ORIGINAL ARTICLE

Bcl-2 expression and frequency of apoptosis correlate with morphogenesis of colorectal neoplasia

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Aim: To investigate whether the expression of apoptosis and cell proliferation related proteins is related to the macroscopic form of colorectal neoplasia.

Methods: The extent of apoptosis, using the 3' end DNA labelling method, and the immunohistochemical expression of cell proliferation (Ki-67) and apoptosis related proteins (Bcl-2, Bak, and p53) were investigated in 64 colorectal adenomas and 22 early carcinomas extending no further than the upper submucosal region. The specimens were classified into three types of macroscopic form (polypoid, flat, and depressed).

Results: The Ki-67 labelling index and the Bak score did not differ significantly among each macroscopic form. In contrast, the apoptotic index and the Bcl-2 score changed significantly according to the macroscopic forms. Compared with polypoid and flat tumours, depressed tumours had a significantly lower apoptotic index (2.84, 2.28, and 1.44, respectively) and a significantly lower Bcl-2 score (3.18, 2.70, and 1.64, respectively). The proliferation/apoptosis ratio was significantly lower in polypoid tumours than in the other two macroscopic forms. The Bcl-2 score became significantly lower as the tumours flattened or took on a depressed form. Immunohistochemical p53 overexpression did not correlate with the macroscopic forms.

Conclusions: These results suggest that differences in both Bcl-2 expression and apoptosis may play an important role in the morphogenesis of colorectal neoplasia.

In the past, most colorectal carcinomas were believed to arise from pre-existing adenomas that are usually recognised as polypoid lesions.¹ However, recent reports on the presence of flat adenomas² and depressed type cancers³ have awakened clinicians to the important index of the macroscopic form of colorectal neoplasia. This index may indicate the degree of malignancy because flat and/or depressed tumours are known to have higher malignant potential than polypoid ones.²⁻⁴

The growth and development of tumours depends on the balance of cell proliferation and loss,⁵ and it is thought that the morphological features of tumours are closely related to their growth pattern.⁶ Numerous studies of colorectal tumours have analysed cell proliferation markers such as Ki-67,⁷ apoptosis,^{8,9} and apoptosis related proteins.¹⁰⁻¹² However, it is not clear why some adenomas of the large intestine grow into polypoid forms and why others remain flat or even depressed. Recently, several studies have investigated the relation between cell proliferation and the macroscopic form of gut tumours.^{6,13} Although differences in apoptotic and proliferative activities have been found in the colorectum between the various macroscopic types of larger carcinomas,¹³ there have been no reports on smaller adenomas, the precursors of carcinomas.

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The purpose of our present study was to clarify the effect of cell proliferation and loss on the macroscopic forms of incipient tumours of the large intestine.

METHODS

Patients

Eighty six colorectal tumours (64 tubular or tubulovillous adenomas and 22 carcinomas extending no further than the

upper submucosal region) were resected surgically or endoscopically from 59 patients (44 men and 15 women; age range, 40–83) at Niigata University Hospital. Histological classification was determined according to the criteria of the Japanese Research Society for Cancer of the Colon and Rectum.¹⁴ Patients with familial adenomatous polyposis, hereditary non-polyposis colorectal cancer, or inflammatory bowel disease were excluded from our study.

Assessment of macroscopic form

The extirpated specimens were stretched on to styrofoam and immersed in 10% buffered formalin. The macroscopic form was determined according to the following three types: polypoid, over 3 mm in height; flat, 3 mm in height or less; and depressed, lower than the surrounding normal mucosa. The specimens were then embedded in paraffin wax, and histological diagnoses were made on routine haematoxylin and eosin stained sections.

Immunohistochemistry

We applied the streptavidin–biotin, technique using a Histofine SAB-PO kit (Nichirei Corporation, Tokyo, Japan) for immunostaining. Serial sections heated in a microwave oven in 0.01M sodium citrate buffer (pH 5.9) for five cycles of three minutes were incubated with the anti-Ki-67 antibody (MIB-1; Zymed Laboratories, San Francisco, USA), anti-Bcl-2 antibody (clone 124; Dako, Copenhagen, Denmark), anti-Bak antibody (TC102; Oncogene Research Products, Cambridge, USA), or anti-p53 antibody (PAb1801; Oncogene Research Products). The colour was developed with 3,3'-diaminobenzidine (DAB) solution. The sections were counterstained with haematoxylin and mounted. Negative controls were established by replacing

Abbreviations: AI, apoptotic index; DAB, 3,3'-diaminobenzidine; KI, Ki-67 index; TUNEL, 3' end DNA labelling

Table 1 Relation between the macroscopic form and clinicopathological parameters of the 86 colorectal tumours investigated

	Macroscopic form		
	Polypoid	Flat	Depressed
No. of patients (men/women)	28 (21/7)	47 (36/11)	11 (9/2)
Tumour size (mm)*	9.4 (5.5)	5.0 (2.3)	5.4 (3.2)
Site of tumour			
Caecum – transverse	10	35	5
Descending – rectum	18	12	6
Histological diagnosis			
Adenoma	17	43	4
Intramucosal carcinoma	10	4	3
Submucosal carcinoma	1	0	4

*Mean (SD).

