

Bcl-2 protein expression and gut neurohormonal polypeptide/amine production in colorectal carcinomas and tumor-neighboring mucosa, which closely correlate to the occurrence of tumor

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Summary. To clarify whether advanced colorectal carcinomas and tumor-neighboring mucosa simultaneously produce both Bcl-2 protein and gut neurohormonal polypeptides and/or amines, and the interrelationship of these phenomenon, we studied retrospective analysis of Bcl-2 protein production and neuroendocrine characteristics in 52 cases of advanced colorectal carcinoma and surrounding mucosa. All of the tumor-neighboring mucosa presented hyperplasia. The rates of enhanced immunoreactivity of the tumor-neighboring mucosa and of positive immunoreactivity of the carcinomas against human Bcl-2 protein and against human vasoactive intestinal polypeptide, pancreatic polypeptide and somatostatin were 78.8% and 94.2%, 82.7% and 59.6%, 78.8% and 67.3%, and 88.5% and 84.6% respectively. Double immunostaining for Bcl-2 protein and each peptide hormone revealed simultaneous expression. In contrast, that of tumor-neighboring mucosa and carcinomas to serotonin and chromogranin-A and to argyrophilia were 11.5% and 1.9%, 32.7% and 17.3%, and 26.9% and 21.2%, respectively. We concluded that tumor-neighboring crypt cells displayed not only hyperplasia but also neuroendocrine characteristics and that enhanced Bcl-2 protein immunoreactivity correlated with tumor occurrence in the wall of the colorectum. The production of Bcl-2 protein by tumor cells and tumor-neighboring crypt cells indicates that the *bcl-2* proto-oncogene may act not only as an inhibitor of apoptosis but also as an inducer of neuroendocrine differentiation from the latent characteristics of the endodermal stem cell.

Key words: Bcl-2 protein, Gut hormone, Neurohormonal polypeptide, Neurohormonal amine, Colorectal carcinoma, Colorectal epithelium, Immunohistochemistry

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Introduction

Colorectal epithelium composed of columnar, mucous, and endocrine crypt cells originates from multipotential endodermal stem cells at the base of the intestinal crypts (Chang et al., 1971) and the endocrine cancer cells found in gastrointestinal carcinomas develop by amphocrine differentiation during carcinogenesis in the same tumor (Cox and Pierce, 1982; Kirkland, 1988). A recent study by us demonstrated that colorectal carcinomas exhibit not only multiendocrine characteristics producing various types of neurohormonal polypeptide, but also amphocrine characteristics of different grades and, interestingly, most tumor-neighboring crypt cells possess those characteristics also (Ohmori et al., 1996).

Most recently, Wang and Reed (1998) reviewed the function of Bcl-2 protein and its mechanisms together with several cellular and viral homologues with particular interest in the regulation of the programmed cell death (apoptosis) pathway in normalcy and disease, and suggested that dysregulation of the process probably plays a major role in carcinogenesis, autoimmune disease and AIDS. Furthermore, there have been a number of studies suggesting that Bcl-2 protein expression has a close correlation with neuroendocrine differentiation in the development of enterochromaffin-like cell gastric carcinoids (Azzoni et al., 1996), small cell lung carcinoma (Jing et al., 1996), and neuroblastoma (Krajewski et al., 1995) and its cell line (Hanada et al., 1993), while others suggest that high levels of Bcl-2 protein can render cells remarkably resistant to killing by a wide variety of cytotoxic drugs (Fannidi et al., 1992; Miyashita and Reed, 1992; Hanada et al., 1993). The *bcl-2* proto-oncogene (*bcl-2* gene) was first detected in follicular B cell lymphomas accompanied by the over-expression of its 26 KD protein (Bcl-2 protein) (Tsujimoto and Croce, 1986), and the *bcl-2* gene initially plays an unambiguous role in prolonging cell survival by blocking programmed cell

death (apoptosis) without affecting cell proliferation (Korsmeyer, 1992). The presence of its protein at high levels prevents cell apoptosis (Sentman et al., 1991). In addition to merely protecting cells from apoptosis, it may also play a role in terminal cell differentiation, possibly in association with neurohormonal cell differentiation, as has been reported not only in neuroectodermal tissue, such as the developing nervous system and adult peripheral nervous system (Merry et al., 1994), and neural-crest-derived tumors in the lung (Jing et al., 1996), but also in endodermal tissue-derived tumors such as gastric carcinoids (Azzoni et al., 1996). Recently, Chen et al. (1997) announced an exciting discovery concerning regeneration in mammalian CNS: the *bcl-2* gene plays a key role in developmental change by promoting the growth and regeneration of severed retinal axons.

In this study we tried to discover whether the expression/production of the Bcl-2 protein by colorectal carcinoma cells and crypt cells in the surrounding mucosa closely correlates to the expression of pancreatic and gut neurohormonal polypeptides (NHPs) such as vasoactive intestinal polypeptide (VIP), pancreatic polypeptide (PP) and somatostatin (SOM), and neurohormonal amines (NHAs) such as Grimelius stain-positive (argyrophilic) neurosecretory granules, serotonin (SER) and chromogranin-A (CG-A).

