Short Communication

Expression of the Anthracycline-Metabolizing Enzyme Carbonyl Reductase 1 in Hearts from Donors with Down Syndrome

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ABSTRACT:

Cancer patients with Down syndrome (DS) are susceptible to developing anthracycline-related cardiotoxicity. The pathogenesis of anthracycline-related cardiotoxicity has been linked to the intracardiac synthesis of alcohol metabolites by carbonyl reductase 1 (CBR1). *CBR1* is located in the DS critical region (21q22.12). The expression of *CBR1* in hearts from individuals with DS has not been characterized. This study documented *CBR1* expression in hearts from donors with DS ($n = 4$) and donors without DS ($n = 15$). The DS samples showed 1.8-fold higher *CBR1* mRNA levels compared to the non-DS samples (levels in DS samples were 3.3-relative fold, and those in non-DS were

1.8-relative fold; $p = 0.012$). *CBR1* protein levels were 1.9-fold higher in DS samples than in non-DS samples (13.5 ± 7.7 versus 7.2 ± 3.9 nmol/g cytosolic protein, respectively; $p = 0.029$). *CBR1* activity for daunorubicin was 1.7-fold higher in DS samples than in non-DS samples (3.8 ± 0.1 versus 2.3 ± 0.2 nmol daunol/min · mg, respectively; $p = 0.050$). *CBR1* 1096G>A (rs9024) affects *CBR1* activity, and one heart trisomic for the variant A allele (A/A/A) exhibited low enzymatic activity. These findings suggest that increased *CBR1* expression in the hearts of individuals with DS may contribute to the risk of anthracycline-related cardiotoxicity.

Introduction

Down syndrome (DS) is the most common genetic cause of mental retardation and originates from the presence of an extra copy of chromosome 21, although in roughly 5% of documented DS cases, a partial trisomy leads to the condition (Amano et al., 2004; Patterson, 2009). Children with DS have a 10- to 20-fold increased risk of developing acute myeloid leukemia (AML) compared to sex- and age-matched children without DS. Treatment regimens for pediatric AML rely heavily on the use of the anthracycline daunorubicin. However, the therapeutic benefit of chemotherapy with daunorubicin is mitigated by the development of cardiotoxicity in some patients. Risk factors for anthracycline-related cardiotoxicity include total cumulative exposure, younger age at cancer diagnosis, radiation therapy to the heart region, and female sex. Epidemiological studies have identified pediatric cancer patients with DS as a population at particularly greater risk for both acute and chronic anthracycline-related cardiotoxicity (Krischer et al., 1997; Grenier and Lipshultz, 1998; Ravindranath et al., 2005). For example, Krischer et al. (1997) found that Trisomy 21 was associated with an increase in the relative risk for anthracycline-related cardiomyopathy of 3.4-fold. A report from the Children's Oncology Group documented clinically symptomatic cardiomyopathy in 17.5% of patients with DS-AML who were treated with anthracyclines (O'Brien et al., 2008).

The pathogenesis of anthracycline-related cardiotoxicity has been linked to the synthesis of anthracycline C-13 alcohol metabolites by

cardiac carbonyl reductase 1 (CBR1) activity (Minotti et al., 2004). Transgenic mice overexpressing human *CBR1* in heart exhibited high levels of intracardiac doxorubicinol and increased signs of myocardial damage after doxorubicin administration (Forrest et al., 2000). In contrast, mice with a null *Cbr1* allele (*Cbr1*^{-/-}) had decreased doxorubicinol plasma levels and significantly decreased incidence of anthracycline-related cardiotoxicity compared to mice with two active *Cbr1* alleles (*Cbr1*^{+/+}) (Olson et al., 2003). The *CBR1* gene is located in the DS critical region (21q22.12), and an early report noted that *CBR1* expression is subjected to gene-dosage effects in lymphoid cells with Trisomy 21 (Lemieux et al., 1993). Elevated *CBR1* protein levels have been noted in several regions of the brain in individuals with DS (Balcz et al., 2001). There is a paucity of reports documenting cardiac *CBR1* expression in donors with DS. Thus, the goal of the present study was to examine cardiac *CBR1* expression in DS samples and non-DS samples. *CBR1* mRNA and *CBR1* protein levels were investigated by quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) and quantitative immunoblotting, respectively. Cardiac *CBR1* activity was measured for the substrate daunorubicin.

We recently identified a relatively common single-nucleotide polymorphism in the 3'-untranslated region (UTR) of *CBR1* (minor allele, $q = 12.5\%$ in whites; *CBR1* 1096G>A, rs9024) that affects the synthesis of doxorubicinol in liver. Liver samples with the *CBR1* 1096G>A homozygous G/G genotype exhibited 1.5-fold higher doxorubicinol synthesis rates than samples with the heterozygous G/A genotype (Gonzalez-Covarrubias et al., 2009). Thus, we explored whether *CBR1* 1096G>A genotype status affects *CBR1* expression in hearts from individuals with DS.

