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The distribution of CD34-positive stromal cells and myofibroblasts in colorectal carcinoid tumors

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Summary. In order to understand the stromal reaction associated with colorectal neoplasms, we examined specimens from 26 patients including normal colorectal tissues (n=15), carcinoid tumors (n=12), well differentiated adenocarcinomas (n=10), and poorly differentiated adenocarcinomas (n=4), using an immunohistochemical method. Myofibroblasts and CD34-positive stromal cells were distributed in the mucosa and in the area between the submucosal and subserosal layers, respectively. However, the distribution of these cells markedly changed with the invasion of neoplasms. Namely, myofibroblasts were abundant in the invasive stroma of all colorectal neoplasms. CD34-positive stromal cells were completely absent from the invasive stroma of colorectal cancers. On the other hand, CD34-positive stromal cells were absent from four out of five carcinoid tumor cases with lesions measuring less than 2 mm in size, but were present in all seven cases of carcinoid tumors measuring more than 2 mm. Double-immunostaining identified stromal cells expressing both ASMA and CD34 in several carcinoid tumor cases. Finally, no CD34-positive stromal cells were observed in the invasive stroma of colorectal cancers. However, the distribution of these cells in carcinoid tumors may depend on the lesion size. Namely, CD34-positive stromal cells existed between neoplastic nests in large-sized carcinoid tumors. Myofibroblasts in the stroma of colorectal neoplasms may originate from CD34-positive stromal cells.

Key words: Carcinoid tumor, CD34, Myofibroblasts

Introduction

CD34, a 110 kDa transmembrane cell-surface glycoprotein, has been identified as a marker of human hematopoietic cells (Van de Rijn, 1994). The stromal cells expressing CD34 antigen are widely distributed in various normal organs or under pathological conditions (Yamazaki and Eyden, 1995, 1996a,b, 1997, Nakayama et al., 1999, 2000a, 2003; Papadas et al., 2001; Barth et al., 2002a-c; Kuroda et al., 2004a-c). Myofibroblasts are stromal cells that are distributed in various organs including normal digestive tract, pancreas, Fallopian tubes and testis (Nakayama et al., 2000b; Barth et al., 2002a; Kuroda et al., 2004a-c), and which express ASMA, vimentin and/or desmin (Schürch et al., 1997; Eyden, 2001). High molecular weight caldesmon (h-CD) is a well-developed smooth muscle actin-specific antibody and generally no myofibroblasts express h-CD (Ueki et al., 1987; Ceballos et al., 2000; Watanabe et al., 2000; Rush et al., 2001). In order to elucidate the stromal reaction associated with colorectal neoplasms, we examined the distribution of CD34-positive stromal cells, myofibroblasts, and type I and III collagens in human normal colorectal tissues, carcinoid tumors and colorectal cancers, using an immunohistochemical method.

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Materials and methods

Tissue specimens and routine histological procedures

Specimens from 26 cases exhibiting normal colorectal tissue (n=15), rectal carcinoid tumors (n=12) and colorectal cancers, including well differentiated adenocarcinoma (n=10) and poorly differentiated adenocarcinoma (n=4), were selected for the present study. The 26 patients (18 men and 8 women) ranged from 38 to 89 years old (mean, 65.8 years old). For light microscopy, all specimens were immediately fixed in 10% neutral formaldehyde solution and embedded in paraffin. Sections from all patients were stained with hematoxylin and eosin.

Immunohistochemistry and its interpretation

Using a streptoavidin-biotin immunoperoxidase technique, 3-μm sections of each specimen were evaluated for the presence of ASMA (1:50 dilution,

clone: 1A4, Dako Cytomation, Glostrup, Denmark), h-CD (1:50 dilution, clone: h-CD, Dako Cytomation, CA, USA), CD34 (1:20 dilution, clone: MY10, Becton-Dickinson, San Jose, CA, USA), CD31 (1:20 dilution, clone: JC/70A, Dako Cytomation, Glostrup, Denmark), type I collagen (1:50 dilution, clone: I-8H5, Daiichi Fine Chemical, Toyama, Japan) and type III collagen (1:50 dilution, clone: III-53, Daiichi Fine Chemical, Toyama, Japan). The sections tested for h-CD and type I and III collagens were microwaved for five minutes three times in 10 mmol/L citrate buffer, pH 6.0. The sections for CD31 were pretreated before immunostaining with 0.1% pronase E for 20 min at 37 °C. Vascular smooth muscle cells and endothelial cells were used as internal positive controls for ASMA and h-CD, and CD34 and CD31, respectively. Additionally, the stromal collagens in the submucosal layer were used as internal controls for type I and III collagens. Appropriate negative controls were implemented for all antibodies.

