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Effect of Preoperative 5-Fluorouracil on Apoptosis of Advanced Gastric Cancer

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Background: Several studies have reported on apoptosis and the effect of anticancer chemotherapy.
Methods: We studied apoptosis induced by 5-fluorouracil (5-FU) given preoperatively to 28 patients with advanced gastric cancer and compared the findings with 101 untreated patients. The expression of *bcl-2* oncoprotein, cell phase fractions, and histological chemotherapeutic effects were also compared with the apoptotic changes.
Results: The apoptotic and S-phase fractions in 5-FU-treated patients (apoptotic fraction: 10.46 ± 6.93%, S-phase fraction: 17.49 ± 11.65%) were significantly greater than those in untreated controls (apoptotic fraction: 6.56 ± 5.06%, S-phase fraction: 12.17 ± 6.78%). A positive correlation was observed between 5-FU-induced apoptosis and accumulation of tumor cells in the S-phase fraction. There was an inverse relationship between *bcl-2* oncoprotein expression and apoptosis in 5-FU-treated patients, but no significant correlation between histological effect and apoptosis. However, two patients with significant histological effects showed no *bcl-2* oncoprotein expression, whereas the histological effects were mild in all the *bcl-2*-positive patients.
Conclusions: Apoptosis may be induced by 5-FU administered preoperatively and *bcl-2* oncogene expression may suppress 5-FU-induced apoptosis.
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KEY WORDS: **apoptotic fraction; bcl-2 oncoprotein; stomach cancer; chemotherapy**

INTRODUCTION

Recently, apoptosis, or programmed cell death, was reported to be an important regulator of various events in the human body, including developmental processes and clonal selection of the immune system [1,2]. In the field of cancer research, apoptosis is also concerned with important processes including tumor progression, tumor growth, and the effects of radiation and several DNA-damaging chemotherapy regimens [3–6].

5-Fluorouracil (5-FU) is widely used for gastrointestinal cancers. Two biochemical mechanisms are considered to be involved in the cytotoxic effects of 5-FU [7–12]. One is the conversion of 5-FU to 5-fluorodeoxyuridine monophosphate (FdUMP), which binds irreversibly to thymidylate synthetase (TS), resulting in inhibition of the conversion of deoxyuridine monophosphate to thymidine monophosphate (dTMP), which leads to dTMP depletion and DNA synthesis inhibition. The other involves the conversion of 5-FU to fluorouridine triphosphate, which is incorporated into RNA, resulting in RNA dysfunction. In our previous study [13], we demonstrated that apoptosis induced by 5-FU was related to the biochemical changes of human tumor xenografts transplanted into nude mice. A study of the effects of 5-FU on apoptosis, TS, and the incorporation of 5-FU into RNA

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and cell cycle fractions in two human gastrointestinal tumor xenografts demonstrated that apoptosis induced by 5-FU occurred after TS inhibition and tumor cells accumulated in the S-phase fraction due to inhibition of DNA synthesis.

In this study, we investigated the apoptosis of advanced gastric carcinomas in patients treated preoperatively with 5-FU. The relationships between apoptosis and the histological chemotherapeutic effects and *bcl-2* oncoprotein expression were also investigated.

MATERIALS AND METHODS

Twenty-eight patients with stage 3 or 4 [14] advanced gastric cancer underwent gastrectomy at Tochigi Cancer Center, from 1986 to 1995. They were all treated with 5-FU until just before gastrectomy and gave their informed consent. 5-FU (350 mg/m^2 per day for 1 week) was given by continuous infusion through a central venous catheter. A total of 101 patients with stage 3 or 4 advanced gastric cancer who had received neither preoperative chemotherapy nor radiotherapy were gastrectomized during the same period and served as a control group. All the tumors in both these groups were adenocarcinomas.

All the surgical specimens were fixed in 20% buffered formalin for 1 or 2 days and embedded in paraffin wax.

HISTOLOGICAL CHEMOTHERAPEUTIC EFFECT AND *bcl-2* ONCOPROTEIN STAINING

Thin sections (3 μm) were cut, mounted on poly-L-lysine-coated glass slides, and dried. The chemotherapeutic effects were assessed histologically by examining hematoxylin and eosin-stained sections, according to the general rules for the study of gastric cancer in relation to surgery and pathology of the Japanese Research Society for Gastric Cancer [14]. The histological response grades were 0: no change, 1: slight change (1a: necrosis or disappearance of the tumor is present in <⅓ of the whole lesion, or only cellular or structural changes are visible in variable amounts, and 1b: necrosis or disappearance of the tumor in no more than ⅔ of the whole lesion) and 2: moderate change (necrosis or disappearance of the tumor in >⅔ of the whole lesion, but viable tumor cells still remain).

