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Apoptosis in Gastric Carcinomas and Its Association with Cell Proliferation and Differentiation

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The significance of apoptosis in human gastric carcinomas was investigated in comparison with proliferative activity and p53 accumulation, using an *in situ* DNA nick end labeling method and immunohistochemistry for both Ki-67 antigen and p53 protein. Apoptotic labeling indices (LI) of 51 differentiated carcinomas (21 of early and 22 of advanced stage) were significantly lower than for 33 undifferentiated tumors (9 of early and 24 of advanced stage) ($P<0.05$). In both types, apoptotic LI of advanced stage lesions were significantly higher than for the early stage cases ($P<0.005$, $P<0.03$). The distribution of apoptotic cells was different from that of Ki-67-positive cells, generally exhibiting an inverse correlation for areas of predominance. In contrast, there was no significant correlation between p53 immunoreactivity and either apoptotic LI or Ki-67 LI. It is concluded that in human gastric carcinomas the susceptibility to apoptosis is related to tumor cell differentiation and depth of invasion, and may play a role in selection of clonal subpopulations with high growth potential.

Key words: Apoptosis — Gastric carcinoma — p53 — Ki-67 — *In situ* DNA nick end labeling

Apoptosis (programmed cell death) plays an important role in modulation of development and differentiation in embryogenesis.[1] For example, in the nervous system approximately half of the neurons generated during neurogenesis are eliminated by apoptosis (naturally occurring neuronal death),[2] and in the thymus, elimination of self-reactive T lymphocytes is related to activation-induced apoptosis (negative selection).[3,4] It is proposed that the occurrence of apoptosis requires expression of several genes, such as c-*myc*,[5,6] p53[6-8] and TRPM-2,[9] suggesting that this is an active gene-directed process.

Information on the correlation between apoptosis and cellular proliferative activity is clearly important in considering tumor behavior since tumor progression is determined not only by cell proliferation but also by cell death. A number of reports have described the significance of proliferative activity in malignant tumors.[10,11] Although the morphologically distinct features of apoptotic cells, such as chromatin condensation and nuclear fragmentation, are frequently observed in tumor tissues, the relation of apoptosis to tumor development is still unknown. Recently, Kasagi *et al.*[12] demonstrated the significance of apoptosis in human gastric carcinomas, suggesting that the higher occurrence of apoptosis in well-differentiated carcinomas may reflect their slow-growing nature, and poorly differentiated carcinomas escape from this process.

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In the present study, we attempted to clarify the significance of apoptosis and tumor cell proliferative activity in human gastric carcinoma, using the *in situ* DNA nick end labeling method[13] and immunohistochemistry for both Ki-67 antigen and p53 protein.

MATERIALS AND METHODS

Cases Eighty-four cases of surgically resected gastric carcinoma were selected from the patients' charts of the Kitasato University Hospital and the Hospital of the Institute of Medical Science, University of Tokyo. Histopathological diagnoses were made by means of the modified classification described elsewhere,[14,15] with the differentiated type (intestinal type) including well- and moderately differentiated adenocarcinomas, and the undifferentiated type (diffuse type) equating with poorly differentiated adenocarcinomas. Cases of both types were subclassified into two groups in accordance with tumor grading, early stage lesions demonstrating invasion of the mucosa and/or submucosa and those of advanced stage exhibiting invasion into or through the muscularis propria. With this classification, 51 of the investigated cases were differentiated (29 of early and 22 of advanced stage), and 33 undifferentiated (9 of early and 24 of advanced stage). Ten samples of non-cancerous gastric mucosa, chosen randomly from the cases examined, were used as controls.

***In situ* DNA nick end labeling** For detection of apoptotic cells in 4 μm thick sections of formalin-fixed and paraffin-

embedded tissue, the *in situ* DNA nick end labeling method (Apop Tag *in situ* apoptosis detection kit-peroxidase, Oncor, USA) was used. Briefly, after routine deparaffinization and blocking of endogenous peroxidase with 0.3% hydrogen peroxidate (H_2O_2) in methanol for 30 min at room temperature, incubation with 100 μg/ml proteinase K (Sigma, USA) for 15 min at room temperature was performed. After prehybridization treatment, the sections were exposed to terminal deoxynucleotidyl transferase with digoxigenin-11-dUTP and dATP, with incubation in a moist chamber for 60 min at 37°C. Anti-digoxigenin-antibody-peroxidase was employed for detecting digoxigenin-11-dUTP labeling for 30 min at room temperature, followed by color development with 3,3′-diaminobenzidine containing H_2O_2 solution. Counter-staining was achieved with 0.3% methyl-green solution.