Stromal cells that were positive for both ASMA and h-CD were identified as smooth muscle cells, and ASMA-positive and h-CD-negative cells as ASMA-positive stromal cells. Furthermore, CD34-positive and CD31-negative stromal cells were identified as CD34-positive stromal cells. Thus, we evaluated the distribution of CD34-and/or ASMA-positive stromal cells in normal mucosal tissues and colorectal neoplasms including carcinoid and carcinoma.

Double immunostaining

Double immunostaining for CD34 and ASMA was conducted on specimens from all patients to clarify the relationship between myofibroblasts and CD34-positive stromal cells. Sections were treated with 0.3% hydrogen peroxide/methanol for 10 min at room temperature (RT) and incubated overnight with anti-CD34 antibody. The sections were then incubated with peroxidase-conjugated mouse IgG and rabbit IgG (Simple stain PO-MAX (multi), Nichirei, Tokyo, Japan) for 1 h at RT and immersed in 0.2% DAB and 0.1% hydrogen peroxide in 0.05 M Tris buffer. After washing with PBS, the sections were incubated for 2 h at RT with anti-ASMA antibody, for 1 h at RT with biotinylated rabbit anti-mouse IgG $F(ab')^2$ fragment (Dako Cytomation, Glostrup, Denmark), and alkaline phosphatase-conjugated streptavidin (Nichirei, Tokyo, Japan) for 30 min at RT. To visualize this reaction the sections were stained with Fast blue.

Results

Distribution of stromal cells in normal colorectal tissue

A small to moderate number of myofibroblasts was observed along the crypt in the mucosa (Fig. 1a), although myofibroblasts were absent from anatomic sites beneath the submucosal layer. CD34-positive stromal cells were observed in the portion between the submucosal and subserosal layers in the reticular network (Fig. 1b). However, these cells were absent from the mucosa.

Distribution of stromal cells in carcinoid tumor and colorectal cancer

The distribution of stromal cells and collagens is summarized in Table 1. Many myofibroblasts were observed in the 10 carcinoid tumors, eight well differentiated adenocarcinomas, and four poorly

