Induction of Apoptosis and Apoptosis-associated Gene Products with Neoadjuvant Chemotherapy for Gastric Cancer

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ABSTRACT

BACKGROUND. It has been reported that induction of apoptosis by anticancer drugs is an important factor in cancer chemotherapy. To evaluate the effect of neoadjuvant chemotherapy for gastric cancer, I examined the correlation between induction of apoptosis and expression of p53, Bcl-2, and Bax.

METHODS. Eighty-five patients with advanced gastric cancer were retrospectively divided into the following two groups: fifty-four patients received 5-fluouracil (5-FU) at 300 mg/body/day for 14 days and cisplatin (CDDP) at 15 mg/body/day for 2 days as group A; thirty-one patients without any preoperative chemotherapy as group B. Apoptotic indices (AI) were calculated by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) method. The expression of p53, Bcl-2, and Bax were examined by immunohistochemical staining. According to histological change of the tumor by neoadjuvant chemotherapy, patients were divided into effective group (major change) and ineffective group (minor or no change).

RESULTS. AI of group A was significantly higher than that of group B (1.12 ± 0.40 vs. 0.67 ± 0.24; p < 0.01). In group A, AI of p53 positive cases was significantly lower than that of negative cases (0.92 ± 0.32 vs. 1.39 ± 0.32; p < 0.01). AI of the effective group was
significantly higher than that of the ineffective group (1.02 ± 0.38 vs. 1.34 ± 0.35; p < 0.01).

There was no significant correlation between AI and expression of Bcl-2 or Bax. In group A, cumulative survival rates of the effective group and Bcl-2 positive patients, or high AI patients were significantly higher than those of the ineffective group, Bcl-2 negative patients, or low AI patients, respectively.

CONCLUSIONS. It was demonstrated that neoadjuvant chemotherapy for gastric cancer enhanced induction of apoptosis. It was suggested that AI might be a factor to evaluate the effect of neoadjuvant chemotherapy.

INTRODUCTION

Apoptosis, one process of cell death, plays important roles in the regulation of tissue development and morphogenesis (1-3). Apoptosis also occurs in tumors and plays a key role in determining tumor growth (4, 5). Apoptosis is induced in cancer cells by anticancer agents (5-11). It has been reported that anti-cancer effects were related to induction of apoptosis (12-15). It is believed that apoptosis might be a predictor of chemotherapeutic effect or outcome.

In the current study, I examined the correlation between induction of apoptosis and the expression of p53, Bcl-2 and Bax by neoadjuvant chemotherapy with continuous infusion of 5-fluorouracil (5-FU) and cisplatin for gastric cancer. Furthermore, I investigated the correlation among apoptosis, chemotherapeutic effect, and survival.
PATIENTS AND METHODS

This study involved the analysis of 85 patients with advanced gastric cancer who had undergone gastrectomy for gastric carcinoma between 1991 and 1998 at the First Department of Surgery, Chiba University School of Medicine. Eighty-five patients were retrospectively categorized into the following two groups: 54 patients who received 5-fluorouracil (5-FU) at 300 mg/body/day for 14 days and cisplatin (CDDP) at 15 mg/body/day for 2 days as group A; the other 31 without any preoperative chemotherapy as group B. Informed consent was contained in all patients. Resected specimens were fixed in 10% formalin and embedded in paraffin. Representative sections were prepared and stained with hematoxylin and eosin to assess histopathologic diagnosis and the response of preoperative adjuvant chemotherapy under light microscope. Serial sections were examined by immunohistochemistry, and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method.

Response assessment of neoadjuvant chemotherapy

To evaluate the therapeutic effects on tumors, I observed histological changes that occurred in the tumors in accordance with Japanese Classification of Gastric Carcinoma (10). Such changes included ballooning or vacuolation of cells, pyknosis of nuclei, disappearance of cells or tissue, disorganization of glandular structures, necrosis of cells or tissue, and so on. According to the amount of necrosis or disappearance of the tumor in the estimated total amount of lesion, two categories, effective group and ineffective group were defined.

