Jpn. J. Cancer Res. 85, 939–945, September 1994

Apoptotic Cell Death in Human Gastric Carcinoma: Analysis by Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin Nick End Labeling

Noriko Kasagi,[1,3] Yoshihito Gomyo,[1] Hiroyuki Shirai,[1] Shunichi Tsujitani[2] and Hisao Ito[1]

[1]*First Department of Pathology and* [2]*First Department of Surgery, Faculty of Medicine, Tottori University, Nishi-machi 86, Yonago, Tottori 683*

We have examined the occurrence of apoptotic cell death in formalin-fixed, paraffin-embedded human gastric carcinoma specimens by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method. The specificity of the TUNEL signals was confirmed by the omission of either TdT or biotinylated dUTP as negative controls, and by pretreatment with DNase I as a positive control. Careful observation of routine hematoxylin and eosin-stained sections showed a few tumor cells with apoptosis, especially in well-differentiated carcinomas. Intense TUNEL signals were frequently observed even in ordinary, non-pyknotic nuclei of tumor cells, and occasionally also in nuclear fragments corresponding to apoptotic bodies. Apoptotic indices (number of apoptotic cells/total number of tumor cells) ranged between 7.7 and 14.5% (mean, 10.9%) in nine well-differentiated carcinomas and between 2.7 and 7.5% (mean, 4.0%) in five which were poorly differentiated, the mean number being significantly higher in the former ($P<0.01$). No apparent correlation was found between apoptosis and the expression of proliferating cell nuclear antigen, P53 or Le^y in the present study. This high frequency of apoptosis, implying cell loss, may be related to the slow-growing nature of well-differentiated carcinomas. Poorly differentiated carcinomas, including scirrhous gastric carcinomas, showed a lower incidence of apoptosis, indicating the existence of an escape mechanism from the process.

Key words: Apoptosis — Gastric cancer — *In situ* end-labeling

Apoptosis is a physiological process for the elimination of specific types of cells,[1–6] occurring extensively in embryogenesis, morphogenesis, hematogenesis, and clonal selection in the thymus. Moreover, this process plays a crucial role in both proliferation and cell turnover in various tumors.[1,6–9] Morphologically, apoptotic cell death is characterized by cell shrinkage, loss of cell-cell contacts, and aggregation of the chromatin into dense, often crescent-shaped masses under the nuclear membrane, followed by formation of membrane-bounded apoptotic bodies.[1,7,10] Because of the short duration of morphological changes and seemingly low incidence, apoptosis can be difficult to detect in routine histological sections, which is why pathologists have generally paid little attention to this process in a variety of human tumors including gastric cancer. At present, there exists very little data concerning rates of apoptosis in neoplastic disorders.[11,12]

Recently, Gavrieli *et al.*[13] introduced a new method termed terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) to detect apoptotic cells in formalin-fixed, paraffin-embedded sections. This procedure is based on the detection of naturally occurring chromatin DNA strand breaks, which are the most characteristic biochemical feature of the process of apoptosis caused by activation of endogenous nuclease activity.[7,8,10,14,15] Signals visualized by this method have been demonstrated to be highly specific to apoptotic cells in histological sections of rat prostate and endometrium after castration, and in rat embryos and human lymph nodes, in which apoptosis is well characterized and easy to detect even in hematoxylin and eosin (HE)-stained sections.[13,16,17]

This method has, however, never been applied to human gastric carcinomas. Given the considerable evidence that proliferation indices of gastric cancer cells may be of prognostic significance and that parameters of cell loss are biologically relevant, enhanced apoptosis may be of considerable clinical significance. Tumor progression should thus be analyzed on the basis of both proliferation and apoptosis (cell loss). Therefore, we investigated apoptotic cell death in gastric carcinomas, in comparison with their histologic type. In addition, immunohistochemistry was conducted to compare the relationship between apoptosis and the expression of apoptosis-related antigens.

