Transcriptional analysis of doxorubicin-induced cardiotoxicity

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Doxorubicin is one of the most widely used antitumor agents. It is effective against a wide spectrum of cancers, including carcinomas, sarcomas, and lymphomas. However, it causes dilated cardiomyopathy in a dose-dependent manner, leading to heart failure in cancer patients treated with this agent (21, 32, 33). Progressive ventricular dysfunction and congestive heart failure may occur after the patients have stopped doxorubicin treatments (32). The precise mechanism of doxorubicin-induced cardiotoxicity remains unclear, but the antitumor activity of doxorubicin is likely to be distinct from the mechanism of its cardiotoxicity. The therapeutic activity of doxorubicin is thought to be due to DNA damage, and it is most effective against highly proliferative tumors (17, 20, 32). By contrast, cardiomyocytes are minimally replicative cells that should be resistant to such antimitotic mechanisms. Much evidence indicates that free radical generation contributes to the cardiotoxic effects of this drug (9, 27). Doxorubicin can generate free radicals directly, and it is also a potent chemical catalyst for the creation of oxygen radicals by redox cycling (8, 27, 28). Moreover, doxorubicin appears to reduce the amount of endogenous antioxidants (10). The oxidative damage caused by doxorubicin is complex, including effects on mitochondria (26), lipid peroxidation (22, 23), sarcoplasmic reticulum, lysosomes, and microfibrils (32). Eventually, these insults lead to increased apoptosis in cardiomyocytes (2, 14, 18, 24). Scavengers of free radicals, such as probucol and vitamin E, or overexpression of antioxidant catalytic proteins, such as manganese superoxide dismutase, heme oxygenase-1, and metallothionein, appear to ameliorate this doxorubicin-induced toxicity (4, 7, 15, 16, 25, 37).

Because of the large number of different free radicals that doxorubicin can generate once it gets into the cell and the complex changes that these different free radicals cause at the molecular, organelle, and cellular levels, the mechanism and key events associated with this cardiotoxicity are still not fully understood (28, 32). However, apoptotic loss of cardiac myocytes is the likely eventual result of these oxidative insults. The loss of cardiac fibers contributes to remodeling of the ventricle with a globular geometry that can increase wall stress and exacerbate cell loss, replacement fibrosis with resulting impairment of relaxation, and insufficient systolic contractility, all of which contribute to the development of symptoms of congestive heart failure. The complexity of the various oxidative insults caused by doxorubicin suggests the utility of a comprehensive analysis of transcriptional regulation to identify pathways that play a role in toxicity.

Most of the previous studies of doxorubicin toxicity have been performed in cell culture with high doses of the drug. A high dose of the drug can elicit profound transcriptional responses in many genes. These findings may implicate pathways that do not operate in patients, who receive lower levels of the drug given over many weeks. Nor do these in vitro models allow the integration of the manifold influences on drug metabolism, intercellular interactions, and physiological responses that occur in vivo. A model of toxicity in the rat does recapitulate the physiological and histological findings in patients (19). However, genomic resources are not as advanced for the rat, and the transcriptional analysis that can be performed is still limited. In this study, we identify genes that are transcriptionally regulated in an acute and chronic murine model and find in the chronic model, which more closely recapitulates chemotherapeutic cardiotoxicity, that specific pathways of apoptotic death and oxidative damage are implicated in the development of dilated cardiomyopathy.
MOUSE MODELS OF DOXORUBICIN-INDUCED CARDIOTOXICITY