Effective group corresponds to the presence of necrosis or disappearance of the tumors in less than 1/3 of the whole lesion, or only cellular or structural changes in variable amounts. Ineffective group corresponds to the presence of necrosis or disappearance of the tumors in more than 1/3 of the whole lesion.

Immunohistochemistry

The deparaffinized and rehydrated slides were boiled in 10 mM citrate buffer (pH 6.0) for 5 minutes an autoclave at 121 °C (17). The sections were immunohistochemically stained by the labeled streptavidin-biotin peroxidase method (18) (LSAB2 Kit; Dako Japan Inc., Kyoto, Japan) with the following primary antibodies: monoclonal antibodies raised against p53 protein (DO-7 [1:100]; Dako Japan Inc.), Bel-2 (100 [1:100]; Immnotech S. A., Marseille, France), and Bax (4F11 [1:100]; Immnotech S.A.). The primary antibody was applied, and the sections were incubated overnight at 4 °C. Diaminobenzidine (DAB) was used as the chromogen. The sections were counterstained slightly with hematoxylin. The presence of > 30% positive cells was defined as p53 positive (19-21). The cases were considered positive
for Bcl-2 or Bax, when positive staining of cytoplasm was observed in > 10% of tumor cells respectively (21-23).

**TUNEL Staining**

Apoptotic carcinoma cells were identified using the Apop Tag in situ apoptosis detection Kit (Oncor, Inc., Gaithersburg, MD), that is based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method (24). Briefly, sections were deparaffinized, rehydrated, and washed with distilled water (DW). The tissues were digested with 20 μg/ml protease K (Boehringer Mannheim, Mannheim, Germany) at 37 °C for 30 minutes. Endogenous peroxidase activity was blocked by incubating them in 3% hydrogen peroxide in phosphate-buffered saline (PBS). The sections then were incubated with terminal deoxynucleotidyl transferase at 37 °C for 60 minutes to add the digoxigenin-conjugated dUTP to the 3'-OH ends of fragmented DNA. Anti-digoxigenin antibody peroxidase was applied to the sections for detecting the labelled nucleotides. The sections were stained with DAB and counterstained slightly with hematoxylin. The percentage of apoptotic cells was calculated as the number of TUNEL positive cancer cells per 2000 cancer cells in the most frequently identified areas and determined as the apoptotic index (AI).

**Statistical Analysis**

The statistical difference was analyzed using the chi-square test and Mann-Whitney U test. Survivor analysis was estimated by the Kaplan-Meier method and examined by the log-rank test. A P value < 0.05 was considered statistically significant.
RESULTS

Clinicopathological features

Clinicopathological features for group A and B are shown in Table 1. There were no differences in the clinicopathological features between them.

Histological evaluation of the therapeutic effect

The patients in group A were divided into the effective group and the ineffective group according to the histological evaluation. Seventeen patients were in the effective group, and 37 in the ineffective group (Table 3).

Expression of p53, Bcl-2, and Bax

p53 immunoreactivity was detected in 57.4% (31 patients) of group A and in 67.7% (21 patients) of group B. Positive expression of Bcl-2 was observed in 38.9% (21 patients) of group A and in 35.5% (11 patients) of group B. Positive expression of Bax was observed in 59.3% (32 patients) of group A and in 61.3% (19 patients) of group B. There were no significant differences in the positive rate of p53, Bcl-2, or Bax expression between group A and group B (Table 2).

Apoptotic index

An example of apoptosis cells detected by the TUNEL method is shown in Figure 1.

Apoptotic index for group A was 1.12 ± 0.40%, compared with 0.67 ± 0.24% for group B; the difference was statistically significant (P < 0.01, Table 2).

Relationship between apoptosis and therapeutic effect or protein expression

The correlation in group A between apoptosis and therapeutic effect or expression of p53, Bcl-2, or Bax are shown in Table 3. In group A, apoptotic index for p53 negative tumors was 1.39 ± 0.32%, compared with 0.92 ± 0.32% for positive tumors, which was statistically different (P < 0.01). AI of the effective group was significantly higher than that of the ineffective group (1.02±0.38 vs. 1.34±0.35; p < 0.01). No significant differences between the apoptotic index and Bcl-2 or Bax expression were found in group A.

