Protection against Daunorubicin Cytotoxicity by Expression of a Cloned Human Carbonyl Reductase cDNA in K562 Leukemia Cells

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ABSTRACT

Carbonyl reductase (CBR) catalyzes the reduction of daunorubicin (DN) to its corresponding alcohol, daunorubicinol (DNOL), and changes the pharmacological properties of this cancer chemotherapeutic drug. The DN reductase associated with CBR reduces the C13 methyl ketone group and does not metabolize the quinone ring of DN. Reports comparing DN and DNOL toxicity have resulted in various conclusions depending on the cell line tested. Differences in toxicity could be due to variations in several enzymes involved in DN metabolism. In this report, the effects of CBR on DN metabolism and cell toxicity were determined by cloning and expressing a human CBR cDNA in DN reductase-deficient myeloid erythroleukemia K562 cells. CBR activity increased 83-fold in the K562-transfected cells and was associated with a 2–3-fold reduction in DN toxicity. Maximum protection occurred at 30 nM DN where 94% of the intracellular DN was converted to DNOL within 2 h. The reduced toxicity was specific for DN. Other CBR substrates such as menadione, phenanthrenequinone, and doxorubicin were equally toxic to both the CBR expressing cells and the control cells under the conditions tested. Our results suggest that high levels of CBR in tumor cells could contribute to drug resistance. The results also suggest that reduction of DN to DNOL protects against DN toxicity by altering interaction of the drug at one or more of the many target sites.

INTRODUCTION

CBR, a NADPH-dependent cytosolic enzyme with both DN reductase and menadione reductase activity, is involved in the metabolism of the anticancer drug daunorubicin (1–4). DN belongs to a class of anticancer drugs called anthracyclines. The DN reductase activity of CBR reduces the C13 methyl ketone group of DN to its C13 alcohol metabolite DNOL. The conversion of DN to DNOL by CBR changes the pharmacological properties of the drug in ways that are not well understood. Initially, it was observed that higher levels of CBR correlated with increased DN toxicity (5, 6), whereas later reports suggested no correlation between DN toxicity and CBR levels (7). Additional in vitro studies using cultured tumor cells demonstrated that DNOL is less toxic than DN (8, 9). Currently, it is generally accepted that conversion of DN to DNOL is an initial step in the detoxification of DN.

However, contrary to the in vitro detoxification data derived using cultured tumor cells, there is evidence suggesting that DNOL may play a significant role in the cardiotoxicity associated with DN chemotherapy (10). Two issues that have been widely investigated in vitro are the role of free radicals and the role of the C13 alcohol metabolites. Although evidence linking free radicals to cardiotoxicity is firmly established, an additional role involving the C13 alcohol metabolites cannot be ruled out (11, 12). Cytotoxic anthracycline-free radicals can be generated by both enzymatic and nonenzymatic mechanisms (11–13). However, in the case of DN, the most likely source of free radicals would originate from one electron reduction of the quinone ring by enzymes other than CBR. Previous conflicting results describing the role of CBR on DN cardiotoxicity could be explained by variations in the levels of other DN-metabolizing enzymes. Thus, the effect of CBR activity on DN toxicity remains unclear. In this report we cloned and expressed human CBR in K562 cells, an established cancer cell line which does not normally express CBR. The introduction of a single DN-metabolizing enzyme affected DN cytotoxicity.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The myeloid erythroleukemia cell line K562 was grown in HEPES-buffered RPMI 1640 with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (50 μg/ml) at 37°C in a humidified atmosphere of 5% CO2.

Expression Vector. The human CBR cDNA (14) was amplified and modified by PCR to generate HindIII and BglII restriction sites on the 5′ and 3′ ends, respectively. The 5′ primer containing the underlined HindIII site (CAC-AAGCTTGGCATGGAACACGCTGCGG) was synthesized to retain most of the 5′ untranslated region. The 3′ primer containing the underlined BglII site (GTGAGATCTGCAACACTGCTCAACTCCTCTC) was synthesized to remove the 3′ untranslated region. After PCR amplification, the PCR product was gel purified and cloned into a Bluescript vector (Stratagene, La Jolla, CA) that was cut with EcoRV and tail with dideoxythymidine according to the method of Holton and Graham (15). The modified CBR cDNA plasmid was amplified and the HindIII-BglII cDNA fragment was cloned into the HindIII-BamHI sites of the expression plasmid pHg Apr-1-neo (16). The expression vector contains a β-actin promoter, splice site, and polyadenylation signal. Orientation of the cDNA and its sequence was verified by DNA sequencing.

