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Frequency of apoptosis relates inversely to invasiveness and metastatic activity in human colorectal cancer

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Abstract The frequency of apoptosis was determined in 102 cases of human colorectal cancer. The results were correlated with the frequency of cell proliferation and with clinicopathological characteristics such as degree of differentiation, invasiveness and metastasis. As a marker of apoptosis, intranuclear DNA strand breaks were localized with in situ nick translation (ISNT). As a marker of proliferation, proliferating cell nuclear antigen (PCNA) was localized immunohistochemically. The numbers of nuclei positive with ISNT and for PCNA per 1,000 nuclei on tissue sections were obtained. The labelling indices were compared with clinicopathological characteristics for each tumour. The ISNT labelling index of well differentiated colon carcinomas was higher than that of poorly differentiated carcinomas. Among similarly differetiated cancers, ISNT L.I. of colon carcinomas classified as Dukes A was higher than Dukes B/C, and L.I. of carcinomas which did not metastasize to lymph node or liver was higher than that of carcinomas which metastasized. The PCNA labelling index did not correlate with any of the clinicopathological characteristics or with the ISNT labelling index. The data suggest that apoptosis indices severe as a marker of tumour progression.

Key words Colorectal cancer · Apoptosis · Cell differentiation · Tumour invasiveness · Metastasis

Introduction

Proliferative activity, invasiveness and metastasis are often used as indicators of tumour behaviour. Some solid tumours, with high proliverative activitiy, such as gastric [19, 32], breast [16, 26] and transitional cell bladder cancers [18], have been demonstrated to have malignant potential. However, previous studies on colon cancers using Ki-67 [15, 25] or proliferating cell nuclear antigen (PCNA) [17] as markers of proliferative activity failed to establish a clear correlation between this activity and tumour stage, which embraces depth of invasion, lymph node metastasis and liver metastasis.

In the regulation of tumour mass, a balance between rates of cell proliferation and cell loss is crucial. Cell loss by cell death from cancers has begun to receive attention as a possible indicator of tumour growth [8, 14]. Among various malignant tissues examined, morphological evidence of apoptosis was commonly found and apoptosis demonstrated in regressing tumours [11, 30, 31]. Moreover, it was reported that in vitro tumour cells can gain a selective growth advantage by blocking the apoptotic pathway [24]. These findings show that apoptosis is a mode of cell death in cancers and its frequency may severe as an useful variable to characterize the behaviour of tumours.

In this study, we addressed the question as to whether the frequency of apoptosis detected by ISNT [12, 30] correlates with malignant characteristics, including depth of invasion and metastasis, in 102 cases of human colorectal cancer. It was found that the ISNT labelling indices were higher in colon carcinomas that were more highly differentiated and did not invade or metastasize than those that were poorly differentiated and invasive or metastasizing. These data suggest that the apoptosis index serves as an indicator of tumour progression.

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Materials and methods

Human primary colorectal cancer specimens were obtained from patients who underwent colectomy in the Second Department of Surgery, Nagasaki University Hospital, Nagasaki, Japan, from 1991 September to 1994 May. The excised colons were opened and carcinomas were processed for histopathological examination. In all cases, diagnosis was made by histopathological examination. Carcinomas in situ and carcinomas less than 1 cm in maximum di-

ameter were excluded from this study as they were too small to obtain adequate labelling indices. Colorectal carcinomas and the adjacent normal colon mucosa from 102 patients were used and were classified according to the WHO classification. Thirteen carcinomas were classified as well differentiated, 84 as moderately differentiated, and 5 as poorly differentiated. The poorly differentiated lesions showed some, but highly irregular, gland formation. There was no carcinoma classified as undifferentiated in this series. The depth of invasion was defined according to Dukes' staging: carcinoma confined to the muscularis propria (Dukes' A) and carcinoma extending beyond the muscularis propria (Dukes' B and C).

Well, moderately and poorly differentiated colorectal cancers demonstrated: lymph node metastasis in 4/13 (36.4%), 42/84 (50%) and 4/5 (80%); invasion through the muscularis propria in 9/13 (69.2%), 68/84 (81.0%) and 5/5 (100%); and liver metastasis in 2/13 (15.4%), 12/84 (14.3%) and 0/5 (0%), respectively. Except for liver metastasis, differentiation correlates inversely with the rate of invasion and metastasis.

For the histochemical examination the specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Then 4-µm-thick sections were cut and placed onto amino-propyl-triethoxysilane-coated glass slides. The frequency of apoptotic cells was assessed by detecting single- and double-stranded breaks (DSB) using in situ nick translation (ISNT) and terminal deoxynucleotidyl transferase using nick end labelling (TUNEL) [7] in nuclear DNA. The incorporated biotin-11 dUTP by ISNT and TUNEL was detected immunohistochemically using HRP-labelled goat anti-biotin. The proliferating cells were identified by localizing PCNA using mouse monoclonal antibody PC10 (DAKO, Santa Barbara, Calif.) as the first antibody, and HRP-labeled sheep $F(ab')^2$ anti-mouse IgG (Amersham) as the second. As a negative control, an equivalent quantity of normal mouse IgG was used as the first antibody. HRP was localized using 3,3′-diaminobenzidine plus $CoCl_2$, $NiSO_4(NH_4)_2SO_4$ and H_2O_2 as a chromogenic solution [1].

