Alteration of Endogenous Glutathione Peroxidase, Manganese Superoxide Dismutase, and Glutathione Transferase Activity in Cells Transfected with a Copper-Zinc Superoxide Dismutase Expression Vector

EXPLANATION FOR VARIATIONS IN PARAQUAT RESISTANCE*

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Transfection of a human pSV2 (copper-zinc) superoxide dismutase expression vector into murine fibroblasts resulted in stable clones producing increased amounts of copper-zinc superoxide dismutase. A marked increase in endogenous glutathione peroxidase activity (up to 295%) and a smaller increase in glutathione transferase activity (up to 18%) also occurred. Manganese superoxide dismutase activity was decreased in all clones, whereas catalase and NADPH reductase activities were not affected. Alterations in glutathione peroxidase and manganese superoxide dismutase activities correlated with increases in copper-zinc superoxide dismutase activity. Whereas all clones were resistant to paraquat, a direct correlation between copper-zinc superoxide dismutase activity and resistance to paraquat did not exist. In agreement with previous reports, clones expressing the highest copper-zinc superoxide dismutase activity did not display the highest resistance to paraquat. However, there was a direct correlation between the increase in glutathione peroxidase activity and paraquat resistance (p < 0.002).

The discovery of the enzyme superoxide dismutase indicated that superoxide anion existed in cells and that its effects might be harmful (1). These harmful effects of superoxide have been extensively documented in vitro (2). Investigating the effects of superoxide in vivo has been elusive due to the instability of the radical and the difficulty of generating the radical intracellularly. Paraquat has been used as an intracellular generator of superoxide because the reduced form of the herbicide transfers an electron to molecular oxygen, producing the superoxide radical (3, 4). Despite intensive studies with paraquat, the mechanism of toxicity remains unclear, and whether superoxide is a necessary vector of paraquat toxicity in mammalian cells remains unsolved (5, 6).

In previous transfection studies (7, 8) of the human copper-zinc gene into mouse and HeLa cells, it was noted that paraquat resistance did not correlate with expression of superoxide dismutase. Cells with the highest intracellular enzyme activity did not display the highest resistance to paraquat (7). A lack of correlation after transfection of copper-zinc superoxide dismutase was again reported with NIH/3T3 and HeLa cells (8). As alterations in copper-zinc superoxide dismutase activity can also result in alterations in glutathione peroxidase activity (9), we considered the possibility that this enzyme may be involved in protecting against the herbicide.

MATERIALS AND METHODS

RESULTS

Eight G418-resistant clones were chosen on the basis of their stability and differences in paraquat resistance for detailed studies of their antioxidant enzyme activities. The antioxidant enzyme activities of these clones were monitored over a 4-month period to ensure that activities were stable. Paraquat resistance was also monitored and quantified during this time.

As expected, the activity of copper-zinc superoxide dismutase was increased in all clones as compared to the parent cells (Table 1). Whereas all clones were resistant to paraquat, cells expressing the highest amount of copper-zinc superoxide dismutase were not always the most resistant to paraquat (whether assayed by the trypan blue or the thymidine incorporation method) (Table 1). This discrepancy is in agreement with previous reports (7, 8). There was no statistical correlation between copper-zinc superoxide dismutase activity and paraquat resistance as measured by either assay (Table 2). There was a marked increase in selenium-dependent glutathione peroxidase activity in all clones when compared to the parental cells. Glutathione peroxidase activity correlated with increased copper-zinc superoxide dismutase activity (Table 2). Resistance to paraquat by either assay correlated more closely to glutathione peroxidase activity than to copper-zinc superoxide dismutase activity (Table 2). Surprisingly, manganese superoxide dismutase activity was markedly decreased in all of the clones. The decrease in manganese superoxide dismutase activity correlated with the increase in copper-zinc superoxide dismutase and glutathione peroxidase activities, but not with paraquat resistance (Table 2). NADPH reductase and catalase activities were equivalent to the parental cell line in all clones (data not shown). Glutathione transferase activity...
Superoxide dismutases are expressed as units required to inhibit cytochrome c reduction by xanthine oxidase under previously published conditions (1). Glutathione peroxidase in nanomoles/min/mg of protein. IC<sub>50</sub> values for paraquat resistance are expressed in micrograms/ml. Results are expressed as mean ± S.E. from three to seven different determinations.