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MATERIALS AND METHODS

Two mouse models of doxorubicin-induced cardiomyopathy. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. In the acute model of doxorubicin-induced cardiotoxicity, 6- to 8-wk-old male C57BL/6J mice are injected intraperitoneally with 15 mg/kg body wt of doxorubicin once. Treated and control mice were euthanized at 1 day and 1, 3, 6, 12, and 18 wk after the first injection, and hearts were harvested. To develop the chronic model, initial dose-ranging studies revealed that 2 mg/kg produced minimal mortality after 18 wk, whereas 4 mg/kg killed all mice by 9 wk. Three dose-ranging studies revealed that 2 mg/kg produced minimal mortality after 18 wk, whereas 4 mg/kg killed all mice by 9 wk. Three milligrams per kilogram for 12 wk produced a total dose equivalent to 2,520 mg/70-kg man, a dose at which cardiomyopathy is expected in milligrams per kilogram for 12 wk. Treated and control mice were euthanized with intraperitoneal injection of tribromoethanol (10 g/l 1.25% Avertin). Echocardiography was performed 10 min after initiation of sedation to limit anesthesia-induced impairment of cardiac function with intraperitoneal injection of ethane. Echocardiography was performed with Avertin. Echocardiography leading edge method (30). Fractional shortening (FS) was calculated as FS% = [(LVEDD − LVESD)/LVEDD] × 100.

Microarray hybridization and analysis. RNA was prepared from whole hearts with the use of TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Equal amounts of all samples in the doxorubicin-treated or control group for a given time point were pooled for microarray hybridization and real-time PCR analysis. A set of dye-reversal array hybridizations was performed to compare the doxorubicin-treated and control group at each time point. RNA was converted to cDNA, labeled, and hybridized to a cardiac-specific array as previously described (38). Slides were scanned with an Axon scanner and GenePix software (Axon Instruments, Union City, CA). The images were analyzed with GeneTraffic software from Iobion Informatics (La Jolla, CA).

Real-time PCR. DNase I-treated RNA was reverse transcribed by using SuperScript (Invitrogen). Equal amounts of cDNA derived from similar experimental animals were pooled, and real-time PCR was performed by using cDNA derived from 5 ng of total RNA. PCR data were analyzed with the Opticon Monitor software package from MJ Research. The transcript of cyclophilin A, a relatively stable housekeeping gene, was used to normalize PCR quantitations (3, 34).

Histology. Tissues for histology were harvested from anesthetized mice after fixation via transcardial perfusion with 10% neutral buffered formalin. Subsequent paraffin processing, embedding, and sectioning were performed by standard procedures (31, 36). Review and photography of all histological preparations were carried out on a Leica Laborlux S photomicroscope with brightfield illumination. Photomicrography was achieved by using this microscope and an Optronics DEI-750 analog charge-coupled device color camera interfaced with a Scion CG-7 frame-digitizer-equipped Macintosh G4 computer. Images were captured by using Image J version 1.23 acquisition and analysis software (Scion and National Institutes of Health) and processed with Adobe Photoshop 7.0.

RESULTS

Chronic mouse model of doxorubicin-induced cardiomyopathy. The LV function of the mice in our chronic model was assessed by echocardiography. At week 1, FS for the control group and the treated group was almost identical, with control group at 63.2 ± 2.5% and doxorubicin-treated group at 62.3 ± 2.7%. The average FS for the control group increased to 73.1 ± 1.4% at week 18, whereas the average FS for the doxorubicin-treated group decreased to 46.2 ± 2.5% at week 18 (Fig. 1).

Histology of the hearts from treated animals revealed the typical findings associated with doxorubicin-induced dilated cardiomyopathy in human patients: myocyte loss, myofibrillar degeneration, and extensive vacuolization (Fig. 2). This dilated cardiomyopathy is associated with high subsequent mortality (Fig. 3).

Transcriptional analysis. We have generated a cDNA array with more than 13,000 features representing more than 5,000 genes cloned from skeletal and cardiac muscle (38). Each pooled DNA sample was analyzed with at least two sets of dye-reversal hybridizations (four slides). For subsequent real-time PCR confirmation, we selected transcripts with regulation >2.4-fold; these were >2.5 SD from the mean for these experiments. These confirmed transcripts are shown in Tables 1 (for the acute model) and 2 (for the chronic model). We have...
divided these dysregulated genes into five groups: 1) oxidative stress-related proteins; 2) signal transduction proteins; 3) apoptotic proteins; 4) transcription factors; and 5) structural proteins and extracellular matrix proteins. All array results may be found at the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE2965.