Small pieces of tissue were dissected out from some carcinomas and fixed in 2% glutaraldehyde and processed for routine electron microscopy.

The labelling index (L.I.) for ISNT was determined by counting ISNT-positive nuclei among 1,000 nuclei of tumour cells on

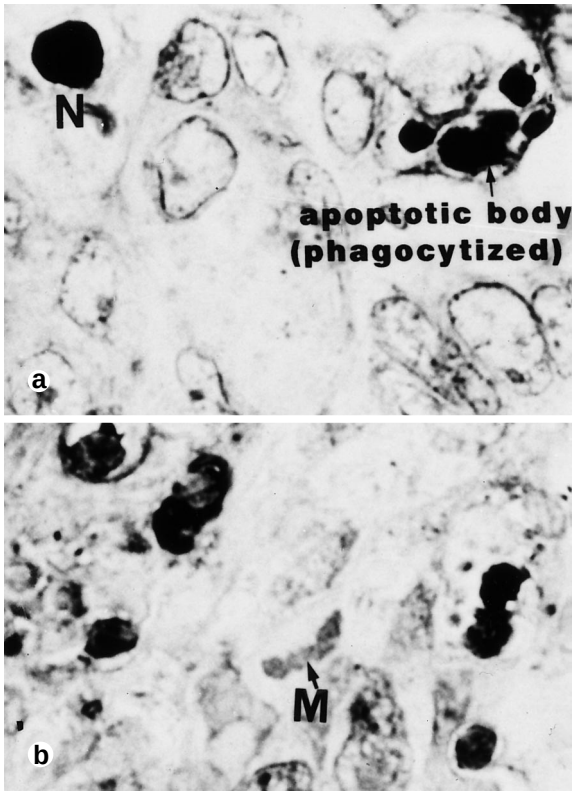

Fig. 2a, b Detection of nuclei with DSB by ISNT. **a** Some nuclei of normal appearance and apoptotic bodies phagocytized by a neighbouring cancer cell were stained. **b** Mitotic cells (M) were not stained

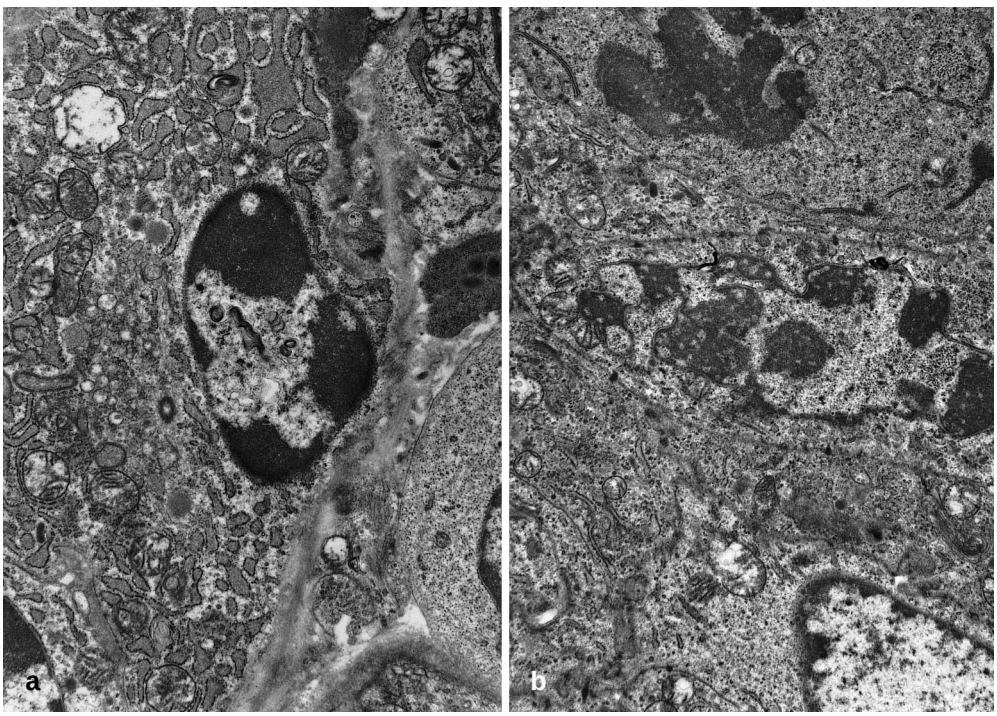

Fig. 1a, b Electron micrographs of colorectal cancer. Note the margination of chromatin to the nuclear membrane (**a**) and focal aggregation of chromatin (**b**), which indicate an early stage of apoptosis. **a** ×8,500, **b** ×7000

photographs by the method used by Mori et al. to determine the PCNA L.I. in gastric cancer [21]. Sträter et al. [27] described two types of apoptotic patterns in colonic mucosa and familial adenomatous polyposis lesions, the so-called engulfment and extrusion pattern. In our study, the extrusion pattern was more frequent than the engulfment pattern, but we did not differentiate between the types as this was difficult in the distorted cancerous glands.

The L.I. for PCNA was determined by counting PCNA-positive nuclei among 1,000 nuclei of tumour cells on photographs by the method used by Mori et al. [21].

Statistical analyses were performed using the Stat View II software (Abacus Concept Inc., Ver. 4.0). The frequency table was tested for association using Student's *t*-test or ANOVA. The correlations between the ISNT L.I. and PCNA L.I. were evaluated using a correlation coefficient.

