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Increased Apoptosis Associated with Depressed Type of Early Intestinal Gastric Cancer

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Early gastric cancer can be macroscopically classified into elevated and depressed types. To clarify the relationship between macroscopic appearance of early gastric cancer and apoptosis or cell proliferation, formalin-fixed paraffin-embedded tissue specimens of 44 intestinal-type early gastric cancers were investigated by the TUNEL method and immunohistochemical techniques. Diffuse type was excluded in this study. When tissue sections of gastric cancer were vertically classified into the 3 compartments of luminar, intermediate and basal, the apoptosis index (%) was significantly higher in the basal compartment of depressed type (1.76±2.04, mean±SD) than in the basal compartment of elevated type (0.63±0.81, P=0.01). In depressed type, the apoptosis index (%) was significantly higher in the basal compartment than in the luminar compartment (0.76±0.85, P=0.03). Apoptosis-inducing protein, Bax, was expressed more in each of the compartments of depressed type than in those of elevated type, while there were no significant differences in expression of anti-apoptotic protein, Bcl-2, between the two types. Moreover, the apoptosis index (%) of Bax-positive gastric cancer was significantly higher in the basal compartment (P=0.03), compared to that of Bax-negative gastric cancer, while there were no significant differences in apoptosis index (%) in any compartment between Bcl-2-positive and Bcl-2-negative gastric cancers. There were no significant differences in Ki-67 expression, either between the two types, or among the compartments of depressed type. These results indicate that increased apoptosis with excessive expression of Bax in the basal compartment is involved in the morphogenesis of the depressed type in intestinal-type early gastric cancer.

Key words: Apoptosis — Early gastric cancer — Ki-67 — Bcl-2 — Bax

Early gastric cancer is defined as that confined to the mucosa or submucosa, regardless of the presence or absence of regional lymph node metastasis.[1)] Early gastric cancer is known to show a more diverse macroscopic appearance than other solid cancers. It can be generally classified into the depressed (excavated) type and elevated (polypoid) type.[2)] The depressed type with excavation in the center accounts for most of the diffuse (poorly differentiated)-type and a part of the intestinal-type gastric cancers.[3–5)] The elevated type extends upward and is characterized by a greater possibility of hematogenous metastasis in an early stage.[6)] Thus, the two types show different biological behaviors.

The mechanisms by which gastric cancers differentiate into these two types, especially in the early stage, have not yet been clarified. However, in lung and colorectal cancer, several investigators have reported that a disturbance in the balance between cell proliferation and cell loss underlies neoplastic development and might result in marked morphological differences.[7, 8)]

Cell death is divided into two types, necrosis and apoptosis. Necrosis is a pathologic form of death resulting from acute cellular injury, which is typified by rapid cell swelling and lysis. In contrast, apoptosis is a type of cell death defined by morphological characteristics including chromatin condensation in the nucleus, vacuolation of the cytoplasm, and appearance of apoptotic bodies.[9)] Oncogenes of the Bcl-2 family are known to control apoptosis. Bcl-2 potently inhibits apoptosis and prolongs cell life,[10, 11)] whereas Bax, which is homologous to Bcl-2, induces apoptosis.[12, 13)] They have a reciprocal relationship in normal tissues and maintain homeostasis.

There have been many reports concerning the occurrence of apoptosis in gastric cancer.[14–22)] As the influence of necrosis is thought to be very small in the early stage of gastric cancer, cell loss is thought to be mainly due to apoptosis. In the present study, we evaluate the role of proliferation and apoptosis in the morphogenetic differentiation of early gastric cancer and we also examine the relationship between apoptosis and the expression of the Bcl-2 family members, Bcl-2 and Bax protein. We focused on intestinal-type gastric cancer in the latter study, as diffuse-type cancers lose polarity in the tumors, leading to diffi-

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culty in compartmentalization of the tumors when determining the most intensive area of apoptosis and proliferation of the tumor cells.

