A reduced COX-2 expression and a reduced number of pericryptal myofibroblasts are associated with depressed adenoma of the colon

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Abstract. The histogenesis of depressed adenoma of the colon has not been sufficiently investigated. Pericryptal myofibroblasts are stromal cells expressing smooth muscle actin, and are involved in the differentiation and multiplication of epithelial cells in the colonic epithelium. COX-2 has been reported to be involved in the development of colon adenoma. We studied the histogenesis of depressed adenoma of the colon by examining the relationship between the presence of pericryptal myofibroblasts and COX-2 expression. Twenty-one depressed adenomas of the colon that had been resected endoscopically between June 1998 and May 2003 (mild-moderate atypia; mean diameter, 6.7 mm) and 23 elevated adenomas that had been resected endoscopically in 2003 (mild-moderate atypia; mean diameter, 11.7 mm), were studied. We performed immunohistochemical staining using α-smooth muscle actin antibody to detect pericryptal myofibroblasts. We also performed immunohistochemical staining for Cox-2. Eighteen (78.3%) of the 23 elevated adenomas and six (28.6%) of the 21 depressed adenomas were positive for pericryptal myofibroblasts immunohistochemically, showing a significant difference (P<0.001). Seventeen elevated adenomas (73.9%) and eight depressed adenomas (38.1%) were positive for COX-2 expression (P=0.016). COX-2 expression was detected in the stroma, and the sites of COX-2 expression coincided with the sites of pericryptal myofibroblasts. The histogenesis of depressed adenomas differs from that of elevated adenomas. Our results suggest that a low number of pericryptal myofibroblasts and a low COX-2 expression are associated with depressed adenomas.

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Introduction

There have been many reports of colon cancers arising from depressed adenomas (1-10). Evidence for the depressed adenoma-carcinoma sequence also comes from experimental models of carcinogenesis (11). Several reports have confirmed that depressed adenomas exist in the human colon and that these depressed adenomas show high malignant potential and frequent submucosal invasion, even when they are small in size (12-24).

We studied the distribution of pericryptal myofibroblasts and COX-2 expression immunohistochemically in both elevated and depressed adenomas in an attempt to clarify the histogenesis of depressed adenoma.

Materials and methods

Twenty-one depressed adenomas that were endoscopically resected from 21 patients who were identified as having polyps by colonoscopy at the Department of Gastroenterology and Hepatology at the Hospital of Yamaguchi University School of Medicine between June 1998 and May 2003 were included in this study. As for the elevated adenomas, a total of 23 pedunculated (Ip) and semipedunculated (Isp) polyps that were resected in 2003 and had a mean diameter of 11.7 mm, were included.

Patients with the diagnosis of familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer syndrome or inflammatory bowel disease were excluded from this study. Among the resected polyps, colon cancers infiltrating deeper than the submucosa and polyps other than adenomas (such as hyperplastic polyps) were excluded, as the purpose of this study was to evaluate the differences between depressed and elevated adenomas of the colon.

The presence of pericryptal myofibroblasts and the expression of COX-2 were evaluated immunohistochemically in formalin-fixed specimens.