Survival

The survival curves in relation to the histological change by chemotherapy in group A are shown in Fig. 2. There was a significant difference in survival between two groups (P <0.05).

The 5-year survival rates for the effective and the ineffective group were 87.9% and 32.9%, respectively. Stratifying patients by Bcl-2 status showed a significant difference in survival (P <0.05), with Bcl-2 positive patients having a better prognosis (Fig. 3). The 5-year survival rates of patients with Bcl-2 positive and negative expression were 69.4% and 38.8%, respectively. Stratification for the frequency of apoptosis in group A showed that patients with
low AI (< 1.33) did worse compared with those with high AI (≥ 1.33), although it was of marginal significance (P = 0.05, Fig. 4). The 5-year survival rates of patients with high and low AI were 70.9% and 36.4%, respectively. There was no significant difference in survival between the patients with p53 positive tumors and those with p53 negative.

DISCUSSION

Although progression of diagnostic methods has declined mortality of gastric cancer, many patients have been diagnosed as having advanced disease. Resectability is one of the important prognostic factors in patients with gastric carcinoma (25). To increase curative resection, neoadjuvant chemotherapy for gastric cancer patients with serosal invasion using by 5-FU and cisplatin has been performed at the First Department of Surgery, Chiba University School of Medicine since 1993. I evaluated the therapeutic effects by observing histological changes that occurred in the tumors by neoadjuvant chemotherapy.

Anti-cancer agents induce apoptosis in cancer cells in vitro (5-11). Some clinical or experimental reports demonstrated that anti-cancer drugs, including 5-FU or cisplatin, induce apoptosis in cancer cells in vivo (12, 13, 26-28). Therefore, I have been interested in relationship between histological change and induction of apoptosis and apoptosis-associated gene. Although all mechanisms of induction of apoptosis by anti-cancer agents have not been clarified, p53-dependent pathway has been showed to play important roles in apoptosis induced by anti-cancer agents (29, 30). p53 is a multifunctional tumor suppressor protein that is charged with the task of monitoring the integrity of the genome (31). p53 arrests the cell cycle in G1 following the detection of DNA damage and repair it (32, 33). When repair of the
damage is not feasible, p53 activate the apoptosis pathway (34). p53 is a positive transcriptional regulator of the apoptosis by inducing Bax protein (35) and a negative regulator of the apoptosis repressor protein, Bcl-2 (36). Bax homodimers accelerate apoptosis, whereas Bax heterodimers with Bcl-2 is inactivated. The Bcl-2/Bax ratio by transcriptional regulation of p53 is a central component of p53-dependent apoptosis (34). My results showed induction of apoptosis of gastric cancer cells after neoadjuvant chemotherapy and moreover, it was significantly induced in p53 mutant tumors compared with p53 wild-type tumors. However, I could not find correlation between the frequency of apoptosis and expression of Bcl-2 or Bax. Immunohistochemistry could not evaluate correctly the activity and status of heterodimer or homodimer of these proteins. Moreover, there are other homologues such as Bad, Bcl-Xs, Bcl-Xl in Bcl-2 family proteins (37-39). They interact with each other and constitute a network of homo- and heterodimers that regulate apoptosis. Bcl-2 heterodimerizes with Bax, Bcl-Xs, Bcl-Xl, Bad, BIK (38-42). To clarify my results, further study of the relationship among these homologues will be required.

Many studies have reported relationship between apoptosis and chemotherapeutic response such as tumor reduction estimated by X-ray and endoscopy (12, 13, 43, 44). In my study, a correlation was showed between apoptosis and histological chemotherapeutic effect.

Furthermore, my results showed that patients with good chemotherapeutic effect, Bcl-2 positive, and high AI had a better prognosis. The results about prognosis of the patients with Bcl-2 positive or high AI seem to be conflict. However, some studies have demonstrated that Bcl-2 expression in some types of cancer was paradoxically associated with favorable outcome for patients (21, 45-54). It has been shown that bcl-2 inhibits apoptosis and also reduces cell proliferation. These facts are consistent with my results.

