Histol Histopathol (1998) 13: 743-749

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Apoptosis in adenoma and early adenocarcinoma of the colon

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Summary. Twenty-six specimens of tubular adenoma and 7 specimens of adenocarcinoma in adenoma of the colon were examined to evaluate apoptosis between adenoma and early adenocarcinoma. Cell proliferation and cell death seemed to be balanced in adenoma with mild and moderate atypia, but unbalanced in adenoma with severe atypia and cancer. Apoptosis was considered to be suppressed at cancer in some cases. However, a number of apoptosis increased at cancer in other cases. Necrosis was seen only in cancer areas. The ratio of cells simultaneously stained by anti-Ki-67 antibody (MIB-1) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) tended to be high from adenoma with moderate atypia to cancer, suggesting the unstableness of DNA. It is possible that cancer cells having highly unstable DNA easily underwent apoptosis as well as necrosis, accidentally. The p53 protein was positive only in cancer areas of three cases. One of these three cases showed decreased apoptosis in a cancer area, but the other two cases showed increased apoptosis. Furthermore, certain numbers of cancer cells were double-stained by p53 immunohistochemistry and TUNEL. These results suggest that the p53 protein may contribute to suppress apoptosis in the last stage of carcinogenesis of the colonic adenocarcinoma, but other factors including extrinsic stimulation may cause apoptosis despite the mutation of p53 protein.

Key words: Apoptosis, p53, Adenoma, Adenocarcinoma, Colon

Introduction

Growth or homeostasis of tissues depends on the balance of cell proliferation and cell death (Kerr et al., 1972; Willie et al., 1980). Apoptosis and necrosis have been considered for cell death (Wyllie et al., 1980; Buja et al., 1993; Cummings et al., 1997), and a cell loss by apoptosis seems to be important for the regulation of cell number in the mammalian gastrointestinal tract (Hall et al., 1994). The number of proliferating cells significantly increases in proportion to the severity of dysplasia in colonic epithelial neoplasms (Johnston et al., 1989; Diebold et al., 1992; Risio and Rossini, 1993). On the other hand, the number of apoptosis in colonic tumors is still obscure, although correlation between mitotic and apoptotic indices has been reported in colonic adenoma (Arai and Kino, 1995). We investigated apoptosis in adenoma and early adenocarcinoma of the colon to clarify whether any difference is seen between adenoma and cancer. For this purpose, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-nick end labeling (TUNEL) (Gavrieli et al., 1992) was used to detect apoptosis in addition to counting apoptotic bodies in hematoxylin and eosin staining (HE). The close relation between cell cycle and apoptosis has been considered, although there may be an another apoptotic pathway occurring without entering cell cycle (Coates et al., 1996). An anti-Ki-67 antibody (MIB-1) detects cells existing in G_1, S, G_2, and M phases of cell cycle (Gerdes et al., 1984; Cattoretti et al., 1992). Cells simultaneously positive for MIB-1 and TUNEL may indicate that cells entering in cell cycle die (Coates et al., 1996), and that these cells have the unstable DNA. Various genes are involved in the regulation of cell cycle, and some of them are also necessary to regulate apoptosis (Coates et al., 1996). The p53 protein, which normally induces apoptosis depending on cell cycle (Kobayashi et al., 1995), is presumed to play a role during carcinogenesis close to the stage of the final malignant transformation in colonic adenoma-carcinoma sequence (Fearon and Vogelstein, 1990; Kikuchi-Yanoshita et al., 1992; Auer et al., 1994). Immunohistochemistry of p53 protein, including double staining with TUNEL, would reveal whether p53 expression is a major factor for the regulation of apoptosis in the last stage of carcinogenesis.

