Enhanced Coexpression of Thioredoxin and High Mobility Group Protein 1 Genes in Human Hepatocellular Carcinoma and the Possible Association with Decreased Sensitivity to Cisplatin

Naoyuki Kawahara, Toshiya Tanaka, Akira Yokomizo, Hiroki Nanri, Mayumi Ono, Morimasa Wada, Kimitoshi Kohno, Kenji Takenaka, Keizo Sugimachi, and Michihiko Kuwano

Abstract

Thioredoxin (TRX), a disulfide-reducing intracellular protein, functions as a cellular defense mechanism against oxidative stress. In this study, we asked whether expression of TRX, glutathione-thiol transferase \( \pi \), and high mobility group protein 1 (HMG-1) genes is enhanced in human hepatocellular carcinoma and whether expression of these genes is associated with sensitivity to cisplatin. Both TRX and HMG-1 were co-overexpressed in almost all cancerous lesions in comparison to normal tissue in surgically resected hepatocellular carcinomas of 20 patients. Tumor sensitivity to cisplatin [cis-diaminedichloroplatinum (II)], but not to mitomycin C or doxorubicin, was correlated with mRNA levels of TRX and HMG-1 in cancer tissue. TRX and HMG-1 may be useful tumor markers, and TRX might be also a useful marker for sensitivity to cisplatin in human hepatocellular carcinomas.

Introduction

TRX, as a cellular thiol like GSH and metallothionein, has multiple functions, including acting as a radical scavenger, defense reactor to \( \text{H}_2\text{O}_2 \) and tumor necrosis factor, and redox-regulating molecule for NF\( \kappa \)B (1–3). Of the multiple biological functions of TRX (4), TRX shows protective activity against \( \text{H}_2\text{O}_2 \) or tumor necrosis factor \( \alpha \)-induced cytotoxicity (5), suggesting that TRX mediates cellular responses to oxidative stress or other environmental stimuli. We have recently demonstrated that cisplatin [cis-diaminedichloroplatinum (II)]-resistant cell lines have increased expression of TRX, and that reduced cellular levels of TRX using an antisense expression plasmid are associated with increased sensitivity to cisplatin in cultured human cancer cells (6). Sasada et al. (7) also have reported that human TRX overexpression induces development of drug resistance to cisplatin in cultured human cancer cells.

Various factors associated with drug sensitivity include GSTs and the high mobility group protein HMG-1 which mediate intracellular defense mechanism against xenobiotic toxicity or DNA damage (8–11). GSTs are multifunctional detoxification enzymes that catalyze the conjugation of GSH to hydrophobic and electrophilic compounds: a GST isozyme, GST-\( \pi \), limits cellular sensitivity to cisplatin, alkylating agents, and anthracyclines (8–11). However, direct involvement of GST-\( \pi \) in drug resistance to cisplatin has been disputed (9–11). On the other hand, formation of cisplatin-DNA adducts is essential for cisplatin-induced cytotoxicity, and a defect in excision repair results in markedly enhanced drug sensitivity to cisplatin and alkylating agents (12). The abundant DNA-binding molecule HMG-1, which affects replication and transcription (13), can bind to cisplatin-DNA adducts with high affinity (14, 15), and levels of HMG-1 protein are increased in cisplatin-resistant cancer cells (16).

One could ask whether levels of GST-\( \pi \), TRX, and HMG-1 are elevated in human tumors and also whether their levels are correlated with cisplatin sensitivity in cancer patients: cisplatin has been applied to the treatment of hepatocellular cancer (17, 18). In this study, we examined mRNA or protein levels of GST-\( \pi \), TRX, and HMG-1 in surgically resected hepatocellular carcinomas. Of these three factors, TRX and HMG-1 were specifically expressed in hepatocellular cancer. Moreover, the levels of TRX appeared to be correlated with cisplatin sensitivity of hepatic cancer.

Materials and Methods

Samples. Twenty patients with hepatocellular carcinoma who underwent hepatectomy in the Department of Surgery II, Kyushu University Hospital were enrolled. No patient had received chemotherapy prior to surgery. Tumor samples were frozen in liquid nitrogen and stored at \(-80°C \) until RNA extraction.