Immunostaining with a murine antihuman *bcl-2* monoclonal antibody (mAb) (DAKO, Tokyo, Japan) was performed using an immunoperoxidase method (LSAB kit, DAKO). After facilitation of antigen recognition by autoclaving for 5 min at 121°C, serial sections were incubated with the anti-*bcl-2* mAb at a dilution of 1:20, for 1 h at room temperature. The *bcl-2* immunostaining intensity was classified as positive or negative according to the staining intensities of the gastric mucosal lymphoid follicles and infiltrating lymphocytes.

DETECTION OF APOPTOSIS AND FLOW-CYTOMETRIC ANALYSIS

Sections, 50 μm thick, from the same paraffin blocks used for *bcl-2* oncoprotein immunostaining were used for detection of apoptosis and flow-cytometric analysis. The samples were deparaffinized, incubated overnight at 37°C in citrate-buffered trypsin, and single-cell suspensions were obtained by the modified method of Hedley et al. [15]. Apoptosis was identified and evaluated by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick and labeling method [16] using flow cytometry, as described previously. Apoptosis was analyzed flow-cytometrically using the MEBSTAIN Apoptosis kit (Medical & Biological Laboratories Co., Nagoya, Japan). The nuclear suspensions were incubated with 40 μg/ml proteinase K for 30 min, followed by TdT buffer, and then TdT (3.0 unit/μl) 5 μl and biotinylated dUTP (250 μM) 5 μl for 60 min, at 37°C. The reaction was terminated by termination buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature and incubated with biotinylated fluorescein isothiocyanate. After incubation with ribonuclease A (Sigma, St. Louis, MO), the suspensions were incubated with propidium iodide, and flow cytometry was performed using a FACScan flow-cytometer (Becton Dickinson, San Jose, CA). The apoptosis-positive fraction was calculated and the cell cycle analyzed using CELL Quest and Mod Fit (Becton Dickinson), respectively.

The data are presented as means ± standard deviation (S.D.) and were analyzed statistically using Student's t-test for unpaired data and the Chi-squared test. Differences at P values <0.05 were considered to be statistically significant.

RESULTS

Histological Chemotherapeutic Effect and *bcl-2* Staining

No apparent histological chemotherapeutic effects were observed in the 28 treated patients: the grades were 0 in 5, 1a in 21, 1b in 1, and 2 in 1. Staining of *bcl-2* protein was observed frequently at the apical portions of tumor cells and was prominent in the nuclear membranes in both the control and treated groups (Fig. 1). Positive bcl-2 protein staining was observed in 9 of 28 (32.1%) 5-FU-treated patients and in 39 of 101 (38.6%) controls and these values were not significantly different.

Apoptosis and cell phase fraction. In the control group, the apoptosis-positive fraction ranged widely from 0.02–21.95%, and the mean ± S.D. was 6.56 ± 5.06% (Fig. 2). The mean ± S.D. apoptosis-positive fraction in the 5-FU treated group was 10.46 ± 6.93% (range: 1.27–27.07%) and was significantly different from the control group value.

Flow-cytometric cell cycle analysis showed that the S-phase fractions of the control and 5-FU-treated groups were 12.17 ± 6.78% (range, 2.21–34.15%) and 17.49 ±

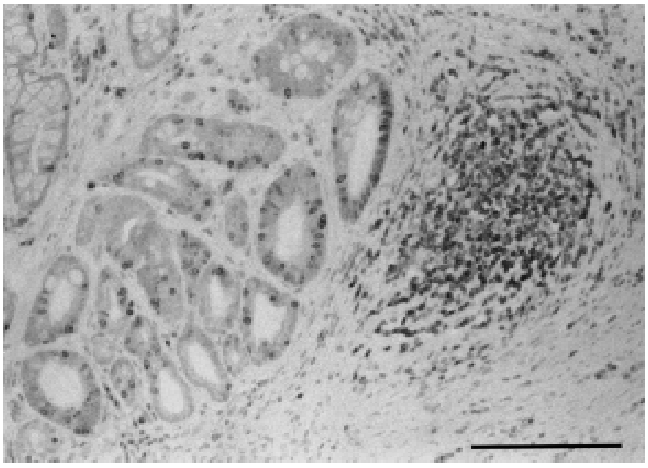

Fig. 1. Immunohistochemical staining of *bcl-2* oncoprotein: Positively stained cancer cells and lymphocytes. Bar = 100 μm.