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Materials and methods

Patients and specimens

Thirty-three specimens of colonic tubular adenoma and adenocarcinoma in adenoma obtained by endoscopic

resection were examined (Table 1). Cases were from the files of the Department of Surgical Pathology, Tokyo Women's Medical College. Specimens were fixed in 10% buffered formalin, embedded in paraffin and cut into serial sections at 3 μm thick. Sections were stained with HE. The grading and mapping of adenoma was performed according to the classification of the Japanese Society for Cancer of the Colon and Rectum (Japanese Society for Cancer of the Colon and Rectum, 1994): adenomas were divided into mild, moderate and severe atypia according to the grade of cytological and structural atypia, and glands consisting of cytologically malignant cells were regarded as cancer (Fig. 1). In 25 cases that contained more than two areas showing different grades of atypia in one specimen (Table 1), analysis was performed in each lesion.

Immunohistochemical staining

Immunostaining for MIB-1 (1:100 dilution, monoclonal; Immunotech, Marseilles, France) and p53 protein (ready-to-use, monoclonal; Immunotech) was performed on 33 cases and 28 cases, respectively. The avidin-biotin complex method was employed. Microwave antigen retrieval for 10 minutes at 95 °C in 0.01M citrate buffer, pH 6.0, was performed after deparaffinization and elimination of endogenous peroxidase activity. Sections were incubated with the primary antibody overnight at 4 °C. Reaction products were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB), and sections were counterstained with hematoxylin. A negative control study was performed by eliminating the primary antibody.

Table 1. Characteristics of Patients and Polyps.

CASES	AGE	SEX	SIZE	CONTAINED LESIONS
1	48	M	0.3	Mild
2	72	M	0.8	Moderate, severe
3	43	M	1.1	Mild, moderate
4	67	F	0.7	Mild, moderate
5	62	M	0.5	Moderate
6	61	M	0.3	Moderate
7	76	M	0.8	Moderate, severe
8	65	F	0.4	Mild, moderate
9	35	F	1.0	Mild, moderate
10	51	M	1.8	Mild, Moderate, severe
11	56	F	0.4	Mild
12	67	M	0.7	Mild, moderate
13	72	M	1.2	Mild, moderate, severe
14	71	M	0.4	Moderate
15	71	M	0.7	Mild, moderate
16	65	M	0.6	Moderate, severe
17	65	M	0.6	Mild, moderate
18	56	M	0.3	Mild
19	40	F	0.8	Moderate
20	69	M	1.3	Mild, moderate
21	61	M	0.9	Mild, moderate
22	60	M	0.5	Mild
23	60	M	0.6	Mild, moderate
24	62	M	0.2	Mild
25	79	F	0.5	Mild, moderate
26	59	M	1.0	Moderate, severe, cancer
27	60	M	1.1	Mild, moderate, severe, cancer
28	59	F	0.7	Mild, moderate, severe, cancer
29	64	M	0.6	Severe, cancer
30	46	M	0.9	Mild, moderate, severe, cancer
31	49	M	0.9	Severe, cancer
32	70	M	1.2	Moderate, severe, cancer
33	53	M	0.7	Severe, cancer

Mild, moderate and severe refer to the degree of atypia in adenoma.

TUNEL

Sections were deparaffinized in a routine manner, digested with 20 μg/ml proteinase K (Sigma Chemical Co. Saint Louis, MO) for 30 min at room temperature (RT), and then immersed in 2% hydrogen peroxide for 5 min at RT to quench endogenous peroxidase. After washing, they were incubated with TdT (0.15 units/μl; GIBCO BRL, Gaithersburg, MD) and digoxigenin-11-dUTP (0.02 nm/μl, Boehringer-Mannheim/Yamanouchi, Tokyo) in TdT buffer (GIBCO BRL) in humidified atmosphere at 37 °C for 60 min. The reaction was stopped by immersing them in TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at 37 °C. Anti-digoxigenin-peroxidase (1:50 dilution, Boehringer Mannheim) was applied and sections were incubated for 30 min, RT. Reaction products were developed with DAB, and sections were counterstained with hematoxylin. Sections digested with DNAse I (10 units/μl, Boehringer Mannheim) after deparaffinization were used as positive controls. Elimination of TdT in the reaction solution was done as a negative control.