For DNA size analysis a portions of some samples were quickly frozen with OCT compound (Miles, USA) and stored at –80° C. High molecular-weight DNA was extracted from the frozen tissues as described elsewhere [13]. Aliquots (5 µg/lane) of the DNA were electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

Results

In various types of colorectal cancer the occurrence of apoptosis was confirmed by electron microscopy. Typical features of apoptosis, such as the margination of chromatin to nuclear periphery, fragmentation of nuclei and apoptotic bodies were remarkable in cancer cells (Fig. 1a, b).

In non-neoplastic colon epithelium, DSB was observed mainly in nuclei of cells facing the lumen (not illustrated). The DSB was detected in the nuclei of cancer cells with an intense signal (Fig. 2a, b). No staining was observed in mitotic cells (Fig. 2b). Most of the label was in nuclei that were normal in appearance, but some were in pyknotic or in apoptotic bodies.

In serial sections of well-differentiated colon cancer, the ISNT-positive nuclei (Fig. 3a) and apoptosis bodies or marginated chromatin detected by H&E staining (Fig. 3b) were distributed in the same area. The sections incu-

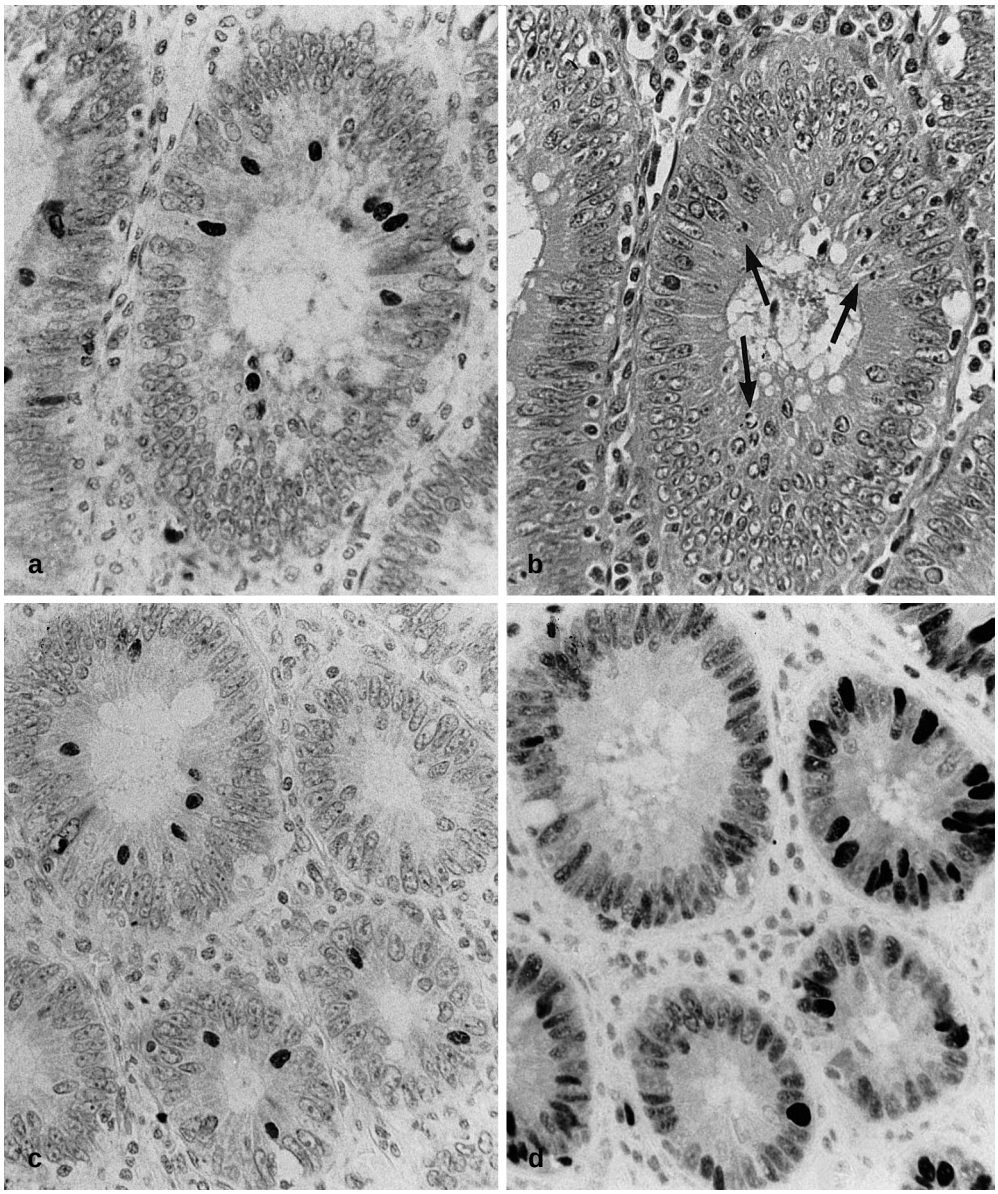

Fig. 3a ISNT and **b** HE staining of a colorectal cancer tissue section. Nuclei labeled with ISNT (**a**) and margination of chromatin (**b** *arrows*) were distributed in the same area. **c** ISNT and **d** PCNA immunostaining of colorectal cancer tissue section. Nuclei labelled with ISNT and those labelled with PCNA were distinct