MATERIALS AND METHODS

Patients and tissue specimens Primary tumor specimens from 44 intestinal-type early gastric cancers were consecutively obtained by surgery or endoscopic mucosal resection[23, 24] from the Hokkaido University Hospital during 1995 and 1997. The patients with intestinal-type early gastric cancers consisted of 32 men and 12 women (mean age at diagnosis, 58 years). None of the patients underwent preoperative or postoperative radiotherapy or chemotherapy. All samples were early gastric cancer classified histologically as being mucosa or submucosa.[1] The macroscopic appearance of the tumor was classified according to the Japanese Classification of Gastric Cancer.[1] To avoid the influence of ulcers, type III early gastric cancer was excluded from this study. Twenty-eight samples were diagnosed as depressed type (type IIc: superficial depressesd), and 16 samples as elevated type (type I: protruded or IIa: superficial elevated). No IIb cancers were observed. The surgically removed samples were fixed with formalin and embedded in paraffin according to routine procedures. Four-micrometer-thick sections were cut from the paraffin-embedded blocks, mounted on poly-L-lysine-coated glass slides, and used in the following experiments. Histopathological assessment was performed according to the criteria of Laurèn[25] after staining with hematoxylin and eosin.

TUNEL staining Apoptotic cancer cells were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) method. The ApoTag *in situ* detection kit (Oncor, Gaithersburg, MD) was used according to the manufacturer's instructions in this study.

Immunohistochemical staining Ki-67, Bcl-2 and Bax were analyzed by immunohistochemical staining using the streptoavidin-biotin technique (Histofine SAB-PO Kit; Nichirei, Tokyo). Briefly, after deparaffinization, tissue sections were placed in a Coplin jar filled with 10^{-3} *M* sodium citrate buffer (pH 6.0) and exposed to microwaves at 500 W for 10 min before treatment. The sections were washed with phosphate-buffered saline (PBS), treated with 1.5% hydrogen peroxidase in methanol for 5 min to block endogenous peroxidase, and incubated with the primary antibody in a humid chamber at 4°C overnight. As primary antibodies, monoclonal antibody MIB-1 (Medac, Hamburg, Germany) for Ki-67 diluted at 1:200, monoclonal antibody chron 124 (DAKO A/S, Glostrup, Denmark) for Bcl-2 diluted at 1:40, and polyclonal antibody against Bax protein (Pharmingen Co., San Diego, California) diluted at 1:2000, were used. Sections were washed three times with PBS, then incubated with biotinylated rabbit antimouse IgG antibody for 30 min, washed again three times with PBS, and incubated with avidin-biotinylated peroxidase complex for 30 min. After three additional washings with PBS, diaminobenzidine tetrahydrochloride working solution was applied. Meyer-hematoxylin was used as a counterstain.

Counting method A single representative tissue section was taken from each tumor. Tissue sections of gastric cancer were vertically classified into luminar (upper 1/3 portion of the whole tumor), intermediate (middle 1/3 portion of the whole tumor) and basal (deeper 1/3 portion of the whole tumor) compartments, by modifying the reported method for colorectal neoplasm.[8, 26] Positively stained cells were counted in each compartment. Cell counts were performed at ×200 in an area of 250×300 μm, using a tablet measure unit for micromeasurements (Olympus Optical Co., Tokyo), coupled with a light microscope. Positive cells on the luminal surface, necrotic areas, and lymphocytes were excluded. The apoptosis index (%) and Ki-67 index (%) were defined as the percentage of tumor cells displaying immunoreactivity in 1000 tumor cells from 3 arbitrary microscopic fields. For Bax and Bcl-2, tissue sections with more than 10% immunoreactive tumor cells in 1000 tumor cells of 3 arbitrary microscopic fields were defined as being positive in each compartment, and those with less than 10% immunoreactive tumor cells were defined as being negative.

Statistical analyses Comparison of the apoptosis indices (%) among various compartments was performed by the Kruskal-Wallis technique, and the test of differences between two compartments was performed by Fisher's PLSD test after logarithmic conversion of the values. The associations between Bcl-2 or Bax expression and categorical variables were evaluated with the χ^2 test. Values of $P<0.05$ were considered to indicate statistical significance.

