Effect of preoperative regional artery chemotherapy on proliferation and apoptosis of gastric carcinoma cells

Hou-Quan Tao, Shou-Chun Zou

Hou-Quan Tao, Shou-Chun Zou, Department of Surgery, Zhejiang Provincial People’s Hospital, Hangzhou 310014, Zhejiang Province, China.

INTRODUCTION
Human gastric carcinogenesis is a multistep and multifactorial process[11-16]. Cell necrosis and apoptosis may result in tumor development and progression[11-16]. Cell necrosis and apoptosis are two fundamental processes of tumor cell death. Apoptosis is the biological process of tumor cell death regulated by genes[17-20]. Many (and perhaps all) agents of cancer chemotherapy effect tumor cell killing in vitro and in vivo through inducing the mechanisms of apoptosis. Many chemotherapy-induced side-effects and mass shrinkage may result from the increase of tumor cell apoptosis and the inhibition of tumor cell proliferation[17-29].

To clarify the relationship between the effects of preoperative regional artery chemotherapy (PRACT) on inhibition and killing of GC cells with apoptosis, methods of terminal-deoxynucleotidyltransferase (TdT)-mediated dUTP-fluorescein and labeling (TUNEL) and immunohistochemical techniques were used to detect the apoptosis and proliferation of GC cells in 110 cases of GC with or without PRACT. Histopathologic changes and prognosis were also observed and compared between the two groups.

MATERIALS AND METHODS
Clinical data
110 patients with GC who underwent curative resections at Zhejiang Provincial Peoples’ Hospital from Dec. 1988 to July 1996 were studied, including 68 cases with PRACT and 42 cases without PRACT. No significant difference was found in the age, sex, and TNM staging between the two groups. The surgical specimens were fixed in 10% formaldehyde solution, and paraffin embedded tissue blocks were cut into 6μm sections and mounted on glass slides. All patients had been followed up at least 5 years after operation.

Scheme of preoperative chemotherapy
Celiac arteriography was performed by precutaneous transfemoral-artery catheters according to Selldinger’s method and superselective catheterization proceeded to the supplying artery of focus of lesion. Antineoplastic agents of FAP(5-FU 1.0g/m², MMC 10mg/m², CDDP 80mg/m²) or FMP(5-FU 1.0g/m², ADR 20mg/m², CDDP 80mg/m²) scheme was infused into regional artery by a single administration and thereafter surgical operation was performed in 10-14 days.

Main reagents
Terminal-deoxynucleotidyl-transferase(TdT)-mediated dUTP-fluorescein and labeling(TUNEL) kits were purchased from Boehringers Inc. and stored in -20°C for use. SP kits and PCNA monoclonal antibody were produced by Maixin Inc.(Fujian).

Histochemical detection of apoptosis
Tumor cell apoptosis was identified by the TUNEL method[35,36]. Briefly, deparaffinized and rehydrated sections were treated with protease K (20mg/L in 10mol/L Tris, pH 8.0) for 20min at room temperature and washed with 1×TBS (20mmol/L Tris, pH 7.6,
140mmol/L NaCl). After, endogenous peroxidase was inactivated by using 30ml/L hydrogen for 5min and washing with 1X TBS. Equilibration buffer was added to each section and samples were incubated at room temperature for 20min. Terminal deoxynucleotidyl transferase (TdT) enzyme in TdT labeling reaction mixture at 1:20 dilution was piped onto the sections, followed by 2h incubation at 37°C. After terminating the reaction by immersing sections into stop solution and washing with blocking buffer for 10min at room temperature, the anti-digoxigenin-peroxidase was added to the sections. NBT/BCIP solution was used for color development. Sections were counterstained by fast red. A positive control was generated by covering a specimen with DNase I(1mg/L) as the first step of the procedure. Specific positive tissue sections were used for negative controls by substituting distilled water for the TdT in the reaction mixture. Positively stained tumor cells were identified as nuclei that were blue-brown in color, and were counted in ten randomly selected fields under high power of microscope to determine the rate of apoptosis cell among all tumor cells. Apoptotic index (AI)=(the number of apoptosis cells/total number of tumor cells)×100‰.