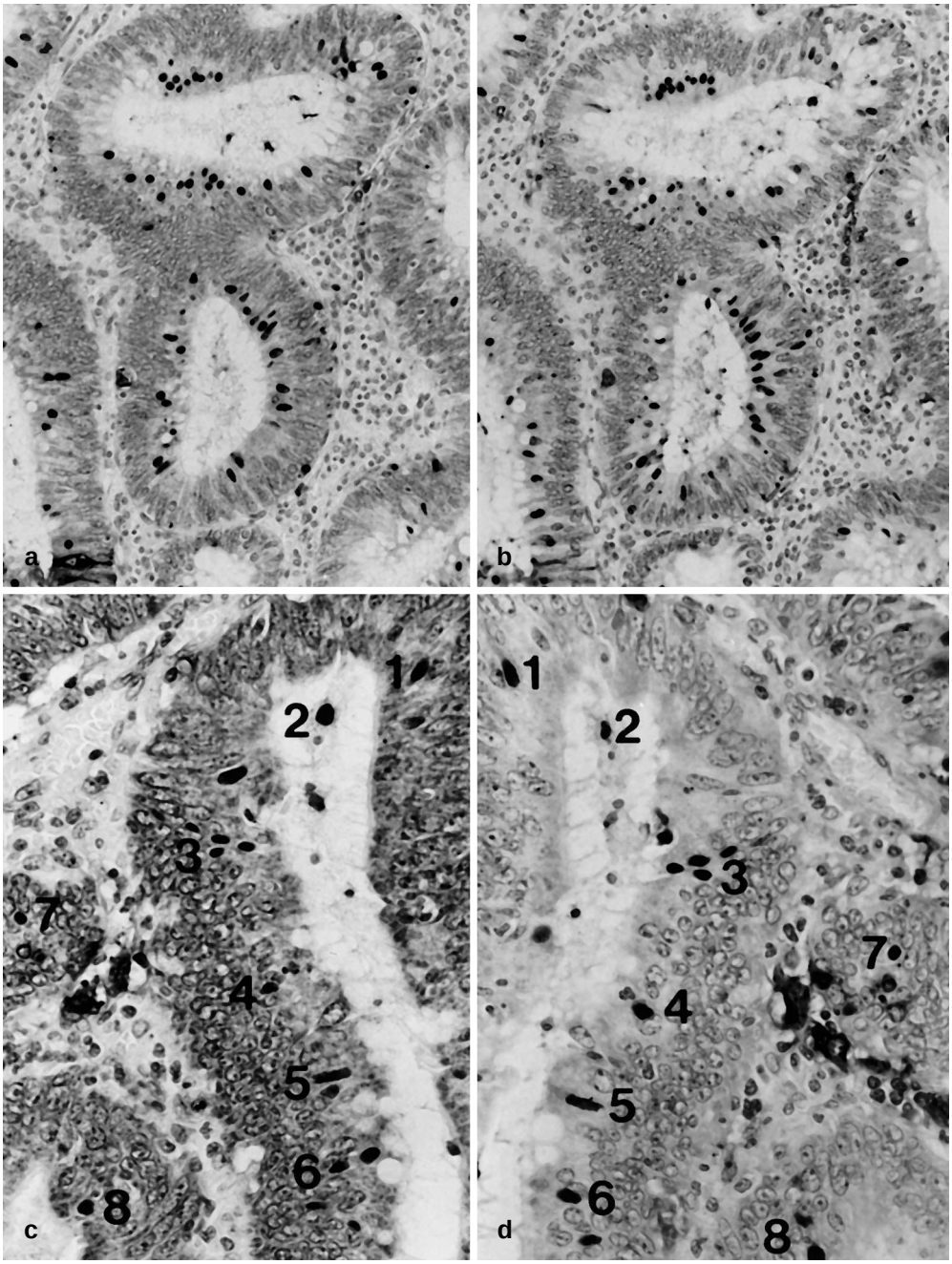

Fig. 4a–d Serial sections labelled with ISNT and TUNEL. The distributions of **a** ISNT- and **b** TUNEL-positive nuclei are the same in the cancer tubules. **c, d** Mirror sections labelled with ISNT (**c**) and TUNEL (**d**); same nuclei (numbered 1–8) are labelled with both ISNT and TUNEL

bated with ISNT or TUNEL solutions minus enzymes or dUTP labelled with biotin were not stained (data not shown).

The PCNA-positive nuclei were negative for ISNT and vice versa, and the distributions of nuclei stained for PCNA and those labelled with ISNT were not the same (Fig. 3c, d).

Although we have reported that ISNT was a more reliable and sensitive tool for detection of apoptosis in sections than TUNEL, TUNEL has been more widely used as a histological marker of apoptosis than ISNT. For this reason, the distribution of nuclei labelled by ISNT and by TUNEL were compared in serial sections and in mirror sections. Their distributions coincided precisely (Fig. 4a, b), and the same cells were stained (Fig. 4c, d); however, the staining intensity tended to vary with TUNEL.