Double staining

TUNEL with successive MIB-1 staining was performed in five cases (cases 10, 12, 23, 28 and 33), and TUNEL with successive p53 staining was done in 11 cases (cases 3, 10, 12, 23, 26-28, and 30-33). Reaction products of TUNEL were developed with DAB, and those of MIB-1 and p53 were developed with nickel DAB. Sections were counterstained with methyl green.

Evaluation

Upon HE staining, the ratio of mitotic figures and that of apoptotic bodies were regarded as the mitotic index and apoptotic index, respectively. The MIB-1 and TUNEL indices were calculated by counting positive cells. To obtain above indices, 1000 to 2000 cells were counted. Each index was counted in the same area that included glands from upper to lower level and showed fine results in both MIB-1 immunohistochemistry and TUNEL. The ratio of double (MIB-1 and TUNEL)-positive cells per TUNEL-positive cells was also calculated in the same area. The result of p53 immunostaining was evaluated as positive when most of the glands were stained, focally positive when a few glands were stained, and negative when no glands were stained.

For double staining of p53 and TUNEL, the ratio of double-positive cells per TUNEL positive cells was calculated in the areas that showed positive immunoreaction against the anti-p53 protein antibody. Apoptotic bodies in the lumen or nuclear debris that could not be identified with certainty as apoptotic bodies were not counted. Although necrosis was positive for TUNEL, this was excluded from counting. Immunostaining to an antibody for leukocyte common antigen was performed to exclude intraepithelial leukocytes. In addition to apoptosis, necrosis was also evaluated. The extent of necrosis in tumor was classified negative (including none and a very little) and positive.

Results

Sixty-seven lesions were mapped in 33 polypectomized specimens (Table 1). Cancer invaded into submucosa in three cases (Table 2). A significant correlation was found between the mitotic and MIB-1 indices, and between the apoptotic and TUNEL indices (data not shown). In specimens with areas showing different grades of atypia, the mitotic and MIB-1 indices increased in proportion to the severity of atypia (Fig 2a,c). Scores of apoptotic and TUNEL indices were diverse in each case (Fig. 2b,d). However, these indices tended to increase between mild and moderate atypia. Scores peaked at moderate or severe atypia in some cases, and either increased or decreased betwen severe atypia and cancer. Necrosis was exceptional in adenoma, and was generally seen in areas where cancer showed submucosal invasion (Table 2). Some cells were doubly stained by both MIB-1 and TUNEL (Fig. 3). MIB-1-TUNEL/TUNEL tended to increase between mild and moderate atypia, and to keep high scores from moderate atypia to cancer (Fig. 4).

Adenoma areas were negative for p53 protein in 22 cases. Only a few glands or cells were positively immunostained in 6 cases. Cancer areas were positive in 3 cases (Fig. 5) and focally positive in 4 cases (Table 2). Among cases showing positive immunoreaction against the anti-p53 protein antibody in cancer areas (cases 27, 30 and 31), apoptotic and TUNEL indices between severe atypia and cancer increased in cases 30 and 31, while they decreased in case 27 (Table 2). Cells double-stained by p53 and TUNEL were hardly found in areas where p53 was focally positive. p53-TUNEL/TUNEL was 47.8 % (case 27), 50.0 % (case 30) and 39.2 % (case 31) in the areas where p53 was positive (Fig. 6).