MATERIALS AND METHODS

Studies were conducted on 14 surgical cases of gastric adenocarcinoma extracted from the files of the Department of Pathology, Faculty of Medicine, Tottori University. All specimens were fixed in 10% formalin and embedded in paraffin wax. Two or three representative

[3] To whom correspondence should be addressed.

blocks were selected, mainly including the greater diameter of the tumors, and serial sections were examined by light microscopy, immunohistochemistry, and the TUNEL procedure.

Immunohistochemistry Dewaxed paraffin sections were immunostained by the avidin-biotin-peroxidase complex (ABC) method of Hsu *et al.*,[18] using the following primary antibodies; monoclonal antibodies (mAbs) raised against P53 (1801, diluted 1:20; Novocatra Laboratories Ltd., Newcastle, UK), Le^y (BM1/JIMRO, 1:200; Japan Immunoresearch Laboratory Co. Ltd., Takasaki) and proliferating cell nuclear antigen (PCNA, 1:200; Medac, Hamburg, Germany). Sections were counter-stained with methyl green or alcian green.

TUNEL DNA breaks were detected *in situ* by nick end labeling according to the method of Gavrieli *et al.*,[13] which is based on the specific binding of TdT to 3′-OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide polymer. Briefly, paraffin sections were dewaxed, rehydrated through a graded alcohol series, and washed with distilled water (DW). Subsequently, tissues were digested with 20 μg/ml proteinase K (Boehringer Mannheim/Yamanouchi, Tokyo) at room temperature (RT) or 0.5% pepsin (Sigma Chemical Co., St. Louis,