Northern Blot Analysis. Total RNA extractions, Northern blots, and hybridization were carried out as described previously (19). Total RNA was obtained from liver tissues and fractioned cells using the acid guanidium thiocyanate-chloroform extraction method. About 20 \( \mu \)g of total cellular RNA was electrophoresed on a formaldehyde-containing agarose gel, transferred to a Hybond N\( @ \)filter, and hybridized with various radiolabeled probes. DNA fragments used as probes were human GST-\( \pi \), human TRX (6, 8), and human HMG-1 cDNA isolated in our laboratory. Radioactivity was detected with a Fuji X BAS 2000 bio-imaging analyzer (Fuji Photo Film Co., Tokyo, Japan; Ref. 12). The expression levels of each gene were normalized to the \( \beta \)-actin mRNA level in each sample.

Immunohistochemistry. Resected specimens of hepatocellular carcinoma were fixed in 10% formaldehyde, processed, and embedded in paraffin (20). Five-\( \mu \)m-thick sections were obtained and stained immunohistochemically using the avidin-biotin-peroxidase complex method with rabbit polyclonal antibodies for TRX. The deparaffinized sections were preincubated in normal goat serum diluted 1:20 in PBS and stained overnight at 4°C with 1:200 anti-TRX polyclonal antibody. Sections were treated with biotinylated goat anti-rabbit antibody and then with the avidin-biotin-peroxidase complex, each for 30 min at room temperature. The peroxidase was developed with 0.02% \( \text{H}_2\text{O}_2 \) and 0.05% 3',3-diaminobenzidine tetrahydrochloride. A rinse with PBS was done between these steps to avoid the interaction of reagents. After the sections were counterstained with Meyer’s hematoxylin, they were dehydrated, rinsed in xylene, and coverslipped.

SDI Test. The SDI test was done using methods described previously (21, 22). In brief, tumor tissues were cut with scissors, and the resulting fragments were put into a sterile flask containing a mixture of Pronase (protease type...
we examined surgically removed liver samples from 20 patients with
Northern blot hybridization. Of the 20 patients, 17 samples (85%) showed simultaneous overexpression of the TRX and HMG-1 genes in hepatocellular carcinoma compared to noncancerous tissue. Examples of experimental data on four cases are shown in Fig. 1. In contrast,

Table 1 Comparison of clinicopathological characteristics of patients with high and low levels of TRX in hepatocellular carcinoma

<table>
<thead>
<tr>
<th>TRX levels</th>
<th>High</th>
<th>Low</th>
</tr>
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<tbody>
<tr>
<td>Mean age (yr)</td>
<td>64.1 ± 7.3</td>
<td>61.7 ± 7.4</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>9/2</td>
<td>8/1</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>6.3 ± 4.8</td>
<td>5.1 ± 3.6</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>6/11 (55%)</td>
<td>5/9 (56%)</td>
</tr>
<tr>
<td>Poor</td>
<td>5/11 (45%)</td>
<td>4/9 (44%)</td>
</tr>
<tr>
<td>Glutamic oxaloacetic transaminase (IU/liter)</td>
<td>79.2 ± 41.5</td>
<td>69.7 ± 28.8</td>
</tr>
<tr>
<td>Glutamic pyruvic transaminase (IU/liter)</td>
<td>81.7 ± 54.0</td>
<td>59.0 ± 26.0</td>
</tr>
<tr>
<td>Indocyanine green dye retention rate at 15 min (%)</td>
<td>15.8 ± 7.2</td>
<td>21.3 ± 11.0</td>
</tr>
<tr>
<td>Hepatitis B surface antigen positive</td>
<td>1/11 (9%)</td>
<td>2/9 (22%)</td>
</tr>
<tr>
<td>Antihapatitis C virus antibody positive</td>
<td>9/11 (82%)</td>
<td>7/9 (78%)</td>
</tr>
</tbody>
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a High level of TRX was defined as patients who had a 2-fold or more increase in TRX levels in cancer lesions than normal tissue. Low level of TRX was defined as a less than 2-fold increase.

b NS, not significant.