Transfections. Transfections were performed in K562 cells using Transfectam (Promega, Madison, WI) according to the supplied protocol. Cells (5 × 106) were grown in 60-mm dishes and transfected using 10 μl Transfectam and 5 μg DNA. The DNA complex remained on the cells for 24 h before the addition of an equal volume of media containing 20% fetal bovine serum. After 72 h, cells were subcloned every 3–4 days in selection media.

RNA Isolation and Northern Blot Analyses. Total RNA was isolated using the method of Kamber and Evans (17). Cells (1 × 106) were collected by centrifugation, suspended in 0.1 ml PBS, and lysed by adding the cells dropwise to 1 ml lysis buffer (8 mM guandine HCl, 0.1 M EDTA, and 0.1% volume of 3 M sodium citrate). The lysates were passed through a 25-gauge needle three times and then centrifuged for 20 min at 16,000 × g. The supernatants were transferred to fresh tubes, and the RNA was precipitated with 0.5 volume of ice-cold ethanol. Samples were placed at −20°C for at least 30 min and then centrifuged at 16,000 × g for 20 min. The pellets were dissolved in 0.5 ml lysis buffer (prewarmed to 60°C) and reprecipitated at −20°C with the addition of 0.25 ml ethanol. After centrifugation the pellets were washed once with 70% ethanol, once with 100% ethanol, dried briefly under vacuum, and dissolved in 20 μl diethyl pyrocarbonate water. Ten μg of RNA were denatured in formaldehyde/formamide buffer, separated on a 1.2% agarose gel, and blotted to nitrocellulose. The nitrocellulose was hybridized with a CBR cDNA probe (14) and quantitatively analyzed using a Phosphor Imager System (Molecular Dynamics, Sunnyvale, CA).
The dried sample was dissolved in 2-butanol and analyzed using TLC (20) and buffer consisting of 0.1 M potassium phosphate (pH 6), 0.2 mM menadione, 1.5 mM cytochrome c, and 0.2 mM NADPH. Rutin (20 μM) was used to inhibit CBR and was added to the reaction mixture 5 min before starting the reaction with NADPH. DN reductase was measured by incubating the cell extracts at 37°C in 0.1 M potassium phosphate (pH 6), 0.65 mM DN, and 0.5 mM NADPH with NADPH. DT-diaphorase activity was determined by measuring the reduction of cytochrome c at 550 nm using a Uvikon 930 spectrophotometer (Kontron Instruments/Research without further purification. Protein content was determined using the Bio-Rad column (Applied Biosystems, Inc., Foster City, CA) and eluted using the isocratic HPLC buffer described above. For HPLC analysis, an aliquot of the sample was dried under vacuum, reconstituted in 200 μl of the isocratic HPLC buffer (30% acetonitrile and 70% 0.32 M phosphoric acid) and filtered through a 0.2-μm Spin-X column (Costar, Cambridge, MA). Fifty μl of the sample were injected onto a 4.6-mm X 25-cm Econosil C18 5μm column (Altech, Deerfield, IL) with a HPLC C18 guard column (Applied Biosystems, Inc., Foster City, CA) and eluted using the isocratic HPLC buffer. The HPLC system consisted of a Spectra Physics 8700 Solvent Delivery System (Spectra Physics, San Jose, CA). Peaks were detected using a Shimadzu RF-551 spectrofluorometric detector (Shimadzu, Columbia, MD) and quantitatively analyzed using a MacLab 4 Data Recording System (ADInstruments, Milford, MA). Doxorubicin or 4'-epidoxorubicin was used as a human CBR. DN toxicity was assayed by measuring K562 cell growth after 72 h in the presence of different drug concentrations. The doubling time of both the control cells and the CBR expresser cells was 18 h. Fig. 2A shows DN cytotoxicity protection of K562 cells expressing CBR after a 72-h exposure to DN. Fig. 2B demonstrates similar protection when the cells were treated for 2 h with the drug. Continual growth of the expresser cells for a period of several months was undetectable in the control and increased significantly in the expresser cells. High levels of CBR mRNA were synthesized in the expresser cells while the CBR message was undetectable in the control cells (Fig. 1). These data demonstrate that increased CBR activity is correlated to synthesis of CBR mRNA.

**Table 1 CBR activity in CBR-transfected K562 cells and controls**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Menadione reductase&lt;sup&gt;a&lt;/sup&gt; (nmol/min/mg), mean ± SD</th>
<th>Doxorubicin reductase&lt;sup&gt;b&lt;/sup&gt; (nmol/min/mg), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562 control</td>
<td>2.5 ± 1.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>K562 human CBR D16</td>
<td>208 ± 2.8</td>
<td>127 ± 1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rutin-inhibitable quinone reductase activity.

<sup>b</sup> Aldo-keto reductase activity.

<sup>c</sup> Transfected with pHβ Apr-1-neo.