<table>
<thead>
<tr>
<th>Clone</th>
<th>CuZnSOD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GSHPX</th>
<th>MnSOD</th>
<th>PQR-TR</th>
<th>PQR-3dThd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>9.2 ± 0.9</td>
<td>49 ± 3</td>
<td>0.85 ± 0.05</td>
<td>4.1 ± 0.9</td>
<td>12.3 ± 0.9</td>
</tr>
<tr>
<td>FD5</td>
<td>13.4 ± 0.7</td>
<td>104 ± 11</td>
<td>0.51 ± 0.09</td>
<td>8.2 ± 0.5</td>
<td>22.3 ± 0.5</td>
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<tr>
<td>FE5</td>
<td>13.4 ± 0.5</td>
<td>78 ± 6</td>
<td>0.50 ± 0.05</td>
<td>7.3 ± 1.0</td>
<td>17.3 ± 1.5</td>
</tr>
<tr>
<td>FC6</td>
<td>13.8 ± 1.4</td>
<td>54 ± 2</td>
<td>0.56 ± 0.08</td>
<td>4.8 ± 0.6</td>
<td>15.0 ± 1.4</td>
</tr>
<tr>
<td>FD4</td>
<td>15.5 ± 0.7</td>
<td>74 ± 4</td>
<td>0.54 ± 0.16</td>
<td>8.1 ± 1.1</td>
<td>16 ± 1.6</td>
</tr>
<tr>
<td>FA1</td>
<td>21.1 ± 0.8</td>
<td>80 ± 6</td>
<td>0.39 ± 0.11</td>
<td>8.9 ± 0.9</td>
<td>18.4 ± 0.6</td>
</tr>
<tr>
<td>FA4</td>
<td>18.8 ± 1.2</td>
<td>124 ± 22</td>
<td>0.30 ± 0.08</td>
<td>7.7 ± 0.6</td>
<td>19.5 ± 1.3</td>
</tr>
<tr>
<td>FC1</td>
<td>29.4 ± 1.8</td>
<td>106 ± 7</td>
<td>0.40 ± 0.04</td>
<td>7.3 ± 0.5</td>
<td>18.0 ± 0.9</td>
</tr>
<tr>
<td>FE4</td>
<td>25.9 ± 0.5</td>
<td>139 ± 20</td>
<td>0.39 ± 0.05</td>
<td>8.5 ± 1.0</td>
<td>30.0 ± 2.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>CuZnSOD, copper-zinc superoxide dismutase; GSHPX, glutathione peroxidase; MnSOD, manganese superoxide dismutase, PQR-TR, IC<sub>50</sub> value for paraquat resistance as measured by trypan blue exclusion (cell count); PQR-3dThd, paraquat resistance as measured by tritiated thymidine incorporation.

**DISCUSSION**

The introduction of an expression vector for copper-zinc superoxide dismutase had a marked effect on two other antioxidant enzymes: manganese superoxide dismutase and glutathione peroxidase. In addition, glutathione transferase was mildly affected, but the alterations in transferase activity correlated with glutathione peroxidase activity (p < 0.02) and not with superoxide dismutase activities. This suggests that increases in glutathione transferase activity occur as a competitive response to increases in glutathione peroxidase activity, that is, both enzymes are competing for the same substrate (7, 8). These results may also explain the previous report (22) of increased sensitivity toward paraquat in *Escherichia coli* transfected with multiple copies of superoxide dismutase. If the bacteria failed to respond to increased superoxide dismutase activity with increased production of endogenous glutathione peroxidase, then they could indeed become more sensitive to paraquat. The ratio of glutathione peroxidase to copper-zinc superoxide dismutase activity varied between clones. As a group, however, the mean ratio for the clones was 5.2 ± 0.5 (X ± S.E.) versus the 5.3 ratio for the parent cell line. The finding that this ratio is conserved and that all clones expressing increased superoxide dismutase responded with increased glutathione peroxidase supports the previous hypothesis (22) that balanced increments of antioxidant enzymes are necessary for cell integrity and growth.