RESULTS

Relationship between apoptosis and macroscopic appearance of early gastric cancer Apoptotic cancer cells were detected by the TUNEL method in all of the tissue specimens of intestinal-type early gastric cancer investigated in this study (Fig. 1A). TUNEL-positive cells had morphological features characteristic of apoptotic cells, as previously reported. The apoptosis index (%) was 0.98±1.24 (mean±SD, median value 0.6) in all of the tissue specimens. When tissue sections of gastric cancer were vertically classified into the 3 compartments of luminar, intermediate and basal, the percentage of TUNEL-positive cells (apoptosis index (%)) was higher in the basal compartment (1.76±2.04) of the depressed type of early gastric cancer, compared to that in the luminal compartment (0.76±0.85, $P=0.01$, Fig. 2). On the other hand, in the elevated type of gastric cancer, the apoptosis index (%)

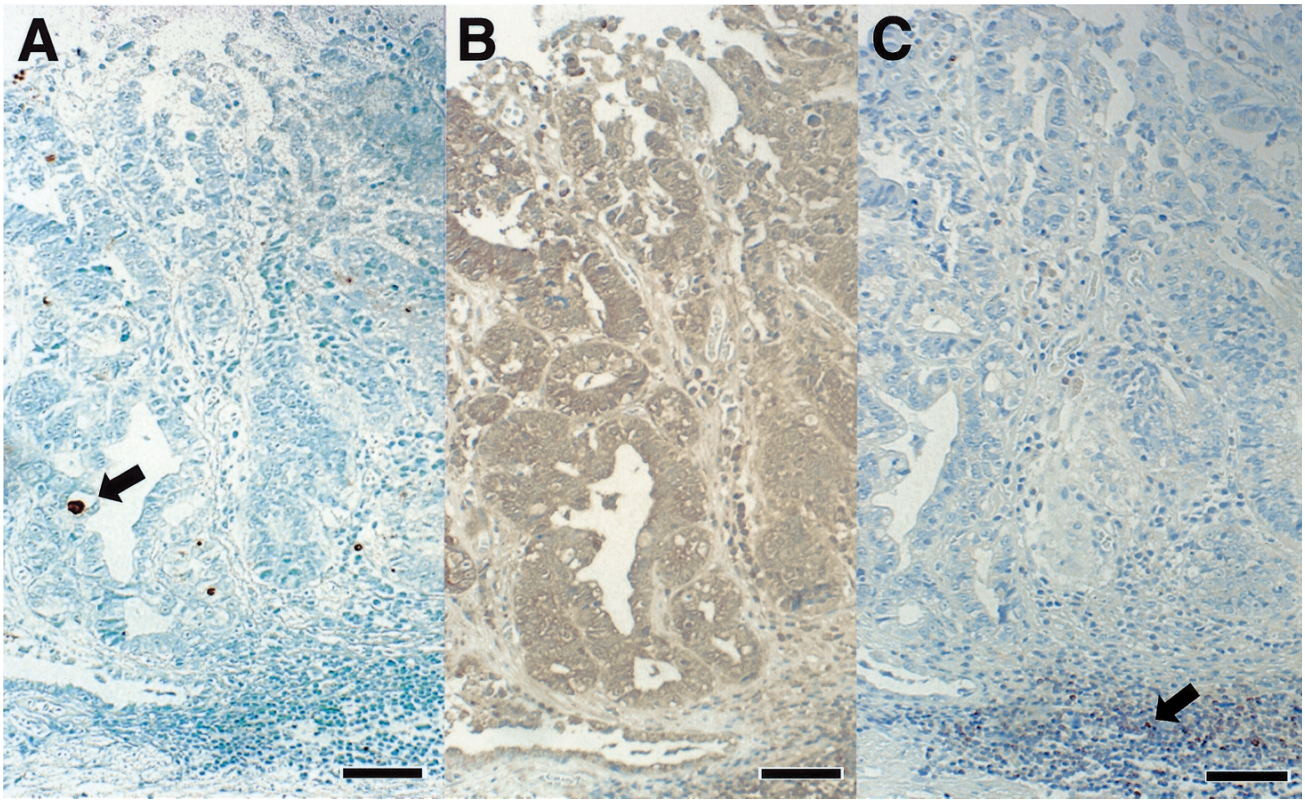

Fig. 1. TUNEL and immunohistochemical staining in the depressed type of early gastric cancer. Bars, 300 μm. A. TUNEL-positive cells are observed in the basal compartment more frequently than in the luminar compartment of the tumor. B. Immunostaining for Bax, showing that most of the tumor cells are positively stained. C. Immunostaining for Bcl-2, showing that most of the tumor cells are negatively stained, and lymphocytes in the stroma are positively stained.

was consistently low, with no significant differences among the compartments. Apoptosis was observed significantly more frequently in the basal compartment of the depressed type than in any compartment of the elevated type of early gastric cancer. When the apoptosis index in the basal compartment was compared between early gastric cancers with (sm) or without (m) submucosal invasion, there was no significant difference between them.