Morphological classification of tumors. The polyps were classified into two groups according to their gross configuration during colonoscopy. Protruding lesions, whether they were sessile or pedunculated, were classified as elevated adenomas.
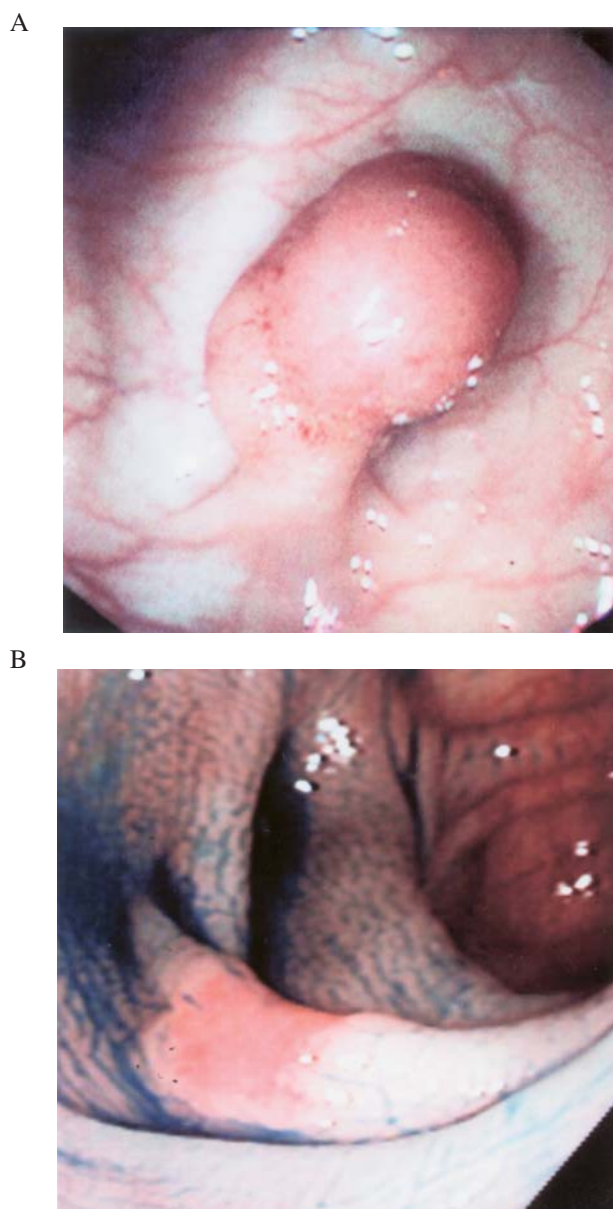
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Figure 1. Endoscopic views of colonic polyps. (A) Elevated adenoma. (B) Depressed adenoma.

A pedunculated polyp was defined as a protruding lesion in which a stalk could be demonstrated (Fig. 1A), while a sessile polyp was defined as a protruding lesion without a stalk. Adenomas that had a minimally depressed area were classified as depressed adenomas (14) (Fig. 1B).

The histological diagnosis of the lesions was made by a pathologist (T.G.) according to the criteria established by the World Health Organization as described previously (25).

Immunohistochemistry. Immunohistochemical staining was performed using the established avidin-biotin-peroxidase complex (ABC) method of Hsu *et al* (26). From the paraffin-embedded specimens, 3-μm-thick sections were prepared. Each section was deparaffinized and incubated in normal rabbit serum (Vector, Burlingame, CA, USA) for 20 min. Mouse anti-human α-smooth muscle actin monoclonal antibody (Dako, Carpinteria, CA, USA) at a dilution of

1:1,000 was added for overnight incubation at 4˚C in a moist chamber. For the staining of COX-2, rabbit anti-COX-2 polyclonal antibody (Cayman Chemicals 160112, Ann Arbor, MI, USA) was used at a dilution of 1:500 for overnight incubation at 4˚C in a moist chamber.

Each section was then incubated in biotinylated anti-mouse immunoglobulin (Dako) for 60 min, followed by incubation in ABC (Vector) for 60 min. Sixty mg 3-3' diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan) and 50 ml 30% H_2O_2 were dissolved in 150 ml phosphate-buffered saline (PBS), and this was applied to each section. For the negative controls, tissue sections were incubated with antibody diluent without the primary antibody. For nuclear counterstaining, 0.2% methyl green or hematoxylin was used.

Evaluation of immunostaining. The staining intensity and distribution were assessed using the scoring method of Yukawa *et al* (27). Distribution was scored according to the number of positive cells: 0, No cells are stained; 1, focal staining (<1/3 of the cells are stained); 2, multifocal staining (1/3 to <2/3 of the cells are stained); or 3, diffuse staining (>2/3 of the cells are stained). The staining intensity was scored as 0, no staining; 1, mild staining (a distribution score between 0 and 2); 2, strong staining (staining is clearly identified at x40 magnification). The distribution and intensity scores were added to obtain the overall grade of α-smooth muscle actin staining or COX-2 staining: 0-2, Negative; or 3-5, positive.

Statistical analyses. The results were analyzed using Fisher's exact probability test. The StatView statistical package (vers. J 4.11; Berkeley, CA, USA), was used. P-values of <0.05 were considered to be statistically significant.

Ethics. This study was approved by the Ethics Committee of the University of Yamaguchi, in Ube, Japan, and was performed in accordance with the principles of the Declaration of Helsinki.