Table 1 summarizes the relationship of ISNT L.I. and PCNA L.I. to the clinicopathological characteristics in 102 colorectal cancers. The ISNT L.I. varied from 3 to

Fig. 5 Scattergrams showing the relation between ISNT labelling index (*L.I.*) and PCNA L.I. of the tumour studied, classified according to **a** histological grade [△ moderately, × poorly, ○ well differentiated) **b** depth of invasion (▼ Dukes' B/C, △ Dukes' A), **c** lymph node metastasis, (● node-positive, ○ node-negative), **d** liver metastasis (▼ positive, ▽ negative for liver metastasis) ▶

Table 1 Relationships between ISNT and PCNA labelling indices (L.I.) and clinicopathological characteristics in 102 patients with colorectal carcinoma (*M* means, *NS* not significant, *diff.* differentiated, *Mod.* moderately, *Poor.* poorly)

Variables	No. of patients	ISNT L.I. M (SD)	Significance of ISNT L.I.	PCNA L.I. M (SD)	Significance of PCNA L.I.
Age of patients					
≤65 years	50	28.1 (18.9)		433 (149)	
>65 years	52	32.4 (27.3)	NS	413 (137)	NS
Gender of patients					
Male	61	28.8 (20.2)		422 (140)	
Female	41	31.3 (25.7)	NS	425 (149)	NS
Tumour location					
Right	23	33.9 (25.9)		455 (133)	
Left	79	29.6 (22.6)	NS	414 (145)	NS
Tumour size					
≤5 cm	48	30.6 (20.2)		427 (146)	
>5 cm	54	30.0 (26.3)	NS	419 (140)	NS
Depth of invasion					
Dukes' A	22	46.2 (25.6)		449 (159)	
Dukes' B/C	80	25.9 (21.1)	$P<0.001$	416 (140)	NS
Lymph node metastasis					
Negative	52	45.0 (21.3)		415 (134)	
Positive	50	15.0 (14.3)	$P<0.001$	432 (151)	NS
Liver metastasis					
Negative	88	33.0 (24.1)		428 (143)	
Positive	14	13.2 (6.5)	$P<0.005$	390 (140)	NS
Histological differentiation					
Well diff.	13	47.7 (30.7)		407 (134)	
Mod. diff.	84	29.3 (21.1)		418 (141)	
Poor. diff.	5	7.4 (6.7)	$P<0.005$	551 (134)	NS

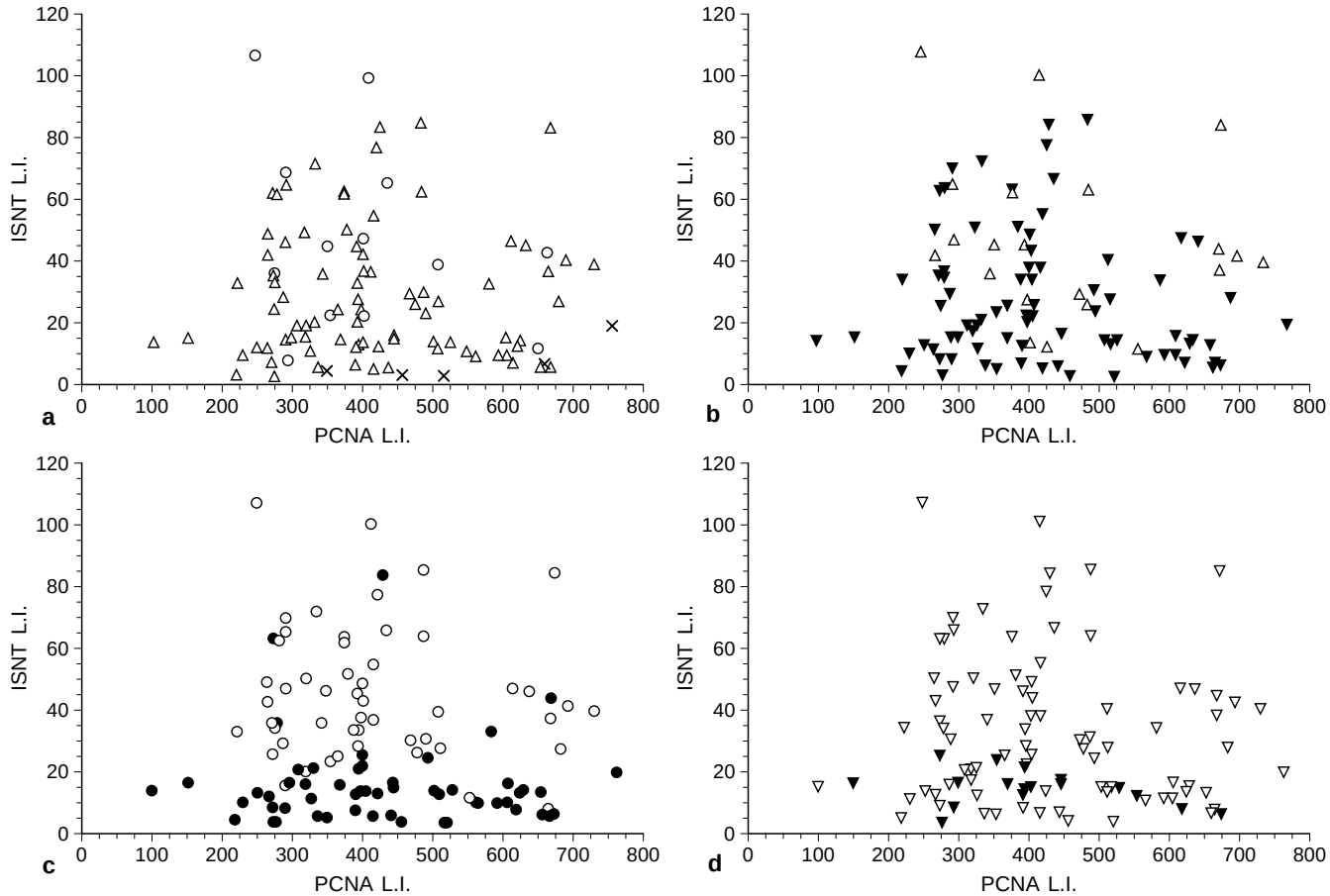

Table 2 Relationships between ISNT L.I. and depth of invasion, lymph node metastasis or liver metastasis among similarly differentiated colon carcinomas

