Virchows Arch (1996) 428:229–235

ORIGINAL ARTICLE

Masato Ishida · Yoshihito Gomyo · Shigeru Tatebe
Satoshi Ohfuji · Hisao Ito

Apoptosis in human gastric mucosa, chronic gastritis, dysplasia and carcinoma: analysis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling

Received: 28 February 1996 / Accepted: 2 April 1996

Abstract We examined the existence and distribution of apoptotic cells in human gastric mucosa, chronic gastritis, adenomatous dysplasias and carcinomas in 15 surgically removed stomachs in which dysplasia and carcinoma were found simultaneously. Serial sections were cut for immunohistochemistry for p53 oncoprotein and Ki-67 antigen, and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL). TUNEL signal-positive apoptotic cells were rare in normal mucosa, while a few apoptotic cells were noted in gastritic mucosa and intestinal metaplasia, intermingled with Ki-67 antigen-positive cells forming a generative cell zone. This suggests the cell-cycle-dependent apoptosis of gastric mucosa. The frequency of apoptotic cells per crypt was higher in incomplete than in complete metaplasia, implying greater underlying DNA damage in the former. TUNEL indices (TI; percentage of TUNEL-positive cells in tumour cells) were slightly higher in adenomatous dysplasias (4.9±2.1) than in carcinoma (3.9±1.1), but there was no no statistical difference. Ki-67 indices (KI; percentage of Ki-67 antigen-positive cells in tumour cells) were significantly ($P<0.05$) higher in carcinomas than in dysplasias. Thus, gastric adenomatous dysplasias were characterized by relatively higher TI and lower KI, which might reflect a more static growth potential. The expression of p53 oncoprotein in cancer cells is thought to be an apoptosis-suppressing event, although its precise role remains to be elucidated. Overall, these results indicate that apoptosis plays a crucial part in the morphogenesis of gastritic mucosa including intestinal metaplasia, and that the process is correlated both with tumourigenesis and with proliferative activity.

Key words Apoptosis · TUNEL · Human gastric mucosa · Carcinoma · Ki-67

M. Ishida · Y. Gomyo · S. Tatebe · S. Ohfuji · H. Ito (✉)
First Department of Pathology, Faculty of Medicine,
Tottori University, 86 Nishi-machi, Yonago, Tottori, 683 Japan
Tel.: (81) 859-34-8016, Fax: (81) 859-34-8273

Introduction

Apoptosis (programmed cell death) is a form of cell death characterized by morphological, biological, and molecular genetic changes [5, 11, 34]. The process is now considered to occur physiologically for cell elimination, extensively in embryogenesis, haematogenesis, clonal selection in the thymus and morphogenesis [19, 26, 33]. Moreover, apoptosis plays a crucial part in proliferation and cell turnover in a variety of benign and malignant tumours, and tumour progression may be considered in the context of both proliferation (mitosis) and cell loss (apoptosis) [2]. For example, the slow-growing basal cell carcinoma shows a high mitotic index but a high rate of apoptosis [18]. 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, 6, 23]. The apoptotic index becomes higher with progression of colorectal cancers, higher values being detected in metastatic foci [36]. Thus, apoptosis might be correlated with a higher proliferative activity of various tumours, although it was initially considered to be a manifestation of programmed cell death and, therefore, a good prognostic sign, in contrast to the number of mitoses [4].

Previously, we examined the occurrence of apoptosis in gastric carcinomas, and our findings can be summarized as follows; (1) a higher frequency of apoptosis in well-differentiated (intestinal type) than in poorly differentiated adenocarcinomas (diffuse type) [15], (2) a lower frequency of apoptosis in Epstein-Barr virus-associated gastric carcinomas with lymphoid stroma and also in carcinomas expressing p53 oncoprotein [30]. In these studies, we visualized apoptotic tumour cells by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL), which is based on the detection of naturally occurring chromatin DNA strand breaks, the most characteristic biochemical feature of the process of apoptosis.

In this study, we examined the distribution of apoptotic cells not only in gastric dysplasias and carcinomas, but

also in the neighbouring gastric mucosa with chronic gastritis and intestinal metaplasia.

