Prevention of Doxorubicin Myocardial Toxicity in Mice by Reduced Glutathione

Yasuhiro Yoda, Masaki Nakazawa, Tsukasa Abe, and Zenji Kawakami

Division of Hematology, Institute of Clinical Medicine [Y. Y., M. N., T. A.], and Department of Research Development [Z. K.], University of Tsukuba, Sakura-Mura, Niihari-Gun, Ibaraki 305, Japan

ABSTRACT

The effect of reduced glutathione (GSH) on acute myocardial toxicity due to doxorubicin (DXR) was investigated. At 20 mg/kg i.p. DXR was 100% lethal to BALB/c × DBA/2 F1 mice. At 500 or 1000 mg/kg i.p. GSH significantly decreased the lethality (P < 0.01). Electron micrographs of the myocardium from DXR-treated mice showed narrowing of myofibrils, edematous cytoplasm, and mitochondrial swelling which were detectable at day 2, was strongest at day 14, but had disappeared by day 56. Light microscopic examination revealed that intercellular spaces between myocardial cells were widened at day 56, indicating shrinking of myocardial cells. These changes were significantly decreased by treatment with GSH (500 mg/kg i.p.). Treatment with DXR (14 mg/kg) significantly decreased myocardial non-protein sulfhydryl content (P < 0.02) and administration of GSH (500 mg/kg) prevented the drop of non-protein sulfhydryl levels due to DXR treatment. Thus it was considered that administration of exogenous GSH contributes to prevention of the acute myocardial toxicity of DXR by increasing extracellular GSH levels and intracellular GSH synthesis, which detoxifies DXR-oxygen metabolites. The administration of GSH did not interfere with the antineoplastic effect of DXR against L1210 mouse leukemia.

INTRODUCTION

DXR is one of the most potent antineoplastic agents of the anthracycline group and is effective against acute leukemia, lymphoma, and solid tumors (1). Despite its potent antitumor effect, clinical use is limited because of serious side effects, such as myocardial toxicity (1). Recent reports suggest that free oxygen radicals induced during the metabolism of DXR may have toxic effects on the heart muscle (2-4). DXR accelerates electron transfer in the membrane of myocardial cells and accumulates oxygen radicals in the mitochondria or endoplasmic reticulum, where damage occurs (2, 5, 6). The accumulated oxygen radicals damage cardiac membrane lipoproteins (4) and accelerate the hexose monophosphate shunt, resulting in damage to the heart muscle (3). These effects are closely related to the metabolism of superoxide, by GSH peroxidase and catalase, to water and molecular oxygen (7, 8).

GSH peroxidase is depleted by the administration of DXR (7). Thus depletion of these enzymes is considered to be responsible for the myocardial toxicity of DXR. GSH is the major source of intracellular sulfhydryl groups and plays an important role in maintaining the acid-base balance in cells by controlling sulfhydryl enzymes (9). Doroshov et al. (10) reported that acute myocardial toxicity of DXR can be prevented in mice by i.p. injection of N-acetyl-L-cysteine which increases sulfhydryl compounds in the heart muscle. It is possible that administration of exogenous GSH would increase extracellular GSH, which could detoxify extracellular DXR metabolites and also increase intracellular GSH, acting as a reservoir of cysteine. GSH is one of the least toxic compounds in the NP-SH group and is clinically available for patients (11). Hence, we hypothesized that administration of GSH could protect against the acute myocardial toxicity of DXR in experimental animals and humans. This is a report of studies on the effects of GSH in preventing acute DXR myocardial toxicity in mice.

MATERIALS AND METHODS

Animals

Male BALB/c × DBA/2 F1 (hereafter called CD2F1) mice, 6 to 8 weeks old, were purchased from Charles River Japan. Inc. The mice were housed seven to a cage on hardwood bedding at a constant temperature of 23 ± 1°C (SD), under an alternating 12-h light-dark cycle. Food and tap water was provided ad libitum.

Drugs

DXR hydrochloride was purchased from Kyowahakko Kogyo Co., Ltd. (Tokyo, Japan). Reduced GSH was kindly supplied by Yamamoto Chemical Co., Ltd. (Tokyo, Japan). The drugs were reconstituted in NS just before use. Concentrations of drugs were adjusted to contain a required dose in 0.2 ml. The drugs were injected i.p. in each experiment.