Results

Twenty-three elevated adenomas and 21 depressed adenomas were subjected to immunohistochemical staining using α-smooth muscle actin antibodies for the detection of pericryptal myofibroblasts. Eighteen (78.3%) of the 23 elevated adenomas were positive for pericryptal myofibroblasts (Fig. 2A and B). However, only six (28.6%) of the 21 depressed adenomas were positive for pericryptal myofibroblasts (Fig. 3A and B). The positive rate of pericryptal myofibroblasts was significantly higher among the elevated adenomas than among the depressed adenomas (P<0.001) (Table I).

The expression of COX-2 was found in 17 (73.9%) of the 23 elevated adenomas. In some elevated adenomas, COX-2 expression was detected in the stroma near the superficial layer (Fig. 4A). In other elevated adenomas with a high level of COX-2 expression, staining was observed widely throughout the stroma (Fig. 4B). Eight (38.1%) of the 21 depressed adenomas were positive for COX-2 (Fig. 5). The positive rate of Cox-2 expression was significantly higher among the
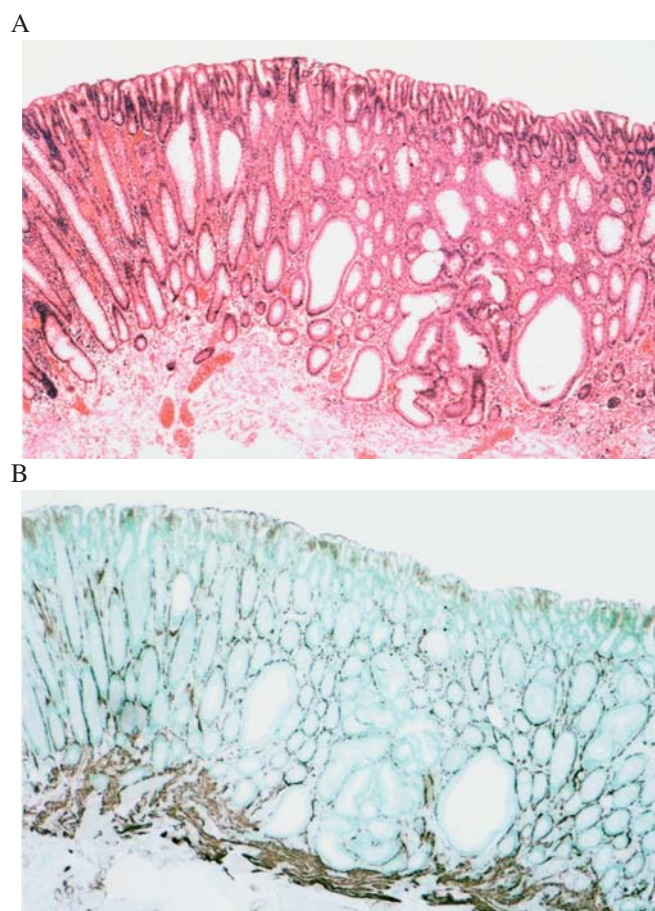
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B

Figure 2. Microscopic views of an elevated adenoma. (A) Elevated adenoma. This is a tubular adenoma (H&E, x40). (B) Immunohistochemical staining with α-smooth muscle actin antibody reveals that pericryptal myofibroblasts surround the adenomatous glands (x40).
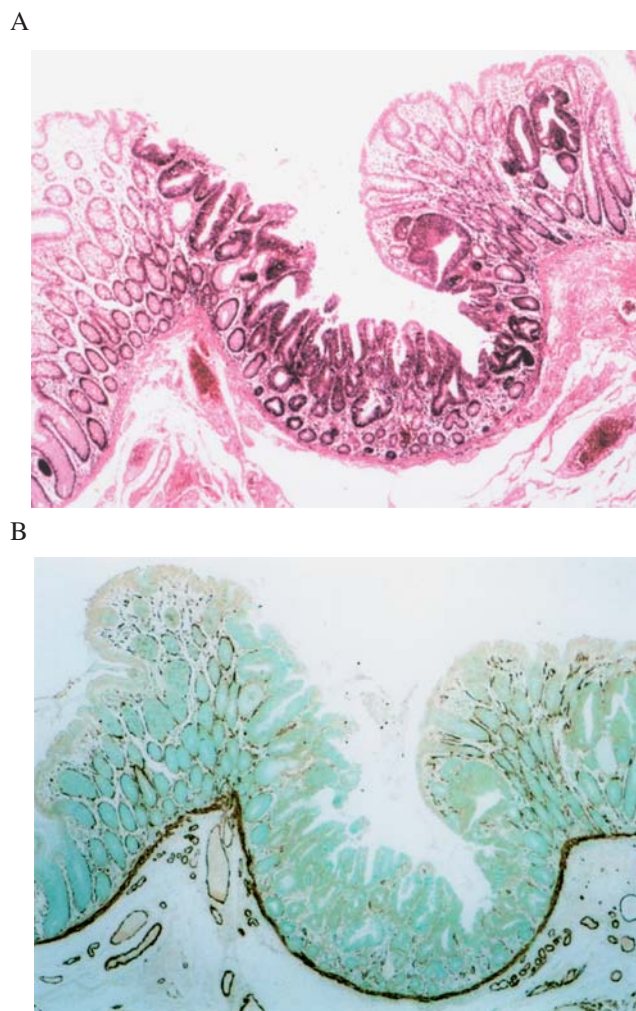
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Figure 3. Microscopic views of a depressed adenoma. (A) Depressed adenoma. The tumor is depressed in comparison with the normal colonic mucosa. Histologically, it is a tubular adenoma. (H&E, x40). (B) Immunohisto-chemical staining with α-smooth muscle actin antibody is negative around the adenomatous glands, indicating that pericryptal myofibroblasts are not present around the adenomatous glands (x40).