Variables	No. of patients	ISNT L.I.	Significance
Well-differentiated colorectal cancer (*n*=13)			
Depth of invasion			
Dukes' A	4	74.1±34.4	
Dukes' B	9	36.1±21.9	*P*<0.05
Lymph node metastasis			
Negative	9	59.4±28.9	
Positive	4	21.6±15.6	*P*<0.05
Liver metastasis			
Negative	11	53.7±29.7	
Positive	2	15.5±10.6	NS
Moderately differentiated colorectal cancer (*n*=84)			
Depth of invasion			
Dukes' A	16	38.2±19.7	
Dukes' B/C	68	27.2±20.9	*P*<0.05
Lymph node metastasis			
Negative	42	42.8±17.8	
Positive	42	15.8±14.5	*P*<0.001
Liver metastasis			
Negative	72	31.8±21.6	
Positive	12	14.0± 6.4	*P*<0.05

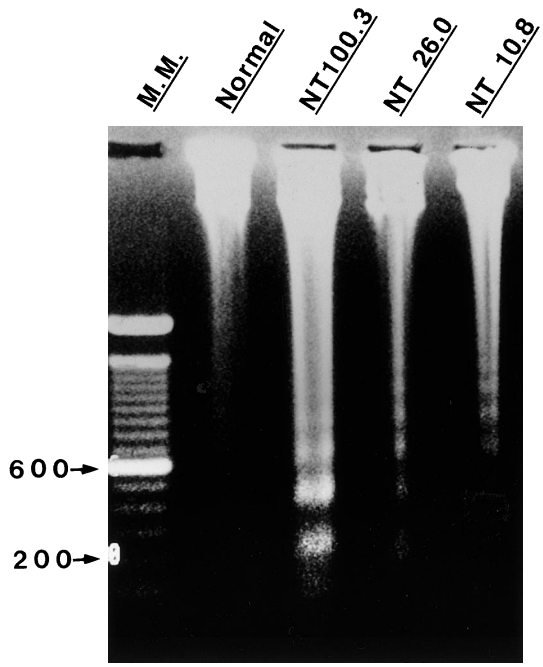

Fig. 6 Gel electrophoresis of DNA extracted from colon mucosa (*Normal* DNA extracted from normal colon mucosa, *NT100.3* DNA extracted from mucosa of colon carcinoma with ISNT L.I. of 100.3, *NT26.0* DNA extracted from mucosa of colon carcinoma with ISNT L.I. of 26.0, *NT 10.8* DNA extracted from mucosa of colon carcinoma with ISNT L.I. of 10.8, *M.M.* molecular marker). Bands of 600 bp and 200 bp were marked. DNA formed ladder proportional to the ISNT L.I.

107 with a mean of 30.6, SD±23.3 (Table 1, Fig. 5a–d). The ISNT L.I. of the carcinomas classified as Dukes' A was significantly higher than that of those classified as B/C; those without lymph nodes and/or liver metastasis had significantly higher L.Is than those with metastasis; and well-differentiated carcinomas had signifiantly higher L.I.s than did poorly differentiated carcinomas. Among cancers with similar levels of differentiation, the ISNT L.I. was higher in colon carcinomas classified as Dukes' A than in Dukes' B/C carcinomas, and those that did not metastasize to lymph node or liver had higher ISNT L.I.s than those that did (Table 2). No significant variation of ISNT L.I. was found with age of patients (more than 65 and less than 65), sex of patients, size of carcinomas (more than 5 cm and less than 5 cm) or location of carcinomas in the colon (proximal and distal to the splenic flexure).

PCNA L.I. varied from 101 to 771 with a mean of 423.1 (SD±142.6). No significant PCNA L.I. difference was found between those with and without lymph node metastasis and/or liver metastasis, those with invasion of sub-serosal areas and beyond and those with tumours limited to the colorectal wall, or those with poorly differentiated and well-differentiated carcinomas. Also, no difference in PCNA L.I. was found between patients in different age groups (more than 65 and less than 65), male and female patients, carcinomas more than 5 cm and less than 5 cm or different locations of carcinomas in the colon (proximal and distal to the splenix flexure). There was no correlation between ISNT L.I. and PCNA L.I.

The DNA extracted from cancer with a high ISNT L.I. (>100) electrophoresed as a typical 180- to 200-bp ladder with a high-molecular-weight band, whereas DNA from cancer with a low ISNT L.I. (<30) electrophoresed as normal colon mucosa (Fig. 6). These results confirm that the ISNT index reflected the incidence of DNA breaks and vice versa.