Materials and methods

Cases

Fifty-two cases of advanced colorectal carcinoma surgically resected from 1983 to 1993 were selected from the files of the Pathology Division of the Central Laboratory of Ehime University Hospital and the Second Department of Pathology of the Ehime University School of Medicine: 21 colonic and 31 rectal, from 26 women and 26 men, aged 36 to 86 years, with an average age of 65.0 years.

Tissue specimens

Three representative blocks of surgically resected colorectal cancer tissue specimen - a carcinoma tissue specimen that included the immediately adjacent (within 3 cm) surrounding mucosa (tumor-neighboring mucosa), a specimen from the proximal side and one from the distal side, with one or both being more than 20 cm from the tumor site (tumor-distant mucosa) - were routinely fixed in 10% buffered formalin solution and embedded in paraffin. Each paraffin block was sliced, the deparaffinized sections were stained with hematoxylin and eosin, and a histopathological diagnosis of the carcinoma was made in accordance with the general rules for clinical and pathological studies on carcinoma of the colon, rectum, and anus of the Japanese Research Society for Cancer of the Colon and Rectum (Kanehara Publisher Ltd., 1994). The tumor-neighboring mucosa

was examined for the presence of elongation of the crypts (hyperplasia) adjacent to the tumor, which corresponds to what some researchers have termed the transitional mucosa immediately adjacent to the tumor (Filipe and Branfoot, 1974; Mori et al., 1995). Other deparaffinized sections were prepared with Grimelius' stain to determine the presence of argyrophil neurosecretory granules in neuroendocrine cells; i.e., argyrophilic cells.

Immunohistochemistry

Immunoreactivity was examined by the ENVISION-labeled polymer reagent method for monoclonal mouse antibody against human Bcl-2 protein (Dako A/S, Glostrup, Denmark), and by the labeled streptavidin-biotin complex method (LsAB) for polyclonal rabbit antibodies against human VIP (antigen source: keyhole limpet hemocyanin-conjugated composite VIP; Nichirei, 6-19-20, Tsukiji, Tokyo, Japan), PP, and SOM (Dako, Carpinteria, Calif, USA) as NHPs, and for monoclonal mouse antibody against human SER and polyclonal rabbit antibody against human CG-A (Dako, Carpinteria, Calif, USA) as NHAs, to determine the immunoreactivities for Bcl-2 protein, NHPs, and NHAs of the colorectal carcinoma cells as well as the mucosal crypt cells. Immunohistochemical staining for NHPs and NHAs by the labeled streptavidin biotin complex method (LsAB) was performed. As the positive control, immunoreactive staining for VIP, PP, SOM, SER, and CG-A was performed on tissue specimens of the human colon or rectum and pancreas containing immunoreactive crypt cells, mural ganglion cells (for VIP, SOM, SER and CG-A), and nerve fibers (for VIP). Immunohistochemical staining for Bcl-2 protein was performed as follows. Deparaffinized sections were treated with phosphate-buffered saline containing 3% hydrogen peroxide for 5 minutes at room temperature to block endogenous peroxidase. Next, they were incubated with a primary antibody prediluted against human Bcl-2 protein for 40 minutes at room temperature and then incubated with an ENVISION-labeled polymer reagent solution for 40 minutes at room temperature. Between each incubation, the sections were rinsed 5 times for 5 minutes each in 0.05M TRIS-buffered saline (pH 7.6) containing 0.1% Tween 20. Immunoreactive sites were visualized with hydrogen peroxide solution of 3-amino-9-ethyl carbazole (AEC) (reddish brown in color) for Bcl-2 protein or 3,3'-diaminobenzidine tetrahydrochloride (DAB) (brown in color) for VIP or new fuchsin (red in color) hydrogen peroxide solution for PP, SOM, SER and CG-A. Finally, the sections were counterstained with Mayer's hematoxylin solution for 10 seconds. As the negative control, normal mouse serum was substituted for the primary antiserum. As the positive control, immunoreactive staining for Bcl-2 protein of the lymphocytes infiltrating in the mucosa propria or outer layer of the mantle of lymph follicles in the mucosa was performed for each tumor-neighboring

or tumor-distant mucosa specimen.

Double staining for Bcl-2 protein and NHPs

Samples from different types of tumor and tumor-neighboring mucosa were subjected to double immunostaining by the same method described above, first for Bcl-2 protein and then for NHP, to examine and identify the individual Bcl-2 protein immunoreactive cells and NHP-immunoreactive cells. Immunoreactive sites for Bcl-2 protein were visualized with DAB (brown in color) and for NHP with new fuchsin (red in color) or AEC (reddish brown in color) hydrogen peroxide solution.

Presence and incidence of neuroendocrine cells immunoreactive for Bcl-2 protein, NHPs, or NHAs and argyrophilic cells in the tumor-neighboring mucosa and tumor

The tumor-neighboring and tumor-distant mucosa in each case were compared for the presence and incidence of Bcl-2 protein- or NHP- or NHA-immunoreactive crypt cells and argyrophilic cells. Tumor-neighboring mucosa in which presence and incidence were similar to tumor-distant mucosa was diagnosed as neuroendocrine-stationary mucosa (grade 1), and that with increased presence and incidence was diagnosed as neuroendocrine-enhanced mucosa (grade 2, positive cells less than 50% of total crypt cells; grade 3, positive cells more than 50% of total crypt cells).