Apoptotic labeling indices (LI) were calculated after counting 5,000 nuclei in randomly selected fields for each gastric carcinoma case under a light microscope (using ×40 objective and ×10 ocular lenses). Statistical analysis was done with the Mann-Whitney U test.

Immunohistochemistry A combination of the ordinary avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, USA) method and microwave oven heating was performed. Antibodies used were rabbit anti-human Ki-67 antigen (×150 dilution, Dako, Denmark) for Ki-67 and CM1 (×1000 dilution, Novocastra Laboratories, UK) for p53 protein. Counter-staining was achieved with 0.3% methyl-green solution.

Cases were defined as positive for p53 immunostaining when over 30% of the cells were stained in each section. Ki-67 labeling indices of apoptotic areas (Ki-67 LI-apo) were determined by counting 5,000 nuclei in the areas for

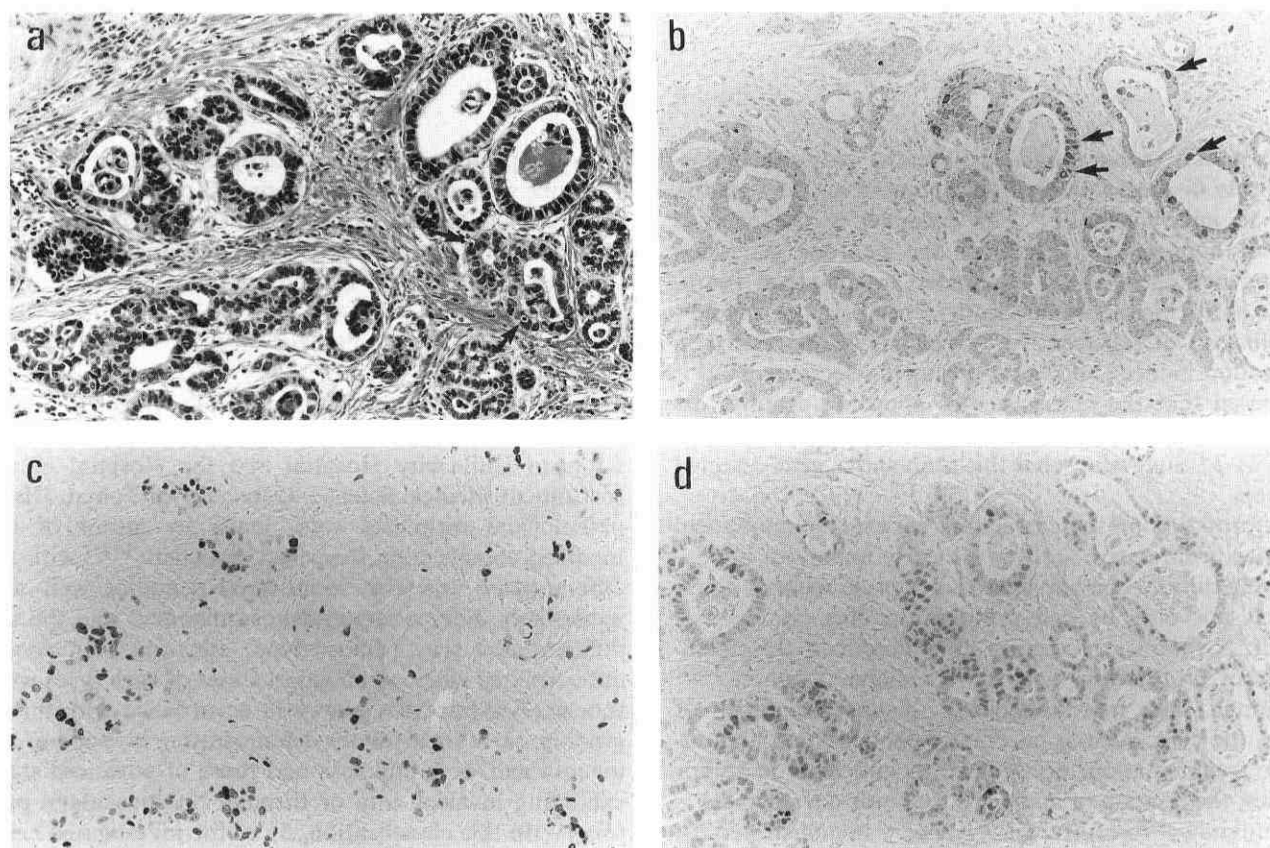

Fig. 1. Correlation between locations of apoptotic cells and Ki-67 or p53 immunoreactivity in differentiated type carcinoma in semi-serial sections. a) Apoptotic cancer cells are sporadically found in cancer foci (arrows). H & E, ×200. b) Apoptotic cancer cells (arrows) showing distinct nuclear staining by the *in situ* DNA nick end labeling method are remarkable in glands on the left side, but are rare in the portion on the right. ×200. c) Immunohistochemistry for Ki-67 antigen. Comparison of the distribution of Ki-67-positive cancer cells with that of apoptotic cells shows an inverse correlation. ×200. d) Immunohistochemistry for p53 protein. Cancer cells demonstrating p53 immunoreactivity are diffusely distributed in glands of both left and right portions, showing no significant correlation with either apoptotic cells or Ki-67 positive cells. ×200.