Discussion

In this study, we demonstrated that apoptosis was more frequent in human colorectal carcinomas that did not invade through muscularis propria or metastasize than in those that invaded or metastasized. Similarly, apoptosis was more frequent in well- and moderately differentiated carcinomas than in poorly differentiated carcinomas.

The ISNT L.I. had clear negative associations with depth of invasion (Fig. 5a) and with occurrence of metastasis (Fig. 5b, c) and a positive association with the degree of differentiation (Fig. 5d). The PCNA L.I., on the other hand, was almost independent of the behaviour of colonic carcinoma with regard to invasiveness (Fig. 5a) and occurrence of metastasis in lymph nodes (Fig. 5b) or liver (Fig. 5c). There was no significant positive or negative correlation between the population of cells in S-phase (PCNA L.I.) and that in the process of apoptosis (ISNT L.I.). In this regard the report by Takano et al. [29] is of interest. They found a positive correlation between the Ki-67 L.I. and TUNEL L.I. in "individual cases" of colon carcinomas. Ki-67 is present throughout

the cell cycle except for G0 [5] and is an indicator of cells in cycle regardless of the length of G1, whereas PCNA appears at the G1/S border [3] and is an indicator of cells in division. These subtle differences in the nature of the indices may contribute to the variance among the correlations between PCNA and ISNT/TUNEL and between Ki-67 L.I. and TUNEL L.I. for "individual cases" of colon carcinoma.

When the ratio between PCNA L.I. and ISNT L.I. (PCNA/ISNT) was compared with the behaviour of the tumours, there was also a negative correlation with the behaviour of colonic carcinomas (PCNA/ISNT vs depth of invasion, $P<0.05$; PCNA/ISNT vs lymph node metastasis, $P<0.05$; PCNA/ISNT vs liver metastasis, $P>0.05$), but the negative association between ISNT L.I. alone with the behaviour of carcinomas was more clearly seen (ISNT L.I. vs depth of invasion, $P<0.001$; ISNT L.I. vs lymph node metastasis, $P<0.001$; ISNT L.I. vs liver metastasis, $P<0.005$).

Our findings on proliferation are in agreement with previous reports that markers of proliferative activity such as Ki-67 L.I. [15, 25] or PCNA L.I. [17] did not correlate with depth of invasion or lymph node and/or liver metastasis. The high rate of apoptosis found in non-invasive or non-metastasizing carcinomas is in agreement with the conclusion drawn by Ikenaga et al. [9], although they found no positive correlation with the degree of tumour differentiation. This difference may have resulted from the sizes of the tumours examined (all sizes in their study and those more than 1 cm in largest diameter in our study) and the unusually low apoptosis indices obtained by them (e.g. 1.36 in well-differentiated tumours in their study vs 4.77 in well-differentiated tumours in ours.

The high frequency of apoptosis in well- to moderately differentiated carcinomas and the low frequency in poorly differentiated carcinomas might have been anticipated, as most neoplastic cells retain various levels of ability to differentiate along the path of normal cells [22]. In normal colon, epithelial cells undergo apoptosis at the luminal surface in the terminal phase of differentiation [4, 6]. Hence, our results may be interpreted as showing that the high frequency of apoptosis in well-differentiated carcinomas is a representation of its normal counterpart. Conversely, poorly differentiated carcinomas failed to reach the level of differentiation at which cells undergo apoptosis, and the frequency of apoptosis was consequently low.

Among similarly differentiated colon carcinomas, apoptosis was more frequent in colon carcinomas that had not invaded or metastasized than in those that had (Table 2). The mechanism of apoptosis is not well understood, and some workers suggest that a specific inducer for apoptosis and receptor for the inducer are required for the cells to undergo apoptosis, such as Fas ligand/Fas system [20]. Whether there is a positive or negative relationship between the invasiveness of carcinomas and the onset of apoptosis is not known, but this question merits further investigation. In view of the finding by Bates et al. [2] that when cell–cell adhesion was interrupted on colon carcinoma cell lines in vitro the cells underwent apoptosis, it may be speculated that if the phenomenon holds true in vivo, some of those cells that underwent apoptosis in colon carcinomas are the ones that have lost contact with the tumour mass and those that have metastasized as cell emboli [10, 23, 28].

Currently the determination of levels of histological differentiation of colon carcinomas is based mainly on their glandular morphology. When the occurrence and frequency of ISNT-/TUNEL-positive nuclei are used as an additional marker of glandular differentiation, the histological differentiation and the behaviour of colon carcinoma may be better delineated.

In summary, this study demonstrated that apoptosis was more frequent in well- and moderately differentiated colon carcinomas than in poorly differentiated types and was more frequent in carcinomas that did not invade or metastasize.