Materials and Methods

Heart Samples. The Institutional Review Board of the State University of New York at Buffalo approved this research. Heart samples (20–100 g, left

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ABBREVIATIONS: DS, Down syndrome; CBR1, carbonyl reductase 1; AML, acute myeloid leukemia; UTR, untranslated region; QRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; aCGH, comparative genomic hybridization arrays.

ventricle) from donors with DS ($n = 4$) and donors without DS ($n = 15$) were procured from the National Disease Research Interchange (Philadelphia, PA). DS status (yes/no) was obtained from medical records. Tissue procurement protocols have been open since December 2007 and February 2008 for non-DS samples and DS samples, respectively, and included the following criteria: no current diagnosis of cancer, myocardial infarction, and/or congestive heart failure, no evidence of sepsis, and no history of chemotherapy and/or radiation within the last year. Postmortem to tissue recovery interval was ≤ 10 h. Heart samples were frozen immediately after recovery and stored in liquid nitrogen until further processing. To process heart samples, we followed standardized procedures to isolate RNA and DNA as described previously (Gonzalez-Covarrubias et al., 2009). Cytosols were obtained by differential ultracentrifugation as described previously (Mordente et al., 2003).

Comparative Genomic Hybridization Arrays. Genomic DNA was used to probe high-resolution comparative genomic hybridization arrays (aCGH) as described previously (Snijders et al., 2001; Cowell et al., 2004). Arrays contained ~ 6000 sequenced bacterial artificial chromosome clones, which provided a resolution of 700 kilobases across the genome. Changes in DNA copy number were determined by evaluating $\log_2$ ratios across whole chromosomes.

QRT-PCR. Total RNA from liver (200 ng) was reverse transcribed and amplified by using one-step QuantiTect SYBR Green RT-PCR kits (QIAGEN, Valencia, CA). *CBR1* mRNA levels were determined by the comparative quantitation method as described previously (Gonzalez-Covarrubias et al., 2009). Individual β -actin mRNA levels were used for normalization. Experimental samples and standards for calibration curves were analyzed in quadruplicate. The relative amount of *CBR1* mRNA in each sample was automatically calculated with a comparative quantitation algorithm (iQ5 Optical System Software, version 2.0; Bio-Rad Laboratories, Hercules, CA).

Quantitative Immunoblotting. Cytosolic CBR1 protein levels were quantitated as described previously (Gonzalez-Covarrubias et al., 2009). In brief, cytosols (150 μ g) and recombinant CBR1 standards (0.03, 0.05, 0.10, 0.15, and 0.30 μ g; Abcam Inc., Cambridge, MA) were loaded into 12% protein gels and separated by electrophoresis. Proteins were probed with a specific polyclonal anti-human CBR1 antibody (1:2500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a secondary rabbit anti-IgG antibody conjugated with horseradish peroxidase (1:10,000; Sigma-Aldrich, St. Louis, MO). Immuno-reactive bands were visualized with the ECL Plus Western blotting detection system (GE Healthcare, Chalfont St. Giles, UK). CBR1 band intensity values (pixels/ mm^2) were quantified with a ChemiDoc XRS gel documentation system (Bio-Rad Laboratories). Cardiac CBR1 levels were estimated by extrapolation from the calibration curves of recombinant CBR1. Detection of CBR1 was linear (range 0.03–0.30 μ g; $r^2 > 0.93$).

Kinetic Analysis. Validation experiments with pooled heart cytosols showed that 400 μ M daunorubicin [S] ensured conditions of $V_{\max}$ and/or maximal CBR1 activity (zero-order kinetics). Thus, maximal CBR1 activities (reaction rates) were directly proportional to the amount of cytosolic CBR1 enzyme (Gonzalez-Covarrubias et al., 2009). Maximal CBR1 activities for the substrate daunorubicin were measured in cardiac cytosols essentially as described previously (Gonzalez-Covarrubias et al., 2009). Incubation mixtures (1.0 ml) contained potassium phosphate buffer (0.1 M; pH 7.4), NADPH (200 μ M; Sigma-Aldrich), and daunorubicin (400 μ M; Sigma-Aldrich). Kinetic reactions were started by the addition of cytosols (100 μ l, total protein concentration: 7.0 ± 2.1 mg/ml; range 4.5–11.7 mg/ml). Reaction mixtures were flash frozen with liquid nitrogen and stored at -70°C until daunorubicinol quantification. Daunorubicinol was quantitated with a validated method of ultrahigh pressure liquid chromatography coupled to tandem mass spectrometry as described previously (Gonzalez-Covarrubias et al., 2009).