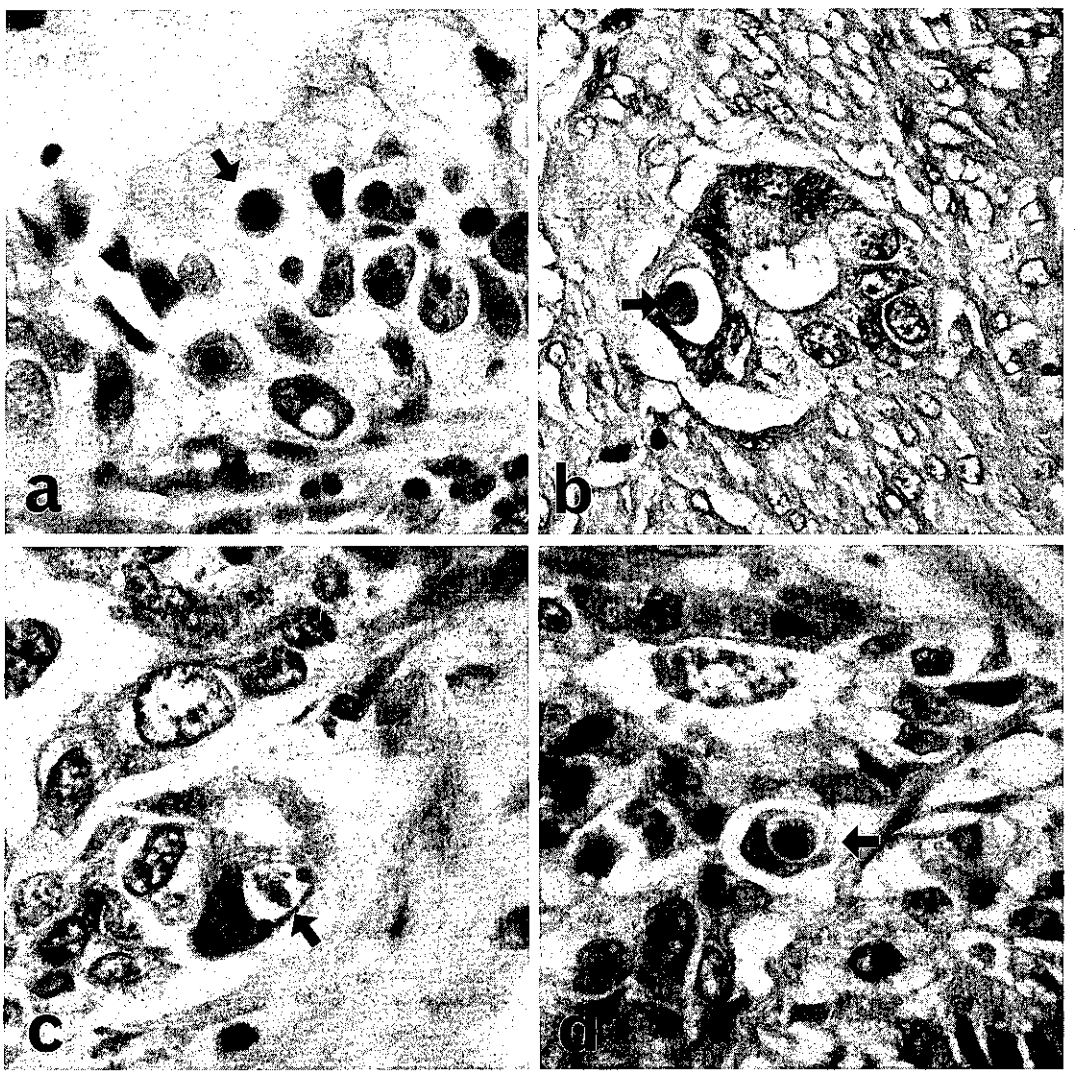

Fig. 1. Apoptotic cells in well-differentiated carcinomas. (a) A tumor cell with loss of cell-cell contacts and cell shrinkage (arrow). (b) A tumor cell shows aggregation of dense crescent-shaped chromatin attached to nuclear membrane (arrow). (c) Apoptotic bodies (arrow) are phagocytized by neighboring tumor cells. (d) So-called pair cells (arrow), one of which showed a lobulated nucleus with dense chromatin. a–d. HE staining, ×1000.

MO) in hydrochloric acid (HCl, pH 2) at 37°C, because proteinase K and pepsin enhanced positive nuclear labeling in apoptotic cells, as described previously.[13, 16, 17] Treatment durations were varied from 0–60 min, and digestion was stopped by washing in running tapwater. Digestion with 20 μg/ml proteinase K or 0.5% pepsin markedly elevated weak staining of a few apoptotic cells observed at 0 min, yielding much stronger staining of apoptotic cells after incubation for 10 min. Intensity was maximal after 20 min proteinase K or 30 min pepsin digestion, and a degree of non-specific staining was seen with both enzymes after digestion for 1 h. Thus, proteolytic pretreatment considerably enhanced the TdT reaction. In the present study, the sections were digested with 20 μg/ml proteinase K at RT for 10–20 min.

Sections were then treated with 2% H_2O_2 solution and washed with DW. TdT buffer solution (100 m*M* potassium cacodylate, 2 m*M* cobalt chloride, 0.2 m*M* dithiothreitol, pH 7.2) containing 0.3 U/μl TdT (Gibco Brl. Life Technologies Inc., Gaithersburg, MD) and 0.04 nmol/μl biotinylated dUTP (Boehringer Mannheim/Yamanouchi) were added to cover the sections, which were then incubated in a humidified atmosphere for 90 min at 37°C. Sections were washed with TB buffer (300 m*M* sodium chloride, 30 m*M* sodium citrate) for 15 min at RT, and then with phosphate-buffered saline (PBS). They were subsequently incubated with peroxidase-labeled streptavidin for 30 min and finally stained with diaminobenzidine-H_2O_2 solution. Sections were counterstained with alcian green.

To confirm the staining specificity, the TUNEL procedure was modified as follows: for the positive control, sections were treated with 0.7 μg/ml DNase I (Stratagene Co., La Jolla, CA) in potassium cacodylate buffer (pH 7.2) for 10 min before treatment with TdT. Negative controls included omission of TdT or biotinylated substrate from the buffer solution.