Animal Studies

Experiment 1: Toxicity of GSH. Eighteen mice were divided into three groups of six. The three groups received GSH, at either 500, 1000, or 2000 mg/kg. Survival in each group was observed daily for 60 days.

Experiment 2: Effects of GSH on the Lethality of DXR. Sixty-four mice were treated with DXR (20 mg/kg) and were divided into 4 groups of 16 animals. Three groups received GSH, at either 1000, 500, or 250 mg/kg, and one group received an equivalent volume of NS. The survival was observed daily for 60 days.

Experiment 3: Effects of GSH on Myocardial Toxicity of DXR. Forty-five mice were divided into three equal groups. Two groups received DXR (14 mg/kg). One of the two groups received GSH (500 mg/kg) and the other one received an equivalent volume of NS after DXR i.p. The control group received an equivalent volume of NS only.

Three mice from each group were killed by cervical dislocation at days 2, 7, 14, 28, and 56, followed by a heart puncture. WBC, GPT levels, and/or electron micrographs of the myocardium were examined. WBC were counted on a Model ZBI Coulter Counter. The glutamic-pyruvic transaminase level was determined by the method of Reitman and Frankel (12). Electron microscopic observation was performed using approximately 1-mm³ cubes of the myocardium excised from the left ventricles. The tissue was fixed by 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h and washed 3 times with the buffer. The samples were postfixed by 1.0% aqueous osmium tetroxide in 0.1 M cacodylate buffer for 2 h, dehydrated through a series of graded ethanol solutions, transferred to propylene oxide, and embedded in epoxy resin. Ultrathin sections of the myocardium were examined with a Model JEOL-100CX electron microscope (Japan Electron Optics Co., Tokyo, Japan). Semithin sections were stained with toluidine blue and analyzed under a light microscope.

Experiment 4: Effects of GSH and DXR on Myocardial NP-SH Level. Seventy-two mice were divided into 6 groups of 12 animals. Three groups received GSH, at either 500, 750, or 1000 mg/kg; 2 groups received DXR at either 14 or 20 mg/kg; and the remaining control group received NS only. The NP-SH of heart was measured at various time points. In the next experiment the effects of GSH (500 mg/kg) and/or DXR (14 mg/kg) on the myocardial NP-SH level were studied. Forty mice were divided into 4 equal groups. Three groups were treated with either DXR (14 mg/kg) plus GSH (500 mg/kg), GSH (500 mg/kg) alone, or NS alone. The fourth group was reproducted by GSH (500 mg/kg) alone.
PREVENTION OF DXR MYOCARDIAL TOXICITY BY GSH

kg) plus NS, or DXR (14 mg/kg) plus NS. The control group was given an equivalent volume of NS only. The myocardial NP-SH was measured at each time point. This experiment was repeated five times. Similar experiments were performed to observe the NP-SH level at days 7 and 14, with DXR (14 mg/kg) or with DXR (14 mg/kg) plus GSH (500 mg/kg). The NP-SH level was determined by the method of Sediak and Lindsay (13). Briefly, a pair of mice were killed by cervical dislocation followed by a heart puncture and excision of the left ventricles. After excess connective tissue was trimmed, the myocardium was washed well in NS and residual water was absorbed by filter paper. A 200-kg myocardial sample was weighed and homogenized in 4 ml 0.02 M EDTA in a Potter-Elvehjem homogenizer. A 2.5-ml aliquot was mixed with 2 ml distilled water plus 0.5 ml 50% trichloroacetic acid and cooled at 4°C for 15 min. After centrifugation at 1500 x g for 15 min, 2 ml of the supernatant were mixed with 4 ml 0.4 M Tris (pH 8.9) and 0.1 ml 0.01 M 5,5′-dithiobis-2-nitrobenzoic acid. The absorbance of the mixture at 412 nm was determined within 5 min, using a Hitachi Model 100-61 spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

Experiment 5: Effect of GSH on the Antineoplastic Effect of DXR against L1210 Mouse Leukemia. L1210 mouse leukemia cells, maintained in our laboratory by sequential i.p. transfer to CD2F1 mice, were obtained from the ascites of leukemic mice. Each of 36 mice received 1.0 x 10⁶ L1210 cells and were divided into 3 groups of 12 animals. Three days later one group was treated with DXR (14 mg/kg) plus 0.2 ml NS, the second group was treated with DXR (14 mg/kg) and GSH (500 mg/kg), and the third group was given an equivalent volume of NS. The number of survivors in each group was counted daily for 70 days.