I demonstrated the correlation between the induction of apoptosis gastric cancer cells after neoadjuvant chemotherapy with continuous infusion of 5-FU and cisplatin and histological chemotherapeutic effect or survival. This result suggests that apoptosis might be a predictor of chemotherapeutic effect for gastric cancer with neoadjuvant chemotherapy. Selection of tumors in which apoptosis expected to occur with high frequency might be available for chemotherapeutic effect.
ACKNOWLEDGMENTS

I thank prof. N. Nakajima, N. Takiguchi, and K. Koda for their constant encouragement and prof. H. Ishikura for giving me an opportunity to perform the study in Second Department of Pathology. I also thank Dr. B. Akikusa and members of Second Department of Pathology for their help.

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<table>
<thead>
<tr>
<th>Clinicopathological Features</th>
<th>group A (n = 54)</th>
<th>group B (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average age (yrs)</td>
<td>66.2</td>
<td>62.9</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>44/10</td>
<td>22/9</td>
</tr>
<tr>
<td>Stage&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ib</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>IIIa</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>IIIb</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Iva</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Ivb</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>intestinal</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>diffuse</td>
<td>20</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Japanese classification of gastric carcinoma
Table 2: Apoptosis and expression of p53, Bcl-2, and Bax

<table>
<thead>
<tr>
<th></th>
<th>group A (n = 54)</th>
<th>group B (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic index (%)*</td>
<td>1.12 ± 0.40</td>
<td>0.67 ± 0.24</td>
</tr>
<tr>
<td>p53 positive cases ^</td>
<td>31 (57.4)</td>
<td>21 (67.7)</td>
</tr>
<tr>
<td>Bcl-2 positive cases §</td>
<td>21 (38.9)</td>
<td>11 (35.5)</td>
</tr>
<tr>
<td>Bax positive cases ¶</td>
<td>32 (59.3)</td>
<td>19 (61.3)</td>
</tr>
</tbody>
</table>

* P < 0.01 compared with group B (Mann Whitney U test)
^ Apoptotic index was calculated as the number of TUNEL positive cancer cells per 2000 cancer cells
§ The presence of > 30% positive cells was defined as positive
¶ The presence of > 10% positive cells was defined as positive

Table 3: Correlation between apoptosis and chemotherapeutic effect and expression of p53, Bcl-2, and Bax in group A

<table>
<thead>
<tr>
<th>Chemotherapeutic effect</th>
<th>No. of patients</th>
<th>Apoptotic index (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective</td>
<td>17</td>
<td>1.34 ± 0.35</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Ineffective</td>
<td>37</td>
<td>1.02 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>p53 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>1.39 ± 0.32</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Positive</td>
<td>31</td>
<td>0.39 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Bcl-2 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>33</td>
<td>1.06 ± 0.36</td>
<td>0.256</td>
</tr>
<tr>
<td>Positive</td>
<td>21</td>
<td>1.20 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>Bax expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>1.02 ± 0.31</td>
<td>0.228</td>
</tr>
<tr>
<td>Positive</td>
<td>32</td>
<td>1.17 ± 0.43</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1  Detection of apoptosis by TUNEL method in a patient received neoadjuvant chemotherapy with 5-FU and cisplatin. Arrows show typical apoptotic cells. TUNEL signals are observed in the nuclei of apoptotic cells. (original magnification X400)

Figure 2  Kaplan-Meier survival curves of patients received neoadjuvant chemotherapy according to the therapeutic effects on tumors. There was a significant difference in survival between patients with effective change and those with ineffective change (p<0.05). The 5-year survival rates for the effective and ineffective group were 87.9% and 32.9%, respectively.
Figure 3  Kaplan-Meier survival curves of patients received neoadjuvant chemotherapy according to Bcl-2 expression. There was a significant difference in survival between the positive and negative patients (p < 0.05). The 5-year survival rates of patients with Bcl-2 positive and negative expression were 69.4% and 38.8%, respectively.

Figure 4  Kaplan-Meier survival curves of patients received neoadjuvant chemotherapy according to high or low apoptotic index (p = 0.05). The 5-year survival rates of patients with high and low AI were 70.9% and 36.4%, respectively.
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