Each tumor specimen was examined for the presence and incidence of both Bcl-2 protein- or NHP- or NHA-immunoreactive tumor cells and argyrophilic tumor

cells, according to the following definitions: grade 0, no immunoreactive/argyrophilic tumor cells; grade 1, few (less than 5% of all tumor cells, sporadic to focal positive cells); grade 2, few to many (5% to less than 50%); grade 3, many to numerous (50% to less than 95%); grade 4, almost all (95% or more) immunoreactive/argyrophilic tumor cells.

Results

The positive reactions to Bcl-2 protein immunostained fine granular in the cytoplasm or occasionally on the nuclear membrane and/or in the nucleus, and those to

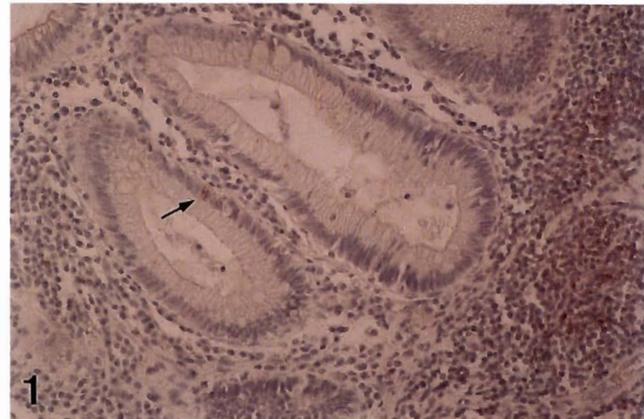


Fig 1. Normal (tumor-distant) colonic mucosa immunostained with ENVISION-labeled polymer reagent method for Bcl-2 (grade 1). A crypt cell (arrow) and many lymphocytes in the propria mucosa stained reddish brown by 3-amino-9-ethyl carbazole (AEC). x 66

Table 1. Immunoreactivity of tumor-neighboring mucosa to Bcl-2 Protein and neurohormonal polypeptides

EXTENT OF IMMUNOREACTIVITY*	BCL-2 PROTEIN	NEUROHORMONAL POLYPEPTIDE			
		VIP	PP	SOM	AVERAGE
Stationary: Grade 1	11(21.2%)	9(17.3%)	11(21.2%)	6(11.5%)	8.7(16.7%)
Enhanced: Grade 2	28	16	20	19	
Grade 3	13	27	21	27	
Grades 2 and 3	41(78.8%)	43(82.7%)	41(78.8%)	46(88.5%)	43.3(83.3%)

VIP: vasoactive intestinal polypeptide; PP: pancreatic polypeptide; SOM: somatostatin. *: Neuroendocrine-stationary mucosa, incidence of immunoreactive crypt cells in the tumor-neighboring mucosa similar to the tumor-distant mucosa (grade 1); neuroendocrine-enhanced mucosa, incidence of immunoreactive crypt cells in the tumor- neighboring mucosa less than 50% (grade 2) and more than 50% of crypt cells (grade 3).

Table 2. Immuno-/historeactivity of tumor-neighboring mucosa to neurohormonal amines and Grimelius' stain

EXTENT OF IMMUNO/HISTOREACTIVITY*	NEUROHORMONAL AMINE			GRIMELIUS' STAIN
	SER	CG-A	AVERAGE	
Stationary: Grade 1	46 (88.5%)	35 (67.3%)	40.5 (77.9%)	38 (73.1%)
Enhanced: Grade 2	6 (11.5%)	17 (32.7%)	11.5 (22.1%)	14 (26.9%)
Grade 3	0	0	0	0

SER: serotonin; CG-A: chromogranin-A. *: Neuroendocrine-stationary mucosa, incidence of immunoreactive/argyrophilic crypt cells in the tumor-neighboring mucosa similar to the tumor-distant mucosa (grade 1); neuroendocrine-enhanced mucosa, immunoreactive/argyrophilic crypt cells in the tumor-neighboring mucosa less than 50% (grade 2) and more than 50% of crypt cells (grade 3).

