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Frequent occurrence of apoptosis is an early event in the oncogenesis of human gastric carcinoma

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Abstract We examined the relationship between apoptosis and the progression of human gastric carcinoma. Studies were conducted on a total of 88 surgically removed stomachs, comprising 26 minute (less than 5 mm in diameter), 29 early (limited to the mucosal and submucosal layer) and 33 advanced carcinomas. Apoptotic cells were visualized by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labelling (TUNEL). Serial sections were immunostained for p53 and Ki-67. The mean apoptotic indices (AI: percentage of TUNEL signal positive cells) of minute, early, and advanced carcinomas were 4.1±0.6, 3.8±1.2, and 4.0±1.2 in 46 well differentiated carcinomas, and 2.1±0.5, 2.7±0.9, and 2.2±1.1 in 42 poorly differentiated carcinomas, respectively. Similarly, the mean Ki-67 labelling indices (KI) were 39.2±7.8, 47.2±12.8, 52.6±13.1 in the former, and 35.0±9.3, 36.9±10.3, and 40.0±9.2 in the latter, respectively. Both mean AI and mean KI were significantly higher in well differentiated than in poorly differentiated carcinomas ($P<0.05$). However, the value of mean AI did not differ among minute, early, and advanced carcinomas in either histological type, while KI increased gradually with tumour progression. The frequency of nuclear *p53* expression did not differ among the three categories, implying that the gene mutation is an early event in gastric carcinogenesis. There was no statistical significance between nuclear *p53* expression and mean AI. These results suggest that the progression of gastric cancer is defined by a gradual increase of proliferative activity and constant occurrence of apoptosis and that naturally occurring apoptosis is induced predominantly via a *p53*-gene-independent pathway.

Key words Apoptosis · Gastric carcinoma · Minute carcinoma · Progression · Oncogenesis

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Introduction

Apoptosis refers to energy-dependent cell death in which individual cells participate in their own fragmentation and deletion from living tissue [14]. It is a natural and active process in normal and neoplastic tissues [20]. Moreover, it plays a crucial part in both proliferation and cell turnover in various tumours [13]. For example, the slow-growing basal all carcinoma shows a high mitotic index, but also a high rate of apoptosis [2]. Both apoptotic and proliferative indices have been demonstrated to be higher in higher grades of malignancy in non-Hodgkin's lymphomas and prostate carcinomas [1, 7, 16]. The apoptotic index increases with progression of colorectal cancer, the values being higher in metastatic foci than in the primary lesion [25]. Thus, tumour progression should be considered in the context of both proliferative activity and cell loss.

Recently, we have demonstrated apoptotic cells in human gastric mucosa, adenomatous dysplasia, and carcinoma. In vivo and in vitro analyses were also conducted to examine the relation between *p53* gene status and the occurrence of apoptosis in the gastric carcinoma. Our findings can be summarized as follows: a significantly higher frequency of apoptosis is found in well-differentiated (intestinal type) than in poorly differentiated (diffuse type) adenocarcinomas ([11], confirmed by another group [21]); a higher mean apoptotic index is found in adenomatous dysplasias than in simultaneously existing well-differentiated adenocarcinomas [12]; and apoptosis occurs in both a *p53*-gene-dependent and a *p53*-independent manner, and expression of a mutated *p53* gene slightly attenuates apoptotic cell death [9, 10, 18].

Here we examined the relationship between apoptosis and tumour progression, particularly in minute gastric carcinomas less than 5 mm in diameter. Our findings give insights into the progression of human gastric carcinoma.