elevated adenomas than among the depressed adenomas (P<0.016) (Table II). The sites of COX-2 expression coincided with the sites of pericryptal myofibroblasts (Fig. 6A and B).

Seventeen elevated adenomas and 6 depressed adenomas were positive for both pericryptal myofibroblasts and Cox-2.

Discussion

The incidence of depressed colonic adenomas among patients who underwent colonoscopic examinations of the entire colon in a population in Japan has been reported to be 0.1% by Kudo *et al* (15). Although the number of cases is small, such adenomas have also been reported in Western countries (15,28-30). Along with these reports, a question arose as to what kind of histopathological changes and biological mechanisms leads to the formation of depressed adenomas. Oncogenic studies of depressed and elevated adenomas have shown that they are genotypically different adenomas (31,32). For instance, the incidence of k-ras mutation among elevated adenomas was very high, while that among depressed adenomas was very low. As to the biological mechanisms of

Table I. The number of tumors in which PMFs were present among elevated and depressed adenomas of the colon.

Type of tumor	Number of tumor specimens	Number of tumors positive for PMFs	PMF-positive rate (%)
Elevated adenomas	23	18	78.3
Depressed adenomas	21	6	28.6[a]

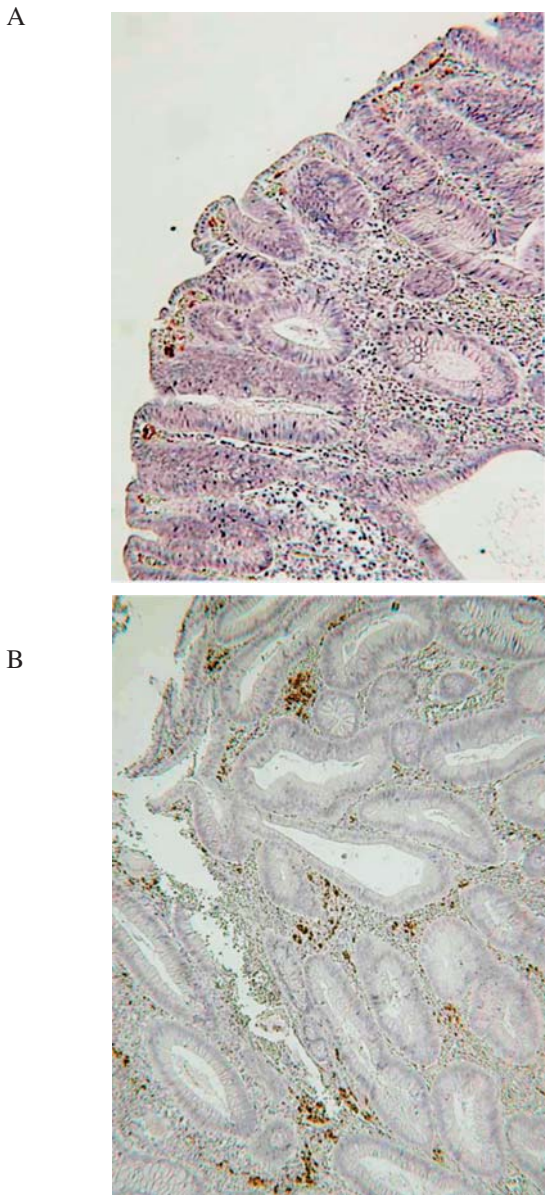PMFs, pericryptal myofibroblasts; [a]P<0.001.