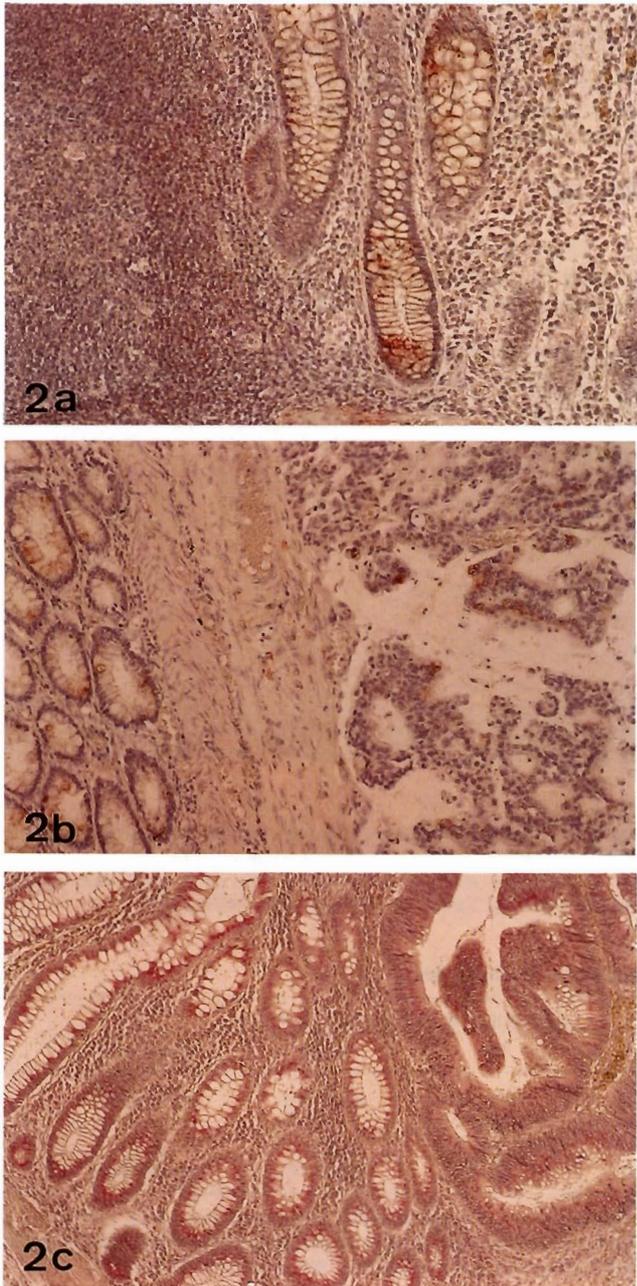


Fig. 2a-c. Bcl-2 protein immunostain of neuroendocrine-enhanced tumor-neighbor mucosa (**a**; grade 3) and immunoreactive tumor (**b**; grade 2). More (**a**) or less (**b**) than 50% of immunoreactive crypt cells reveal immunoreactivity for Bcl-2 protein stained reddish brown by AEC. A few to many (5% to less than 50%) tumor cells (grade 2) reveal Bcl-2 protein immunoreactivity (**b**). **c.** Somatostatin (SOM) immunostain of immunoreactive tumor and neuroendocrine-enhanced tumor neighboring mucosa reveal almost all (>95%) immunoreactive tumor cells (grade 4) and more than 50% of immunoreactive crypt cells (grade 3) stained red by new fuchsin. ENVISION-labeled polymer reagent method for Bcl-2 protein and labeled streptavidin-biotin complex method for SOM. a, x 50; b, x 40; c, x 66

NHP and argyrophilic neurosecretory granules were coarse to fine in the cytoplasm, possibly at the site of the Golgi area of the immunoreactive cells.

Table 1 shows the extent and incidence of immunoreactivity of stationary and enhanced tumor-neighbor mucosa to Bcl-2 protein, VIP, PP, and SOM. From among the 52 samples of tumor-neighbor mucosa, we judged the number (and incidence) of stationary mucosa exhibiting a positive reaction to be 11 out of 52 cases (21.2%) for Bcl-2 protein (Fig. 1), 9/52 (17.3%) for VIP, 11/52 (21.2%) for PP, and 6/52 (11.5%) for SOM; for the NHPs, a total of 26 cases and an average of 8.7 cases (16.7%). The figures for enhanced mucosa were 41/52 (78.8%) for Bcl-2 protein (Fig. 2a,b), 43/52 (82.7%) for VIP, 41/52 (78.8%) for PP, and 46/52 (88.5%) for SOM (Fig. 2c); for the NHPs, a total of 130 cases and an average of 43.3 cases (83.3%). Table 2 shows the extent and incidence of immunoreactivity and historeactivity of stationary and enhanced mucosa to SER and CG-A for the NHAs, and Grimelius' stain for argyrophilic