Immunohistochemical staining for PCNA
SP immunohistochemical staining techniques were used. The primary antibody was PCNA monoclonal antibody (diluted 1:50). Before staining, the sections were microwave heated in 0.05mol·L⁻¹ citric acid solution for antigen retrieval. PBS was substituted for primary antibodies as negative control. PCNA-positive cells (proliferative bodies) were observed. The proliferative index (PI) was obtained by calculating the percentage of positively stained cells evaluated for each tissue section after counting 1000 cells at ten high power fields randomly.

Comparison of pathologic histology change
In H&E staining sections, tumor cell necrosis and degeneration, endothelium change, and the degree of fibrosis were observed and compared between the PRACT group and untreated group. The degree of histopathologic change was divided into four grades from 0 to III.

Statistical analysis
Data were expressed as mean±s, and the t test or Wilcoxin test were used for statistical analysis. Survival rate was calculated by using Kaplan-Meier method and analyzed by the log-rank test. The level of significance was P<0.05.

RESULTS
Comparison of tumor cell proliferation and apoptosis between PRACT and untreated groups
The main morphological characteristics of apoptosis cell consist of cell shrinkage, cytoplasmic condensation, nuclear pyknosis, cytomembrane blebbing or fragmentation, and formation of apoptotic bodies. More apoptosis and less proliferation were detected in the untreated group compared with the PRACT group. The apoptosis index (AI) of the untreated group (6.6‰±3.3‰), AI was significantly different between the two groups (P<0.01). However, the PI of 32 untreated groups of the differentiation type was (14.8‰±4.9‰), while that of undifferentiated type tumor was only (6.6‰±3.3‰). AI was significantly different between the two groups (P<0.001). However, the PI of 32 tumors of the differentiation type (29.6‰±7.4‰) was lower than that in the undifferentiated group (38.5‰±11.2‰, P<0.01).

Effect of PRACT on proliferation and apoptosis of metastatic lymph node GC cells
Among the 62 cases with lymph node metastasis, AI of metastatic lymph node GC cells in 34 cases with PRACT was (7.9‰±3.41‰), and that of 28 cases without PRACT was (7.9‰±2.93‰). The t test indicated that there was a significant difference between two groups (P<0.01). On the contrary, the PI in the metastatic lymph node GC cells in 34 cases with PRACT (17.2‰±6.8‰) was significantly lower than that of 28 untreated cases (26.7‰±9.3‰, P<0.01).

Comparison of histopathologic changes between PRACT and untreated groups (Table 1)
The data are shown in Table 1. No change was marked as grade 0, I to III grade was defined change. The Wilcoxin test showed a significance difference between the two groups.

Table 1 Comparison of histopathologic changes between the PRACT group and the untreated group

| Histopathologic grade | PRACT group (n=68) | Untreated group (n=42) |
|-----------------------|--------------------|-----------------------|
| 0                     | 26                 | 26                    |
| I                     | 22                 | 12                    |
| II                    | 16                 | 4                     |
| III                   | 4                  | 0                     |

P<0.05, vs I+II+III

Effect of PRACT on the survival rate of GC patients
All patients underwent curative resection and had been followed up for at least 5 years. 49 died of tumor recurrence. A postoperative survey demonstrated that the 5-year survival rate of patients with PRACT (63.2%, 43/68) was significantly higher than that of patients without PRACT (42.8%, 18/42, P<0.01).