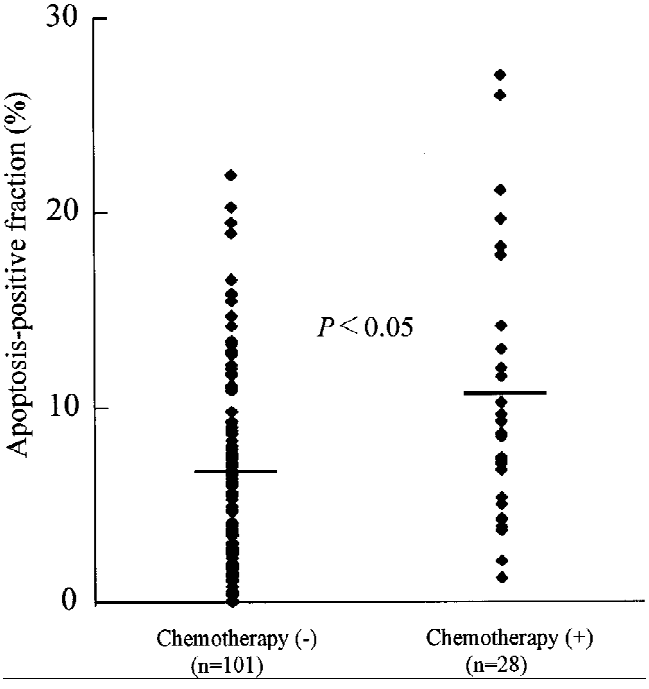

Fig. 2. Apoptotic-positive fraction with reference to preoperative chemotherapy.

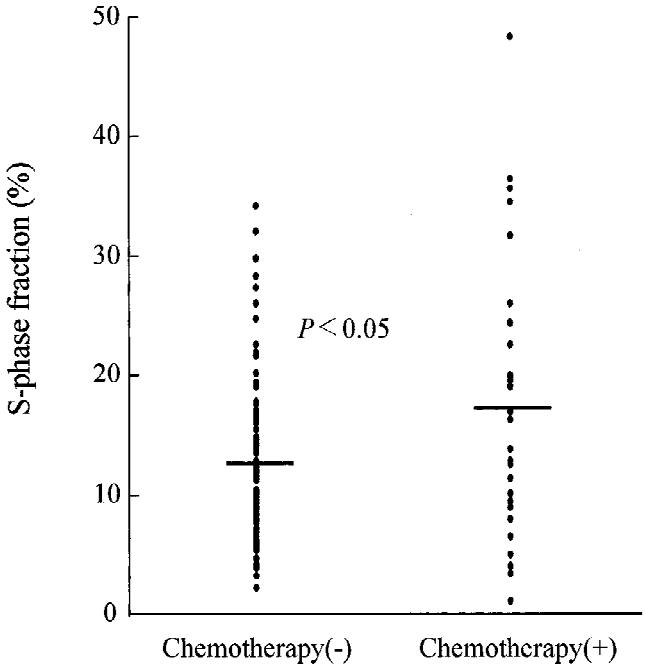

Fig. 3. S-phase fraction with reference to preoperative chemotherapy.

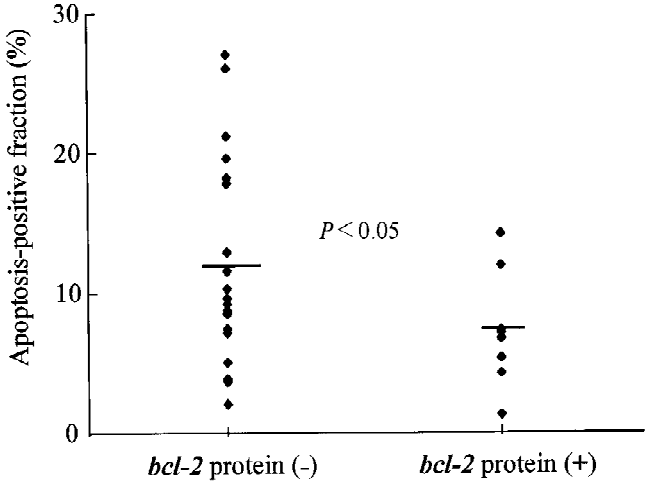

Fig. 4. The relationship between bcl-2 oncoprotein and apoptosis in preoperatively 5-FU-treated patients.