Discussion

The significant relationship between the number of proliferating cells and the severity of dysplasia has been reported with regard to colonic neoplasms (Johnston et al., 1989; Diebold et al., 1992; Risio and Rossini, 1993), and similar results were obtained in our study. In contrast, the apoptotic or TUNEL index showed no positive relation like the mitotic or MIB-1 index. However, some interesting findings were observed as

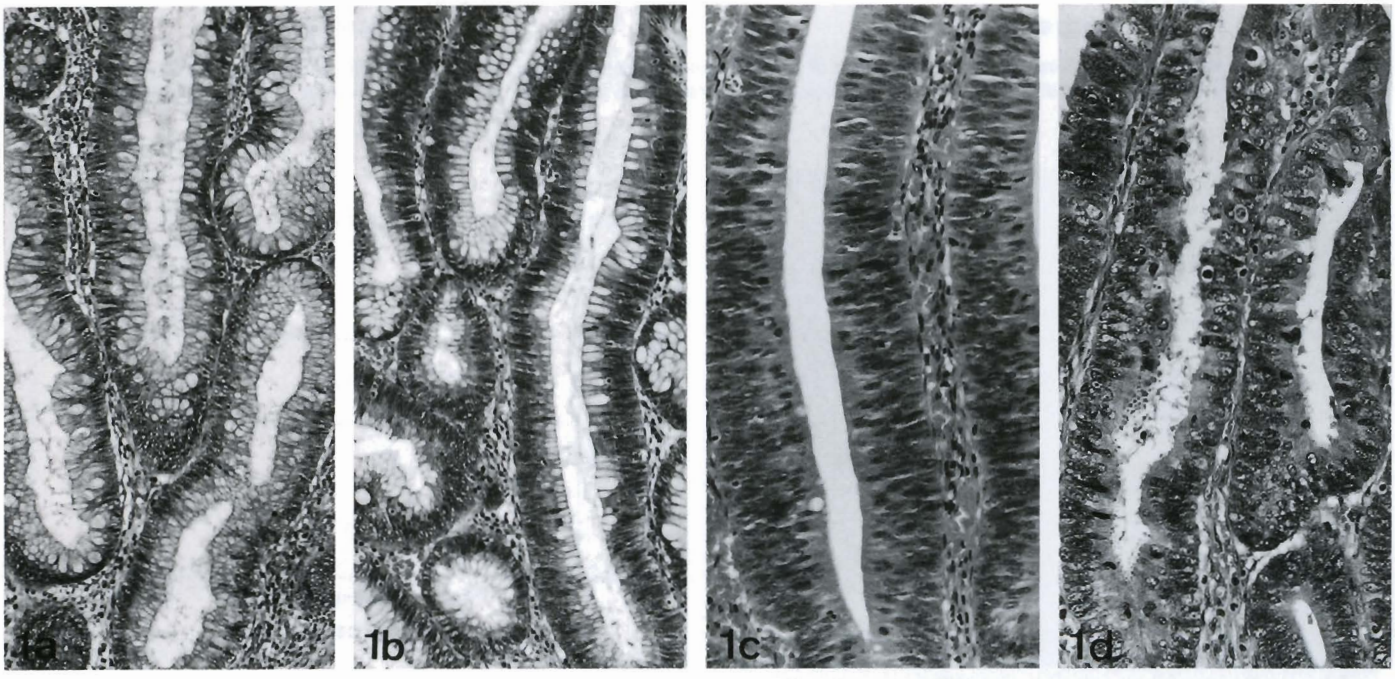

Fig. 1. Adenoma with mild **(a)**, moderate **(b)** and severe **(c)** atypia, and cancer **(d)**. Cells are tall columnar and have an elongated nucleus in the adenoma. Cytoplasmic mucin is reduced and nuclei are more stratified in adenoma with higher grades of atypia. Cancer cells have large, oval shaped nucleus sometimes with conspicuous nucleoli . a, b, x 125; c, d, x 200