which the apoptotic labeling indices were examined using serial sections. Ki-67 labeling indices of the highest labeling areas (Ki-67 LI-high) were also determined for 5,000 nuclei in areas of sections with the highest labeling rates. Ki-67 labeling ratios were then calculated by dividing the Ki-67 labeling indices of the highest labeling areas by those for apoptotic areas (Ki-67 LI-high/Ki-67 LI-apo). Statistical analysis was performed by using the Mann-Whitney U test.

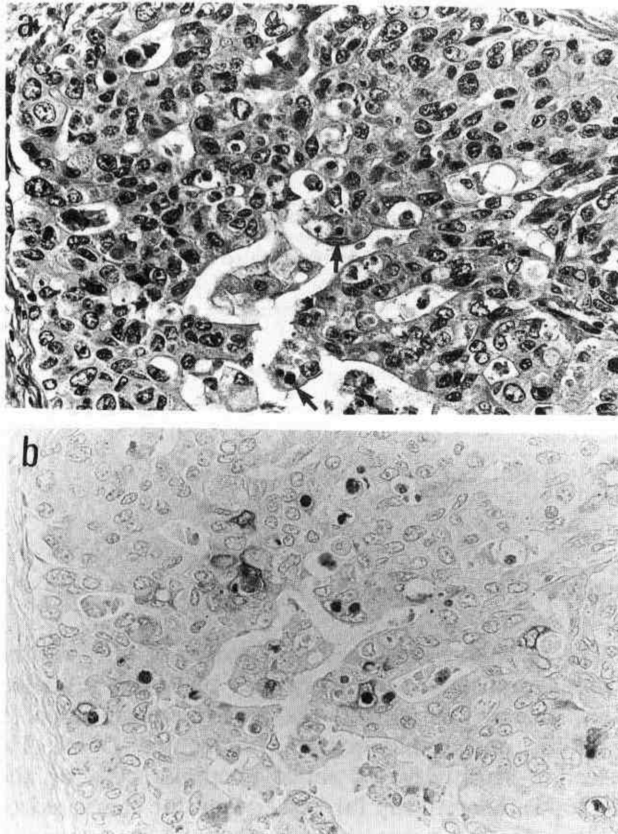

Fig. 2. Serial sections of undifferentiated type carcinoma. a) Note the pyknotic nuclei and isolation from neighbors by breakdown of cell-cell interactions of apoptotic cells (arrows). H & E, ×400. b) The *in situ* DNA nick end labeling method reveals numerous apoptotic cells in the cancer cluster. ×400.

RESULTS

Staining patterns of apoptotic cells and Ki-67-positive cells On conventional hematoxylin and eosin (H & E)-stained sections, apoptotic cells showed pyknotic nuclei together with nuclear fragments (apoptotic bodies) and were isolated from their neighbors by the breakdown of cell-cell interactions (Figs. 1a and 2a). By the *in situ* DNA nick end labeling method, cells undergoing apoptosis, including morphologically viable cells, can be distinguished on the basis of distinct nuclear staining (Figs. 1b and 2b). Care to avoid false-positive results is necessary, however, since some neutrophils and a small number of necrotic cells were also labeled by this method. Comparative observation of serial histological sections was needed for this purpose.