Materials and methods

Specimens

The studies were conducted on 15 surgically removed stomachs in which adenomatous dysplasia and carcinoma were present simultaneously. The histological type of gastric cancer in all cases was tubular adenocarcinoma, corresponding to the intestinal type according to Lauren's classification [22]. Of the 15 gastric cancers, 5 were intramucosal, 2 had invaded the submucosa and 8 had invaded into or beyond the muscularis propria. Adenomatous dysplasias were classified into two categories: lesions with low-grade dysplasia and those with high-grade dysplasia. The former category is atypical but benign, while the latter is categorized as possible carcinoma as defined by the Pathology Panel of the International Study Group on Gastric Cancer (ISGGC) in 1984 [28]. Histological findings indicating definite carcinoma were severe cellular atypia with prominent nucleoli and structural atypia, such as gland-in-gland appearance or back-to-back arrangement, as described previously [12].

We regarded gastric mucosa with no atrophy and only slightly inflammatory cells in lamina propria as "normal" in this study. The intestinal metaplasias were classified into two groups; complete and incomplete [25]. The complete type is characterized by the absence of sulfomucin-containing goblet cells and the presence of Paneth cells as in the mucosa of small intestine; the incomplete type is categorized by the appearance of sulfomucin-containing goblet cells and the absence of Paneth cells. The specimens were fixed in 10% formalin solution and embedded in paraffin wax. Serial sections 3 μm thick were stained with haematoxylin and eosin, and were analysed by immunohistochemistry and TUNEL.

Immunohistochemistry

Dewaxed paraffin sections were immunostained by the streptavidin-biotin-peroxidase complex (SAB) method, with use of the following primary antibodies; monoclonal antibodies (mAbs) raised against Ki-67 antigen (MIB1, diluted 1:75; Novocastra Laboratories, Newcastle, UK) [38] and p53 (BP53-12, diluted 1:50; Novocastra Laboratries). As pretreatment, microwave-based antigen retrieval was performed for both Ki-67 antigen and P53 immunostaining [10]. Trypsin pretreatment was performed before microwave treatment only for the former. Sections were visualized with diaminobenzidine (DAB) and counterstained with methyl green or alcian green.

In situ nick end labelling

To detect DNA breaks in situ, TUNEL was performed according to the method of Gavrieli et al. [7]. Briefly, paraffin sections were dewaxed, rehydrated through a graded alcohol series, and washed in distilled water (DW) three times. Subsequently, to strip proteins from nuclei, sections were incubated in 20 μg/ml proteinase K (Boehringer Mannheim, Germany/Yamanouchi, Tokyo, Japan) solution for 10–20 min at room temperature (RT), and then washed in running tapwater. This digestive process was important for enhancement of positive nuclear labelling in apoptotic cells. Endogenous peroxidase was inactivated by incubating sections in 2% H_2O_2/methyl alcohol solution for 20 min at RT, followed by washing with DW.

TdT buffer solution (100 mM potassium cacodylate, 2 mM cobalt chloride, 0.2 mM dithiothreitol, pH 7.2) containing 0.3 U/μl TdT (Gibco BRL, Life Technologies, Gaithersburg, Md) and 0.04 nmol/μl biotinylated dUTP (Boehringer Mannheim/Yamanouchi) was added and the sections covered with micro cover slides or parafilm, followed by incubation in a humidified atmosphere for 60–180 min at 37°C. To stop the synthesis reaction of TdT, sections were incubated in TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at RT, and then washed with phosphate-buffered saline (PBS). They were subsequently incubated with peroxidase-labelled streptavidin for 30 min at RT, and finally stained with DAB-H_2O_2 solution. Sections were counterstained with methyl green or alcian green.

To confirm the staining specificity, TUNEL was modified as follows: as positive controls, sections were treated with 0.7 μg/ml DNase I (Stratagene, La Jolla, Calif.) in potassium cacodylate buffer (pH 7.2) for 10 min before treatment with TdT. For negative controls, TdT or biotinyated substrate was omitted from the buffer solution.

Counting of TUNEL- and Ki-67 antigen-positive cells

In the 15 adenomatous dysplasias and carcinomas, the TUNEL index (TI) was defined as the percentage of TUNEL-positive cells relative to counted tumour cells following cell counting in the well-labelled areas, as determined by scanning at low magnification. Actual counts were made at ×200 magnification in 10 fields until more than 10,000 different tumour cells were counted. In intestinal metaplasia, TUNEL-positive cells were quantified as the number per metaplastic gland.

The number of TUNEL-positive cells was calculated by the two authors independently (M.I. and Y.G.). When a different number was reported, the area was reexamined until the results coincided.