A

B

Figure 4. Immunohistochemical expression of COX-2 in many elevated adenomas. (A) COX-2 expression is not present in the epithelium of the mucosa, although it is present in the subepithelial stroma close to the surface in immunohistological studies. (B) In elevated adenomas with a very high COX-2 expression, staining for COX-2 is widely observed in the stroma.

Figure 5. Immunohistochemical expression of COX-2 in depressed adenomas. Many depressed adenomas were immunohistologically negative for COX-2.

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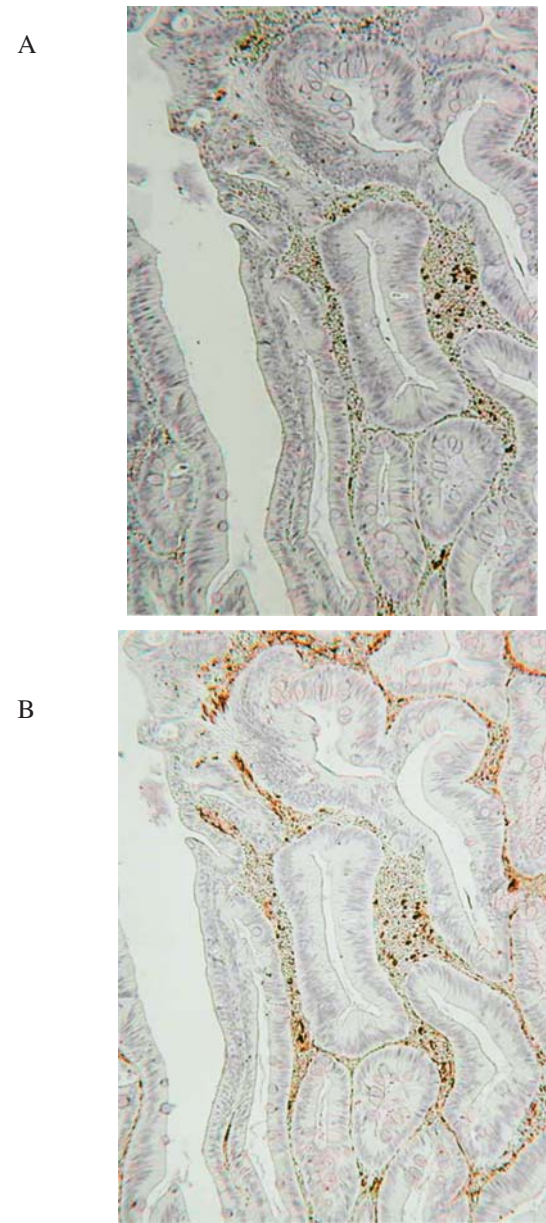
Figure 6. Co-localization of pericryptal myofibroblasts and COX-2 expression in adenomas. The area where COX-2 is expressed coincides with the area where pericryptal myofibroblasts are present. (A) COX-2 (Color is brown). (B) Pericryptal myofibroblasts (Color is brown).

the formation of depressed adenomas, Suzuki *et al* (33) reported that the apoptotic index and level of Bcl-2 expression were higher in the cells of depressed adenomas than in those of elevated adenomas. Watari *et al* (34) studied the relationship between the proliferative/apoptotic index ratio and the morphological changes of flat adenomas over time, and found that tumors with a high proliferative/apoptotic index ratio showed exophytic changes. On the contrary, tumors with a low proliferative/apoptotic index ratio either converted to a depressed adenoma or disappeared. A cell proliferation study also suggested that depressed and elevated adenomas are different types of adenomas (34).