Materials and methods

Studies were conducted on 88 gastric adenocarcinomas. Surgically removed specimens were selected from the files of the Department

Table 1 Apoptotic and Ki-67 labelling index in human gastric carcinomas

Histology	Depth of invasion	Number of lesions	Apoptotic index (AI) (mean±SD)	Ki-67 labelling index (KI) (mean±SD)	Ratio of AI/KI (mean±SD)
Well differentiated	Minute (mucosal)	16	4.1±0.6	39.2±7.8	0.104±0.012
	Early (submucosal)	19	3.8±1.2	47.2±12.8	0.081±0.033
	Advanced	18	4.0±1.2	52.6±13.1	0.089±0.046
	Total	53	3.9±1.1[a]	46.6±12.4[b]	0.091±0.035[c]
Poorly differentiated	Minute (mucosal)	10	2.1±0.5	35.0±9.3	0.064±0.023
	Early (submucosal)	10	2.7±0.9	36.9±10.3	0.058±0.020
	Advanced	15	2.2±1.1	40.0±9.2	0.074±0.027
	Total	35	2.3±0.7[a]	37.7±7.0[b]	0.064±0.023[c]

[a, b, c] Comparison between each labelled pair: $P<0.05$

Table 2 Expression of nuclear p53 and apoptotic index in gastric carcinomas. Values are means±SD

	Minute carcinomas		Early carcinomas		Adavanced carcinomas	
	Well differentiated	Poorly differentiated	Well differentiated	Poorly differentiated	Well differentiated	Poorly differentiated
AI of p53-positive cases	(*n*=13) 4.0±0.7	(*n*=6) 1.9±0.5	(*n*=12) 3.7±1.1	(*n*=5) 2.8±1.2	(*n*=11) 4.2±1.3	(*n*=8) 2.1±0.4
AI of p53-negative cases	(*n*=3) 4.2±0.1	(*n*=4) 2.3±0.5	(*n*=7) 3.9±1.5	(*n*=5) 2.6±0.7	(*n*=7) 3.8±1.0	(*n*=7) 2.4±0.3

of Pathology, Faculty of Medicine, Tottori University. These cases were classified into three categories: minute gastric carcinomas less than 5 mm in diameter, early gastric carcinomas demonstrating invasion into the submucosa; and advanced gastric carcinomas exhibiting invasion beyond the submucosa. None of the patients received anticancer agents preoperatively. Histologically, tumours were classified into two categories: well-differentiated and poorly differentiated (including signet-ring-cell carcinoma). The classification corresponds to the intestinal type in the former and the diffuse type in the latter (Lauren's classification [15]). All specimens were fixed in 10% formalin and embedded in paraffin wax. Serial sections were stained by routine H&E, immunohistochemistry, and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick end labelling (TUNEL).

A combination of the avidin-biotin peroxidase complex method and microwave oven heating was performed. Primary antibodies used were MIB-1 (Immunotech, Marseille, France) reacting with Ki-67 antigen expressed in proliferating cells, and BP53 (Novocastra Laboratories, Newcastle, UK) for p53 protein. Counterstaining was achieved with methylgreen solution. Cases were defined as positive for p53 immunostaining when over 20% of the cells were stained in each section.

The TUNEL procedure was conducted for the detection of apoptotic cells using an Apop Tag Plus in situ apoptosis detection kit (Oncor, USA) as described elsewhere. Briefly, after deparaffinization and blocking of endogenous peroxidase with 2% hydrogen peroxidate (H_2O_2) in methanol for 30 min at room temperature, incubation with 20 mg/ml proteinase K (Boehringer Mannheim/Yamanouchi, Tokyo) for 10 min at 37°C 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 90 min at 37°C. Anti-digoxigenin antibody peroxidase was used for detecting digoxigenin-11-dUTP labelling for 30 min at room temperature, followed by colour development with 3,3′-diaminobenzidine containing H_2O_2 solution. Methylgreen was used for counterstaining.

The apoptotic indices (AI) were obtained as the ratio of TUNEL-positive cells to total number of cancer cells and were calculated in at least 5,000 cells. Ki-67 labelling indices (KI) were obtained as AI. KI were counted at the front of the infiltrative growth.

Statistical analysis was performed using Student's *t*-test. A *P* value below 0.05 was considered to be significant.