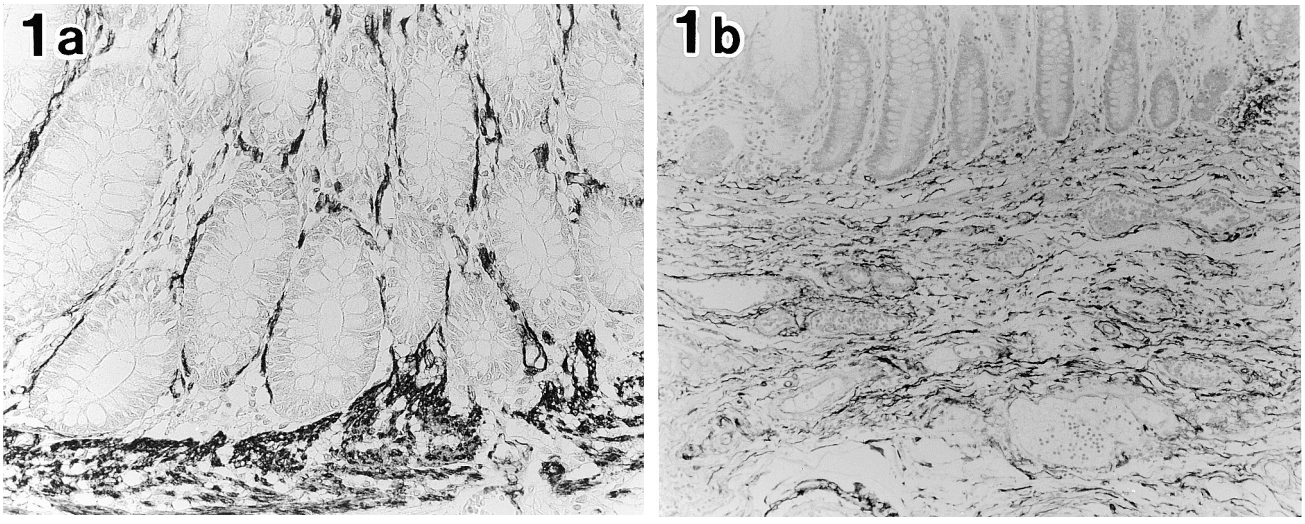


Fig. 1. The distribution of myofibroblasts and CD-34 positive stromal cells in normal colorectal tissue. a. Immunohistochemical results for ASMA. AMSA-positive stromal cells are observed along the glands (x 50). b. Immunohistochemical results for CD34. CD34-positive stromal cells are present in a reticular network beneath the submucosal layer. x 25

Table 1. Intensity of distribution of stromal cells and collagens in colorectal neoplasm.

	CARCINOID TUMOR (n=12)	WELL DIFFERENTIATED ADENOCARCINOMA (n=10)	POORLY DIFFERENTIATED ADENOCARCINOMA (n=4)
Myofibroblasts			
-~+-	0/12	0/10	0/4
+	0/12	0/10	0/4
++	2/12	2/10	0/4
+++	10/12	8/10	4/4
CD34-positive stromal cells			
-~+-	4/12	10/10	4/4
+	5/12	0/10	0/4
++	1/12	0/10	0/4
+++	2/12	0/10	0/4
Type I collagen			
-~+-	10/12	7/10	0/4
+	2/12	3/10	1/4
++	0/12	0/10	2/4
+++	0/12	0/10	1/4
Type III collagen			
-~+-	0/12	0/10	0/4
+	0/12	1/10	0/4
++	1/12	3/10	0/4
+++	11/12	6/10	4/4

-: negative; +-: scarce; +: small number; ++: a moderate number; +++: a large number