Assessment of P53 immunostaining

Assessment of P53 staining was conducted on the same 15 gastric adenomatous dysplasias and carcinomas. These were classified as follows; (–): negative, no positive cells, (+): ≦5% positive cells, one or a few scattered positive cells without any clustering, (++): 6–25% positive cells, (+++): 26–50% positive cells, (++++): ≧51% positive cells.

Results

The omission of either TdT or biotinylated substrate gave completely negative results. TUNEL signals were detectable in nuclei of all cells including stromal cells, pretreated with DNase I for 10 min at RT.

Non-neoplastic mucosa

In normal gastric mucosa, TUNEL-positive cells were rarely observed in the neck zone of gastric mucosa, where Ki-67 antigen-positive cells were located. The number of Ki-67 antigen-positive cells was higher than that of TUNEL-positive cells. With increasing degree of severity of atrophic gastritis, TUNEL-positive cells moved downwards and were frequently detected in the basal zone of the mucosa (Fig. 1), while Ki-67 antigen-positive cells were observed more widely in the middle and lower thirds. Apoptotic cells were noted even in HE-stained sections (Fig. 1A). In intestinal metaplasia, apoptotic cells were seen in the lower half of the glands, mainly in basal portions where Ki-67 antigen-positive

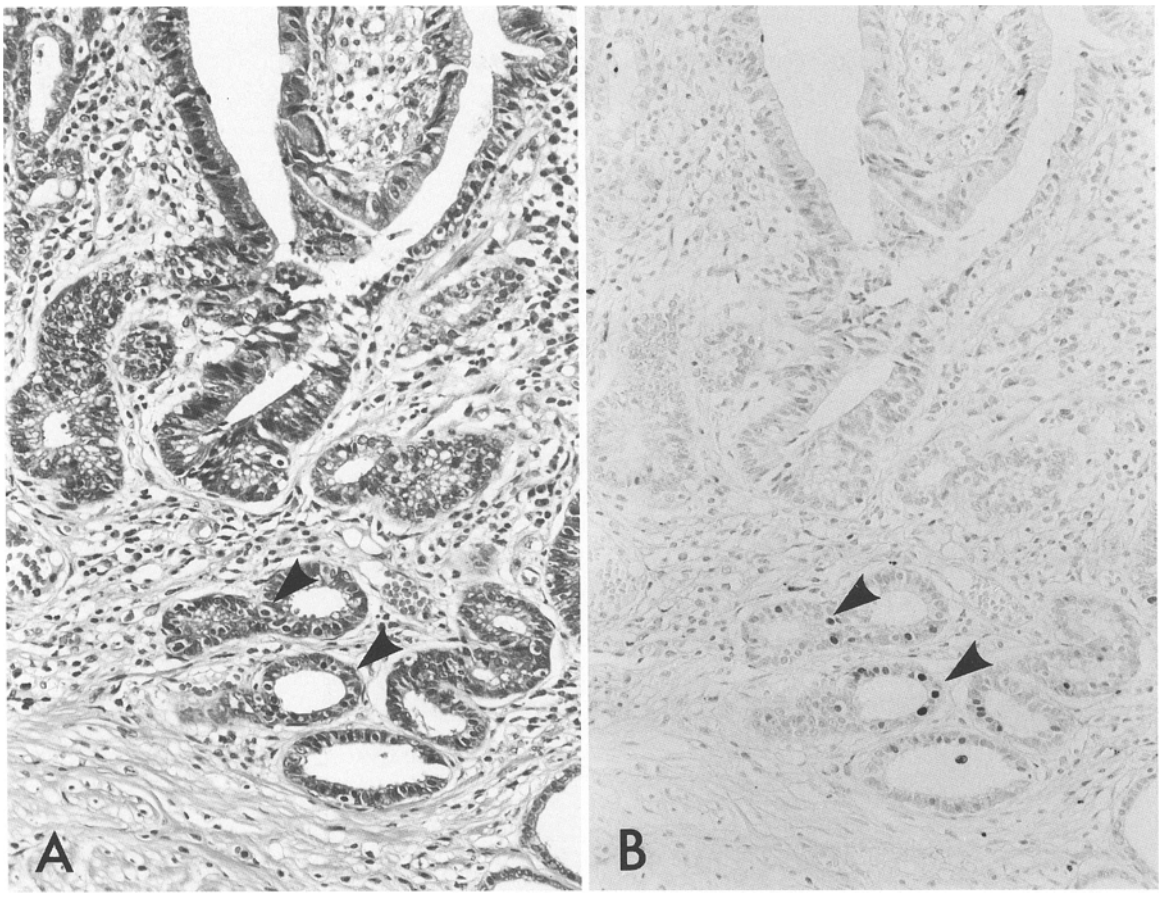

Fig. 1A, B Serial sections of chronic atrophic gastritis with regenerative changes. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL)-positive cells were noted in deeper portions of glands (*arrowheads*). **A** HE, ×170; **B** TUNEL, ×170