Results

To compare the levels of TRX, HMG-1, and GST-π in human hepatocellular carcinoma and the neighboring noncancerous tissue,
Fig. 4. A, correlation between TRX and HMG-1 levels in hepatocellular carcinoma (n = 20). The mRNA levels of TRX and HMG-1 were derived from Northern blot analyses of hepatocellular carcinoma (Fig. 1). TRX and HMG-1 expression indices were normalized to the β-actin mRNA level, and the expression index of each probe was plotted. The regression line $Y = 72.410 + 4.4176(X)$ had a correlation coefficient of $r = 0.829$. B, correlation between TRX levels in hepatocellular carcinoma (n = 20) and cisplatin sensitivity. Sensitivity to cisplatin, mitomycin C, and doxorubicin was determined using the SD! test on hepatic cancer cells derived from 20 patients (see “Materials and Methods”). The regression line $Y = 43.835 \log(X) + 83.323$ had a correlation coefficient of $r = 0.675$.

There were no increases in GST-α or β-actin mRNA levels in cancerous versus noncancerous lesions in hepatocellular carcinoma (also the four cases depicted in Fig. 1). We observed significant ($P < 0.05$) increases in mRNA levels of both TRX (Fig. 2A) and HMG-1 (Fig. 2B) in hepatocarcinoma in comparison to adjacent noncancerous tissue in all 20 patients. However, there was no significant correlation between the degree of TRX expression and any clinicopathological factors (Table 1). GST-α was overexpressed in the hepatocellular carcinoma of only six patients, and there was no significant correlation between overexpression of GST-α and clinicopathological factors (data not shown).

Fig. 3 shows an example of an immunostaining experiment with anti-TRX antibody in cases 1 and 2. Positive staining for TRX was found primarily in the nuclei and cytoplasm of cancer cells. Both cases also had enhanced expression of TRX in the cancerous lesion compared with adjacent noncancerous tissue (Fig. 3). Immunostaining with anti-TRX antibody in 10 other samples demonstrated that TRX mRNA levels were comparable to TRX protein levels (data not shown).

Since both TRX and HMG-1 levels were simultaneously higher in hepatocellular carcinoma lesions than in adjacent noncancerous tissue, we examined whether TRX levels were correlated with HMG-1 levels in hepatocellular carcinoma. Surprisingly, TRX levels in carcinoma were significantly ($P < 0.05$) correlated with HMG-1 levels (Fig. 4A).

These 20 samples from hepatocellular carcinoma patients were then examined for drug sensitivity to cisplatin, doxorubicin, and mitomycin C using SD! tests (21, 22). There appeared to be a correlation between the levels of TRX and cisplatin sensitivity in hepatocellular carcinoma cells (Fig. 4B). Higher expression of TRX resulted in decreasing sensitivity to cisplatin in hepatocellular carcinoma. Although TRX levels were correlated significantly ($P < 0.05$) with sensitivity to cisplatin, there were no significant differences in drug sensitivity to mitomycin C or doxorubicin based on TRX levels.4 Expression of HMG-1 tended to correlate with sensitivity to cisplatin, but statistical significance was not observed. We did not find any significant correlation between GST-α levels and sensitivity to cisplatin, mitomycin C, or doxorubicin.

Discussion

In the present study, we compared the levels of TRX, HMG-1, and GST-α in hepatocellular carcinoma lesions and adjacent noncancerous liver tissue in 20 patients with hepatocellular cancer. We found that (a) TRX and HMG-1, but not GST-α, were co-overexpressed in cancer lesions in 85% of the samples; (b) TRX levels were significantly correlated with HMG-1 levels in most hepatocellular carcinomas; and (c) TRX levels were inversely correlated with sensitivity to cisplatin in most hepatocellular carcinomas.

4 N. Kawahara, unpublished data.

GSTs are thought to be marker enzymes for preneoplastic/neoplastic states during hepatocarcinogenesis; GST-P, a rat homologue of human GST-α, is overexpressed in preneoplastic lesions arising during chemical carcinogenesis in the rat liver (23). During hepatocellular carcinogenesis in rats, enhanced coexpression of GST-P and multidrug resistance genes were observed (24). However, we did not observe overexpression of either GST-α (this study) or the human multidrug resistance 1 gene in human hepatocellular carcinomas. The discrepancy of GST expression in hepatocarcinogenesis in the rat and our data remains unclear at present.