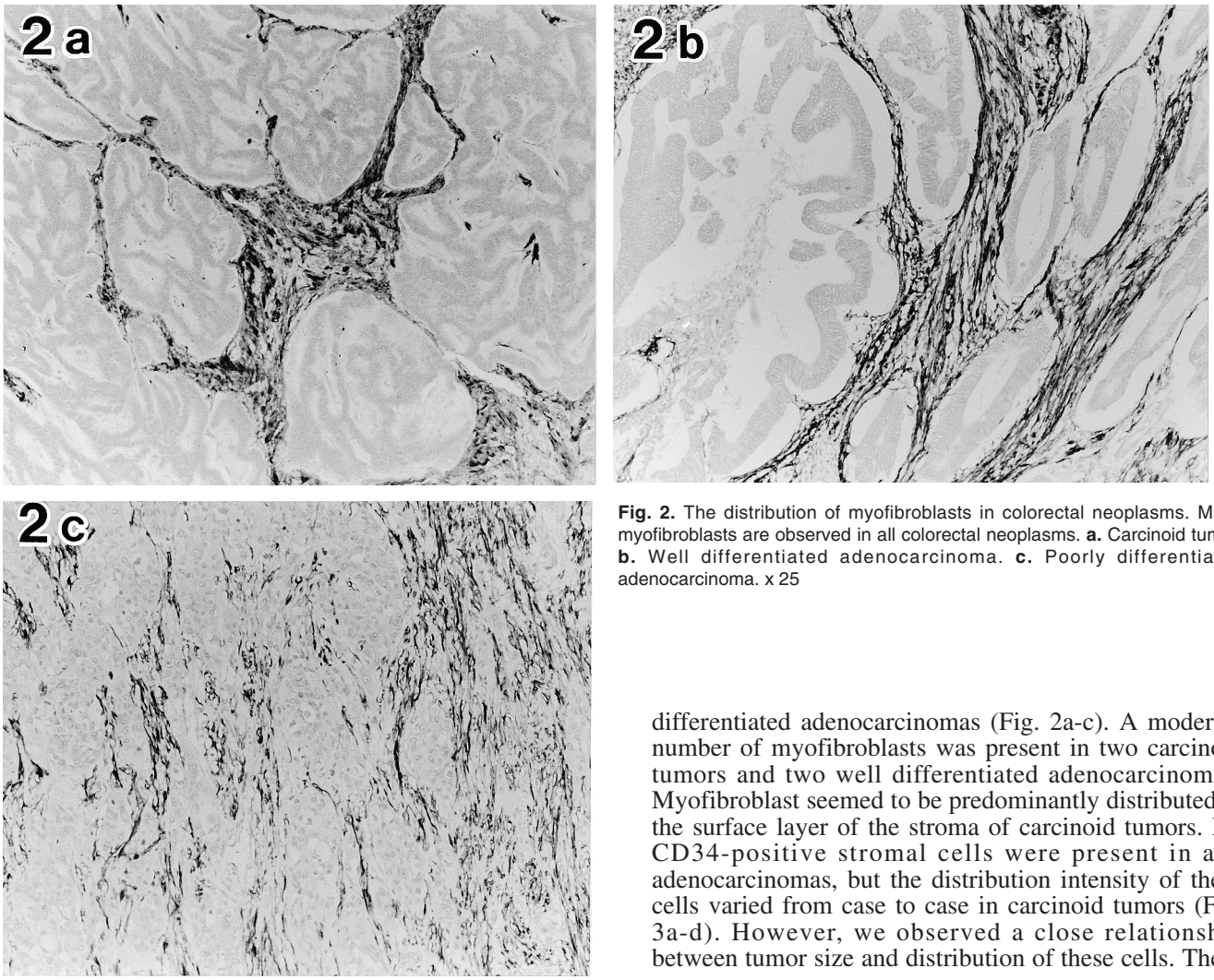


Fig. 2. The distribution of myofibroblasts in colorectal neoplasms. Many myofibroblasts are observed in all colorectal neoplasms. **a.** Carcinoid tumor. **b.** Well differentiated adenocarcinoma. **c.** Poorly differentiated adenocarcinoma. x 25

differentiated adenocarcinomas (Fig. 2a-c). A moderate number of myofibroblasts was present in two carcinoid tumors and two well differentiated adenocarcinomas. Myofibroblast seemed to be predominantly distributed in the surface layer of the stroma of carcinoid tumors. No CD34-positive stromal cells were present in any adenocarcinomas, but the distribution intensity of these cells varied from case to case in carcinoid tumors (Fig. 3a-d). However, we observed a close relationship between tumor size and distribution of these cells. These

Table 2. Relationship between the tumor size and CD34(+)-stromal cells in carcinoid tumor.

CD34-POSITIVE STROMAL CELLS	CARCINOID TUMOR (n=12)	
	2mm> (n=5)	2mm< (n=7)
-~+-	4/5	0/7
+	1/5	4/7
++	0/5	1/7
+++	0/5	2/7

-: negative; +-: scarce; +: small number; ++: a moderate number; +++: a large number

results are shown in Table 2. Specifically, CD34-positive stromal cells were absent from four out of five tumors that were less than 2 mm in maximum diameter, whereas all tumors larger than 2 mm in maximum diameter had a significant number of CD34-positive cells in the stroma (Fig. 3a,b). When observed in detail, CD34-positive stromal cells appeared in large stromal bundles between nests of carcinoid tumors and were present more predominantly in the deep layer than in the superficial layer of the tumor nest (Fig. 3b). Type I collagen was relatively scarce in carcinoid tumors (Fig. 4a) and well differentiated adenocarcinomas, whereas it was relatively abundant in poorly differentiated adenocarcinomas (Fig. 4b). Type III collagens were relatively abundant in all colorectal neoplasms (Fig. 4c).