Results

The cases examined consisted of 26 minute (16 well and 10 poorly differentiated), 29 early (19 well and 10 poorly differentiated) and 33 advanced (18 well and 15 poorly differentiated) carcinomas (Table 1). Careful observation of haematoxylin and eosin (H&E)-stained preparations revealed a few apoptotic cells in all three categories. These cells showed nuclear condensation and eosinophilic cytoplasms forming a clear halo or fragmentation of the nucleus, corresponding to apoptotic bodies with no predominant localization within tumour tissue (Fig. 1a). A few apoptotic cells were detected in the luminal portion. Apoptotic cells were occasionally shed into the glandular lumen, after which apoptotic bodies formed.

TUNEL staining clearly demonstrated cells undergoing apoptosis, on the basis of distinct nuclear signals (Fig. 1b). A few TUNEL-positive cells were found at the basal site of the carcinomatous glands and in the luminal portion, as in the H&E stained sections. A few normal-looking tumour cells (with nonpyknotic nuclei) occasionally showed TUNEL signals. Table 1 shows the rela-

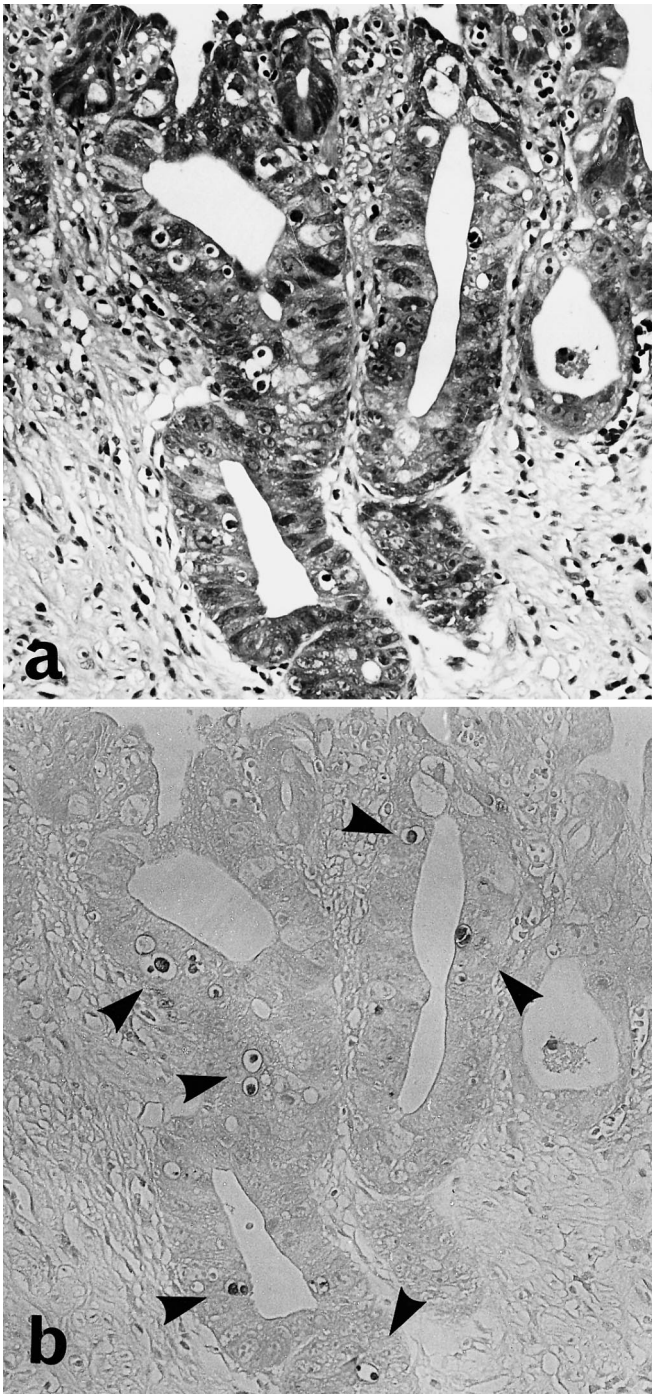

Fig. 1a, b Serial section of well-differentiated adenocarcinoma of the stomach. **a** A few apoptotic cells are present. H&E, ×170. **b** Apoptotic cancer cells (*arrows*) showing distinct nuclear staining by in situ DNA nick end labelling. TUNEL ×170