Adenomas include tubular or tubulovillous adenomas with mild to moderate atypia; intramucosal carcinomas include both adenoma with severe atypia and carcinoma in situ.

the primary antibodies with phosphate buffered saline. As internal positive controls, the proliferative zone cells of normal crypts were used for Ki-67, lymphocytes within the lamina propria for Bcl-2, and epithelial cells of normal crypts for Bak. For p53, a colon cancer sample with a high degree of nuclear immunoreactivity for this oncoprotein was used as a positive control.

Detection of apoptosis

DNA fragmentation associated with apoptosis was identified by means of the 3' end DNA labelling (TUNEL) method using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, USA), according to the method described by Gavrieli *et al.*¹⁵ The steps were performed according to the manufacturer's instructions, with minor modifications. The DAB reaction, counterstaining, and setting up of negative controls were performed using the same procedures as those used for immunohistochemistry. Positive controls were obtained by DNase treatment of the sections.

Evaluation

Cytoplasmic staining was considered positive for Bcl-2 and Bak. A semiquantitative evaluation was performed, the reproducibility of which was confirmed by two of the authors. Because various degrees of staining were found, the proportion of positive to whole tumour cells on each section was scored as follows: 0, < 1%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, > 75%. For p53, only nuclear staining was considered positive; it was evaluated by a slightly modified method (previously described by Kobayashi and colleagues⁸) as follows: –, negative; 1+, sporadic positive; and 2+, diffuse positive.

Cells showing nuclear staining were considered positive for Ki-67, and those with homogeneous or granular nuclear staining, in addition to nuclear fragments located inside the epithelial cells, were defined as apoptosis positive. Positive cells to a total of at least 1000 tumour cells were counted in several fields of specimens in which several longitudinally sectioned crypts could be evaluated. The apoptotic index (AI) and Ki-67 index (KI) were defined as the percentage of apoptosis and Ki-67 positive cells, respectively.

Statistical analysis

The unpaired *t* test was used to compare tumour sizes in each group. The AIs, KIs, and the Bcl-2 and Bak scores in groups classified for macroscopic form were compared using the Mann-Whitney U test. The χ^2 test was used to compare the results of p53 staining. *p* Values < 0.05 were regarded as significant.

RESULTS

Table 1 details the clinicopathological features of each macroscopic form of the tumours investigated. The mean size of

polypoid tumours was significantly greater than that of flat and depressed ones ($p < 0.01$ and $p < 0.05$, respectively). The ratio of carcinoma in depressed tumours was higher than that in polypoid and flat ones.

Ki-67

Increased numbers of Ki-67 positive cells were seen in neoplastic areas, mainly in the upper part of the crypts (superficial pattern, fig 1B), or throughout the crypts (diffuse pattern, fig 2B). The distribution of Ki-67 positive cells differed slightly among the three macroscopic forms. The superficial pattern was shown in 60.7%, 87.2%, and 72.7% of polypoid, flat, and depressed tumours, respectively. Tumours with a diffuse pattern of Ki-67 positivity were significantly larger ($p < 0.01$) than those with a superficial pattern (data not shown).

Apoptotic cells

Apoptotic cells were found throughout the neoplastic crypts and were also found sporadically in the tumour epithelium (fig 1C). The AIs tended to decrease with the progression of the tumours (2.48, 2.06, and 1.76 in adenoma, intramucosal carcinoma, and submucosal carcinoma, respectively), but the difference was not significant.

Bcl-2 and Bak

The proportion of Bcl-2 positive cells varied in each tumour. The following scores were obtained among the 86 tumours: a score of 0 was found in four cases; a score of 1 in 15 cases; a score of 2 in 17 cases; a score of 3 in 15 cases; and a score of 4 in 35 cases (figs 1D and 2C). The positive cells were mainly located in the basal side of the tumour in cases with low scores. The percentage of Bak positive cells was high in most of the tumours (fig 1E), having a score of 4 in 63 of the 86 tumours.

p53

Table 2 shows the p53 immunoreactivity in each macroscopic form. The overexpression of p53 did not correlate with any of the macroscopic forms.