cells were present (Fig. 2). A few apoptotic cells were, however, seen in the luminal portion. TUNEL-positive cells were counted in 1,100 glands of incomplete type and in 450 glands of complete type. Average number of the cells per gland were 2.2 in the former and 1.0 in the latter (Table 1).

Adenomatous dysplasias

Gross examination revealed 13 elevated, 1 flat, and 1 depressed tumours. The greatest diameters of the adenomatous dysplasias were 6.9±3.9 (3–15 mm) for low-grade and 13.1±9.6 (3–33 mm) for high-grade tumours.

TUNEL-positive cells were noted to variable degrees in the glands of adenomatous dysplasia, most frequently in their upper third. They were present in the basal portion adjacent to the basement membrane (Fig. 3B). Histologically, TUNEL-positive cells appeared as single structures separated from the surrounding intact cells by a clear halo (Fig. 3A). Occasionally, apoptotic bodies were noted in the viable epithelial cells or those shed into the gland lumen. Although the mean TUNEL indices were higher in high-grade than in low-grade dysplasias, there was no statistically significant difference between the two (Table 2). Ki-67 antigen-positive cells were distributed most frequently in the upper portions of dysplastic glands.

Gastric carcinomas

TUNEL-positive carcinoma cells were observed diffusely with no predominant localization within tumour tissues. Careful observation of HE-stained sections showed tumour cells with apoptosis or apoptotic bodies, both of which demonstrated TUNEL signals. Nuclear fragments in cancerous gland luming also revealed TUNEL signals (Fig. 4B). TI was 3.6±1.1, ranging from 2.1 to 5.6, in the 7 early carcinomas localized in the mucosa or submucosa, and 4.1±1.2, ranging from 2.9 to 6.5, in the 8 advanced carcinomas. There was no significant difference in TI between early and advanced carcinomas. Overall, the TI of the 15 carcinomas was 3.9±1.1, this value being lower than that for gastric dysplasias. There were, however, no significant differences between groups.

Ki-67 antigen-positive cancers were observed diffusely throughout the tumours (Fig. 4C). KI was 45.1±13.4, ranging from 28.7 to 63.9, in the 7 early carcinomas, and 53.6±13.4, ranging from 40.2 to 79.4, in the 8 advanced