Pericryptal myofibroblasts surround glandular crypts and are closely related to the adjacent epithelial cells in the colonic epithelium. Epithelial cell - myofibroblast inter-
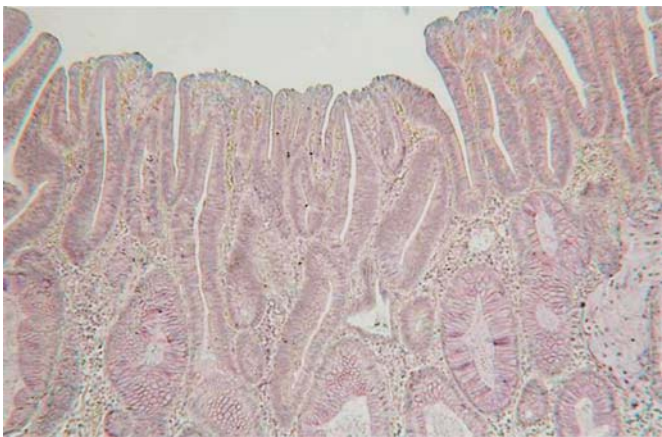
Table II. The number of tumors that expressed COX-2 among elevated and depressed adenomas of the colon.

Type of tumor	Number of tumor specimens	Number of tumors positive for COX-2	COX-2-positive rate (%)
Elevated adenomas	23	17	73.9
Depressed adenomas	21	8	38.1[a]

[a]P=0.016.

actions affect the synthesis of the basement membrane and regulate the growth and differentiation of adjacent epithelial cells (21,35-39).

Cyclooxygenase (COX) is a key enzyme that functions as the rate-limiting step in the conversion of arachidonic acid to prostaglandins, prostacyclines and thromboxane (40). There are two isoforms of Cox, and these are encoded by different genes. COX-1 is constitutively expressed in many human tissues and is viewed as having a housekeeping function. COX-2 expression is induced by several stimuli including bacterial endotoxins, cytokines such as phorbol esters, and growth factors such as the epidermal growth factor (41).

It has been demonstrated that COX-2 is involved in tumor formation at an early stage, namely at the stage of adenoma, in the process of the adenoma-carcinoma sequence. For instance, the level of prostaglandin-E2 synthesis, which is induced by COX-2, progressively increases during the adenoma-carcinoma sequence (42,43). The continued oral intake of a NSAID such as piroxicam, resulted in decreases in the size and number of adenomas in patients with familial adenomatous polyposis (43,44), and also resulted in decreases in the activities of prostaglandin-E2 and COX-2 in adenomas (45). Since prostaglandin-E2 caused an increase in the proliferation rate of intestinal epithelial cells in rats (46), COX-2 participates in the proliferation of tumors via prostaglandin-E2. COX-2 also regulates apoptosis. For instance, intestinal epithelial cells that overexpressed COX-2 were resistant to apoptosis induced by butyrate and this effect could be reversed by sulindac sulphide (47).

In our study, immunohistochemically, the rates of positivity for pericryptal myofibroblasts or Cox-2 among the elevated adenomas were high. On the contrary, the rates of positivity for pericryptal myofibroblasts or Cox-2 among the depressed adenomas were significantly lower.

Taking the functions of pericryptal myofibroblasts and COX-2 which are described above into consideration, we propose that in the histogenesis of depressed adenoma, the proliferation of tumor cells is suppressed due to the absence of pericryptal myofibroblasts and COX-2, and accordingly the tumor cannot assume a protruded shape as an elevated adenoma. Our data support the results of Suzuki et al (33) and Watari et al (34).

It is not known whether COX-2 is produced by epithelial cells or stromal cells (48-53). Although fibroblasts, endothelial cells and macrophages in the stroma have been reported to produce COX-2, Adegboyega et al (54) reported that pericryptal myofibroblasts produced COX-2. We found that in elevated adenomas, the area that was positive for pericryptal myofibroblast and the area that was positive for COX-2

staining were in the stroma of the lamina propria mucosa. On the contrary, the levels of both the myofibroblasts and COX-2 were reduced in depressed adenomas. This finding suggests that a reduction in the number of pericryptal myofibroblasts results in a decrease in COX-2 expression in depressed adenomas. The study of Adegboyega et al (54) was performed on elevated adenomas. The present study was designed to clarify the relationship between COX-2 expression and the presence of pericryptal myofibroblasts in depressed adenomas compared with elevated adenomas.

In summary, depressed adenomas had a reduced number of pericryptal myofibroblasts and a reduced COX-2 expression compared with elevated adenomas. Our finding that the level of COX-2 expression in depressed adenomas was markedly lower than that in elevated adenomas suggests that the histogeneses of the two kinds of adenomas differs.

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