The apoptotic cells in early stage gastric carcinomas were evident as single cells apparently separated from neighboring cancer cells, and in more advanced stages they frequently appeared in clusters without associated inflammation or necrosis in the surrounding areas (Figs. 1 and 2). Apoptotic cancer cells were not detected as positive for Ki-67 immunoreactivity in serial sections (Fig. 1).

Correlation between apoptosis and proliferative activity Data on the relation between apoptosis and proliferative activity in gastric carcinomas are summarized in Table I with reference to tumor cell differentiation. The apoptotic LI of differentiated type cancer cells was $0.94\pm0.69\%$, compared to the value of $1.36\pm0.06\%$ for the undifferentiated type, the difference being statistically significant ($P<0.05$). The values of Ki-67 LI in apop-

Table I. Apoptosis and Proliferative Activity in Differentiated and Undifferentiated Types of Gastric Carcinoma

Histological type	Differentiated (n=51)	Undifferentiated (n=33)	Normal epithelium (n=10)
Apoptotic labeling (%)	$0.94\pm0.69^{a)}$	$1.36\pm1.06^{a)}$	ND
Ki-67 labeling (%)			
apoptotic areas (A)	$44.80\pm16.53^{b,d)}$	$34.33\pm13.74^{b,e)}$	ND
highest labeling area (H)	$55.30\pm15.45^{d,f)}$	$55.20\pm16.29^{e,g)}$	$27.71\pm8.89^{f,g)}$
Ki-67 labeling ratio (H/A)	$1.34\pm0.45^{c)}$	$1.78\pm0.79^{c)}$	ND

a–g) Comparison between each labeled pair: *a*; $P<0.05$, *b*; $P<0.003$, *c*; $P<0.0003$, *d*; $P<0.0001$, *e*; $P<0.0001$, *f*; $P<0.006$, *g*; $P<0.006$.
ND: Not done.

Table II. Correlation between Depth of Invasion and Apoptosis or Proliferative Activity

	Differentiated		Undifferentiated	
	early (n=29)	advanced (n=22)	early (n=9)	advanced (n=24)
Apoptotic labeling (%)	0.69±0.41[a]	1.29±0.84[a]	0.64±0.27[f]	1.60±1.10[f]
Ki-67 labeling (%)				
apoptotic areas (A)	49.80±14.48[b,c]	37.92±16.91[b,d]	40.77±19.01[g]	31.92±10.72[h]
highest labeling areas (H)	57.44±15.36[c]	52.75±15.34[d]	62.16±11.61[g]	52.63±17.27[h]
Ki-67 labeling ratio (H/A)	1.17±0.22[e]	1.56±0.56[e]	1.84±0.91	1.76±0.77

a–h) Comparison between each labeled pair: *a*; $P<0.005$, *b*; $P<0.02$, *c*; $P<0.001$, *d*; $P<0.0002$, *e*; $P<0.005$, *f*; $P<0.003$, *g*; $P<0.02$, *h*; $P<0.001$.

Table III. Correlation between p53 Protein Expression and Apoptosis or Proliferative Activity

	Differentiated		Undifferentiated	
	p53 (+) (n=23)	p53 (−) (n=28)	p53 (+) (n=15)	p53 (−) (n=18)
Apoptotic labeling (%)	0.94±0.47	0.95±0.84	1.62±1.28	1.14±0.81
Ki-67 labeling (%)				
apoptotic areas (A)	48.22±18.68[a]	41.77±14.19[b]	32.03±9.81[c]	36.25±16.37[d]
highest labeling areas (H)	56.55±19.88[a]	54.50±10.68[b]	71.56±56.40[c]	55.17±18.41[d]
Ki-67 labeling ratio (H/A)	1.22±0.31	1.44±0.52	1.87±0.80	1.71±0.80

a–d) Comparison between each labeled pair: *a*; $P<0.002$, *b*; $P<0.0001$, *c*; $P<0.002$, *d*; $P<0.001$.

totic areas (Ki-67 LI-apo) for the differentiated type of 44.80±16.53% versus 34.33±13.74% for the undifferentiated type also demonstrated a statistically significant difference ($P<0.003$). In contrast, there was no statistically significant difference for Ki-67 LI in the highest labeling areas (Ki-67 LI-high) between differentiated and undifferentiated type lesions. In both types, the values of Ki-67 LI-high were significantly higher than that of Ki-67 LI-apo ($P<0.0001$, $P<0.0001$, respectively) and the ratio (Ki-67 LI-high/Ki-67 LI-apo) increased with loss of differentiation ($P<0.0003$).