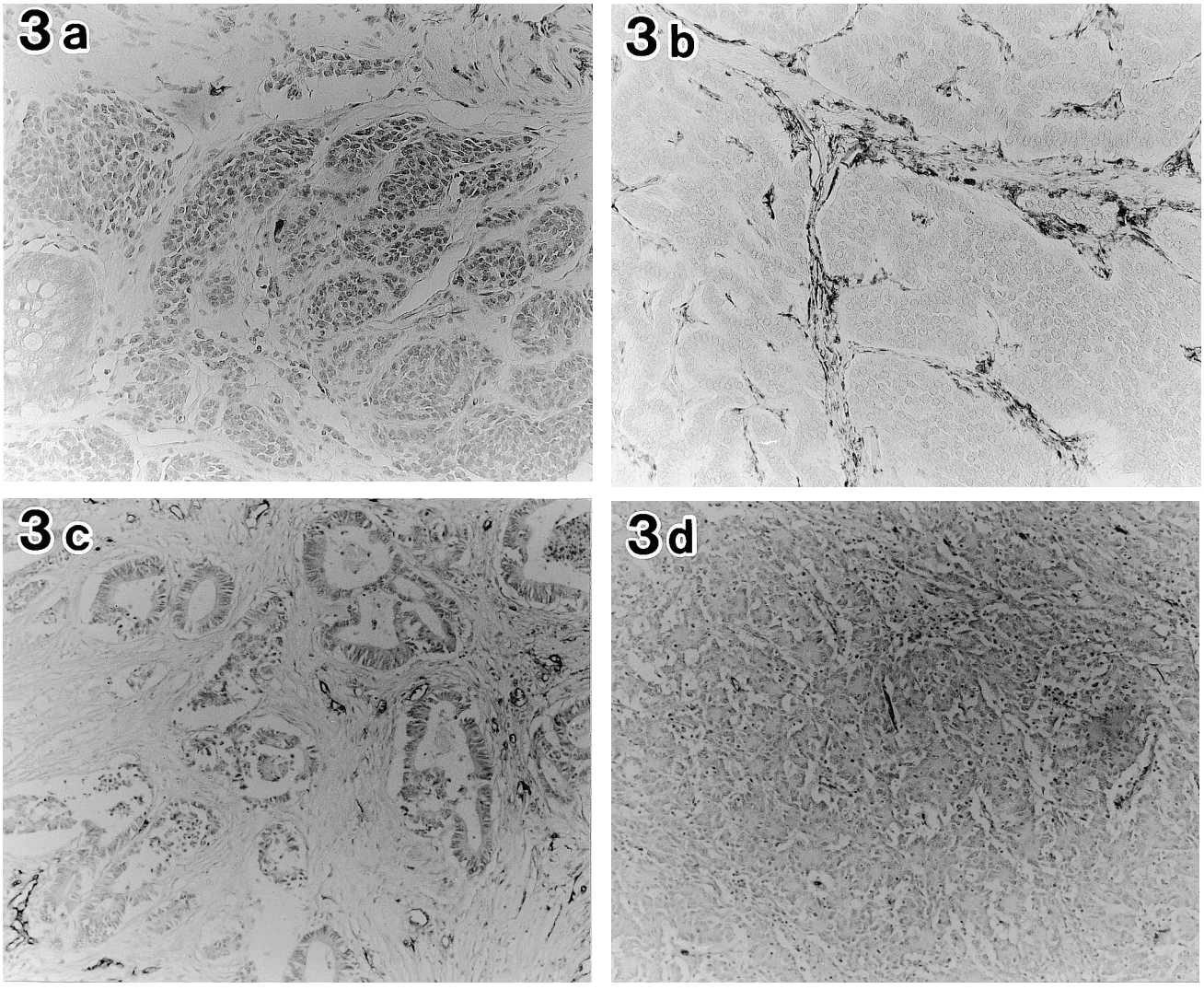
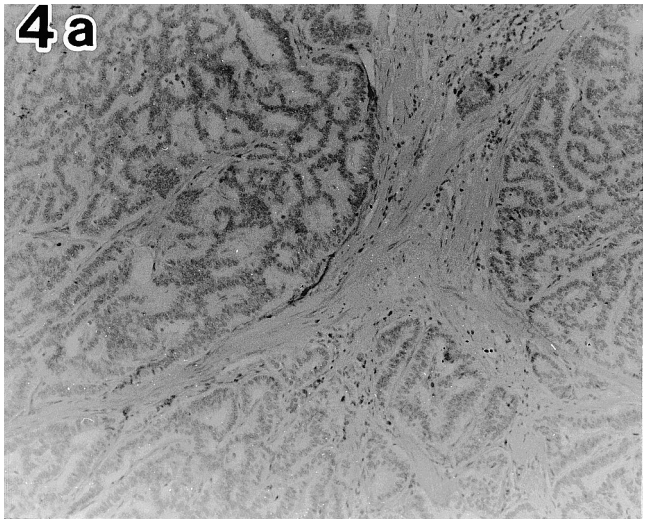
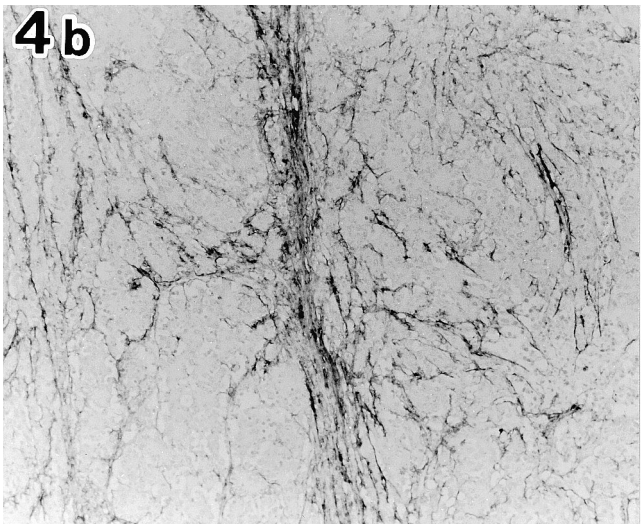