11.65% (range, 1.09–48.38%), respectively, and these values also differed significantly (Fig. 3).

There was an inverse relationship between the apoptosis-positive fraction and *bcl-2* oncoprotein expression in the 5-FU-treated group (Fig. 4), in which 9 *bcl-2*-positive patients had a mean apoptosis-positive fraction of 6.97 ± 4.00%, which was significantly lower than the mean value of 12.11 ± 7.48% for the 19 *bcl-2*-negative patients.

Histological Chemotherapeutic Effect and Apoptosis

As no histological chemotherapeutic effect was apparent in the majority of the treated patients, there was no significant correlation between the histological chemotherapeutic effect and apoptosis (Fig. 5). However, the *bcl-2* immunostaining experiment showed that *bcl-2*-positive patients showed only grades 0 and 1a responses.

DISCUSSION

The efficacy of anticancer chemotherapy against solid tumors, particularly gastrointestinal tumors, is generally low [17]. Many combined chemotherapy regimens that include 5-FU are widely used for gastric cancer [17,18], although their efficacy rates are limited to <50%, even

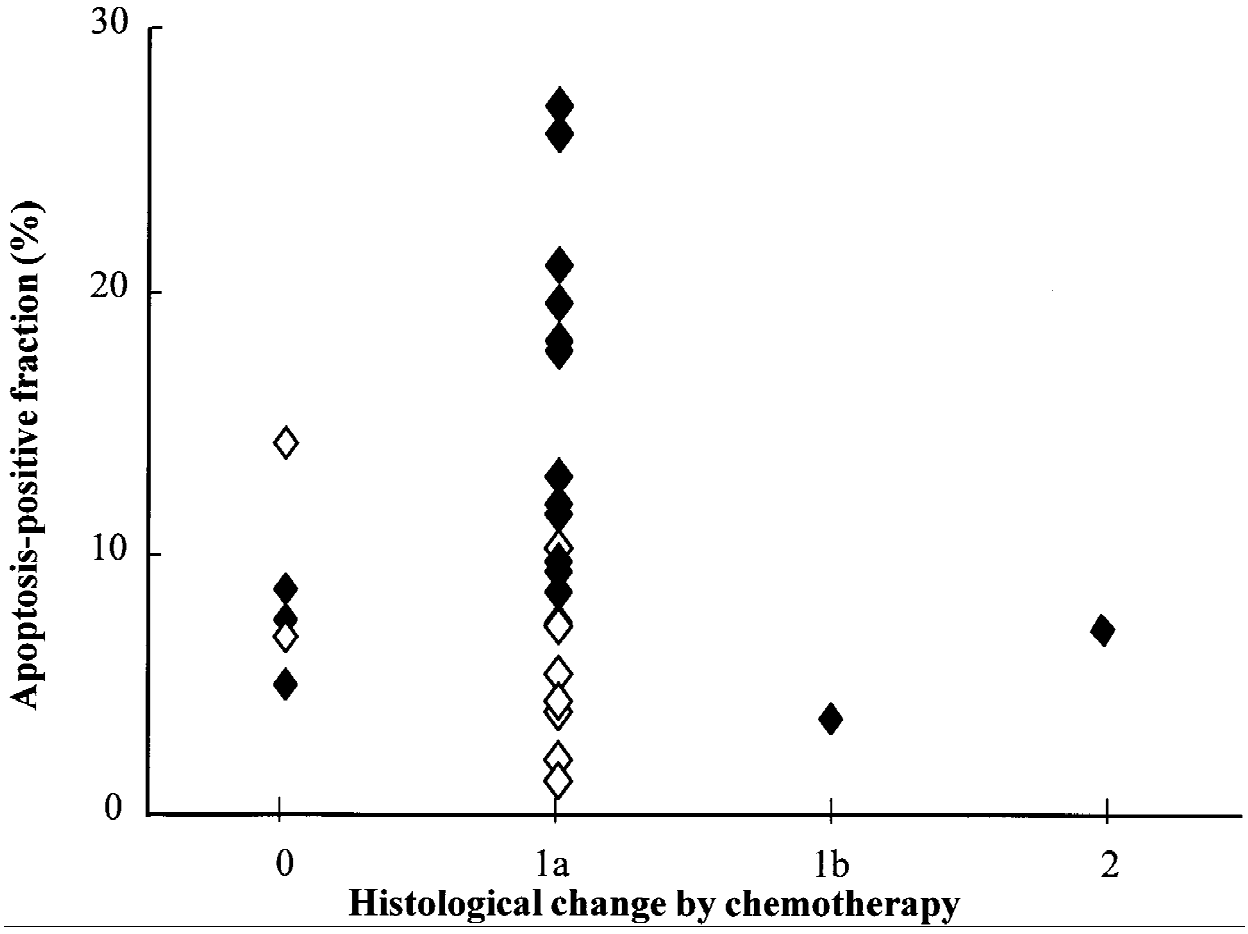

Fig. 5. Histological changes and apoptosis induced by preoperative chemotherapy. ◆: bcl-2 oncoprotein (−) ◇: bcl-2 oncoprotein (+)

with the most potent regimens. In this study, we treated patients with advanced gastric cancer preoperatively with 5-FU (350 mg/m^2/day by continuous intravenous infusion for 1 week). The histological effect of this chemotherapy was mild in most of the patients, probably due to the low intensity and short duration of this regimen. However, the mean apoptosis-positive fraction of the treated group was significantly higher than that of the control group, suggesting that apoptosis was induced in the treated group by preoperative 5-FU infusion.

In our previous study, apoptosis was also induced by 5-FU, together with accumulation of tumor cells in the S-phase fraction, in two human tumor xenografts. The mean S-phase fraction of the preoperatively 5-FU-treated group was larger than that of the control group in the present clinical study. One of the biochemical mechanisms of 5-FU cytotoxicity involves the conversion of 5-FU to FdUMP, which binds irreversibly to TS, leading to inhibition of the conversion of deoxyuridine monophosphate to dTMP, resulting in dTMP depletion and DNA synthesis inhibition. Therefore, the accumulation of tumor cells at the S-phase in the treated group may have been due to inhibition of DNA synthesis by 5-FU.