<sup>d</sup> n.d., not detected.

**RESULTS**

Carboxyl Reductase cDNA Expression in K562 Leukemia Cells. K562 cells were transfected with the human CBR cDNA driven by a β-actin promoter in the expression plasmid pHβ Apr-1-neo (16). Clones were selected by dilution cloning in medium containing the antibiotic G418 and by clonal selection in soft agarose. CBR was assayed for both menadione reductase and DN reductase activities in cell extracts. Table 1 shows that rutin-inhibited menadione reductase activity increased 83-fold in cells transfected with the CBR cDNA expression plasmid compared to the control cells transfected with the expression plasmid without the CBR cDNA. DN reductase activity was undetectable in the control and increased significantly in the expresser cells. High levels of CBR mRNA were synthesized in the expresser cells while the CBR message was undetectable in the control cells (Fig. 1). These data demonstrate that increased CBR activity is correlated to synthesis of CBR mRNA.

Doxorubicin Toxicity Assays in K562 Cells Expressing Human CBR. DN toxicity was assayed by measuring K562 cell growth after 72 h in the presence of different drug concentrations. The doubling time of both the control cells and the CBR expresser cells was 18 h. Fig. 2A shows DN cytotoxicity protection of K562 cells expressing CBR after a 72-h exposure to DN. Fig. 2B demonstrates similar protection when the cells were treated for 2 h with the drug. Continual growth of the expresser cells for a period of several months was undetectable in the control and increased significantly in the expresser cells. High levels of CBR mRNA were synthesized in the expresser cells while the CBR message was undetectable in the control cells (Fig. 1). These data demonstrate that increased CBR activity is correlated to synthesis of CBR mRNA.

**Fig. 1.** Northern blot analysis using total RNA (10 μg) isolated from K562 control cells and from K562 human CBR (HCBR) D16 cells. Probe was a 32P-labeled human CBR cDNA.
resulted in the loss of DN toxicity protection as shown in Fig. 2C. This loss of protection correlated with a reduction in rutin-inhibitable menadione reductase activity from 208 nmol/min/mg protein down to 10 nmol/min/mg protein. There was also a loss of CBR mRNA that correlated with the loss of DN reductase activity (data not shown).

The original clones of transfected cells were thawed from nitrogen storage, cultured, selected in G418, and assayed for CBR activity. A lower CBR activity assayed as rutin-inhibitable menadione reductase was detected at a level of 80 nmol/min/mg protein. Activity at this level was stable for at least 9 months. Fig. 2D shows that DN protection was restored, although at a lower level, in the CBR expresser cells. Maximum protection against DN toxicity in the CBR expresser cells occurred at a concentration of 30 nM DN, where the expressers were 2-3-fold more resistant than the control cells.

Growing the original transfected clones in nonselective medium resulted in the loss of CBR activity within 4 weeks (data not shown). CBR expression appears to be unstable in K562 cells. The loss of CBR expression could occur by several mechanisms including recombinational events or methylation. The CBR gene contains a GC island that extends into the first exon (19), presenting an opportunity for down-regulation by methylation. DN toxicity was also measured in the presence and absence of the selective agent, G418, with similar results (data not shown).

**Intracellular Metabolism of Daunorubicin to Daunorubicinol.** The intracellular conversion of DN to DNOL in the CBR expresser and control cells was measured by extraction and HPLC analyses after a 2-h treatment with 30 nM (maximum protection) DN. There was no measurable conversion to DNOL in the control cells while 94% of DN was converted to DNOL in cells expressing CBR (data not shown). Extracellular and intracellular DN and DNOL concentrations were measured each hour over a 4-h period in the presence of 10 μM DN. The results are shown in Fig. 3, A and B. The extracellular concentrations of DN presented in Fig. 3A show a decrease in DN as cellular uptake increased. Excretion of DNOL was observed only in the CBR expresser cells, beginning 1 h after treatment. Fig. 3B shows that the uptake of DN in the control cells reached a plateau at 2 h, and there was no detectable metabolism to DNOL over 4 h. Uptake of DN in CBR expresser cells also reached a plateau after 2 h. However, the amount of DN was much lower due to its conversion to DNOL, which was detected at 30 min and continued to increase for 2 h.

**Cytotoxicity of Menadione, Phenanthrenequinone, Mitomycin C, and Doxorubicin in CBR-expressing K562 Cells.** Cytotoxicity assays were performed using other known or possible xenobiotic substrates of CBR. The results shown in Fig. 4 demonstrate that no differences were observed between the CBR expresser and control cells. Menadione, phenanthrenequinone, and doxorubicin are known...
although activity levels differed by 150-fold, the maximum amount of chromosome 21 (the location of CBR) was not present in the clones.