Data on the relations between depth of tumor invasion and apoptosis or proliferative activity for differentiated and undifferentiated types are shown in Table II. In both categories, the apoptotic LI in advanced lesions was significantly higher than in early lesions ($P<0.005$, $P<0.003$, respectively), having an inverse correlation with Ki-67 LI-apo. In the differentiated category, the Ki-67 LI ratio in advanced carcinomas was significant higher than for the carcinomas in the early stage ($P<0.005$).

Correlation between p53 expression and cell kinetics Expression of p53 protein was demonstrated in 38 out of the total of 84 cases of gastric carcinoma (45.2%); the figure for differentiated type lesions was 23/51 (45.1%) (Fig.1d), and for the undifferentiated type, 15/33 (45.5%). There was no significant correlation between the distribution of p53-positive cancer cells and either that of apoptotic cells detected by *in situ* nick end labeling method, or Ki-67 LI (Table III).

DISCUSSION

The technique described by Gavrieli *et al.*[13] utilizes terminal transferase to incorporate biotinylated nucleotides, thereby permitting the *in situ* visualization of apoptotic nuclei that contain fragmented DNA. The detection sensitivity for single apoptotic cells by this method is theoretically far higher than for characteristic DNA laddering in agarose gels, obtained by electrophoresis of extracted DNA. Although care must be taken in evaluating staining results since necrotic cells sometimes appear to give a similar staining pattern when formalin-fixed and paraffin-embedded materials are used, the reliability of the method has been confirmed by other researchers.[16]

A relationship has been proposed to exist between apoptosis and regulation of cell proliferation in various malignant tumors.[17,18] In addition, it has been proposed that the occurrence of apoptosis in slowly renewing tissues is a very rare event, while in tissues with a high turnover rate the probability of identifying cells undergoing apoptosis is comparable with that of capturing cells in mitosis.[18,19] Our finding of a clear link between apoptosis and either differentiation or progression (depth of invasion) in gastric carcinomas provides supportive evidence for this hypothesis.

The distribution of apoptotic cells in the present cases was different from that of Ki-67-positive cells, with the apoptotic LI showing an inverse correlation with Ki-67

LI-apo. The Ki-67 antigen is present in proliferating cells, but absent in quiescent cells during the G_0 and long G_1 phases,[20, 21] and the cells undergoing apoptosis are in the G_0 phase.[22] In response to an apoptotic stimulus, these cells initiate apoptosis.[22] Similarly, our finding is supported by this correlation between characteristic expression of Ki-67 and initiation of apoptosis in the cell cycle. The statistically significant differences in Ki-67 LI between apoptotic and highest labeling areas indicated that induction of tumor cell suicide through apoptosis may be closely related to a change in proliferative activity, resulting in selection of clonal subpopulations with a high growth potential.

Mutation of the p53 gene may be an early event in gastric carcinogenesis.[23] Baas *et al.*[24] demonstrated that high expression (p53 labeling index $>30\%$) observed immunohistochemically is strongly specific (90%) for the presence of a mutation in the p53 gene. Another study also indicated that the immunohistochemical detection of nuclear p53 protein accumulation is closely associated with p53 gene mutations.[25] Accordingly, it is conceivable that the majority of p53-positive cases in our series involved accumulation of mutant-type p53 protein, although DNA sequencing of the p53 gene was not performed in this study.

It is accepted that wild type p53 protein, but not mutant type, can lead to the initiation of apoptosis,[6–8, 26] and that apoptosis can be subdivided into at least two distinct pathways, one requiring p53 and one that is p53-independent.[27] Lowe *et al.*[28] found that p53 is not required for cell death in many, perhaps most, instances on the basis of reports concerned with the apparently normal development of mice homozygous for p53 mutation. In our study, no apparent correlations among accumulation of p53 protein, apoptotic LI and proliferative activity were noted. However, further investigations are required to confirm the relationship between p53 gene abnormality and apoptosis during the development and progression of gastric carcinomas.

In conclusion, our results indicate that susceptibility to apoptosis in gastric carcinomas is clearly related to tumor cell differentiation and depth of invasion, and may play a role in selection of clonal subpopulations with high growth potential.

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