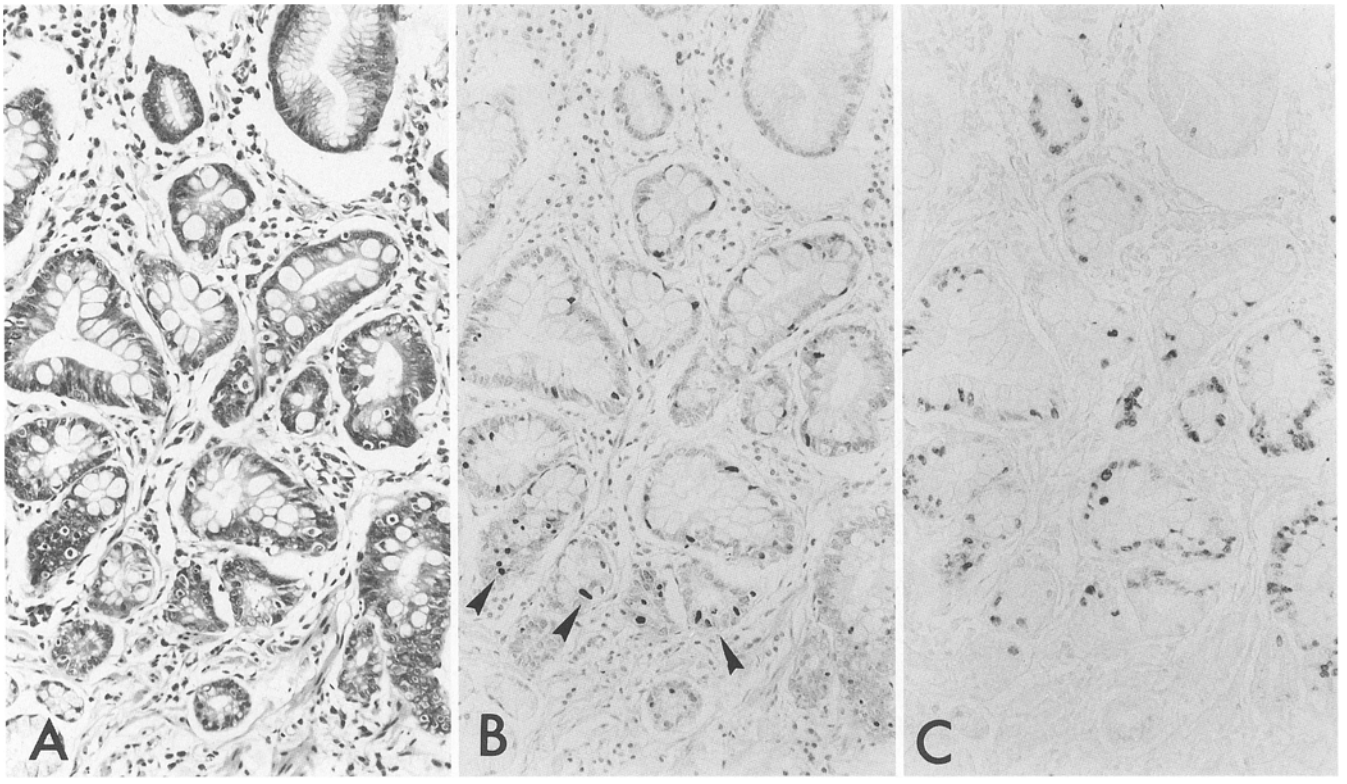

Fig. 2A–C Serial sections of intestinal metaplasia (incomplete type), deeper portion. **A** A few apoptotic cells are detected even at this magnification. HE, ×140. **B** Apoptotic cells indicated in **A** show positive TUNEL signals. TUNEL-positive cells (*arrowheads*) are seen in the basal portion of the glands. TUNEL, ×140. **C** A large number of Ki-67-positive cells is present. Note the positive TUNEL signals and Ki-67 antigen immunoreactivity in different metaplastic cells. ×140

Table 1 Frequency of apoptotic cells in intestinal metaplasia

Histological type of intestinal metaplasia	Number of glands	Number of glands with TUNEL-positive cells	Average number of TUNEL-positive cells per gland
Incomplete type	1100	539 (49%)	2.2
Complete type	450	167 (37%)	1.0

carcinamas. The overall KI of the 15 carcinomas was 49.6±13.7, this value being significantly ($P<0.05$) higher than those for low- and high-grade gastric dysplasias (Table 2). Thus, a nonsignificantly lower TI and a significantly higher KI in gastric carcinomas resulted in an overall lower value of TI/KI in these lesions than in gastric dysplasias.

Table 2 Frequencies[a] of apoptotic cells in gastric adenomatous dysplasias and gastric carcinomas

Histological type	Number of lesions	TUNEL index[b]	Ki-67 index[c]	TUNEL index versus Ki-67 index
Adenomatous dysplasia	15	4.9±2.1	29.5±9.2	0.18±0.88
Low grade	7	4.6±1.4	29.3±11.4	0.18±0.89
High grade	8	5.2±2.7	29.6±7.7	0.18±0.93
Gastric carcinoma	15	3.9±1.1	49.6±13.7	0.09±0.04
Early	7	3.6±1.1	45.1±13.4	0.09±0.05
Advanced	8	4.1±1.2	53.6±13.4	0.08±0.04

[a] Values expressed as mean±SD (%)
[b] No statistically significant difference in TUNEL index between adenomatous dysplasia and gastric carcinoma
[c] Ki-67 index is significantly higher ($P<0.05$) in gastric carcinoma than in adenomatous dysplasia

P53 Immunohistochemical findings

Immunoreactivity for P53 was localized exclusively in the nuclei of positive cells. No nuclear P53 staining was observed in normal gastric mucosa. A few cells showing nuclear P53 staining were noted scattered in the basal portion of both chronic atrophic gastritis and intestinal metaplasia. Cells were occasionally observed showing nuclear staining for P53 in 8 (53%) of the 15 adenomatous dysplasias. Nuclear P53 staining was observed in variable numbers of tumour cells in 10 (67%) of 15 carcinomas. A number of P53-positive cells was noted in 9 carcinomas, the mean TI being 3.8±1.0. The mean TI of the other 6 cases, classified as (-) or (++), was 4.0±1.4.