CBR1 Genotyping. The *CBR1* 1096G>A (rs9024) polymorphism was investigated by allelic discrimination with fluorescent probes and real-time PCR (Assays-by-Design; Applied Biosystems, Foster City, CA). The two possible heterozygous conditions (G/G/A and G/A/A) in DS samples were detected by allele quantification. DNA samples with known *CBR1* 1096G>A genotypes were mixed to generate standards for calibration curves. The theoretical ratios of the variant A allele were 0, 12.5, 20.0, 33.3, 50.0, 66.7, 80.0, 87.5, and 100% (Cheng et al., 2005). Genotyping reactions were run as described previously (Gonzalez-Covarrubias et al., 2009). *CBR1* 1096G>A genotype status was extrapolated from the calibration curves depicting normalized fluorescent signal versus percentage of allele A.

Data Analysis. Statistics were computed with Excel 2007 (Microsoft, Redmond, WA) and GraphPad Prism, version 4.03 (GraphPad Software Inc., San Diego, CA). The Mann-Whitney test was used to compare group means. Differences were considered to be statistically significant at $p < 0.05$.

Results

First, aCGH analysis was performed on DNA isolated from DS samples to confirm Trisomy 21. An analysis of $\log_2$ ratios demonstrated that all DNA samples from donors with DS exhibited a third copy of chromosome 21 (Fig. 1A). Further examination revealed that all DS DNA samples carried three copies of *CBR1* (Fig. 1B). Next, cardiac *CBR1* mRNA expression in DS and non-DS samples was examined by QRT-PCR. On average, cardiac *CBR1* mRNA levels were 1.8-fold higher in DS samples than in non-DS samples (*CBR1* mRNA_{DS}, 3.3 ± 0.5 relative fold, versus *CBR1* mRNA_{non-DS}, 1.8 ± 0.2 relative fold; $p = 0.012$) (Fig. 1C). Increased cardiac *CBR1* mRNA expression dictated higher CBR1 protein levels in cytosols from DS samples compared to non-DS samples (CBR1 protein_{DS}, 13.5 ± 7.7 nmol/g cytosolic protein, versus CBR1 protein_{non-DS}, 7.2 ± 3.9 nmol/g cytosolic protein; $p = 0.029$) (Fig. 1D).

To further examine the impact of Trisomy 21 on cardiac *CBR1* expression, we measured CBR1 activity for the anthracycline substrate daunorubicin. Cardiac CBR1 activity demonstrated typical one-site Michaelis-Menten kinetics for daunorubicin ($V_{\max}$, 1.8 ± 0.2 nmol/min $\cdot$ mg; K_m , 202.0 ± 50.6 μ M; Fig. 1G). In line with the *CBR1* mRNA and CBR1 protein expression data, maximal CBR1 activity, expressed as the rate of synthesis of daunorubicinol, was 1.7-fold higher in DS samples than in non-DS samples (CBR1 activity_{DS}, 3.8 ± 0.1 nmol daunol/min $\cdot$ mg, versus CBR1 activity_{non-DS}, 2.3 ± 0.2 nmol daunol/min $\cdot$ mg; $p = 0.050$) (Fig. 1H). *CBR1* expression in hearts from donors with DS varied between individuals at the mRNA (13.3-fold, range 0.3–4.0 relative fold), protein (5.5-fold, range 3.0–16.4 nmol/g cytosolic protein), and activity levels [3.5-fold, range 1.1–3.9 nmol daunol/min $\cdot$ mg]. Likewise, non-DS samples showed variable *CBR1* expression at the mRNA (1.9-fold, range 2.2–4.1 relative fold), protein (3.9-fold, range 6.0–23.6 nmol/g cytosolic protein), and activity levels [3.1-fold, range 1.6–4.9 nmol daunol/min $\cdot$ mg].

Next, we examined *CBR1* 1096G>A genotype status in the DS samples. The calibration curve of normalized fluorescent signal versus ratio of the variant allele A showed a linear relationship ($r^2 = 0.83$). Table 1 shows cardiac *CBR1* expression stratified by *CBR1* 1096G>A genotype status.