The observation that manganese superoxide dismutase activity is decreased in clones overexpressing copper-zinc superoxide dismutase is an intriguing result and was noted qualitatively in a previous report (manganese superoxide dismutase bands were noted to be decreased on a gel superoxide dismutase assay) (8). There are two probable explanations for the decrease in manganese superoxide dismutase activity. The first explanation is that the presence of increased copper-zinc superoxide dismutase activity leads to a reduction in the intracellular steady-state superoxide concentrations, and manganese superoxide dismutase is not produced when not required by the cell. Alternatively, the overproduction of copper-zinc superoxide dismutase may be harmful to the cell by producing increased intracellular concentrations of hydrogen peroxide, and the response of endogenous glutathione peroxidase supports this hypothesis. If this were true, the cell may try to respond by decreasing superoxide dismutase production. As the introduced human copper-zinc superoxide dismutase is under control of the SV40 promoter and not the cell, the cell could only reduce production of endogenous superoxide dismutases (copper-zinc and manganese isoforms). Further studies are required to determine how manganese superoxide dismutase is controlled intracellularly.

Whereas these results strongly indicate that glutathione peroxidase protects against paraquat in cells under oxidative stress, it remains to be proven that an isolated increase in glutathione peroxidase activity (without an accompanying increase in superoxide dismutase activities) can protect against paraquat toxicity. The increase in endogenous glutathione peroxidase activity in these cells is actually a secondary activity was as follows: chlorodinitrobenzene, 45 ± 3 nmol/min/mg of protein; dichloronitrobenzene, 0.3; cumene hydroperoxide, 2.9; and ethacrynic acid, not detected (sensitivity < 0.1). The activity of glutathione transferase, with chlorodinitrobenzene as a substrate, was mildly increased in all clones, ranging from 47 to 52 ng/min/mg of protein (data not shown). Alterations in glutathione transferase activity, however, were small compared to alterations in glutathione peroxidase activity. The increase in glutathione transferase activity correlated with glutathione peroxidase activity (r = 0.745, p < 0.02), but not with copper-zinc superoxide dismutase or manganese superoxide dismutase activity.
response to increased copper-zinc superoxide dismutase activity. Similarly, the finding that manganese superoxide dismutase activity is decreased, despite the cells being resistant to paraquat, should not be taken as evidence that manganese superoxide dismutase is not involved in protecting against paraquat.

Paraoquat Resistance and Glutathione Peroxidase

Results are expressed as the mean ± standard deviation unless otherwise indicated. Comparison of results was performed using analysis of variance followed by a post-hoc Tukey’s test. Statistical significance is known as the P value and probability values (P value) were calculated from t-values. Probability values of less than 0.05 were considered significant.

Figure 1: The PXR-dependent copper-zinc superoxide dismutase (CuZnSOD) expression vector used to transfect the HeLa/753 cells. The 2.7 kb SOD promoter fused to the SV40 promoter (SV40 POI) was engineered to transfect the HeLa/753 cells. The expression vector was processed using the nucleotide transfer method to transfer the DNA into the cells. The results are shown in the form of DNA replication and the efficiency of the transfection.

Table 1: Statistical analysis

<table>
<thead>
<tr>
<th>Statistical analysis</th>
<th>X value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZnSOD expression</td>
<td>0.76</td>
</tr>
<tr>
<td>vs. POI-TR</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>vs. POI-TE</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>vs. SV40-TR</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>vs. SV40-TE</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>vs. POI-TR-POI</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>vs. SV40-TR-SV40</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>vs. POI-TR-SV40</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>vs. SV40-TR-POI-SV40</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

* Indicates a p value considered significant (p < 0.05)
Alteration of endogenous glutathione peroxidase, manganese superoxide dismutase, and glutathione transferase activity in cells transfected with a copper-zinc superoxide dismutase expression vector. Explanation for variations in paraquat resistance.

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