Studies used DN and DNOL along with other anthracyclines and their C13 alcohol analogues to analyze toxicity by adding the metabolites to the cell medium. DNOL is transported into cells much slower than DN, suggesting that transport may affect the results. Our report supports the evidence that DNOL is less toxic than DN and that differential transport of DN and its metabolite DNOL is not a factor. Doxorubicin, a structural analogue of DN, has been shown to be toxic to cells without entering the cells, suggesting that some target sites may be associated with the membrane (24). The results reported here do not rule out differential toxicity at the membrane level.

Protection against cytotoxicity was specific for daunorubicin. Menadione, phenanthrenequinone, mitomycin C, and doxorubicin were equally toxic to the CBR expresser and control cells. The lack of differential toxicity for menadione, phenanthrenequinone, and mitomycin C may be explained by the presence of DT-diaphorase activity (200 nmol/min/mg) in K562 cells. These three xenobiotics are better substrates for DT-diaphorase than for CBR; therefore, any effects due to CBR expression could be masked. At this time there is no evidence that mitomycin C is a substrate for CBR. Doxorubicin is not a substrate for DT-diaphorase and is a poor substrate for CBR (25).

The suggestion that reduction of DN at the C13 group generates a less toxic compound is supported by the data presented in this report. In this study, conversion of all of the intracellular DN to DNOL in the CBR expressers resulted in a 2–3-fold decrease in 50% inhibitory concentration. This difference is small but may be significant in tumor cells expressing CBR by contributing to drug resistance in cells exposed to lower levels of the drug. The protection against DN toxicity by overexpression of CBR may be the result of protecting one or more potential target sites. Nuclear DNA is a major binding site for DN and for other anthracycline analogues. Inhibition of DNA synthesis correlates with the binding strength of anthracyclines to DNA (26). DNOL appears to bind less tightly to DNA than DN (27–29), suggesting that the lower toxicity of DNOL could be accounted for by lower drug binding. However, in addition to DNA binding, anthracyclines also bind to several proteins such as topoisomerase II and mitochondrial proteins, and these protein interactions, especially the

DISCUSSION

Human CBR was cloned and expressed in K562 cells to study drug metabolism in cells by overexpressing a single enzyme in the same enzymatic background as the control cells. Previous work describing DN metabolism in cells with high and low levels of CBR suggested that DN metabolites may be more toxic than DN (6, 20). The correlations of DN toxicity to CBR metabolism in these early experiments were performed on cells from different sources and did not take into account possible differences in other DN-metabolizing enzymes or possible cellular differences that could have altered DN toxicity.

Somatic cell hybrids were used in one of the first attempts to modulate CBR levels in cells and to correlate DN toxicity to CBR levels (7). Human myeloblasts were fused with Chinese hamster ovary cells, and clones were isolated and analyzed for DN reductase activity. Although activity levels differed by 150-fold, the maximum amount of DNOL formation was only 16%. No significant differences in toxicity were observed. It is questionable whether human CBR was active in these clones because the CBR activity level never increased above the endogenous Chinese hamster ovary cell level, and human chromosome 21 (the location of CBR) was not present in the clones.

More current evidence on DN toxicity suggests that conversion to DNOL may be a first step in the detoxification of DN (8, 9, 23). These studies used DN and DNOL along with other anthracyclines and their C13 alcohol analogues to analyze toxicity by adding the metabolites to the cell medium. DNOL is transported into cells much slower than DN, suggesting that transport may affect the results. Our report supports the evidence that DNOL is less toxic than DN and that differential transport of DN and its metabolite DNOL is not a factor. Doxorubicin, a structural analogue of DN, has been shown to be toxic to cells without entering the cells, suggesting that some target sites may be associated with the membrane (24). The results reported here do not rule out differential toxicity at the membrane level.

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Fig. 4. Cytotoxicity assays of selected quinones on K562 control and CBR expresser cells. Cells were exposed to quinones for 72 h. ■, K562 CBR expressers; ○, K562 control. Cell counts were ± 10%.

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substrates for CBR. Doxorubicin is a poor substrate compared to DN, while menadione and phenanthrenequinone are very good substrates. Menadione, phenanthrenequinone, and mitomycin C are also substrates for another quinone reductase enzyme, DT-diaphorase. Its activity was measured at 200 nmol/min/mg protein in both control and CBR expresser cells. Effects of CBR metabolism may be masked by the presence of DT-diaphorase activity.

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topoisomerase II interaction, may be more significant than DNA binding alone (30, 31).

We have expressed a human CBR cDNA in human K562 leukemia cells which do not normally express this DN-metabolizing enzyme. Expression of CBR resulted in complete conversion of DN to DNOL within 2 h and provided protection against DN toxicity.

REFERENCES


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