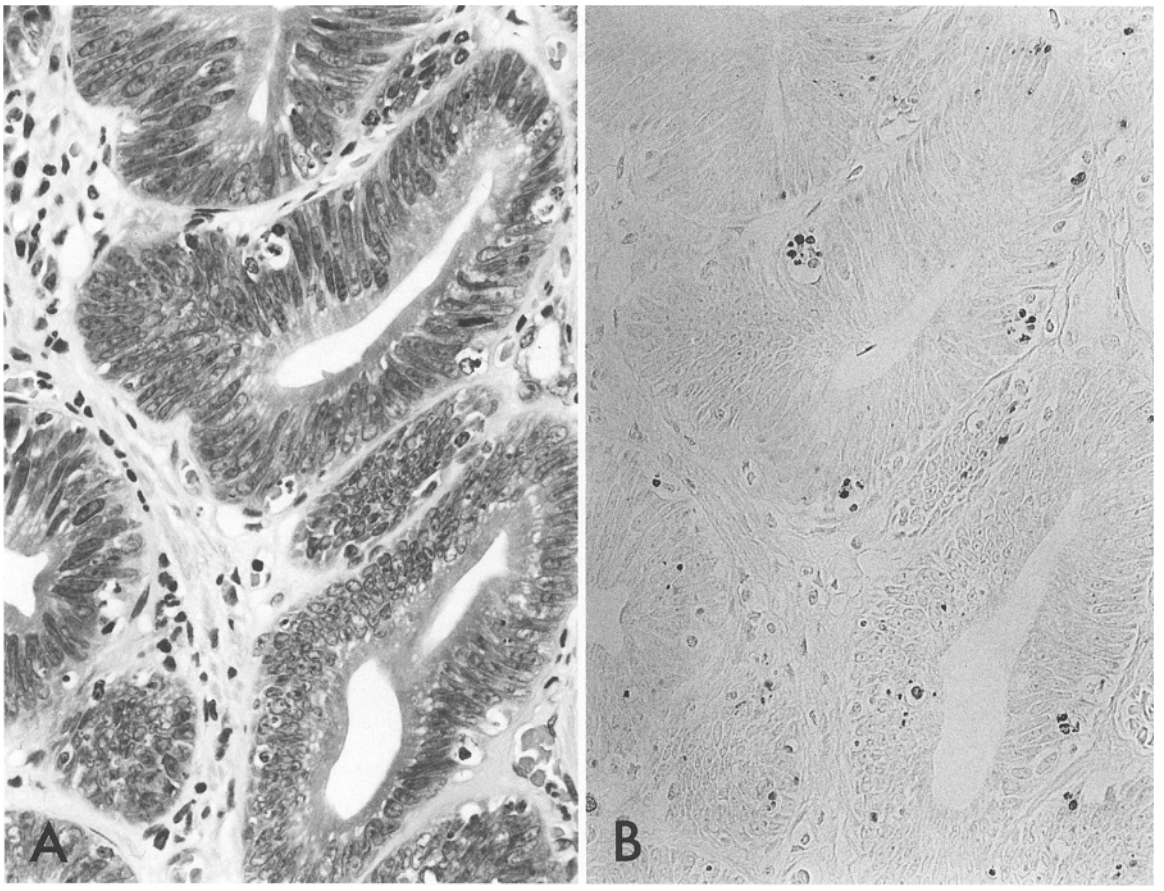

Fig. 3A, B Semi-serial section of adenomatous dysplasia, low grade. Dysplastic glands contain a few apoptotic cells or bodies (**A**), which showed obvious positive TUNEL signals (**B**). **A** HE, ×340; **B** TUNEL, ×340