In contrast, most human hepatocellular carcinomas have increased levels of TRX and HMG-1. Consistent with the present study, the immunostaining study by Nakamura et al. (25) demonstrated expression of TRX in hepatocellular carcinoma, chronic hepatitis, and liver cirrhosis. Hagen et al. (26) reported that hepatocytes adjacent to Kupffer cells produce oxygen radicals and also 8-oxoguanine, a potent mutagen/carcinogen. TRX shows a protective activity against oxygen radicals, and it mediates cellular response to oxidative stress (5). The inflammatory states in liver might induce oxygen radicals, resulting in enhanced expression of TRX. In Fig. 3, inflammatory state and cirrhosis in noncancerous tissue adjacent to a cancerous lesion might be associated with increased TRX levels. Overexpression of TRX may be a self-defense mechanism against inflammation or hepatocellular damages during hepatocellular carcinogenesis, resulting in continued overexpression of the TRX gene in hepatocellular carcinoma. The present study also found co-overexpression of both the TRX and HMG-1 genes in hepatocellular carcinomas. TRX activates the transcription factor NFκB and AP-1 (Jun/Fos; Refs. 2 and 3), and it may activate HMG-1 gene expression through transactivation of these transcription factors. However, further characterization of the HMG-1 promoter is required to elucidate this possibility.

Cisplatin as well as doxorubicin, mitomycin C, and mitoxantrone have been used to treat hepatocellular cancer (17, 18). TRX levels appeared to be closely correlated with sensitivities to cisplatin, but not to mitomycin C or doxorubicin, when drug sensitivities were assayed with the SD! test of hepatic cancer cells. The SD! test in vitro often predicts drug sensitivity of cancer cells in patients, but drug sensitivity determined using the SD! test does not always reflect the in vivo drug sensitivity (21, 22). The present study thus only suggests that TRX might be a drug sensitivity marker for cisplatin in patients with hepatocellular carcinoma. Okuyama et al. (20) have previously reported that expression of GST-α is correlated with sensitivity to cisplatin in human gastric cancer cells in patients. GST-α levels are also correlated with cisplatin sensitivity in some cultured cancer cell lines (8). However, GST-α levels in this study were not associated with the degree of cisplatin sensitivity in human hepatocellular carcinomas. In contrast, Yokomizo et al. (6) have demonstrated that cisplatin-resistant cancer cell lines have increased levels of TRX and
that TRX antisense transfection induces cellular sensitization to cisplatin, doxorubicin, mitomycin C, H$_2$O$_2$, and UV light irradiation. Sasada et al. (7) have further demonstrated that cisplatin enhances expression of TRX in T cells and also that TRX cDNA transfection induces acquisition of cisplatin resistance. TRX thus appears to detoxify cytotoxic insults by cisplatin or other anticancer agents, possibly through a scavenger pathway of toxic oxidative stress. However, it remains unknown why TRX levels are not correlated with drug sensitivity to doxorubicin or mitomycin C in hepatocellular cancer.

On the other hand, HMG-1 levels tended to correlate with the degree of cisplatin sensitivities. HMG-1 inhibits repair of 1,2-deoxy(GpG)-cisplatin cross-linking (15). A yeast mutant missing the HMG-domain protein Ixr1 is more sensitive to cisplatin than its wild-type counterpart (27). However, there was no correlation between the human HMG domain protein SSRP1 levels and sensitivity to cisplatin in various human tissues or cell lines (28). At present, it remains unclear whether HMG-1 is directly involved in the repair of cisplatin-DNA adducts in human hepatocellular carcinomas.

Both TRX and HMG-1 were increased in most hepatocellular carcinomas. TRX/HMG-1 may function as self-defense mechanisms against environmental stimuli by carcinogenesis-associated insults, including inflammation or hepatic cell damage. Moreover, it appeared likely that TRX might have a key role in drug sensitivity to cisplatin in hepatocellular carcinomas in patients, suggesting the TRX-mediated defense mechanism against the potent DNA-interacting anticancer drug. TRX/HMG-1 may provide a new tumor marker, and TRX may also provide a marker for cisplatin sensitivity in hepatocellular carcinoma.

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References

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