Histological classification of gastric carcinomas was made according to the criteria of the Japanese Research Society for Gastric Cancer.[19] The apoptotic indices of the gastric carcinomas were obtained as the ratio of TUNEL-positive cancer cells/total number of cancer cells, and were calculated in at least ten 10×20 microscopic fields. Statistical analysis was performed using Student's *t* test.

RESULTS

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

Careful observation of HE sections showed a few tumor cells with apoptosis or apoptotic bodies, and apoptotic bodies phagocytized by other cells could be seen. Fig. 1 exemplifies possible apoptotic tumor cells in routine sections. Apoptosis was more easily observed in well-differentiated carcinomas than in those which were poorly differentiated. An intense TUNEL signal was observed even in ordinary, non-pyknotic nuclei of tumor cells (Fig. 2), and occasionally in nuclear fragments corresponding to apoptotic bodies. Necrotic foci of tumor cells and nuclear "ghosts" showed faint, diffuse staining, implying non-specific incorporation of nucleotides. No signal was detectable in mitotic cells or inflammatory cells in the tumor stroma.

Table I shows the overall results including clinical data, macroscopic and histologic type, and apoptotic indices. Tumor cells showing apoptosis often appeared in small groups in well-differentiated carcinomas (Fig. 3a) including papillary and tubular adenocarcinomas, in which the mean apoptosis index was 10.9%, ranging

Fig. 2. Intense TUNEL signals of a few tumor cells in a well-differentiated carcinoma. TUNEL signals are demonstrated in both apoptotic cells (arrows) and normal-looking cells. TUNEL method, ×200.

Table I. Relationship between Number of Apoptotic Cells and Histological Types in Human Gastric Cancer

Patient No.	Age	Sex	Gross type	Stage	Histological type[a]	Apoptosis index[b] (%)	Average[c] (%)
1	81	F	3	IIIa	pap	14.5±1.8[d]	
2	72	M	5	Ib	pap	7.7±0.5	
3	81	F	3	IVa	tub1	10.4±0.6	
4	66	M	4	IVb	tub1	14.0±2.9	
5	69	M	3	Ib	tub2	11.8±0.9	10.9±0.5
6	67	M	3	IIIa	tub2	12.2±0.6	
7	68	M	3	IVb	tub2	8.9±0.7	
8	65	M	2	IVb	tub2	8.4±0.6	
9	47	M	3	II	tub2	10.7±0.7	
10	46	M	3	III	por1	2.7±0.3	
11	68	F	4	Ib	por2	2.7±0.5	
12	74	F	4	IIIb	por2	7.5±0.6	4.0±0.3
13	62	F	4	IIIb	por2	4.5±0.5	
14	78	M	4	IVb	por2	2.7±0.5	

a) Classification of the Japanese Research Society for Gastric Cancer.
b) Occurrence of TUNEL-positive nuclei of gastric cancer cells within 10 pictures at ×200.
c) Student's *t* test showed a significant difference ($P<0.01$) in the Apoptosis index between well-differentiated and poorly differentiated carcinomas.
d) Mean±SE.