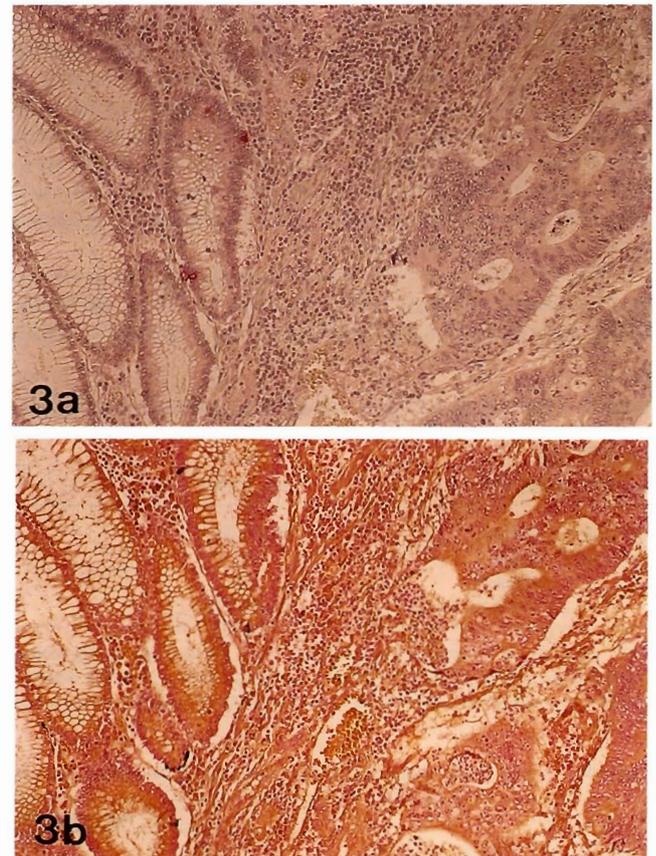


Fig. 3. Serial sections of adenocarcinoma (right) and neighboring mucosa (left). Tumor cells reveal no immunoreactivity for chromogranin-A (CG-A) (**a**; grade 0) and no argyrophilia (**b**; grade 0), and neighboring mucosa reveals a few CG-A immunoreactive crypt cells (**a**) stained red by new fuchsin and argyrophilic crypt cells (**b**) reacted black by argyrophilia (neuroendocrine-stationary, grade 1). Labeled streptavidin-biotin complex method for CG-A (**a**) and Grimelius' stain (**b**). x 40

neurosecretory granules in the crypt cells. The figures for stationary mucosa were 46 out of 52 cases (88.5%) for SER and 35/52 (67.3%) for CG-A (Fig. 3a); for the NHAs, a total of 81 cases and an average of 40.5 cases (77.9%), and 38/52 (73.1%) for argyrophilia (Fig. 3b). The figures for enhanced mucosa were 6/52 (11.5%) for SER and 17/52 (32.7%) for CG-A (Fig. 4); for the NHAs, a total of 23 cases and an average of 11.5 cases (22.1%), and 14/52 (26.9%) for argyrophilia. The statistical differences by the χ^2 test between the results for NHP and NHA and between those for NHPs and argyrophilia were significant at $p < .005$ ($\chi^2 = 39.0$ and 33.4, respectively).

Table 3 shows the extent (number) and incidence of immunoreactivity of the tumors to Bcl-2 protein and VIP, PP, and SOM. The number of immunoreactive tumors was 49/52 (94.2%) for Bcl-2 protein (Figs. 2b, 5a,b) and 31/52 (59.6%), 35/52 (67.3%), and 44/52 (84.6%) (Fig. 5c) for the NHPs (total 110 cases, average 36.7 cases). The number of non-immunoreactive tumors was 3 out of 52 cases (5.8%) for Bcl-2 protein, 21/52 (40.4%), 17/52 (32.7%), and 8/52 (15.4%) for the NHPs (total 46 cases, average 15.3 cases). Table 4 shows the number and incidence of NHA-immunoreactive tumors and argyrophilic tumors. The number of immunoreactive tumors for SER and CG-A was 1/52 (1.9%) and 9/52 (17.3%) (total 10 cases, average 5 cases) (Fig. 6a) and 11/52 (21.2%) for argyrophilia (Fig. 6b). The number of non-immunoreactive tumors for SER and CG-A was 51/52 (98.1%) and 43/52 (82.7%) (total 94 cases, average 47 cases) and 41/52 (78.8%) for argyrophilia. The statistical differences by the χ^2 test between the results for NHP and NHA and between those for NHP and argyrophilia were significant at $p < .005$ ($\chi^2 = 40.2$ and 25.6, respectively).

Double immunostaining for Bcl-2 protein and each

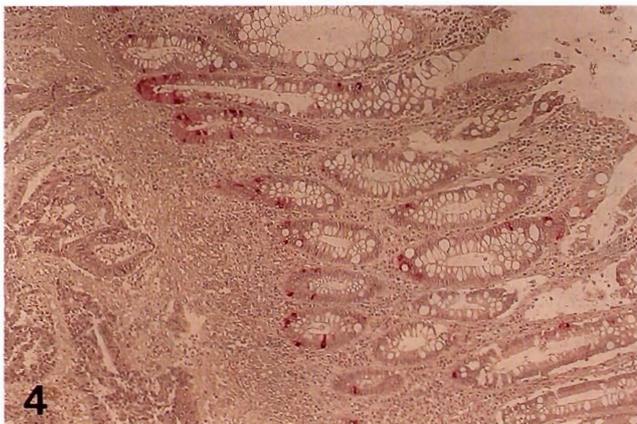


Fig. 4. Neuroendocrine-enhanced tumor-neighbor mucosa (right two-thirds) and non-immunoreactive adenocarcinoma (left one-third). Tumor-neighbor mucosa exhibits many chromogranin-A immunoreactive crypt cells (enhanced but less than 50%, grade 2) stained red by new fuchsin but tumor cells reveal no immunoreactivity (grade 0). Labeled streptavidin-biotin complex method. x 20

NHP revealed the simultaneous presence of Bcl-2 protein (brown by DAB) and each NHP (red by new fuchsin) in both carcinoma cells and epithelial cells (Fig. 7) in most cases.