DISCUSSION
PRACT can effectively inhibit or kill cancer cells by a single administration of high concentration antineoplastic agent into the main supplying artery of the cancer focus. It can not only limit and reduced the tumor mass and improve the curative rate, but it can also act on the peri-operative area by means of drug infiltration to kill subclinical tumor foci which may exist before the operation as well as the invisible micrometastatic foci so as to increase the opportunity of curative resection[13,30]. Many in vitro and in vivo experiments indicated that the induction of apoptosis and inhibition of proliferation are the main mechanisms of eliminating tumor cells by most chemotherapeutic agents[17,29-34,38]. To explore the effect of PRACT on human GC cell apoptosis, TUNEL, a combined molecular biological and morphological technique, was used to investigate and compare the number of apoptotic cells in GC tissue sections as well as that in metastatic lymph node sections of the PRACT and untreated groups. This method, using an in situ staining technique, demonstrates not only the distribution pattern of apoptotic cells, but also the...
sensitivity of the technique: it can detect very small amount of apoptotic cells, so it is wildly used in cell apoptosis studies[19-36]. Moreover, PCNA expression was detected by using an immunohistochemical technique in order to count the proliferation index.

Cell apoptosis is different from cell necrosis; the latter is a pathological form of extensive cell death under strong cell damage and it is not under gene regulation, while cell apoptosis is a normal physiological phenomenon for the active elimination of surplus cells or defective cells under strict genetic control[36]. It plays an important role in regulating total cell amount and also in malignant disease. After gene mutation and formation of malignancy, the rate of cell apoptosis lowers significantly. It is this depletion of cell apoptosis contributing to the expansion of tumor mass; hence it is possible to treat the cancer by means of increasing the proportion of tumor cell apoptosis. Recent studies have shown that 5-Fu, MMC, CDDP, ADR, and many other chemotherapeutic drugs treat cancer by inhibiting proliferation and inducing apoptosis[20-34,39-41]. So induction of tumor cell apoptosis has already been used as an important indicator to detect the ability of chemotherapeutic drugs to inhibit tumor growth. FMC or FAP schemes composed of the aforementioned drugs are now frequently used for pre-operative chemotherapy of GC.

Our results demonstrate that: (1) The apoptosis index of GC cells in the PRACT group is significantly higher than that of the untreated group, and PI of GC cells in the PRACT group is significantly lower than that of the untreated group, indicating that PRACT has an obvious inhibition effect on GC cells. We also found that no significant necrosis was found in the rich blood supply area around the blood vessels, but instead much apoptosis was observed there, indicating that induction of apoptosis by PRACT is the main mechanism of inhibition of tumor growth. (2) Apoptosis rate is correlated with tumor differentiation degree in the PRACT group. AI of differentiated type of GC is significantly higher than that of undifferentiated type, but PI of differentiated type of GC is significantly lower than that of undifferentiated type. This may be due to the better blood supply of the differentiated type of GC[42], allowing more chemotherapeutic drugs to be delivered to the tumor tissue to increase the induction of tumor cell apoptosis so it is more sensitive to chemotherapy. (3) AI of GC cells in metastatic lymph nodes is significantly higher in the PRACT group than that of the untreated group, and PI of GC cells in metastatic lymph nodes is significantly lower in the PRACT group than that of the untreated group, suggesting that PRACT is able to inhibit proliferation and induce apoptosis of metastatic tumor cells. This is very interesting, because lymph node metastasis and recurrence of GC are main factors influencing the overall postoperative survival rate. If the apoptotic cell proportion in metastatic lymph nodes can be increased by effective measures, the prognosis of postoperative GC patients can be improved. Our results suggest that PRACT may approach this goal. (4) With respect to the histopathologic change of GC, including cancer cell reactions, endothelium changes, and the degree of fibrosis, the degree of severity is higher in PRACT group than that in untreated group, suggesting that PRACT can lead to more structural changes of GC tissue so as to enhance the killing effect of cancer cells by chemotherapy drugs. (5) With regard to prognosis, we have showed that PRACT can increase the relapse-free survival rate of GC patients[43]. In fact, altering the balance between apoptosis and proliferation may contribute to the improvement of prognosis of cancer[44-46]. The results of this paper indicate that PRACT can induce apoptosis and inhibit proliferation of GC cells. So we suggest that PRACT is a useful therapeutic scheme for GC.

In conclusion, this study of detecting the proliferation and apoptosis of GC with or without PRACT showed significant inhibition of GC cell growth by PRACT, with its mechanism mainly through inducing tumor cell apoptosis. PRACT can increase the survival rate of GC patients after operation.

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