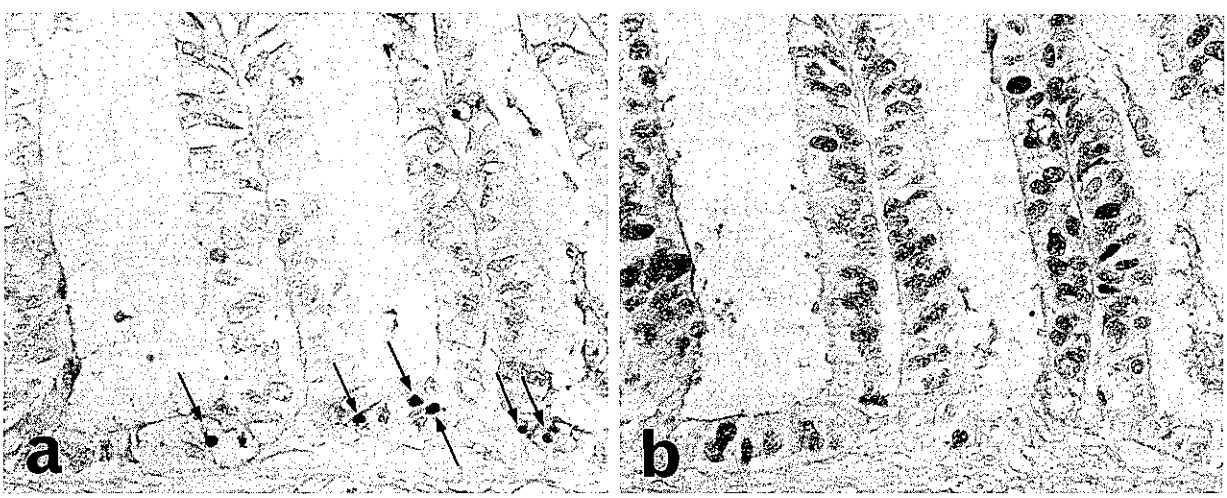

Fig. 3. Serial sections of a well-differentiated carcinoma (case No. 1). Tumor cells with TUNEL signals (arrows in a) are seen in a scattered manner, while most tumor cells show PCNA immunoreactivity in their nuclei (b). a and b, ×150.

between 7.7 and 14.5%. On the other hand, TUNEL-stained nuclei were scattered in poorly differentiated carcinomas (Fig. 4a) including poorly differentiated adenocarcinoma and signet ring cell carcinoma, with apoptosis indices ranging between 2.7 and 7.5% (mean, 4.0%). Case Nos. 11, 12, 13, and 14 were macroscopically Borrman type 4 (scirrhous carcinoma) with vast firbrous stroma, in which only a few tumor cells showed apoptosis. Thus, the mean apoptosis index was significantly higher in well-differentiated carcinomas than in those which were poorly differentiated ($P<0.01$).

Next, immunohistochemistry was conducted to investigate the expression of apoptosis-related antigens. P53-immunoreactive tumor cells were scarcely detected (Fig. 5a) in four well-differentiated carcinomas (case Nos. 1, 2, 6, and 9) and two poorly differentiated carcinomas (case Nos. 10 and 13). PCNA and Le^y (Fig. 5b) immunoreactivities were observed in more than 50% of all