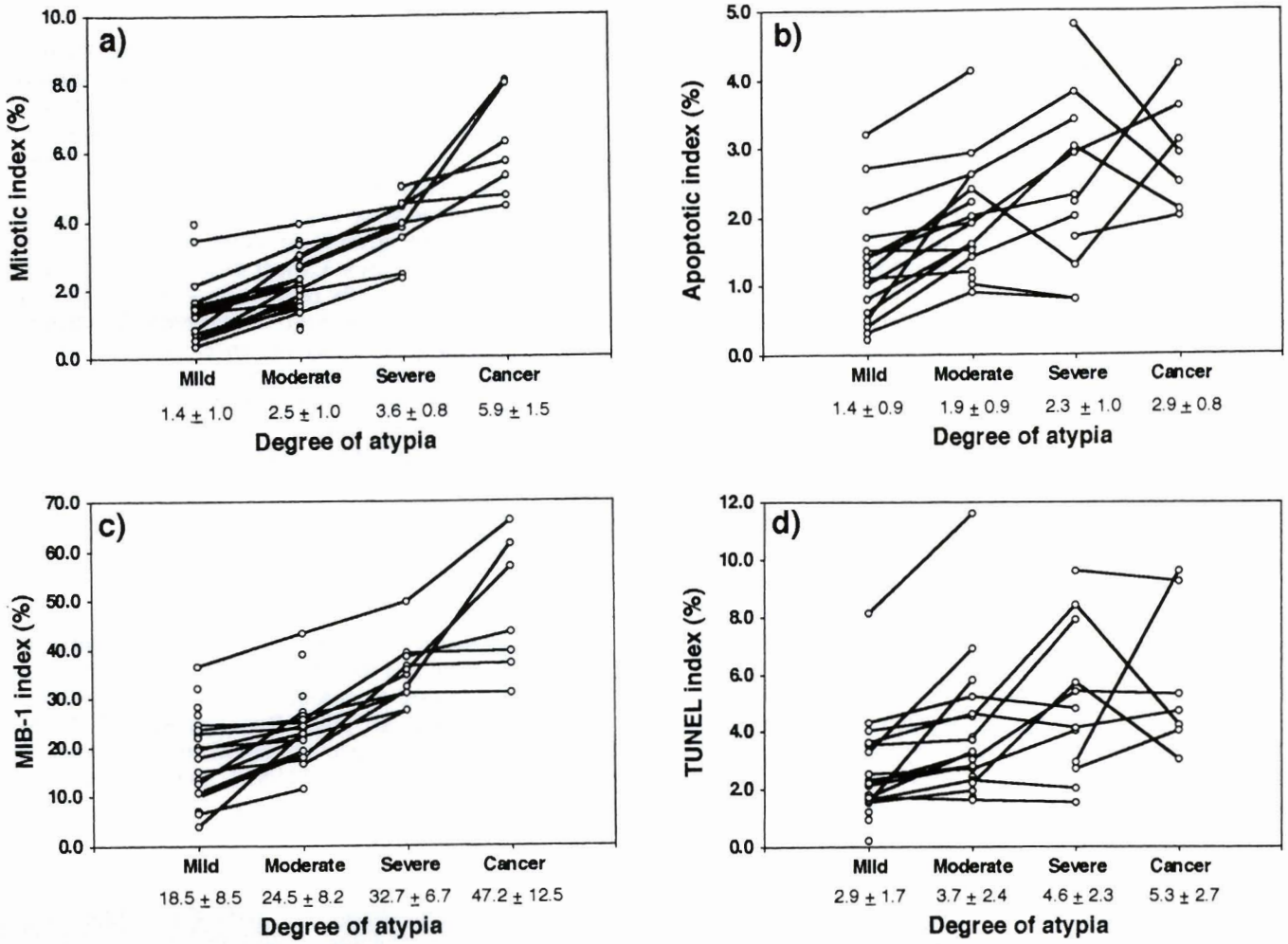

Fig. 2. a and c. Mitotic and MIB-1 indices in various grades of atypia. Scores increase in proportion to the severity of atypia. **b and d.** Apoptotic and TUNEL indices in various grades of atypia. Changes of scores are diverse. Solid lines indicate changes of indices in the same polypectomysed specimen.