Fig. 3. The distribution of CD34-positive stromal cells in colorectal neoplasms. **a.** Carcinoid tumor, small size. No CD34 reactivity is observed in the stroma. x 50. **b.** Carcinoid tumor, large size. CD34-positive stromal cells are distributed in the stromal bundles between neoplastic nests. x 50. **c.** Well differentiated adenocarcinoma. No CD34-positive stromal cells are observed. x 25. **d.** Poorly differentiated adenocarcinoma. CD34-positive stromal cells are completely absent. x 25





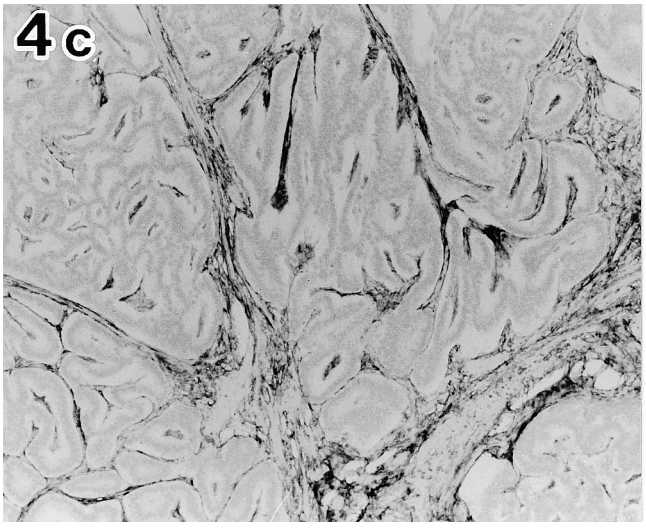


Fig. 4. Immunohistochemical analysis of collagens. **a.** Type I collagen is scarce in the stroma of carcinoid tumors. **b.** Type I collagen is pervasive throughout the stroma of poorly differentiated adenocarcinomas. **c.** Type III collagen is abundant in the stroma of carcinoid tumors. x 25

Double immunostaining

Stromal cells expressing both ASMA and CD34 antigens were observed in the stroma of several carcinoid tumor cases (Fig. 5).