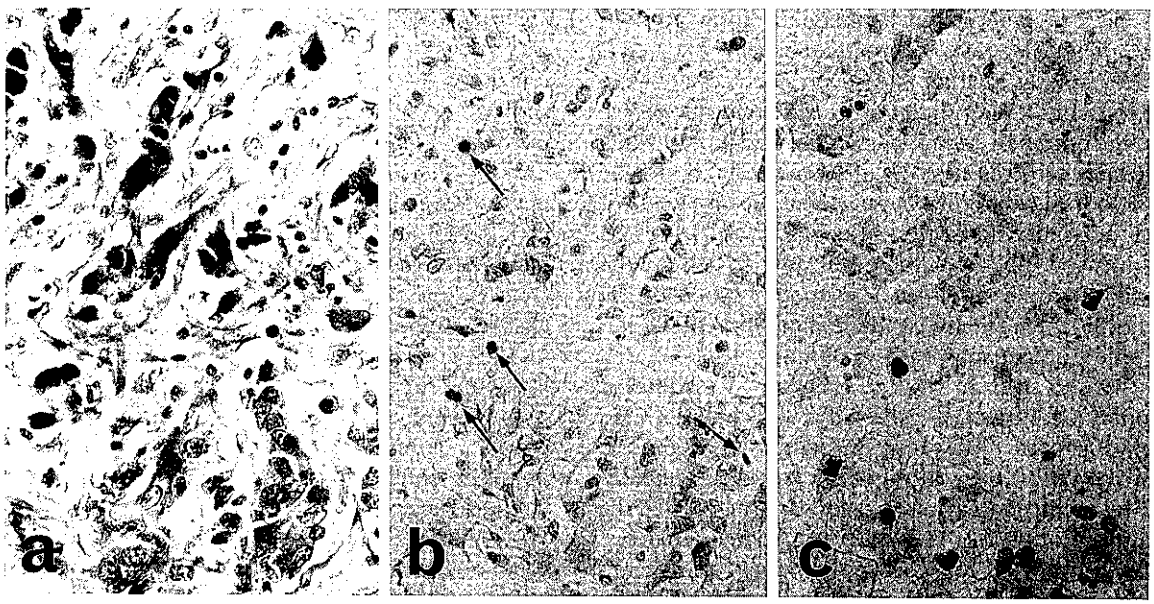

Fig. 4. Semi-serial sections of a poorly differentiated scirrhous carcinoma (case No. 12). (a) HE staining. The TUNEL signal is found in a few tumor cells (arrows in b), while other tumor cells show PCNA-immunoreactivity in their nuclei (c). a–c, ×150.

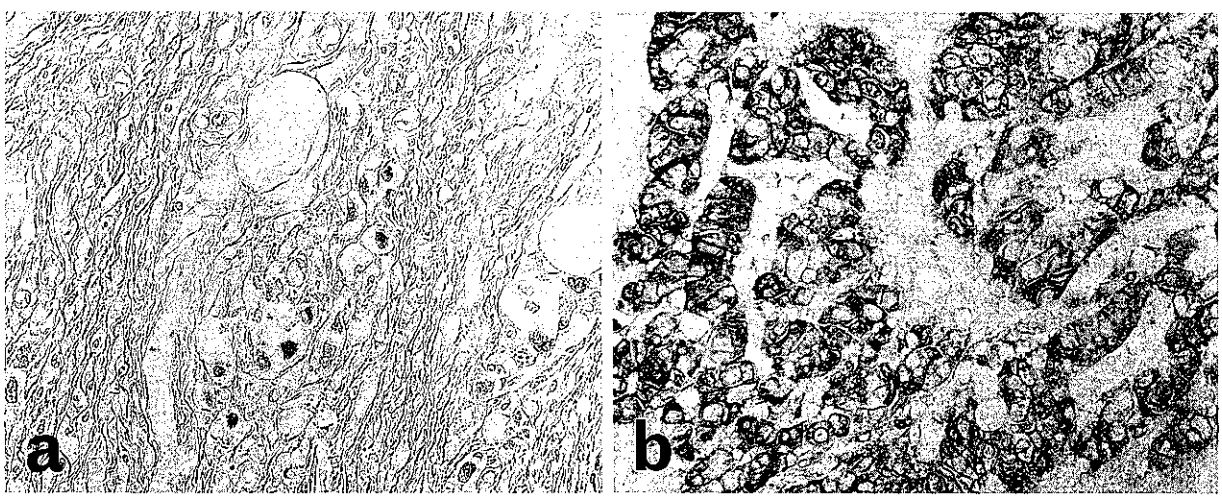

Fig. 5. P53 and Le^y immunostaining. (a) Tumor cells of poorly differentiated scirrhous type show weak P53-immunoreactivity in their nuclei. (b) Most tumor cells show Le^y-immunoreactivity. a and b, ×150.

tumor cells. There was no obvious correlation between apoptosis and immunoreactivity for any of these antigens, but PCNA-positive tumor cells showed no TUNEL signals in either well-differentiated (Fig. 3a and b) or poorly differentiated carcinomas (Fig. 4b and c).