Discussion

In the present study, we demonstrated the existence of apoptotic cells in both gastric dysplasias and carcinomas, and also in non-neoplastic mucosa, including chronic gastritic and intestinalized mucosa. Apoptosis was initially defined by morphological observations recorded by Kerr, who noticed a discrete drop-out of rat liver cells in a model of ischaemia, which became manifest as a sequence of changes that were initially referred to as shrinkage necrosis and later as apoptosis [19]. Apoptotic cells and apoptotic bodies were noted even on routinely HE-stained sections, in variable numbers. They were, however, easy to overlook especially in non-neoplastic mucosa, because of their relatively small number and their resemblance to intraepithelial lymphocytes or mitoses. Careful observation of serial sections disclosed that TUNEL signals were positive not only in apparently apoptotic cells but also in normal-looking cells without condensed chromatin, corresponding to apoptotic cells in the initial stage of the process. We previously reported positive TUNEL signals in normal-looking HL-60 cells induced to undergo apoptosis by UV irradiation [16]. Thus, serial sections stained by TUNEL and by routine staining might provide the most reliable information in the semi-quantitative assessment of apoptotic cells.

TUNEL-positive apoptotic cells were rare in the glandular neck region of normal gastric mucosa, where the generative cell zone lies and Ki-67 antigen-positive cells are present. With progression of atrophic gastritis, the generative cell zone shifted downwards and a relatively large number of apoptotic cells were seen in the areas in which Ki-67 antigen-positive cells were distributed. This is well exemplified by intestinalized glands, in which both apoptotic cells and Ki-67 antigen-positive cells were present in deeper portions of the glands corresponding to the generative cell zone. This implies that apoptosis in non-neoplastic mucosa occurs in a manner that is at least partially cell cycle dependent. It is of interest that apoptosis occurred more frequently in incomplete-type than in complete-type intestinal metaplasia. In other words, DNA-damaged cells appear more frequently in the former (incomplete type) and are presumably more frequently eliminated by apoptosis to avoid cell transformation. This is consistent with the previous report that incomplete type metaplasia has a closer relationship to gastric cancer than the complete type [25]. Thus, gastric carcinomas of the well-differentiated type might develop on a basis of incomplete intestinal metaplasia, which could contain a few DNA-damaged cells, some of which may escape the process of apoptosis. In fact, some authors, including ourselves, have found P53-positive cells and even p53 gene point mutations in in-