Relationship between proliferative activity and macroscopic appearance of early gastric cancer Next, we examined the difference of proliferative activity between the depressed type and the elevated type of intestinal-type early gastric cancer. The immunohistochemical staining by MIB-1 produced a clear expression of Ki-67 nuclear antigen, showing proliferative activity. There were no significant differences in Ki-67 index (%) between the depressed type and the elevated type of intestinal-type early gastric cancer, or among the compartments of the depressed type of intestinal-type early gastric cancer (Fig. 3). Moreover, there was no significant association between the Ki-67 index (%) and the apoptosis index (%).

Relationship between apoptosis and Bax or Bcl-2 expression in early gastric cancer As Bcl-2 family members such as Bcl-2 and Bax are known to control apoptosis, we next examined the expression of these proteins in intestinal-type early gastric cancer by immunohistochemical techniques. Bax was positively stained in at least one compartment in 35 (79.5%) of the 44 tumors (Fig. 1B), whereas Bcl-2 was positively stained in at least one compartment in 20 (45.5%) of the 44 tumors (Fig. 1C). Bax-positive cells were observed more frequently in each of the compartments in the depressed type than they were in the elevated type (Table I). On the other hand, no significant differences in Bcl-2 immunoreactivity were observed in any of the compartments between the depressed type and the elevated type.

Moreover, as more apoptotic cells were seen in the Bax-positive areas when sequential sections were analyzed, the relationship between Bax expression and apoptosis was examined. The apoptosis index (%) of Bax-positive gastric cancers was significantly higher in the basal compartment (1.75±2.09), compared to Bax-nega-

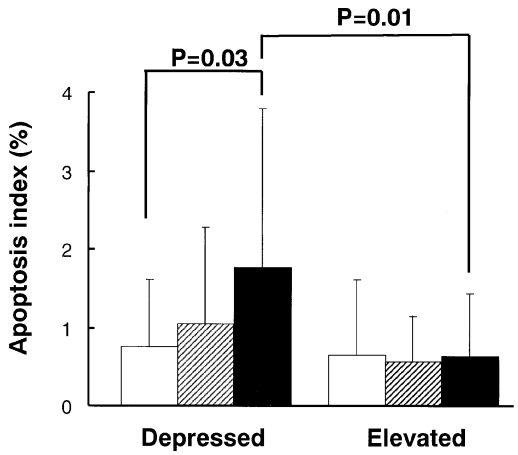

Fig. 2. Apoptosis index (%) in luminar, intermediate and basal compartments of the depressed and elevated types of early gastric cancer. Bars are mean±SD. □ luminar compartment, ▨ intermediate compartment, ■ basal compartment.

