal) was 16.2%, 32.4%, and 65.4% at cumulative doxorubicin doses of 300, 400, and 550 mg/m^2, respectively. Even at relatively low doses (cumulative doses of 200–250 mg/m^2), the risk of cardiotoxicity was found to be as high as 8–9%.

However, with improved methods of detecting subtle changes in cardiac function [5], the incidence of doxorubicin cardiotoxicity is now thought to be much higher, occurring in up to 65% of long-term survivors of childhood cancer, even at doses as low as 228 mg/m^2 [6–8]. Using echo indices such as wall stress rather than symptoms, 90% of children manifest subtle abnormalities in cardiac function during their first year of doxorubicin therapy [9]. These data also suggest that patients develop subclinical cardiotoxicity rather than true "latency" between drug exposure and onset of symptoms [6]. As many as 16% of children with these abnormalities will develop subsequent clinical heart failure [2,3,10] with a mortality rate of as high as 72% [1,2,10]. The incidence of heart failure in adults receiving anthracyclines appears to be lower than in children, although similar high-sensitivity echo studies (e.g. wall stress) have not been performed in adults. This greater risk for cardiotoxicity in children suggests that the immature heart may be more susceptible to anthracycline damage [5] which may correlate with studies showing increased susceptibility of cardiac progenitor cells to anthracyclines [11,12]. With 1 in 750 adults now being survivors of childhood cancer, this problem represents a major challenge in the newly emerging field of cardio-oncology.

2. Mechanisms of Doxorubicin-induced Cardiotoxicity

Despite more than 50 years of research, the mechanism of doxorubicin cardiotoxicity is still incompletely understood, but can be grouped into three interrelated subgroups: (i) generation of reactive oxygen species (ROS), both dependent and independent of iron; (ii) mitochondrial dysregulation; and (iii) topoisomerase II inhibition causing double stranded break-induced apoptosis and transcriptional modulation of the mitochondrial and nuclear genomes. Several studies have implicated oxidative stress, as evidenced by ROS formation, as a central mechanism of doxorubicin cardiotoxicity [13,14]. Doxorubicin can undergo a one- or two-electron reduction by several oxidoreductases to doxorubicin-

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semiquinone or doxorubicinol [15]. In the presence of iron, re-oxidation of the doxorubicin-semiquinone or doxorubicinol radical back to doxorubicin leads to the formation of O_2^- and H_2O_2. These ROS can establish redox cycling, producing more free radicals. Doxorubicin can also induce Ca^{2+} release from the sarcoplasmic reticulum [16] leading to sarcomeric disarray, myofibril deterioration and, as we have shown, increased likelihood of opening of the mitochondrial permeability transition (MPT) pore [17].

Cardiomyocytes may be uniquely at risk of doxorubicin toxicity due to their high number of mitochondria, representing 30% of cardiomyocyte volume [18]. The majority of ROS producing enzymes such as NAD(P)H oxidase are located in the mitochondria and in the closely adjacent sarcoplasmic reticulum, so the major sites of ROS production are located there. Doxorubicin also nearly irreversibly binds to cardiolipin on the mitochondrial membrane, maintaining localization [19] and also disrupting electron transport chain function. This localized production of ROS induces mitochondrial DNA (mtDNA) depletion [20] and mitochondrial membrane lipid peroxidation, leading to reduced mitochondrial function, reduced ATP production and ultimately apoptosis. Doxorubicin can also induce apoptotic pathways independent of ROS through the caspase-3 pathway [21].

Finally, doxorubicin binds DNA and topoisomerase II to form the ternary doxorubicin–DNA cleavage complex, which triggers cell death. Doxorubicin's effect on topoisomerase was initially felt to be responsible only for its anti-cancer effects (mediated through TOP2α), not its cardiotoxic effects (since myocytes only express TOP2β). However, a recent report shows that doxorubicin cardiotoxicity can be attenuated by cardiac-specific deletion of TOP2β, potentially mediated via altered transcription of PGC-1α and PGC-1β [22].

3. Single Nucleotide Polymorphism (SNP) Association Studies of Doxorubicin Cardiotoxicity

At present, it is not possible to predict clinically which patients will be affected by doxorubicin cardiotoxicity [23], so the standard practice has been to limit the cumulative dose of doxorubicin for all patients, thereby reducing its anti-tumor efficacy. In an attempt to discover if there is a genetic basis for the predilection to doxorubicin cardiotoxicity, several gene variant association studies have been performed. One of the largest to date is a multi-center doxorubicin cardiotoxicity analysis involving children enrolled at 11 sites participating in The Canadian Pharmacogenomics Network for Drug Safety. A discovery cohort of 344 Canadian patients (78 cases and 266 controls), was genotyped for 2977 drug biotransformation (absorption, distribution, metabolism and elimination or ADME) genetic variants [24]. An in-depth clinical characterization of patients was followed by grading of cardiac dysfunction as measured by echocardiogram and/or symptoms requiring intervention. A stringent fractional shortening (FS) threshold of 26% or less after anthracycline therapy was used to define cardiotoxicity, rather than alterations in wall stress. Control patients were defined as those having normal echocardiograms (FS of 30% or greater) for at least 5 years after completion of therapy.