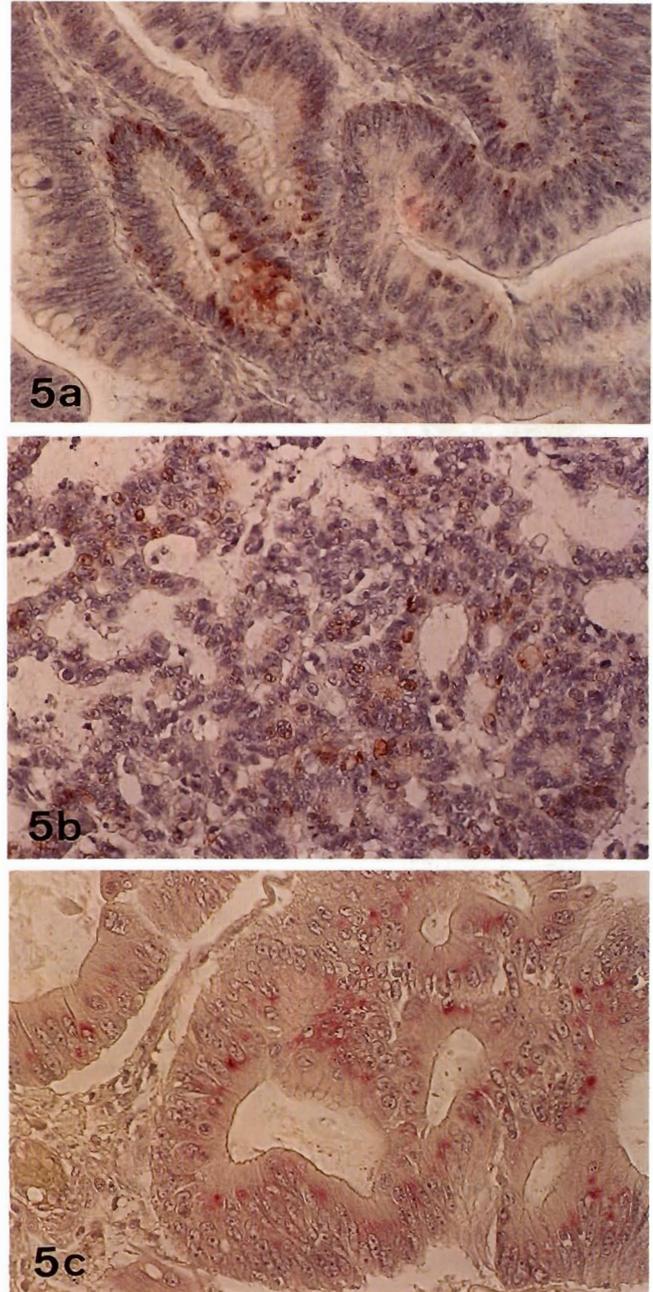


Fig. 5. Bcl-2 protein immunostain of immunoreactive well-differentiated (a; grade 3) or moderately differentiated (b; grade 2) adenocarcinoma stained reddish brown by AEC, and somatostatin (SOM) immunostain of serial section of well-differentiated adenocarcinoma (c; grade 4) stained red by new fuchsin. ENVISION-labeled polymer reagent method for Bcl-2 protein, and labeled streptavidin-biotin complex method for SOM. a and c, x 100; b, x 80

Discussion

It has been reported that the colorectal tumor-neighborhood mucosa, i.e., the transitional mucosa immediately adjacent to a colorectal carcinoma/adenoma, commonly exhibits hyperplasia (Filipe and Branfoot, 1974; Mori et al., 1990). However, the

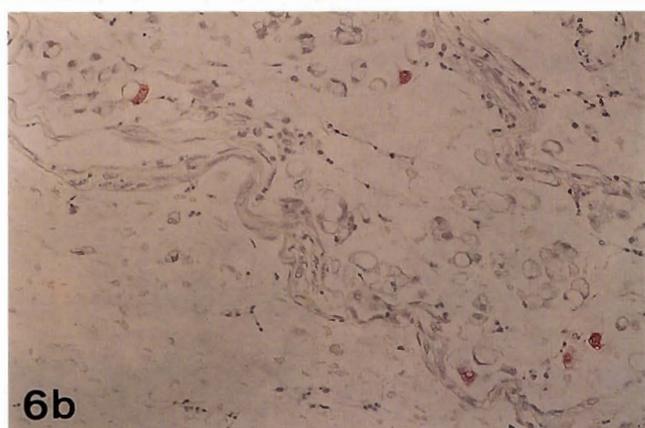
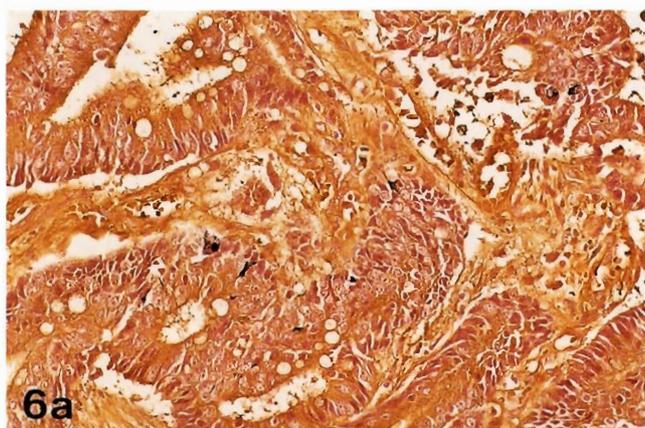