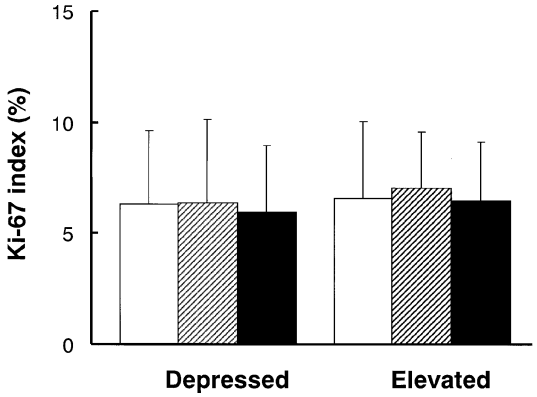

Fig. 3. Overall positive rates of Ki-67 in luminar, intermediate and basal compartments of the depressed and elevated types of early gastric cancer. Bars are mean±SD. □ luminar compartment, ▨ intermediate compartment, ■ basal compartment.

Table I. Frequency of Bax and Bcl-2-positive Cases in Depressed and Elevated Types of Early Gastric Cancer

	Depressed type (%)	Elevated type (%)	*P* value[a)]
Bax			
Luminar	15/28 (53.6)	3/16 (18.8)	0.02
Intermediate	16/28 (57.1)	5/16 (31.3)	0.09
Basal	21/28 (75.0)	5/16 (31.3)	<0.01
Bcl-2			
Luminar	9/28 (32.1)	8/16 (50.0)	0.9
Intermediate	9/28 (32.1)	8/16 (50.0)	0.9
Basal	10/28 (35.7)	6/16 (37.5)	0.9

a) χ^2 test.

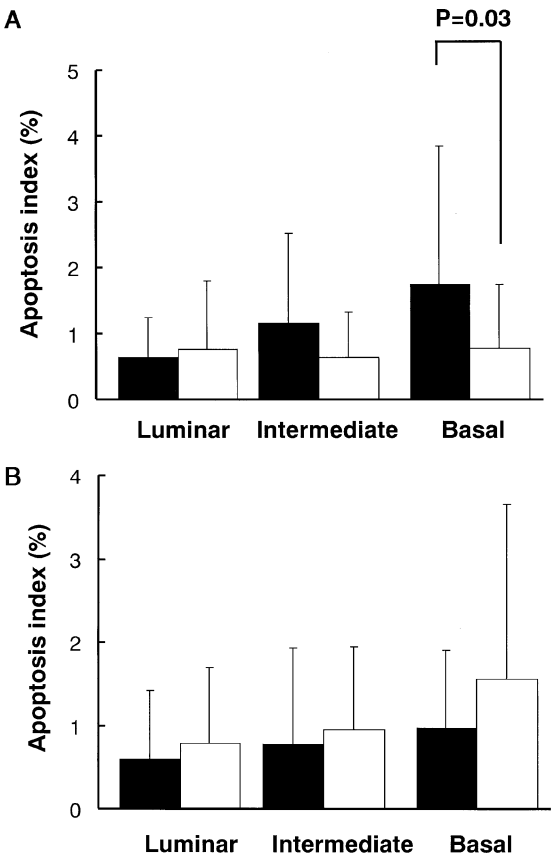

Fig. 4. Apoptosis index (%) in luminar, intermediate and basal compartments of Bax-positive and Bax-negative early gastric cancers (A) and Bcl-2-positive and Bcl-2-negative early gastric cancer (B). Bars are mean±SD. ■ Bax (A)- or Bcl-2 (B)-positive early gastric cancer, □ Bax (A)- or Bcl-2 (B)-negative early gastric cancer.

tive gastric cancers (0.77±0.97, P=0.03, Fig. 4A). However, there was no difference in apoptosis index (%) in any of the compartments between Bcl-2-positive gastric cancers and Bcl-2-negative gastric cancers (Fig. 4B).