Using this targeted approach, three significantly associated genetic variants were identified in two different genes: the nucleoside/anti-cancer drug transporter *SLC28A3* (rs7853758 and rs885004, which are in high linkage disequilibrium [LD]) and UDP glucuronosyltransferase 1A6 *UGT1A6* (rs17863783). The *SLC28A3* variants, one of which is a synonymous coding variant (L461L), are highly protective against the development of anthracycline-induced cardiotoxicity. In contrast, the highly associated variant in *UGT1A6* (a tag SNP for the reduced enzyme activity *UGT1A6**4 haplotype) significantly enhanced patient susceptibility to anthracycline-induced cardiotoxicity. These associations were then replicated in two additional patient cohorts [24,25]. In the combined cohorts, the associations with *SLC28A3* (rs7853758) and *UGT1A6* (rs17863783) have extremely high odds ratios of 0.36 and 4.30 and extremely low *P*-values of 1.6×10^{-5} and 2.4×10^{-4}, respectively.

SLC28A3, a sodium-coupled nucleoside transporter is broadly selective for both pyrimidines and purines, plays a key role in the cellular uptake of a variety of anti-cancer drugs, and has been implicated in doxorubicin intracellular transport [26]. SLC28A3 is widely expressed in the body, including in cardiac tissue [27]. Although the rs7853758 variant is synonymous at the amino acid level (L461L), it is associated with a 46% decrease in mRNA expression in carriers of the variant ($P = 4.68 \times 10^{-10}$) [28,29]. Several studies provide supportive evidence for the functional importance of rs7853758 or a linked variant in chemotherapy response [30]. There are also several loci in high linkage disequilibrium ($r^2 > 0.8$) with rs7853758, several of which may provide putative mechanisms for influencing doxorubicin cardiotoxicity: rs885004 which resides in a DNase hypersensitive and open chromatin region, with evidence of binding the insulator CTCF and disrupting transcription factor binding sites for NKX6-2; and rs4877835, which is in an open and active chromatin region with evidence of binding the activator protein 1 (AP-1) complex (c-JUN and c-FOS) and is predicted to disrupt binding by the myogenic transcription factor, MYF. Together, these functional annotations of the haplotype at *SLC28A3* suggest that variants in high LD with the variant rs7853758 could explain *cis*-regulatory mechanisms of doxorubicin cardiotoxicity through altered *SLC28A3* gene expression.

To uncover novel genetic associations with anthracycline cardiotoxicity, a GWAS study is in progress, and preliminary results show 25 significant SNPs, of which, only two were located in gene coding regions (*RARG*, encoding the γ retinoic acid receptor and *WDR4*, encoding a WD repeat domain). Further exploration of these two SNPs is currently in progress. Retinoic acid receptors (RARs) act as ligand-dependent transcriptional regulators which activate transcription by binding to retinoic acid response elements (RARE) found in the promoter regions of their target genes. In their unbound form, RARs repress transcription of these target genes. RARγ plays an important role in early cardiac development [31], in stem cell transcriptional regulation [32] and, importantly, in regulation of topoisomerase 2β [33], a recently described regulator of doxorubicin cardiotoxicity [22].

Other doxorubicin cardiotoxicity studies concentrating on smaller numbers of SNPs and, in some, fewer patients (with reduced statistical power) have been completed. Of the larger studies, Blanco et al. performed a 487 patient/two variant study on children with doxorubicin cardiotoxicity and identified a carbonyl reductase 3 (*CBR3*) SNP as a predictor of cardiotoxicity [34]. Carbonyl reductases are involved in the formation of the alcohol metabolite of doxorubicin, doxorubicinol. The in vitro studies of this variant are conflicting, with some showing decreased activity of the variant protein with doxorubicin [35,36] and others showing increased activity using menadione [37]. A second study of SNPs of 82 genes from 1697 patients, 3.2% of whom developed either acute or chronic doxorubicin cardiotoxicity, found five significant associations in the NAD(P)H oxidase complex (*CYBA*, *NCF4*, and *RAC2*) [38]. Consistent with this finding, mice deficient in NAD(P)H oxidase were resistant to doxorubicin cardiotoxicity [38]. An additional 108 patient study showed a similar association [39]. Smaller screens have identified SNPs in *ABCB1* [40], *ABCC2* [41], *NADPH oxidase* [42], catalase (*CAT*) [43], and the hemochromatosis gene *HFE* [41,44] associated with doxorubicin cardiotoxicity.

Although these studies represent a potential major advance in applying a pharmacogenomic approach to the field of cardio-oncology, the true connection between these SNPs and cardiotoxicity is far from proven. Given the difficulty that has been experienced in the past in transitioning GWAS data into clinical practice, it is vital that each of these candidate SNPs be