Fig. 6a, b. Argyrophilic tumor cells of well-differentiated adenocarcinoma reacted black by argyrophilia (a; grade 1) and chromogranin-A (CG-A) immunoreactive signet ring cells of mucous carcinoma stained red by new fuchsin (b; grade 1). Grimelius' stain (a) and labeled streptavidin-biotin complex method (b). x 66

etiology of this phenomenon is still unknown. In the present study, we observed not only hyperplasia but also neuroendocrine characteristics, such as a very high

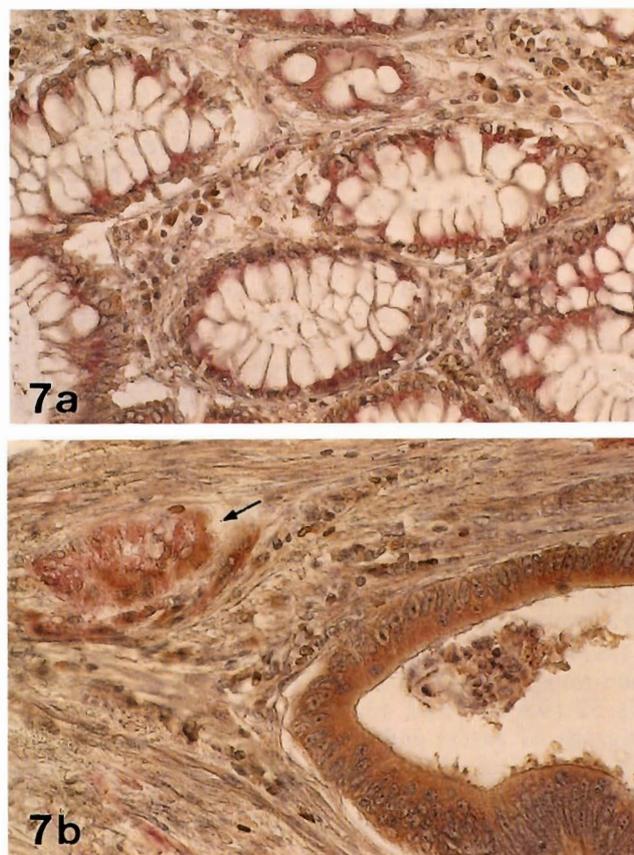


Fig. 7. Double immunostain for Bcl-2 protein and vasoactive intestinal polypeptide (VIP). Almost all crypt cells of tumor-neighborhood mucosa (a; grade 3) and well-differentiated adenocarcinoma cells (b; grade 4) show simultaneous presence of intense immunoreactivity for Bcl-2 protein stained brown by DAB (3,3'-diaminobenzidine tetrachloride) and for VIP stained red by new fuchsin. B cells in the mucosa propria show a positive stain only for Bcl-2 protein but not for VIP (a). Ganglion cells and nerve fibers also show positive double stain (b; arrow). ENVISION-labeled polymer reagent method for Bcl-2 protein and labeled streptavidin-biotin complex method for VIP. a, x 80; b, x 100

Table 3. Immunoreactivity of colorectal tumors to Bcl-2 protein and neurohormonal polypeptides

EXTENT OF BCL-2 IMMUNOREACTIVITY	PROTEIN	NEUROHORMONAL POLYPEPTIDE			
		VIP	PP	SOM	AVERAGE
Reactive:	49 (94.2%)	31 (59.6%)	35 (67.3%)	44 (84.6%)	36.7 (70.6%)
Grade 1	32	6	8	2	
Grade 2	15	15	12	16	
Grade 3	2	8	10	13	
Grade 4	0	2	5	13	
Non-reactive: (Grade 0)	3 (5.8%)	21 (40.4%)	17 (32.7%)	8 (15.4%)	15.3 (29.4%)

VIP: vasoactive intestinal polypeptide; PP: pancreatic polypeptide; SOM: somatostatin. *: incidence of immunoreactive tumor cells; tumors with few (<5%) immunoreactive cells (grade 1), tumor with a few many (5% to less than 50%) immunoreactive cells (grade 2), tumors with numerous (50% to less than 95%) immunoreactive cells (grade 3); and tumors with almost all (>95%) immunoreactive cells (grade 4).

Gut-hormone and Bcl-2 in colorectal carcinoma/mucosa

Table 4. Immuno/Historeactivity of colorectal tumors to neurohormonal amines and Grimelius' stain

EXTENT OF IMMUNO/HITOREACTIVITY*	NEUROHORMONAL AMINE			GRIMELIUS' STAIN
	SER	CG-A	AVERAGE	
Reactive:	1 (1.9%)	9 (17.3%)	5 (9.6%)	11 (21.2%)
Grade 1	1	9		8
Grade 2	0	0		3
Grade 3	0	0		0
Grade 4	0	0		0
Non-reactive: (Grade 0)	51 (98.1%)	43 (82.78%)	47 (90.4%)	41 (78.8%)