tionship among histopathological type, the depth of invasion and apoptotic indices (AI) of the cancer cells. Of the well-differentiated adenocarcinomas, AI was 4.1±0.6 ranging from 3.3 to 5.3 in the 16 minute carcinomas, 3.8±1.2 ranging from 2.1 to 5.8 in the 19 early carcinomas, and 4.0±1.2 ranging from 1.8 to 6.5 in the 18 advanced carcinomas. Similarly, AI was 2.1±0.6 ranging from 1.2 to 2.9, 2.7±0.9 ranging from 1.4 to 4.6 and 2.2±1.1 ranging from 1.1 to 3.2, in the 10 minute, 10 early and 15 advanced poorly differentiated adenocarcinomas, respectively. The mean AI was significantly higher in well-differentiated than in poorly differentiated types ($P<0.05$). No difference in AI was noted among the three categories, regardless of histological type.

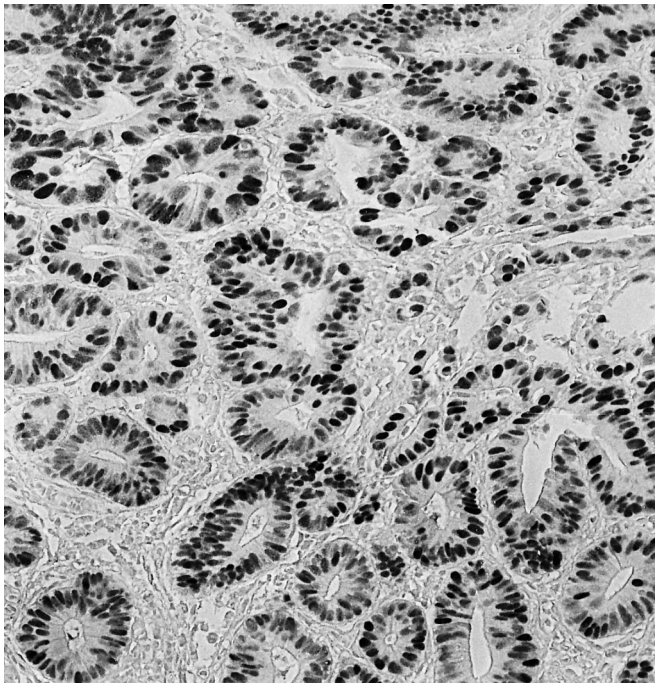

Fig. 2 Immunohistochemistry for p53 protein. Cancer cells with nuclear p53 immunoreactivity are diffusely distributed. ×170

Ki-67 antigen-positive carcinoma cells were observed diffusely throughout the tumours. In the well-differentiated adenocarcinomas, KI was 39.9±5.8 ranging from 30.2 to 50.3 in the 16 minute carcinomas, 47.2±12.8 ranging from 25.3 to 65.5 in the 19 early carcinomas, and 52.6±13.1 ranging from 37.5 to 79.4 in the 18 advanced carcinomas. However, KI was 34.7±8.3 ranging from 22.7 to 48.3, 36.9±8.3 ranging from 27.7 to 48.1, 40.0±9.2 ranging from 30.1 to 49.3 in the 10 minute, 10 early and 15 advanced poorly differentiated carcinomas (Table 1). KI was significantly higher in well-differentiated than in poorly differentiated carcinomas ($P<0.05$). KI did not differ among the minute, early and advanced carcinomas, in spite of a tendency to increase with tumour progression in both histological types.

Table 1 shows the ratio of apoptotic to Ki-67 labelling indices (AI/KI). AI/KI values were significantly higher in well-differentiated than in poorly differentiated types ($P<0.05$).