Discussion

In normal colorectal tissue, the distribution of myofibroblasts and CD34-positive stromal cells is contrastive. Namely, myofibroblasts were distributed only in the colorectal mucosa, and CD34-positive stromal cells were present in the area between submucosal and subserosal layers, where they formed a dense reticular network. In the invasive stroma of colorectal adenocarcinomas, myofibroblasts were relatively abundant and CD34-positive stromal cells were completely absent. These results confirm the results of reports by Nakayama et al. (2000a). The disappearance of CD34-positive stromal cells appears to be characteristic for the stroma of colorectal cancers, as Nakayama et al. (2000a) suggested in a previous study. Type III collagen was relatively abundant in all cases of carcinoid tumors and colorectal cancers. In contrast, type I collagen was relatively abundant in poorly differentiated adenocarcinomas, although in carcinoid tumors and well-differentiated adenocarcinomas, type I

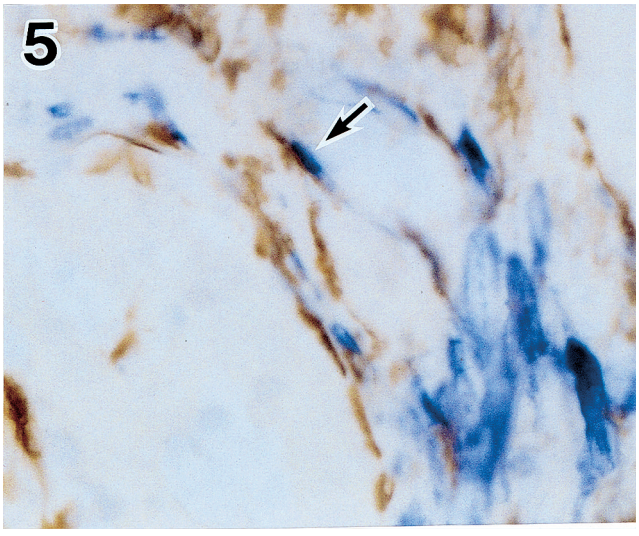


Fig. 5. Double immunostaining of ASMA and CD34 in carcinoid tumors. Several stromal cells expressing both antigens are observed (arrow). x 400

collagen was relatively scarce. We suggest that these findings may depend on the degree of desmoplasia. Therefore, CD34-positive stromal cells may disappear in colorectal cancers, irrespective of the degree of desmoplasia.

Interestingly, the location of myofibroblasts and CD34-positive stromal cells markedly changed beneath the submucosal layer of colorectal cancers. Namely, CD34-positive stromal cells were completely absent from the stroma of colorectal cancers. Taking the normal distribution of myofibroblasts into consideration, we suggest that many myofibroblasts in the invasive area may originate in CD34-positive stromal cells or migrate from the mucosal layer. Recently, several reports have investigated a common origin between myofibroblasts and CD34-positive stromal cells (Barth et al., 2002c; Kuroda et al., 2004b). Furthermore, we identified several individual cells expressing both ASMA and CD34 antigens in several carcinoid tumor cases in the present study. Therefore, it is possible that CD34-positive stromal cells completely transform into myofibroblasts in the stroma of colorectal cancers. We also identified CD34-positive stromal cells in the stroma of the deep layer of large carcinoid tumors. In carcinoid tumors, myofibroblasts seem to be rich in the surface layer stroma full of collagen type III. Therefore, we suggest that myofibroblasts in the stroma of the surface layer of carcinoid tumors may transform from CD34-positive stromal cells of the submucosal layer or migrate from the mucosal layer, and CD34-positive stromal cells may enter into the stroma of deep layer of carcinoid tumors with the formation of thick bundle. Additionally, the use of CD34 immunohistochemistry may be useful for distinguishing carcinoid tumors from colorectal cancers in the diagnosis of large neoplasms. Certain cytokines, such as TGF-ß1, may play an important role in the phenotypic changes of CD34-positive stromal cells. Further examinations will be required to elucidate the relationship between myofibroblasts and CD34-positive stromal cells.

In conclusion, myofibroblasts were abundant in the stroma of carcinoid tumors and colorectal cancers. CD34-positive stromal cells disappeared during the invasion of colorectal cancers, whereas the distribution of these cells in carcinoid tumors may depend on the tumor size.

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