SER: serotonin; CG-A: chromogranin-A. *: incidence of immunoreactive tumor cells; tumors with few (<5%) immunoreactive cells (grade 1), tumor with a very many (5% to less than 50%) immunoreactive cells (grade 2), tumors with numerous (50% to less than 95%) immunoreactive cells (grade 3); and tumors with almost all (>95%) immunoreactive cells (grade 4).

incidence of enhanced immunoreactivity to Bcl-2 protein (78.8% of 52 specimens of tumor-neighbor mucosa) and to three types of NHP (82.7% for VIP, 78.8% for PP, and 88.5% for SOM) but a remarkably to relatively low incidence of immunoreactivity to two types of NHA (11.5% for SER and 32.7% for CG-A) and to argyrophilia (26.9%) in the tumor-neighbor mucosa, along with a very high incidence of positive immunoreactivity to Bcl-2 protein (94.2% of 52 cases of advanced carcinoma) and a relatively to very high incidence to NHP (59.6% for VIP, 67.3% for PP, and 84.6% for SOM) but a remarkably to relatively low incidence to NHA (1.9% for SER and 17.3% for CG-A) and to argyrophilia (21.2%) in advanced colorectal carcinomas.

The high incidence of NHP-positive immunoreactivity of colorectal carcinomas corresponds to the results of our previous study (Ohmori et al., 1996). The low incidence of immunoreactivity to NHA is lower than that described in some reports; i.e., SER immunoreactive carcinoma cells were found in 24 out of 301 colorectal carcinomas (8.0%) by Arends et al. (1986) and CG-A immunoreactive carcinoma cells were found in 38 out of 108 colorectal carcinomas (35.2%) by Mori et al (1995). The incidence of argyrophilic carcinoma cells in 19 out of 94 colorectal carcinomas (20.2%) reported by Smith and Haggitt (1984) is similar to our data.

Moreover, in the present study, the crypt cells in the tumor-neighbor mucosa displayed similar immuno/historeactivity to NHP, NHA, and Grimelius' stain as the colorectal carcinomas. To our knowledge, there have been no reports comparing the immuno/historeactivity of colorectal tumors with that of neighbor mucosa.

The most interesting finding in the present study is the very high incidence of Bcl-2 protein expression in colorectal carcinomas (94.2%) and the enhanced expression in the tumor-neighbor mucosa (78.8%), which suggests a phenotypic progression based on the amphocrine nature of the tumor-neighbor mucosa as well as the tumors (colorectal adenocarcinomas), as reported and discussed in our previous study (Ohmori et al., 1996). Furthermore, although we have confirmed in the present study that not only tumors but also tumor-neighbor crypt cells produce much more NHP than

NHA, the reason for this phenomenon in both carcinomas and neighbor crypt cells is still unclear. One possibility is that NHPs have a simpler molecular structure and more primitive beginnings as gut-neurohormonal polypeptides in coelenterates than NHAs, and may participate in the overexpression of Bcl-2 protein.

Recent studies provide some interesting data for Bcl-2 protein: Ilyas et al. (1996) studied Bcl-2 expression in sporadic colorectal carcinomas and ulcerative-colitis-associated colorectal carcinomas, and found a higher incidence of Bcl-2 overexpression in sporadic colorectal carcinomas than in ulcerative-colitis-associated carcinomas, which suggests a divergence of the carcinogenic pathways. In a study of three types of thymomas, noninvasive, invasive, and malignant, Chen et al. (1996) suggested the expression of Bcl-2 protein as a reliable marker of tumor aggressiveness. In a comparative study of the expression of Bcl-2 protein and Bax protein, which respectively repress and promote apoptosis, based on an immunohistochemical analysis and Western blot analysis of thyroid tumors, Manetto et al. (1997) suggested that an excess of Bcl-2 appears to be associated with benign and less aggressive malignant tumors, while that of Bax relates to more aggressive thyroid neoplasms. The amount of Bcl-2 protein and Bax protein appears to be balanced in malignant thyroid tumors of intermediate aggressiveness.

Therefore, further study is needed to confirm the hypothesis induced by our previous and present study: the high incidence of Bcl-2 protein expression in sporadic advanced colorectal carcinomas and tumor-neighbor mucosa not only prolongs cell survival (i.e., immortalization) by protecting them from or blocking apoptosis (Korsmeyer, 1992; Sentman et al., 1991), it also promotes the neuroendocrine phenotype in both carcinoma cells and tumor-neighbor crypt cells, which may well correspond to the aggressiveness and amphocrine nature of the tumor and its development and the amphocrine nature of the transitional epithelium (i.e., hyperplastic tumor-neighbor mucosa) (Filipe and Branfoot, 1974; Mori et al., 1990). In other words, the enhancement of tumor cell proliferation and the induction of tumor-neighbor crypt cell hyperplasia

associated with a phenotypic adaptation to an amphocrine nature as a pathway of neuroendocrine differentiation (Hanada et al., 1993; Krajewski et al., 1995; Azzoni et al., 1996; Jing et al., 1996) may be caused by aberrant *bcl-2* gene expression during the step-by-step acquisition of mutation proposed by Fearon and Vogelstein (1990) in multistep colorectal carcinogenesis and by an unknown mechanism due to kinetic and/or physiological dysfunction in the tumor-neighboring mucosa in response to the occurrence of tumor.

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