Immunoreactivity for p53 was localized exclusively in the nuclei of positive cells (Fig. 2). A total of 55 (62.5%) of the 88 tumours showed expression of *p53*. Of these, diffuse nuclear expression of *p53* (more than 80%) was noted in 7 well-differentiated and 3 poorly

differentiated carcinomas. AI was 3.3±0.7 in the former and 1.7±0.4 in the latter. Table 2 shows the relationship between the number of cases with nuclear-p53-positive cancer cells and the apoptotic index. There was no relationship between p53-positive cancer cells and mean apoptotic indices.

Discussion

Apoptotic cells in tissue are inconspicuous and can easily be overlooked. Careful observation of routine H&E sections, however, can allow detection of apoptotic cancer cells by means of their characteristic features: cell shrinkage, loss of cell–cell contact, and aggregation of the chromatin into dense, often crescent-shaped masses under the nuclear membrane, followed by the formation of apoptotic bodies, which have occasionally been detected in tumour gland lumina. TUNEL signals were also positive in a few normal-looking gastric cancer cells. These might be apoptotic cells in the initial stage of the process of cell death. We have previously confirmed the nonrandom and diffuse cleavage of DNA at the initial stage of apoptosis and the validity of TUNEL for the identification of apoptosis in normal-looking HL-60 cells in which apoptosis has been induced by UV irradiation [12]. Therefore, it is necessary to analyse serial sections stained by TUNEL and routine H&E simultaneously in a semi quantitative manner [9].

From a precise histological study of gastric carcinomas, Fujita found that the rate of growth was greatly accelerated when early gastric carcinomas infiltrated deep into the gastric wall or metastasized [6]. The doubling time is shortened from 2–3 years to 2–10 months, or even to 0.6–2 months, in metastatic lesions. Clinical reports of early gastric carcinomas with very slow progression have been accumulating [8, 17, 26]. Minute gastric carcinomas are concordant with early gastric carcinomas. Our findings might be partly explained by the slower growth of early gastric carcinomas, including minute carcinomas, than of advanced lesions.

We compared the apoptotic index (AI) and the Ki-67 labelling index (KI) with histological type. The value of AI did not differ among the minute, early and advanced carcinomas of well-differentiated type, but the value of KI was highest for advanced carcinomas, followed by early and minute carcinomas in order, with a significant difference between minute and advanced carcinomas. This implies a more rapid growth of advanced carcinomas caused by gradually increased proliferative activity and constant cell loss by apoptosis during tumour progression. In other words, as the gastric carcinoma begins to infiltrate further into the gastric wall, genetic alterations to the process of multistep gastric carcinogenesis accumulate [24]. Comparison of the AI in metastatic foci and primary lesions is needed to confirm this.

Similar results were also noted in the poorly differentiated carcinoma, although both AI and AI versus KI were consistently lower in these among the three categories. This indicates the rapidly growing nature of poorly differentiated carcinomas.

Apoptosis is regulated by a variety of oncogenes and suppresser genes, including *p53*, *bcl-2*, *c-myc*, *ras*, and *c-fos* [14]. Many studies have focused on *p53* and *bcl-2* expression [4, 22, 23]. The expression of nuclear *p53* almost matches that of the mutant [5]. In the present study, the frequency of nuclear p53 positive cases did not differ among the minute, early and advanced carcinomas, regardless of histological type. This implies that *p53* gene mutation is an early event in gastric carcinogenesis, as suggested previously [8, 19]. Although cases with diffuse nuclear *p53* expression tended to have lower AI, we could not demonstrate that diffuse *p53* expression attenuates apoptotic cancer cells. Thus, naturally occurring apoptosis appears to be induced predominantly via a *p53*-gene-independent pathway.

More recently, Lu and Tanigawa have clearly demonstrated that naturally occurring apoptosis is inversely related to intratumoural microvessel density in gastric carcinoma [3]. They discussed the “paracrine” effect of various factors produced by endothelial cells and hypoxia as putative apoptosis-regulating factors. The precise mechanism, however, remains to be elucidated.

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