Confirmation of dysregulated genes by real-time PCR. We confirmed our array results by real-time PCR (Figs. 4 and 5). Greater than 90% of transcripts showed the same direction of regulation as the array (Tables 1 and 2).

DISCUSSION

Comparison of acute and chronic models. With the use of a relatively rigorous definition of regulation (>2 SD from the mean), the acute model, which used a high dose of doxorubicin, showed a greater number of dysregulated genes than did the chronic model (90 genes in the acute model and 23 in the chronic model). These transcripts rapidly reverted to baseline abundance with a time course similar to the clearance of the drug (6). By contrast, with the use of a low dosage of doxorubicin in the chronic model that more closely reflected the clinical treatment of patients, transcriptional responses in the cardiomyocytes were lower compared with those of the acute model. We think that the chronic model, which demonstrates histological and physiological characteristics similar to patients with doxorubicin cardiomyopathy, provides a more reliable context for identifying pertinent gene regulation.

Table 1. Highly regulated genes in acute model

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change by Array</th>
<th>Fold Change by Real-Time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative stress related</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequestosome 1</td>
<td>2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase 4</td>
<td>4.8</td>
<td>30</td>
</tr>
<tr>
<td>Glutathione S-transferase mu 1</td>
<td>2.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Ferritin heavy chain</td>
<td>2.7</td>
<td>5.1</td>
</tr>
<tr>
<td>Glutathione peroxidase 3</td>
<td>2.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Thioredoxin interacting protein</td>
<td>2.9</td>
<td>15</td>
</tr>
<tr>
<td>Carbonic anhydrase 3</td>
<td>0.46</td>
<td>0.37</td>
</tr>
<tr>
<td>Adenylate kinase 3 alpha like</td>
<td>0.38</td>
<td>0.66</td>
</tr>
<tr>
<td>Cardiac responsive adriamycin protein</td>
<td>2.62</td>
<td>4.53</td>
</tr>
<tr>
<td>Signal transduction/transcription protein</td>
<td>0.37</td>
<td>0.66</td>
</tr>
<tr>
<td>TNF receptor-associated factor 4</td>
<td>3.3</td>
<td>38</td>
</tr>
<tr>
<td>B-cell translocation gene 2</td>
<td>20</td>
<td>2.2</td>
</tr>
<tr>
<td>Tumor differentially expressed 1</td>
<td>0.52</td>
<td>0.36</td>
</tr>
<tr>
<td>Cyclin G</td>
<td>2.09</td>
<td>2.4</td>
</tr>
<tr>
<td>MK gene for TRAIL receptor 2</td>
<td>2.65</td>
<td>19</td>
</tr>
<tr>
<td>SH3-binding glutamine-rich protein</td>
<td>0.41</td>
<td>0.7</td>
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<tr>
<td>Zinc finger protein, multitype 1</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>HELG (helicase)</td>
<td>0.26</td>
<td>0.53</td>
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<tr>
<td>Apoptotic protein</td>
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<tr>
<td>Apoptotic factor GRIM19</td>
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<tr>
<td>Programmed cell death protein 11</td>
<td>0.17</td>
<td>0.54</td>
</tr>
<tr>
<td>p53 apoptosis effector related to Pmp22</td>
<td>2.84</td>
<td>8.1</td>
</tr>
<tr>
<td>Other</td>
<td>3.15</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Real-time PCR results corresponding to maximal regulation on arrays. Real-time PCR results for multiple time points are shown in Figs. 4 and 5.
Nonetheless, many of the regulated genes in both models fall into similar functional categories: 1) metabolism and oxidative stress response proteins; 2) signal transduction proteins; 3) apoptotic factors; and 4) cardiac muscle structural proteins.