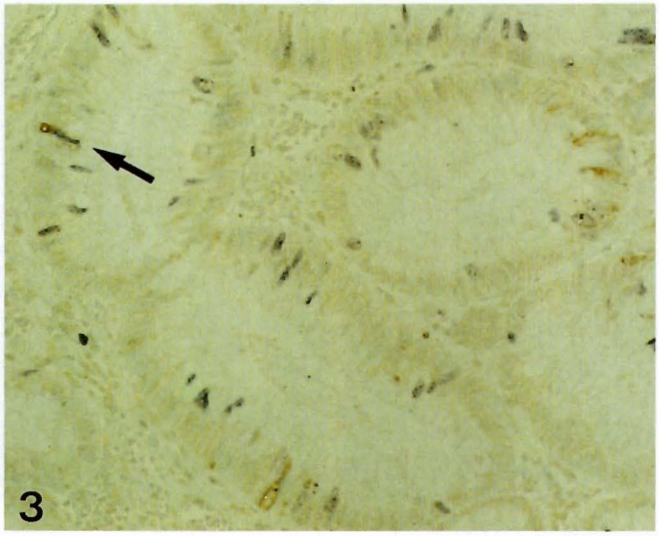

Fig. 3. Double staining of MIB-1 (blue) and TUNEL (brown) in adenoma. An arrow indicates a doubly- stained cell. x 200

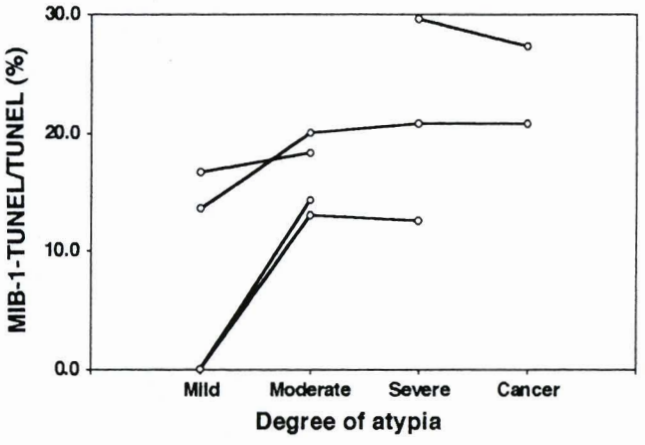

Fig. 4. MIB-1-TUNEL/TUNEL in various grades of atypia. Scores tended to be higher from moderate atypia to cancer.

follows.

All four indices tended to increase between mild and moderate atypia, indicating that cell renewal is accerelated, but that the balance of cell proliferation and cell death is still preserved. On the other hand, changes of these indices were rather diverse from moderate atypia to cancer suggesting unbalanced cell proliferation and cell death in adenoma with severe atypia and cancer. Apoptotic and TUNEL indices peaked at severe atypia in some cases containing cancer. Apoptosis may be suppressed in acute leukemia (Mundle et al., 1994) and malignant lymphoma (Hollowood and Macartney, 1991)

Table 2. Apoptosis and p53 expression in cases containing cancer.