Statistical Analysis

Mean values were compared using Student’s t test and the difference between survival rates, using the χ² test.

RESULTS

Experiment 1. As summarized in Table 1, the mice treated with GSH (500 or 1000 mg/kg) survived for more than 60 days. Two of the six mice treated with GSH (2000 mg/kg) died within 14 days and the rest of the mice survived for more than 60 days.

Experiment 2. The incidence of death at various intervals and the cumulative mortality rates of DXR-treated mice, with or without administration of GSH, are also summarized in Table 1. The DXR-treated mice, with or without the administration of GSH, were equally emaciated (not weighed). The mice treated with DXR (20 mg/kg) were all dead by 46 days. The mice treated with both GSH (500 mg/kg) and DXR (20 mg/kg) survived for a significantly longer period than the mice treated with DXR alone. The difference between the mortalities is statistically significant at day 14 (P < 0.02 and thereafter P < 0.01). The mice treated with GSH (1000 mg/kg) lived longer than the mice treated with DXR alone as tested at days 28 and thereafter (P < 0.01). The mice treated with GSH (250 mg/kg) showed a lower mortality rate during days 8 to 14 but no significant improvement of long term mortality rate was noted.

Experiment 3. WBC and GPT levels are summarized in Table 1. WBC are elevated in both DXR-treated and DXR- plus GSH-treated mice. The maximum WBC was at day 7 and it remained above normal level thereafter. The GPT level was highest on day 2 and returned to the normal range by day 7 in both groups. Average degrees of histological changes in the myocardium of DXR- or DXR- plus GSH-treated (14 mg/kg) mice are summarized in Table 3. As shown in Fig. 1, a and b, the electron micrographs in the DXR-treated mice revealed scattered regions with narrowed myofibrils, cytoplasmic edema, and swelling of mitochondria. These changes appeared at day 2 (Fig. 1a) and almost disappeared by day 56 (Fig. 1c). The narrowing of myofibrils was most significant at day 14 (Fig. 1b), and the cytoplasmic edema with vacuolization was mainly seen from days 7 to 14 (Fig. 1f). The mitochondrial swelling persisted to the same degree from days 2 (Fig. 1a) to 28. In the DXR- plus GSH-treated mice, the histological changes were significantly reduced, as shown in Fig. 2 in which mitochondrial swelling is minimal at days 2 (Fig. 2a) to 7 as compared to the normal control in Fig. 3. The narrowing of myofibrils stayed unchanged from days 7 to 28. The cytoplasmic edema was also minimal at day 2 (Fig. 2a) and although more advanced by day 14 (Fig. 2b) it had disappeared by day 28. This experiment was repeated and similar results were obtained. Semithin sections of the myocardium from DXR-treated mice at day 56 showed widening of intercellular spaces, as compared with GSH-treated and NS-treated control mice (Fig. 4).

Experiment 4. Fig. 5 shows that DXR at 14 or 20 mg/kg decreased the NP-SH level and GSH increased the NP-SH level dose dependently. The NP-SH levels reached a peak 30 min after the injection of DXR and returned to their initial levels by 210 min. The means of NP-SH level after administration of DXR (14 mg/kg), GSH (500 mg/kg), or DXR (14 mg/kg) plus GSH (500 mg/kg) are summarized in Table 4. DXR (14 mg/kg) significantly decreased the NP-SH level at 30 min (P < 0.01). The administration of GSH alone or DXR plus GSH caused a significant elevation of NP-SH level at 30 and 60 min (P < 0.01). No significant difference of NP-SH level was found at days 7 and 14.

Experiment 5. As shown in Fig. 6, the untreated leukemic mice died by day 11 and the DXR- or DXR- plus GSH-treated mice lived significantly longer than the untreated mice. There is no significant difference in survival between the DXR-treated and the DXR- plus GSH-treated mice.

DISCUSSION

The mortality of CD2F1 mice treated with DXR (20 mg/kg) is 100% (Table 1). The administration of GSH (250 mg/kg) reduced the mortality at 7 to 14 days. The mice treated with GSH (500 or 1000 mg/kg) survived for a significantly longer period than mice not treated with GSH. At day 14, GSH (1000 mg/kg) did not appear as effective as GSH (500 mg/kg). This is probably because of the synergistic toxicity of an excess dose of GSH and DXR, since GSH (2000 mg/kg) alone showed lethal effects at days 8 to 14 (Table 1).