DISCUSSION

The TUNEL method applied in the present study is based on the detection of naturally occurring chromatin DNA stand breaks caused by endogenous nuclease activity.[7, 8, 10, 14, 15] After pretreatment of histological sections with proteinase K or pepsin, the 3′ ends of DNA molecules are labeled with biotinylated nucleotides, introduced by TdT, and the signal is subsequently amplified and visualized by avidin-conjugated peroxidase.[13] The tissue digestion step was shown to have a crucial influence on the staining results in the present study. Because the duration of digestion is tissue-dependent, for each tissue type an optimal duration for incubation with the

enzymes must be determined heuristically to avoid non-specific staining. This may be a consequence of the inconsistency of formalin-fixation. However, good results were obtained in many tissues digested for different durations. The TUNEL method seems particularly valuable when apoptotic cells are present at low frequencies and may be easily overlooked when they lie scattered in solid fields of cells with polymorphic nuclei. Wijsman *et al.*[16] stressed that this method may contribute not only to cell kinetic analysis by quantification, but also to evaluation of the effects of different hormonal and chemotherapeutic compounds which induce apoptosis of tumor cells.

Careful observation of routine HE-stained sections of gastric carcinoma tissues, especially well-differentiated types, have shown a few tumor cells with apoptotic bodies in the late stages of apoptosis. DNA fragmentation precedes morphological changes, and in cells at the early stages of apoptosis before morphological changes, DNA breaks were detected at the periphery of the nucleus.[20] In fact, the TUNEL signal was detectable not only in apoptotic bodies, but also in far greater numbers of ordinary, normal-looking nuclei of tumor cells. Thus, apoptosis seems to occur more frequently in gastric carcinomas. In addition, most gastric carcinoma cells showed either a positive TUNEL signal or PCNA immunoreactivity, implying the near absence of cancer cells at the G_0 stage of the cell cycle. This was especially obvious in the well-differentiated carcinomas.

Scirrhous gastric carcinomas have vast fibrous stromata with rapid and extensive growth, and exhibit high malignancy. Interestingly, the number of apoptotic cells was significantly lower in scirrhous carcinomas than in well-differentiated adenocarcinomas. Tumor cells in scirrhous carcinomas may escape from the process of apoptosis, resulting in the rapid growth of the tumor despite low numbers of cells.

Recently, Yanagihara and Tsumuraya[21] investigated the effects of exogenous transforming growth factor (TGF)-β1 on the proliferation of seven human gastric carcinoma cell lines. Proliferation of HSC-39 and HSC-43, both lines derived from human scirrhous carcinomas, was strongly inhibited via apoptotic processes, whereas the other five non-scirrhous gastric carcinoma cell lines showed no response. The discrepancy between lower incidence of apoptosis *in vivo* and selective induction of the process *in vitro* for gastric scirrhous carcinomas may be explained by the presence of TGF-β1 receptors in HSC-39 and HSC-43. Ito *et al.*[22] demonstrated that TGF-β-resistant cell lines, e.g. MKN-1, express extremely low levels of TGF-β receptor. Moreover, most gastric carcinomas showed reductions in the level of type I TGF-β receptor, resulting in escape from the inhibitory effect of TGF-β. Reduced levels of the receptor in tumor tissues were more frequently detected in poorly differentiated carcinomas than in well-differentiated carcinomas,[22] consistent with the lower incidence of apoptosis in the former. Type I TGF-β receptors are expressed in HSC-39 and HSC-43 at reduced levels (Yanagihara; personal communication).

Le^y expression has been reported to be an important marker for predisposition to apoptosis.[23] Le^y expression was observed in more than 50% of all cancer cells, whereas TUNEL-positive cells amounted to less than 15% of all cancer cells. Therefore, no relationship was apparent between apoptosis of gastric cancer cells and expression of Le^y in the present study.

In summary, we have successfully demonstrated apoptotic cell death in human gastric carcinomas. The higher occurrence of apoptosis in well-differentiated carcinomas may reflect their slow-growing nature. Apoptosis should be further investigated in normal and gastritis mucosa, adenoma, and early carcinomas.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan and by a grant from the Research Fund for Digestive Molecular Biology.