DISCUSSION

The present study demonstrated that cell loss due to apoptosis in the basal compartment was significantly greater in the depressed type than in the elevated type of intestinal-type early gastric cancer, but there were no differences in proliferative activity between the depressed and elevated types. These results indicate that the increase in apoptosis near the crypt in the basal compartment plays an important role in the morphogenetic differentiation into the depressed type of intestinal-type gastric cancer in the early stage. It is speculated that the increased apoptosis near the crypt in the basal compartment is what is respon-

sible for inhibiting the growth to thick and elevated tumor, and that this inhibition is not due to lower proliferation of cancer cells in the tumor. To our knowledge, this is the first report showing the relationship between the compartmentalization of apoptosis and the macroscopic appearance of gastric cancer in the early stage.

Several investigators have reported the compartmentalization of apoptosis in colorectal tumors. Moss *et al.* reported that apoptosis was more frequent in the basal layer of adenoma, a precancerous lesion, in the colon, and speculated that increased apoptosis near the basement membrane prevents deep infiltration and suppresses the progression of cancer, which is compatible with our results for gastric cancer.[26] However, Keller *et al.* reported that Sulindac regression of colorectal adenomas is accompanied by a relative increase in apoptosis in surface cells compared to the deeper crypt.[27] Further studies are required to investigate the compartmentalization of apoptosis and its relationship with morphogenetic differentiation in other types of cancer.

The reason for apoptosis being greater in the basal compartment in the depressed type of intestinal-type early gastric cancer is not clear. One possibility is that cancer cells in the basal compartment might be attacked by immunocompetent cells in the peritumoral stroma or damaged by cytokines produced by normal submucosal cells.

We focused on Bcl-2 and Bax as apoptosis-related factors. Bcl-2 is an apoptosis-suppressive factor discovered as a oncogene from t(14:18) translocation in human follicular B-cell lymphoma.[28] Bcl-2 protein is expressed in the proliferative zone in normal gastric tissue and is considered to protect cells that initiate mucosal regeneration.[29, 30] According to early reports, Bcl-2 was frequently expressed in the intestinal type in particular.[31] However, other investigators reported that it was expressed more frequently in the diffuse type.[32, 33] Recently, Saegusa *et al.* found that Bcl-2 decreased in the course of carcinogenesis and speculated that expression of Bcl-2 is a phenomenon that occurs in an early period in the development of gastric cancer.[34] Bax is a protein that constitutes an isomeric dimer with Bcl-2. It antagonizes the action of Bcl-2 in the presence of various cell death signals and induces apoptosis. Wu *et al.*[35] proposed that errors occur in the repair of Bax when the cell turnover is accelerated by gastritis due to *Helicobacter pylori* infection and that the resultant abnormality in Bax plays an important role in the development of gastric cancer. Bcl-2 and Bax are in an inverse relation in various organs, and cell death is controlled by their balance.[13] Korsmeyer *et al.* proposed the hypothesis that the Bcl-2/Bax ratio determines the sensitivity of tissue to apoptosis-inducing stimuli.[36] In gastric cancer, Koshida *et al.* reported that the two proteins were distributed in a reverse pattern in gastric cancer tissue.[37] This kind of imbalance between Bcl-2 and Bax occurring in the process of carcinogenesis may be responsible for the marked morphological differences in gastric cancers.

In the present study, the Bcl-2-positive rate was 45.5% and the Bax-positive rate was 79.5%. These results are not contradictory to the earlier reports that Bax expression remains relatively unchanged while Bcl-2 expression is markedly reduced in gastric cancer. The present study also demonstrated that the frequency of Bax-positive cells was significantly higher in the depressed type of intestinal-type early gastric cancer, and that Bax expression was associated with apoptosis in the depressed type of intestinal-type early gastric cancer. However, the relationship between Bcl-2 and apoptosis is still unclear. Our results indicate that apoptosis in early gastric cancer seems to be more dependent on Bax. However, factors that induce apoptosis are complex, and we must also consider and evaluate the effects of other members of the Bcl-2 family such as Bak, Bcl-X, and Mcl-1.

In conclusion, the increase in apoptosis near the crypt in the basal compartment, associated with excessive expression of Bax, plays an important role in the morphogenetic differentiation into the depressed type in intestinal-type of gastric cancer in the early stage. Further study might be required to examine the association between macroscopic appearance, apoptosis and clinical or biological behaviors.

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