Relation between macroscopic form and apoptosis

The KIs and the Bak scores for each macroscopic form were similar (table 3 and fig 3). In contrast, the mean AI of depressed tumours was significantly lower than that of polypoid ($p < 0.01$) and flat ($p < 0.05$) type tumours. The ratio of KI to AI (KI/AI) in polypoid tumours was significantly lower than that in the other types of tumour ($p < 0.01$). The Bcl-2 score became significantly lower as the tumours became flatter or took on a depressed form.

Figure 1 (A) Histology of a polypoid intramucosal carcinoma in adenoma. (B) The increased Ki-67 positive cells are seen mainly in the upper region of the crypts. (C) The apoptotic cells (arrow) occur in the neoplastic epithelium. Diffuse staining for (D) Bcl-2 and (E) Bak is seen in both the epithelial cells and the intramucosal lymphocytes.

DISCUSSION

In our present study, we focused on the possible role of cell proliferation and loss in early colorectal tumorigenesis, particularly in terms of macroscopic form. There are several proteins in the Bcl-2 family, and their expression differs according to the histological type of the tumour.¹⁶ Among them, antiapoptotic Bcl-2 and proapoptotic Bak are major proteins that have been reported to show characteristic expression patterns in colorectal neoplasia.^{10-12 17} To clarify how they are involved in the morphogenesis of colorectal adenomas and early carcinomas, we investigated the immuno-

histochemical expression of these two proteins, together with Ki-67 and apoptosis.

"Antiapoptotic Bcl-2 and proapoptotic Bak are major proteins that have been reported to show characteristic expression patterns in colorectal neoplasia"

We found that both the AI and the Bcl-2 score were significantly higher in polypoid tumours than in the other two macroscopic forms. Although a direct inverse relation between

Figure 2 (A) Histology of a depressed submucosal carcinoma (the asterisks indicate the edge of the tumour). (B) Ki-67 positive cells are seen throughout the crypts. (C) Bcl-2 staining is absent in the neoplastic cells, whereas it is positive in the intramucosal lymphocytes.

Table 2 p53 staining for each macroscopic form of the 86 colorectal tumours investigated				
p53 staining	Macroscopic form			
	Polypoid	Flat	Depressed	
– (N=74)	21 (6)	44 (3)	9 (5)	
1+ (N=10)	6 (4)	3 (1)	1 (1)	
2+ (N=2)	1 (1)	0	1 (1)	

Values in parentheses are the numbers of cancers.

Bcl-2 expression and apoptosis (which would be expected in view of the fact that the Bcl-2 protein can block apoptosis)¹⁸ has been found in some organs/tissues,^{19–20} this may not hold true during the morphogenesis of colorectal tumours, as has been reported in the lung²¹ and ovary.²² The results of our present study showing that the KI/AI ratio is lowest in polypoid tumours suggests that polypoid adenomas and carcinomas do not grow simply because of increased proliferation and reduced apoptosis. As hypothesised for gastric neoplasia,⁶ the diffuse expression of Bcl-2 and a superficial distribution of proliferating cells may contribute to the formation of a

polypoid configuration in colorectal tumours. This is supported by our results of a higher Bcl-2 score in polypoid tumours, 60.7% of which showed a superficial distribution of Ki-67 positive cells, than in the other types of tumour. Although flat and/or depressed tumours of the large intestine have become apparent in recent years, the mechanism by which the non-polypoid configuration is formed is unknown. Unlike previous reports,^{9–13} the proliferative index did not vary significantly with the different macroscopic forms or histopathological scores in our study. This may be because the tumours investigated in our study were smaller and earlier than those in previous reports. Consequently, our results might suggest that, in the extremely early phase of colorectal tumorigenesis, the relative increase of the KI/AI ratio following a significant reduction of AI contributes to the growth of flat and, especially, depressed tumours with high malignant potential, perhaps through some unknown mechanisms unrelated to Bcl-2,⁹ or through the accumulation of genetic changes. We used the TUNEL technique in our present study to identify apoptotic cells. One known shortcoming of this method is that any type of DNA fragmentation, including that of necrotic cells, might be detected.²³ However, because extensive necrosis caused by severe inflammation or ischaemia rarely occurs in

Table 3 KIs and AIs for each macroscopic form of the 86 colorectal tumours investigated				
Macroscopic form	KI	AI	KI / AI	
Polypoid (n=28)	22.7 (7.45)	2.84 (1.53)	9.56 (4.76)	**
Flat (n=47)	25.6 (8.24)	2.28 (1.23)	15.6 (1.61)	
Depressed (n=11)	24.5 (8.09)	1.44 (0.63)	20.8 (11.3)	

Each pair of values is a mean (SD).
*p<0.05; **p<0.01.
KI, Ki-67 index; AI, apoptotic index.