The previous studies have indicated that apoptosis is regulated by oncogenes and tumor suppressor genes [19,20], and the *bcl-2* proto-oncogene has been reported to inhibit apoptosis in several experiments [19,21]. Alterations of the *bcl-2* gene were first described in follicular and diffuse B-cell lymphomas [21,22], and the *bcl-2* protein was also detected by immunohistochemical methods in some nonlymphoid tissues, including organized epithelia of the skin, gastrointestinal mucosa, and glandular epithelium [23,24].

Other studies suggested that *bcl-2* can influence the clinical behavior of certain human cancers, including follicular lymphoma [25], nonsmall cell lung [26], colorectal [27], and breast [28] cancers. Furthermore, cells expressing *bcl-2* have been found to be resistant to inducers of apoptosis, such as radiation and several DNA-damaging anticancer drugs, including 5-FU [29–32]. In the present report, the mean apoptosis-positive fraction of the *bcl-2* oncoprotein-positive patients was lower than that of the *bcl-2*-negative patients, suggesting that the *bcl-2* oncogene may have suppressed apoptosis in some of the treated patients.

There was no significant correlation between the histological chemotherapeutic effect and apoptosis-positive fraction, as most of the patients were classified as grade 0 or 1a. This suggests that the histological grading based on the findings of tumor cell necrosis bears little relation to apoptosis, which was observed in the present study. In this study, apoptosis was identified by the TdT-mediated dUTP-biotin nick end labeling method [16], the aim of which is to detect the DNA fragmentation that appears during the early stage of apoptosis. As the appearance of apoptotic cells, and apoptotic bodies in diverse forms are seen for only a few hours before they are phagocytized [33,34], the timing is crucial to detect apoptosis by the present method.

Necrosis detected by histological criteria differs from the apoptotic cell death detected in this study, and the discrepancy between these two types of effect suggests the same antitumor agent can produce different results. However, two patients in whom tumor necrosis or disappearance was evident in >⅓ of the whole lesion and with estimated grade 1b or 2, responses showed no *bcl-2* oncoprotein expression. These findings suggest that the effect of 5-FU can be estimated more precisely by detecting *bcl-2* oncoprotein expression and apoptosis, rather than by conventional histological examination.

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