Patients typically receive multiple treatments of doxorubicin of 50–75 mg/m² over several months with a cumulative maximum of 450 mg/m², equivalent to ~12 mg/kg. The treated mice in the acute group received one doxorubicin injection of 15 mg/kg, and the treated mice in the chronic group received a cumulative dosage of 36 mg/kg. These dosages are greater than those patients would receive, although the cardiotoxic dose may differ in mice because of distinct absorption, metabolism, and excretion of the drug. We selected our dose for the acute exposure on the basis of previous studies and our dose for the chronic exposure on the basis of our preliminary dose-ranging experiments that established a predictably cardiotoxic but not rapidly fatal dose. Functional and histological analysis suggests that the chronic model closely mimics the situation of human patients.

Markers of doxorubicin-induced cardiac toxicity in chronic model. The survival curve of the treated group (in Fig. 1) shows that most of the treated mice (7 out of 9) died during the 6 wk after the last doxorubicin treatment, with ventricular dysfunction documented premortem, even though doxorubicin was not given to these mice during this time period. This suggests progressive cardiac decompensation after sufficient exposure to doxorubicin, as is the situation in human patients who may die from heart failure months or even years after chemotherapy has been stopped. This supports the chronic treatment as a valid pathophysiological model for human doxorubicin-induced cardiomyopathy.

To further investigate the genes that might be involved in the progression of cardiac dysfunction, we identified genes in the signal transduction/transcription factor category that maintained regulation after doxorubicin treatment was stopped. These include STARS, SNF1-kinase, AXIN1-upregulated protein 1 (AXUD1), and B-cell translocation gene 2 (BTG2). At week 18, the expression levels of these genes have increased to 3.5-, 5.5-, 30-, 6.9-fold, respectively, compared with that of the control group (Table 2 and Fig. 5).

STARS (striated muscle activator of Rho signaling), a cardiac- and skeletal muscle-specific protein, was found to be an upstream regulator of serum response factor through RhoA signaling. Serum response factor is stimulated by STARS, and this stimulation leads to transcriptional activation of serum response factor-regulated genes. STARS expression is maintained in adult cardiac and skeletal muscle and is dramatically upregulated during hypertrophic growth of the heart. AXIN is an important negative regulator of β-catenin, a transcription factor and important member in the Wnt pathway. Upregulation of AXIN1 suppresses growth and induces apoptosis in a variety of cell types. AXUD1 was cloned during a screen for downstream targets of AXIN1, and it was found to be downregulated in a variety of tumors, suggesting that AXUD1 may have tumor-suppressor activity (13). SNF1-kinase, also known as AMP-activated protein kinase in vertebrates, is stimulated by AMP, and it is a key regulator of ATP production in the cell (11, 12). There is evidence of a defect in ATP production in doxorubicin-treated hearts, although this has been thought to be due to ROS-mediated damage to mitochondria. BTG2 is a member of a gene family that has antiproliferative properties (35). BTG2 causes cell cycle arrest after DNA damage by ionizing radiation or doxorubicin to allow DNA repair. In addition, overexpression of BTG1, a homologue of BTG2, leads to apoptosis in a variety of cell types (5).

An important limitation of our study is that it is confined to evaluation of mRNA abundance, which we assume to result most commonly from changes in gene expression (rather than mRNA stability, for instance). Nor have we extended these findings to the level of protein expression or physiological evaluation of the role of the regulated genes. Thus this study serves only to implicate specific genes in the cardiac response to doxorubicin. Substantial further work will be needed to determine the functional role, if any, of these genes in the development, progression, or amelioration of doxorubicin cardiotoxicity.

In summary, we have developed a chronic mouse model of doxorubicin-induced cardiomyopathy that closely mimics the situation in human patients. We have analyzed the transcriptional profile of the doxorubicin-treated mouse hearts and identified several genes that are dysregulated during the development of doxorubicin-induced cardiomyopathy. We have shown that in the acute model, on doxorubicin injection, there is an immediate transcriptional change in a large number of genes that are involved in antioxidation pathways, transcription factors, and apoptotic factors. However, these transcriptional changes quickly subside as doxorubicin is metabolized and excreted. In the chronic model, we identified regulation of genes that correlate with the development of dilated cardiomyopathy. It is likely that these transcripts identify biochemical pathways that contribute to doxorubicin-induced cardiotoxicity.

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GRANTS

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REFERENCES