Figure 3 The Bcl-2 and Bak scores for each macroscopic form of the investigated tumours. * $p < 0.05$, ** $p < 0.01$. The horizontal bars indicate the median value for each group of samples.

Take home messages

- The Ki-67 labelling index and the Bak score did not differ significantly among each macroscopic form
- Depressed tumours had a significantly lower apoptotic index than polypoid and flat tumours, (1.44, 2.84, and 2.28, respectively) and a significantly lower Bcl-2 score (1.64, 3.18, and 2.70, respectively)
- The proliferation/apoptosis ratio was significantly lower in polypoid tumours compared with the other two macroscopic forms
- The Bcl-2 score became significantly lower as the tumours flattened or took on a depressed form
- Immunohistochemical p53 overexpression did not correlate with the macroscopic forms
- Thus, differences in both Bcl-2 expression and apoptosis may play an important role in the morphogenesis of colorectal neoplasia

the background mucosa without inflammatory bowel disease, we think that there were very few necrotic cells in the very small tumours investigated in our study. Furthermore, to exclude contaminating necrotic cells as much as possible, the positive cells that were sloughed off from the epithelial tubules were not evaluated.⁹

The wild-type p53 protein prevents cells that have DNA damage from entering the cell cycle, which results in apoptosis in some cells,²⁴ and the p53 gene often undergoes mutation in the transition from large adenoma to carcinoma of the colorectum.²⁵ Our previous report indicated that sporadically expressed p53 protein is probably wild type and induces apoptosis in response to active cell proliferation.⁸ Because most (97.7%) of the tumours investigated in our present study showed no or sporadic expression of p53, the activation of apoptosis and those proteins involved in the apoptotic pathway in early colorectal tumorigenesis may be mediated directly by wild-type p53. However, more detailed molecular analysis would be necessary to determine the involvement of p53.²⁶

In conclusion, to investigate the possibility that cell proliferation and death are related to the early morphogenesis of colorectal neoplasia, we demonstrated that the frequency of

apoptosis and Bcl-2 expression differed significantly according to the macroscopic form. Further investigations may be required once more detailed mechanisms of apoptosis are elucidated.

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ECHO

Keratan sulphate MAB 5-D-4 and β ig-h3 protein accumulation in corneas

Please visit the *Journal of Clinical Pathology* website [www.jclinpath.com] for link to this full article.

Build up of keratan sulphate and β ig-h3 protein in the cornea causes opacity in Maroteaux-Lamy syndrome (MLS) type B, as shown for the first time by researchers in Cardiff, UK. MLS is one of the mucopolysaccharide diseases. A deficiency in a lysosomal enzyme causes build up of glycoaminoglycans (mucopolysaccharide) within the cornea, causing it to become opaque. In MLS type B the deficient enzyme is aryl sulphatase B. Recently, mutations in the β ig-h3 gene, which codes for β ig-h3 protein of the extracellular matrix, have been shown in four autosomal dominant corneal dystrophies. Ahktar *et al* looked at how proteoglycans, keratan—a byproduct of incomplete breakdown of proteoglycan of the cell envelope and extracellular matrix—and β ig-h3 were distributed in diseased corneal tissue from a 16 year old girl with MSL VI type B undergoing a corneal transplant and a normal cornea obtained from necropsy.

In MLS epithelial cells, intercellular spaces, and cell junctions contained proteoglycans and the stroma was heavily loaded. Keratocytes were vacuolated and their lysosomes packed with proteoglycan filaments. β ig-h3 protein was localised around electron lucent spaces in the stroma, and keratan sulphate densely located in the posterior stroma and in keratocytes, their vacuoles, and lysosomes. In the normal cornea β ig-h3 protein appeared evenly throughout, save in keratocytes and endothelial cells, and keratan sulphate uniformly within the stroma.

Aryl sulphatase deficiency leads to build up of keratan sulphate within cells, causing keratocytes to degenerate and abnormal deposits of proteoglycan to form. This, the authors conclude, increases light scattering and the opacity of the cornea.

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Immunolocalisation of β ig-h3 and keratan sulphate 5-D-4 in MLS type B cornea showing (A): intense label around lucent spaces (L) in the stroma (S) and (B): strong labelling of keratan sulphate MAB 5-D-4 in the lysosome (LY) of an empty keratocyte.