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References

1. Adams JC (1981) Heavy metal intensification of DAB-based HRP reaction product. J Histochem Cytochem 29:775
2. Bates RC, Buret A, Helden DF van, Horton MA, Burns GF (1994) Apoptosis induced by inhibition of intercellular contact. J Cell Biol 125:403–415
3. Bravo R, MacDonald-Bravo M (1987) Existence of two population of cyclin/proliferating cell nuclear antigen during the cell cycle. Association with DNA replication sites. J Cell Biol 105:1549–1554
4. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
5. Gerdes J, Schwab U, Lemke H, Stein H (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13–20
6. Gerschenson LE, Rotello RJ (1992) Apoptosis: a different type of cell death. FASEB J 6:2450–2455
7. Hashimoto S, Koji T, Niu J, Kanematsu T, Nakane PK (1995) Differential staining of DNA strand breaks in dying cells by non-radioactive in situ nick translation. Arch Histol Cytol 58:161–170
8. Hattori T, Helpap B, Gedigk P (1984) Cell proliferation and growth of gastric carcinoma induced in inbred wister rats by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Cancer Res 44:5266–5272
9. Ikenaga M, Takano Y, Saegusa M, Ohtani Y, Hiki Y, Kakita A, Okayasu I (1996) Apoptosis of colon cancers assessed by in situ DNA nick end-labeling method. Pathol Int 46:33–37
10. Kerbel RS (1994–1995) Impact of multicellular resistance on the survival of solid tumors, including micrometastasis. Invasion Metastasis 14:50–60
11. Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
12. Koji T (1996) Non-radioactive in situ nick translation: a useful molecular histochemical tool to detect single-stranded DNA breaks. Acta Histochem Cytochem 29:71–79
13. Kunkel LM, Smith KD, Boyer SH, Borgaonkar DS, Wachtel SS, Miller OJ, Breg WR, Jones HW Jr, Rary JM (1977) Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. Proc Natl Acad Sci USA 74:1245–1249

14. Landström M, Damber J, Bergh A (1994) Prostatic tumor regrowth after initially castration therapy may be related to a decreased apoptotic cell death rate. Cancer Res 54:4281–4284
15. Lanza G Jr, Cavazzini L, Borghi L, Ferretti S, Buccoliero F, Rubbini M (1990) Immunohistochemical assessment of growth fractions in colorectal adenocarcinomas with monoclonal antibody Ki-67. Pathol Res Pract 186:608–618
16. Leong A S-Y, Milios J, Tang SK (1993) Is immunolocolization of proliferating cell nuclear antigen (PCNA) in paraffin sections a valid index of cell proliferation? Appl Immunohistochem 1:127–135
17. Linden MD, Ma CK, Kubus J, Brown RD, Zarbo RJ (1993) Ki-67 and proliferating nuclear antigen tumor proliferative indices in DNA diploid colorectal adenocarcinomas. Correlation with histopathologic characteristics and cell cycle analysis with two color DNA flowcytometry. Am J Clin Pathol 100: 206–212
18. Lipponen PK, Eskelinen MJ (1992) Cell proliferation of transitional cell bladder cancer determined by PCNA/cyclin immunostaining. A histological description. Anticancer Res 12: 577–584
19. Maeda K, Chung Y, Onoda N, Kato Y, Atsunori N, Arimoto Y, Yamada N, Kondo Y, Sowa M (1994) Proliferating cell nuclear antigen labeling index of preoperative biopsy specimens in gastric carcinoma with special reference to prognosis. Cancer 73:528–533
20. Möller P, Koretz K, Leithäuser F, Brüderlein S, Henne C, Quentmeier A, Krammer PH (1994) Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int J Cancer 57:371–377
21. Mori M, Kakeji Y, Adachi Y, Moriguchi S, Maehara Y, Sugimachi K, Jessup JM, Chen LB, Steele GDJ (1992) The prognostic significance of proliferating cell nuclear antigen in clinical gastric cancer. Cancer 113:683–690
22. Pierce GB, Nakane PK, Mazurkiewicz JE (1974) Natural history of malignant stem cells. In: Nakahara W, Ono T, Sugimura T, Sugano H (eds) Differentiation and control of malignancy of tumor cells. (Proceedings of the Princess Takamatsu Cancer Research Fund, Tokyo, 1973) University of Tokyo Press, Tokyo, pp 453–469
23. Radinsky R (1993) Paracrine growth regulation of human colon carcinoma organ-specific metastasis. Cancer Metastasis Rev 12:345–361
24. Reed JC (1994) Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6
25. Shephered NA, Richman PI, England J (1988) Ki-67 derived proliferative activity in colorectal adenocarcinoma with prognostic correlations. J Pathol (Lond) 155:213–219
26. Siitonen SM, Kallioniemi OP, Isola JJ (1993) Proliferating cell nuclear antigen immunohistochemistry using monoclonal antiboy 19A2 and a new antigen retrieval technique has prognostic impact in archival paraffin-embedded node-negative breast cancer. Am J Pathol 142:1081–1089
27. Sträter J, Korez K, Günthert AR, Möller P (1995) In situ detection of enterocytic apoptosis in normal colonic mucosa and in familial adenomatous polyposis. Gut 37:819–825
28. Sugino T, Kawaguchi T, Suzuki T (1993) Sequential process of blood-borne lung metastases of spontaneous mammary carcinoma in C3H mice. Int J Cancer 55:141–147
29. Takano Y, Suenaga M, Ikenaga M, Mitomi H, Okayasu I (1996) Apoptosis of colon cancer: comparison with Ki-67 proliferative activity and expression of p53. J Cancer Res Clin Oncol 122:166–170
30. Wijsman JH, Johnker RR, Keijzer R, De Velde CJH, Cornelisse CJ, Dierendonck JH (1993) A new method to detect apoptosis in paraffin sections: In situ end-labeling of fragmented DNA. J Histochem Cytochem 41:7–12
31. Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306
32. Yonemura Y, Kimura H, Fushida S (1993) Analysis of proliferative activity using anti-proliferating cell nuclear antigen antibody in gastric cancer tissue specimens obtained by endoscopic biopsy. Cancer 71:2448–2453