The administration of DXR significantly elevates the WBC (Table 2). This indicates that DXR (20 mg/kg) does not suppress myelopoiesis but causes leukocytosis, and probably generalized tissue damage. The GPT level is transiently elevated before the rise of WBC. This indicates that administration of DXR causes some hematocellular damage, inducing leukocyto-
### Table 2 WBC and GPT levels after treatment with DXR or DXR plus GSH

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>WBC (cells/mm³)</th>
<th>GPT (Karmen units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DXR (14 mg/kg)</td>
<td>DXR (14 mg/kg) + GSH (500 mg/kg)</td>
</tr>
<tr>
<td>2 days</td>
<td>21.789 ± 1.875</td>
<td>15.549 ± 4.428</td>
</tr>
<tr>
<td>7 days</td>
<td>32.552 ± 12.147</td>
<td>29.861 ± 2.575</td>
</tr>
<tr>
<td>14 days</td>
<td>18.891 ± 1.501</td>
<td>24.058 ± 5.769</td>
</tr>
<tr>
<td>28 days</td>
<td>20.406 ± 1.936</td>
<td>17.810 ± 7.890</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GPT (Karmen units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXR (14 mg/kg)</td>
</tr>
<tr>
<td>DXR (14 mg/kg) + GSH (500 mg/kg)</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

* Mean ± SD (n = 3).

### Table 3 Electron microscopic changes in the myocardium of mice treated with DXR or DXR plus GSH

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>2 days</th>
<th>7 days</th>
<th>14 days</th>
<th>28 days</th>
<th>56 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXR (14 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial swelling</td>
<td>+*</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Narrowing of myofibrils</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+w</td>
<td>+w</td>
</tr>
<tr>
<td>Cytoplasmic edema</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DXR (14 mg/kg) + GSH (500 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial swelling</td>
<td>+w</td>
<td>+w</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Narrowing of myofibrils</td>
<td>+</td>
<td>+</td>
<td>+w</td>
<td>+w</td>
<td>+w</td>
</tr>
<tr>
<td>Cytoplasmic edema</td>
<td>+w</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* +, weakly positive; +, positive; ++, strongly positive; −, negative.

**Discussion**

The rise of the GPT level is slightly higher in GSH-treated mice (Table 2), suggesting that a high dose of GSH could enhance the hepatotoxicity of DXR. The DXR-treated mice were emaciated probably because of the hepatotoxicity as well as the toxicity to the mucosal membrane of the intestinal tract (6, 10). Hence, the causes of death in this study could include acute myocardial toxicity, hepatotoxicity, and damage to the digestive system. However, there is no evidence that the administration of GSH reduced the hepatocellular damage or emaciation. Thus, we attribute the major cause for improvement in mortality rate to protective effects of GSH against the acute myocardial toxicity of DXR.

Electron micrographs of the myocardium from DXR-treated mice showed definite abnormalities, such as cytoplasmic edema, mitochondrial swellings with vacuolization, and narrowing of myofibrils (Fig. 1, a and b), which are consistent with the changes described by others (4, 6, 10). Those changes are already detectable at day 2 (Fig. 1a). Overall changes are most apparent at day 14, reduced by day 28, and almost absent by day 56 (Fig. 1c; Table 3). It is possible that the initial mitochondrial damage is related to the later advance of myofibrillar narrowing or cytoplasmic edema. These changes were prevented by GSH administration. In particular, mitochondrial swelling at day 2 and myofibrillar narrowing, as well as cytoplasmic edema at day 14, were significantly reduced. Thus GSH administration protects not only against acute toxicity within 2 days of treatment but also against delayed toxicity.
but also against the subacute process of myocardial damage occurring by day 14.