CASES	DEGREE OF ATYPIA	APOPTOTIC INDEX (%)	TUNEL INDEX (%)	NECROSIS	p53	INVASION TO SUBMUCOSA	AREA OF CANCER
	Cancer	2.1	3.0	-	±		
26	Severe	3.0	5.7	-	±	-	<50%
	Moderate	1.6	2.2	-	-		
	Cancer	2.5	4.2	-	+		
27	Severe	3.8	8.4	-	±	-	<50%
	Moderate	2.9	4.5	-	-		
	Mild	2.7	4.0	-	-		
	Cancer	3.1	4.7	-	±		
28	Severe	1.3	4.1	-	±	-	<50%
	Moderate	2.4	4.6	-	-		
	Mild	1.2	3.6	-	-		
29	Cancer	2.5	2.3	-	NE	-	>50%
	Severe	2.2	1.8	-	NE		
	Cancer	2.0	4.0	-	+		
30	Severe	1.7	2.7	-	±	-	<50%
	Moderate	3.7	6.5	-	-		
	Mild	2.0	3.1	-	-		
31	Cancer	4.2	9.6	+	+	+	>50%
	Severe	2.2	2.9	-	-		
	Cancer	3.6	5.3	+	±		
32	Severe	2.9	5.4	-	-	+	>50%
	Moderate	1.9	3.0	-	-		
33	Cancer	2.9	9.2	+	±	+	>50%
	Severe	4.8	9.6	-	±		

NE: not examined, +: positive, ±: focally positive, -: negative.

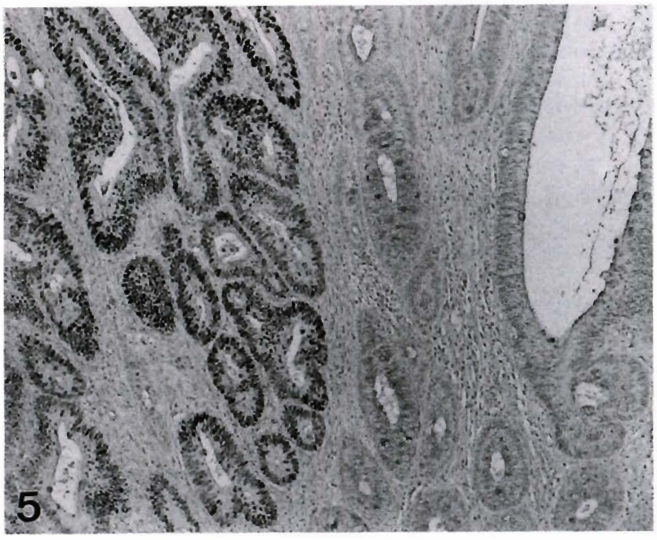

Fig. 5. p53. Positive immunoreaction is seen in a cancer area, but not in an adenoma area. x 80

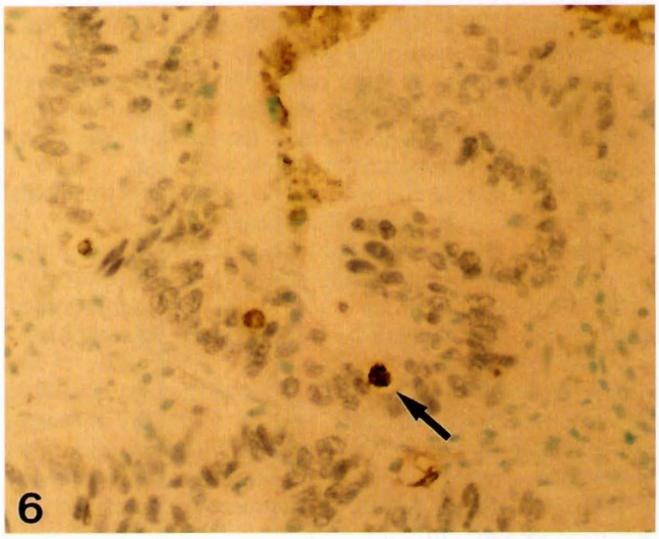

Fig. 6. Double staining of p53 (blue) and TUNEL (brown) in cancer. An arrow indicates a doubly-stained cell. x 250

in comparison with the pre-malignant or non-neoplastic state. Similar conditions can be suspected in adenoma with severe atypia and cancer of the colon. Furthermore, apoptosis is supposed to be already supressed in adenoma with severe atypia in a couple of cases that showed the peak of apoptotic and TUNEL indices at moderate atypia. By histology it is sometimes difficult to distinguish adenoma with severe atypia from cancer. The lesions classified as severe atypia might have acquired cell kinetics close to cancer.