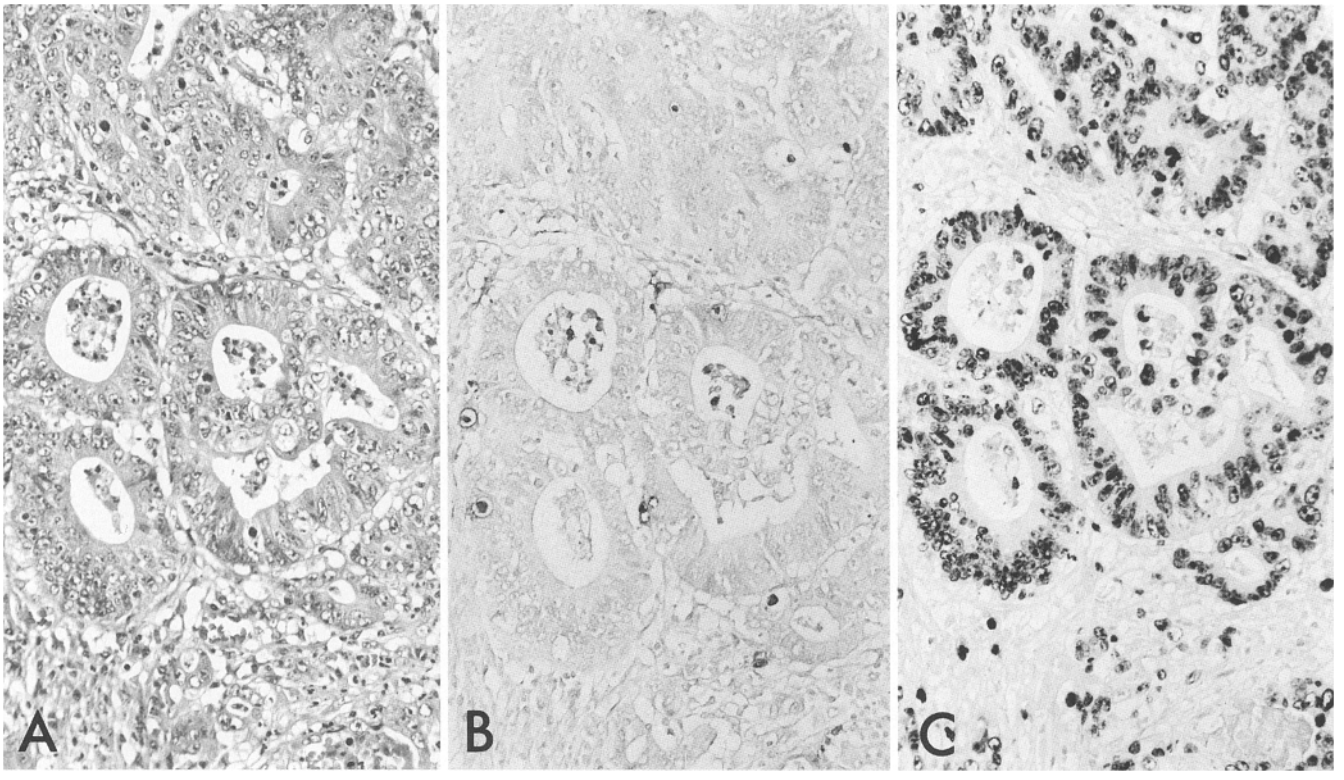

Fig. 4A–C Semi-serial section of gastric tubular adenocarcinoma. **A** A few apoptotic cells can be noted. Fragmented nuclei are seen in the lumen of cancer glands. HE, ×170. **B** TUNEL discloses a few apoptotic cells and even apoptotic bodies in the glandular lumen. TUNEL, ×170. **C** Most cancer cells and a few stromal cells show Ki-67 immunoreactivity in their nuclei. ×170

complete, but not in complete-type, intestinal metaplasia [9, 32, 35].

A higher frequency of apoptosis was noted in gastric dysplasia than in the coexisting gastric carcinomas. There was, however, no statistically significant difference in the average number of apoptotic cells between the two categories of tumour. This was probably due to the relative small number of cases examined in this study. The Ki-67 index, however, was significantly higher in the gastric carcinomas than in the dysplasias. Thus, gastric adenomatous dysplasias might be characterized by higher TI and significantly lower KI than carcinoma. This may explain the rather static nature of gastric dysplasias or adenomas, which has been demonstrated by long-term endoscopic follow-up studies [14]. A higher risk of cancer development was found in high-grade dysplasias [20, 29]. The number of apoptotic cells was higher in the high-grade than in the low-grade dysplasias. A higher apoptotic index (AI) was also confirmed in colonic adenomas with severe atypia than in those with mild atypia [3]. The high-grade dysplasias might eliminate DNA-damaged or unnecessary cells by apoptosis, thus obtaining higher proliferative activity. Gastric cancer may arise from dysplasia when the apoptotic process fails, leading to increased survival of cells with DNA damage.

As mentioned above, apoptosis occurs in either a cell-cycle-dependent manner or independently of cell cycle. If the former applies, apoptosis occurs in late G1 or G2 [24, 37, 39]. Expression of p21 induced by wild-type p53 protein supresses the cell cycle in late G1 and consequently induces apoptosis [8]. In fact, recent studies have demonstrated that the stability of wild-type p53 protein increases transiently in response to DNA damage by irradiation, mediating arrest of the cell cycle in G1 [17, 21]. Nevertheless, apoptosis is suppressed by mutant P53, which can be demonstrated by immunohistochemistry, as shown here. The greater number of cases with diffuse nuclear P53-positive tumour cells might result in a lower TI in gastric carcinomas than in gastric dysplasias. However, the small number of cases examined here did not allow us to reach a definitive conclusion regarding the role of p53 oncoprotein in the process of apoptosis. There might be some forms of apoptosis induced by other pathways not involving wild-type p53 [13, 27, 31, 40]. Further studies will need to be done to clarify the roles of apoptosis-related genes, including *bcl-2*, *c-myc*, and *ras*, for example, in gastric tumourigenesis.

In summary, we examined apoptotic cells in normal and gastritic mucosa, adenomatous dysplasias and carcinomas. Apoptosis plays a crucial part in the morphogenesis of gastritic mucosa, including intestinal metaplasia, eliminating unnecessary or possibly DNA-damaged cells. Frequent apoptosis in gastric dysplasia may reflect the rather static nature of the lesion. In addition, apoptosis is correlated with proliferative activity and tumourigenesis of gastric carcinomas.

Acknowledgements This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare of Japan. The authors would like to thank Mr. N. Itaki for his skilful technical assistance.

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