Since GSH plays an important role as an oxygen radical scavenger, the drop of NP-SH level after the administration of DXR (Table 4) suggests consumption of intracellular GSH due to the influx of DXR and its metabolites. The administration of exogenous GSH promptly elevated myocardial NP-SH level dose dependently (Fig. 5). The NP-SH level reached a peak at 30 min and returned to its initial level in 3.5 h (Table 4, Fig. 5). This rise of NP-SH level may represent an increase of both...
intra- and extracellular GSH. GSH in the plasma converts plasma cystine to cysteine, which rapidly enters cells and accelerates intracellular GSH synthesis (14). Thus administration of GSH increases intra- and extracellular GSH levels, which detoxifies intracellular and, possibly, extracellular DXR-oxygen metabolites and free oxygen radicals. This explains the mechanism of the protective effect of GSH administration at early time intervals.

The histological changes, such as myofibrillar narrowing and cytoplasmic edema, appeared at day 2 and increased by day 14 (Table 3). Since plasma DXR is cleared very rapidly, with a triphasic plasma disappearance curve (15), DXR or its metabolites retained in the plasma are less likely to be responsible for the subacute process of myocardial damage. The myocardial tissue concentration of DXR or its metabolites declines more slowly. Doroshow et al. (7) reported that 48 h after administration of DXR (15 mg/kg), the myocardial tissue DXR is still about 30% of the maximum level, indicating that the turnover of myocardial tissue DXR is much slower than that in plasma. As shown by us in Fig. 1, a and b, and by others (4, 6, 10), DXR damages mitochondria. Since DXR is metabolized in mitochondria (2–4) the damage to mitochondria may lead to a delay of DXR metabolism. Administration of DXR depletes GSH peroxidase and superoxide dismutase which are the major enzymes responsible for detoxification of reactive oxygen metabolites in myocardium (16). Thus, GSH and GSH peroxidase systems in myocardial cells must be seriously impaired by DXR treatment and therefore the detoxification of DXR oxygen radicals must be further delayed. Hence, it is possible for a small amount of DXR or its metabolites to remain in the myocardial cells for more than 1 week. The retained DXR and

---

**Table 4 NP-SH levels (µmol/100 g wet tissue) after administration of therapeutic doses of GSH, DXR plus GSH, or DXR alone**

<table>
<thead>
<tr>
<th></th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>150 min</th>
<th>210 min</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (500 mg/kg)</td>
<td>179 ± 4.5</td>
<td>160 ± 6.1</td>
<td>158 ± 6.2</td>
<td>153 ± 3.4</td>
<td>150 ± 5.0</td>
<td>149 ± 2.1</td>
<td>148 ± 2.8</td>
</tr>
<tr>
<td>DXR (14 mg/kg) + GSH</td>
<td>171 ± 6.6</td>
<td>165 ± 1.4</td>
<td>153 ± 9.6</td>
<td>152 ± 9.4</td>
<td>147 ± 10.2</td>
<td>147 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>DXR (500 mg/kg)</td>
<td>146 ± 5.7</td>
<td>148 ± 8.9</td>
<td>148 ± 6.4</td>
<td>147 ± 6.0</td>
<td>148 ± 6.6</td>
<td>151 ± 9.6</td>
<td>150 ± 3.5</td>
</tr>
<tr>
<td>Control</td>
<td>153 ± 1.5</td>
<td>150 ± 1.2</td>
<td>151 ± 3.5</td>
<td>150 ± 3.0</td>
<td>154 ± 3.2</td>
<td>149 ± 3.5</td>
<td>151 ± 2.1</td>
</tr>
</tbody>
</table>

*Mean ± SD (n = 5 at 30 to 210 min and n = 3 at days 7 and 14).
The administration of GSH does not impair the antineoplastic effect of DXR against L1210 leukemia in mice (Fig. 6). This result is consistent with that of others (10). Thus GSH can protect the heart muscle from the toxicity of DXR without reducing its antitumor effect. GSH is commercially available as a detoxifying drug and is without significant untoward effects (11). An extremely high dose, of up to 200 mg/kg, can be given i.v. to patients (11). Hence, it may be possible to prevent acute DXR myocardial toxicity by administration of adequate doses of GSH to patients. The relationship between acute and chronic DXR myocardial toxicity should be further investigated to clarify whether prevention of acute DXR toxicity could contribute to prevention of chronic DXR myocardial toxicity.

ACKNOWLEDGMENTS

We wish to thank Yamanouchi Pharmaceutical Co., Ltd., which kindly supplied GSH.

REFERENCES

Prevention of Doxorubicin Myocardial Toxicity in Mice by Reduced Glutathione

Yasuhiro Yoda, Masaki Nakazawa, Tsukasa Abe, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/5/2551

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.