(Received March 17, 1994/Accepted May 27, 1994)

REFERENCES

1) Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239–257 (1972).
2) Wyllie, A. H. Cell death: a new classification separating apoptosis from necrosis. *In* "Cell Death in Biology and Pathology," ed. I. D. Bowen and R. A. Lockshin, pp. 9–34 (1981). Chapman and Hall, London.
3) Umansky, S. R. The genetic program of cell death. Hypothesis and some applications: transformation, carcinogenesis, ageing. *J. Theor. Biol.*, **97**, 591–602 (1982).
4) Brusch, W., Kleine, L. and Tenniswood, M. The biochemistry of cell death by apoptosis. *Biochem. Cell Biol.*, **68**, 1071–1074 (1990).
5) Bursch, W., Paffe, S., Putz, B., Barthel, G. and Schulte-Hermann, R. Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. *Carcinogenesis*, **11**, 847–853 (1990).
6) Ucker, D. S. Death by suicide: one way to go in mammalian cellular development? *New Biol.*, **3**, 103–109 (1991).
7) Kerr, J. F. R., Searle, J., Harmon, B. V. and Bishop, C. J.

Apoptosis. *In* "Perspectives on Mammalian Cell Death," ed. C. S. Potten, pp. 93–128 (1987). Oxford University Press, Oxford.
8) Martz, E. and Howell, D. M. CTL: virus control cells first and cytolytic cells second? *Immunol. Today,* **10,** 79–86 (1989).
9) Williams, G. T. Programmed cell death: apoptosis and oncogenesis. *Cell,* **65,** 1097–1098 (1991).
10) Wyllie, A. H., Morris, R. G., Smith, A. L. and Dunlop, D. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.,* **142,** 67–77 (1984).
11) Sarraf, C. E. and Bowen, I. D. Proportions of mitotic and apoptotic cells in a range of untreated experimental tumours. *Cell Tissue Kinet.,* **21,** 45–49 (1988).
12) Hollowood, K. and Macartney, J. C. Reduced apoptotic cell death in follicular lymphoma. *J. Pathol.,* **163,** 337–342 (1991).
13) Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.,* **119,** 493–501 (1992).
14) Shi, Y., Szalay, M. G., Paskar, L., Boyer, M., Singh, B. and Green, D. R. Activation-induced cell death in T cell hybridomas is due to apoptosis. Morphologic aspects and DNA fragmentation. *J. Immunol.,* **144,** 3326–3333 (1990).
15) Tian, Q., Streuli, M., Saito, H., Schlossman, S. F. and Anderson, P. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell,* **67,** 629–639 (1991).
16) Wijsman, J. H., Jonker, R. R., Keijzer, R., Van De Velde, C. J. H., Cornelisse, C. J. and Van Dierendonck, J. H. A new method to detect apoptosis in paraffin sections: *in situ* end-labeling of fragmented DNA. *J. Histochem. Cytochem.,* **41,** 7–12 (1993).
17) Ansari, B., Coates, P. J., Greenstein, B. D. and Hall, P. A. *In situ* end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. *J. Pathol.,* **170,** 1–8 (1993).
18) Hsu, S.-M., Raine, L. and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.,* **29,** 577–580 (1981).
19) Japanese Research Society for Gastric Cancer. "The General Rules for the Gastric Cancer Study," 12th Ed. (1993). Kanehara Shuppan, Tokyo (in Japanese).
20) Gorczyca, W., Gong, J. and Darzynkiewicz, Z. Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.,* **53,** 1945–1951 (1993).
21) Yanagihara, K. and Tsumuraya, M. Transforming growth factor β1 induces apoptotic cell death in cultured human gastric carcinoma cells. *Cancer Res.,* **52,** 4042–4045 (1992).
22) Ito, M., Yasui, W., Nakayama, H., Yokozaki, H., Ito, H. and Tahara, E. Reduced levels of transforming growth factor-beta type I receptor in human gastric carcinomas. *Jpn. J. Cancer Res.,* **83,** 86–92 (1992).
23) Hiraishi, K., Suzuki, K., Hakomori, S. and Adachi, M. Le^y antigen expression is correlated with apoptosis (programmed cell death). *Glycobiology,* **3,** 381–390 (1993).