Discussion

This study documents increased CBR1 expression in DS samples. On average, DS samples showed increased *CBR1* mRNA (1.8-fold), CBR1 protein (1.9-fold), and CBR1 activity (1.7-fold) compared to non-DS samples. These observations are in agreement with the expected 1.5-fold increase in *CBR1* expression dictated by the gene-dosage effect (Lemieux et al., 1993; Amano et al., 2004). Our findings may provide insights concerning the higher incidence of anthracycline-related cardiotoxicity in cancer patients with DS compared to that in patients without DS (Krischer et al., 1997; Ravindranath et al., 2005; O'Brien et al., 2008). Studies have demonstrated that the development of cardiomyopathy correlates with myocardial accumulation of alcohol metabolites (Forrest et al., 2000; Olson et al., 2003; Minotti et al., 2004). Thus, increased *CBR1* expression in hearts from donors with DS would affect the pathogenesis of cardiotoxicity due to the synthesis of relatively high levels of cardiotoxic daunorubicinol.

The relatively small number of DS samples is the main limitation of this study. Heart tissue from donors with DS is scarce, and procurement rates are low (≈ 1 sample/7.3 months), even for cooperative resources

FIG. 1. Characterization of cardiac *CBR1* expression. A, aCGH of a representative DS sample. B, segment of chromosome 21 encompassing *CBR1*. C, cardiac *CBR1* mRNA expression in non-DS samples ($n = 15$) and DS samples ($n = 4$). Relative *CBR1* mRNA levels were determined with a comparative quantitation method. Individual β -actin mRNA levels were used as normalizers. Each circle depicts the average of four replicates. Horizontal lines indicate group means ($p = 0.012$). D, cardiac CBR1 protein expression in non-DS samples ($n = 15$) and DS samples ($n = 4$). Horizontal lines indicate group means ($p = 0.029$). E, immunodetection of β -actin and recombinant CBR1 standards from a typical calibration curve (range 0.03–0.3 μ g). F, immunodetection of CBR1 in heart cytosols. G, maximal CBR1 activities with the substrate daunorubicin in heart cytosols from non-DS samples ($n = 15$) and DS samples ($n = 4$). Each circle depicts the average of duplicates for individual samples. Group means are indicated by horizontal lines ($p = 0.050$). H, typical one-site Michaelis-Menten kinetics for cardiac CBR1 activity with the substrate daunorubicin.

such as the National Disease Research Interchange. Stringent criteria were used for tissue procurement and analysis to minimize experimental variability. Thus, our findings may provide a representative picture of cardiac *CBR1* expression in the hearts of individuals with DS.

We recently identified a relatively common polymorphism in the 3'-UTR of *CBR1* (*CBR1* 1096G>A, rs9024) that affects hepatic *CBR1* expression at the protein and activity levels. CBR1 protein expression levels were 1.7-fold higher in liver samples with the

TABLE 1

Cardiac CBR1 expression in DS samples stratified by CBR1 1096G>A genotype status

	G/G/G	G/G/A	G/A/A	A/A/A
Number of samples	1	2	0	1
CBR1 mRNA (relative fold)	2.2	3.9, 2.9	N/A	4.1
CBR1 protein (nmol/g cytosolic protein)	23.6	9.3, 6.0	N/A	15.3
CBR1 activity (nmol daunol/min · mg)	3.8	4.0, 4.8	N/A	1.6

N/A, not applicable.

homozygous G/G genotype compared to those in samples with one copy of the A allele, and CBR1 activity, assessed as the rate of synthesis of doxorubicinol, was 1.5-fold higher in liver samples that were G/G compared to the activity in those that were G/A (Gonzalez-Covarrubias et al., 2009). In agreement, a recent report described an association between CBR1 1096G>A genotype status and the risk of cardiomyopathy in pediatric cancer survivors without DS exposed to low-dose (1–250 mg/m²) anthracyclines (odds ratio, 5.33; 95% confidence interval, 1.4–20.6; $p = 0.01$; G/G versus GA/AA) (Blanco et al., 2010). In this study, we aimed to extend these observations to heart samples from donors with DS harboring an extra copy of CBR1. The heart from a donor with DS and CBR1 1096G>A genotype homozygous for the variant A allele (A/A/A) showed the lowest CBR1 activity for daunorubicin (Table 1). This individual finding highlights the potential contribution of the polymorphism in dictating variable CBR1 expression in hearts from individuals with DS. We hypothesized that CBR1 1096G>A affects the binding of specific microRNA to the polymorphic site at the 3'-UTR of CBR1. Functional studies are ongoing to evaluate whether candidate microRNAs regulate CBR1 expression in various cell types. Additional genotype-phenotype studies with larger sample sizes are needed to define the impact of CBR1 1096G>A on cardiac CBR1 expression.

This pilot study represents the first characterization of cardiac CBR1 expression in samples from individuals with DS. Our findings suggest that increased CBR1 expression and activity dictated by the gene-dosage effect would affect the risk of anthracycline-related cardiotoxicity in cancer patients with DS.

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