On the other hand, some cases showed increased apoptosis between severe atypia and cancer. Necrosis was seen in cancer areas showing submucosal invasion, whereas it was hardly observed in adenomas. Double staining of MIB-1 and TUNEL reveals that both cell cycle-related and unrelated apoptosis exist in the colonic adenoma and adenocarcinoma. Generally, proliferating cells are located at the lower part of the crypt (Johnston et al., 1989; Diebold et al., 1992), while apoptotic cells are at the upper part of crypt in the normal colonic mucosa (Hall et al., 1994). Double-stained cells were exceptional in the normal colonic mucosa (data not shown). MIB-1-TUNEL/TUNEL tended to increase between mild and moderate atypia and to show high scores between moderate atypia and cancer. Aneuploidy and multiple genetic alterations, more frequent in colonic neoplasms with higher grades of dysplasia, suggest the increase of genomic instability (Fearon and Vogelstein, 1990; Auer et al., 1994). Thus, the existence of double-stained cells may indicate abnormal cell kinetics, probably the unstableness of DNA in the colonic epithelial cells. Apoptosis can occur in physiological and pathological conditions (Kerr et al., 1972; Wyllie et al., 1980) and extrinsic stimulation can cause either apoptosis or necrosis (Wyllie et al., 1980; Hockenbery, 1995; Cummings et al., 1997). The degree of extrinsic stimulation would affect whether cells undergo apoptosis or necrosis (Lennon et al., 1990; Hockenbery, 1995). It is possible in this context that cells showing remarkable genomic instability such as cancer cells easily undergo apoptosis accidentally, even though intrinsic regulatory mechanisms would direct cells to reduce apoptosis, because the colon is one of the organs that has extrinsic stimulation with ease.

The p53 protein normally induces apoptosis depending on cell cycle (Kobayashi et al., 1995), and mutations of the p53 gene have been frequently observed in colonic adenocarcinoma (Fearon and Vogelstein, 1990; Kikuchi-Yanoshita et al., 1992). Accumulation of mutant p53 proteins can be visualized by immunohistochemistry (Kikuchi-Yanoshita et al., 1992; Auer et al., 1994; Kobayashi et al., 1995) and diffusely positive immunoreaction probably results from the mutants (Kobayashi et al., 1995), although not all p53 gene mutations can be detected by immunohistochemistry (Wadayama et al., 1993). Immunoreaction against the anti-p53 protein antibody turned positive in cancer areas of three cases, suggesting that p53 gene mutations concern the last stage of carcinogenesis in adenoma-carcinoma sequence in some cases (Fearon and Vogelstein, 1990; Kikuchi-Yanoshita et al., 1992; Auer et al., 1994). Apoptosis was decreased between severe atypia and cancer in one of these three cases that showed positive immunoreaction against the anti-p53 protein antibody in cancer areas, but increased in the other two cases. Furthermore, certain numbers of cells were doubly stained by TUNEL and p53 immunohistochemistry. These results indicate that apoptosis can occur in cells having the mutant p53 protein. Other intrinsic mechanisms besides p53, such as bcl-2 (Nakamura et al., 1995; Kaklamanis et al., 1996) and adenomatous polyposis coli (APC) genes (Morin et al., 1996) are known to contribute to the regulation of apoptosis in colonic tumors, and it is also reported that sodium butylate derived from dietary fibers can cause apoptosis (Hague et al., 1993). Accumulation of the mutant p53 protein may relate to the suppression of apoptosis in the last stage of carcinogenesis, but this may not be the only factor for the regulation of apoptosis.

Acknowledgments. The authors thank F. Muramatsu, and H. Takeiri for excellent technical assistance. We also thank all the members of the Department of Surgical Pathology for their kind collaboration in this study.

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Accepted January 5, 1998