Virchows Arch (2006) 448: 127–134
DOI 10.1007/s00428-005-0062-8

ORIGINAL ARTICLE

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Endoglin (CD105) expression in angiogenesis of colon cancer: analysis using tissue microarrays and comparison with other endothelial markers

Received: 11 April 2005 / Accepted: 3 August 2005 / Published online: 22 September 2005

Abstract Some markers of angiogenic endothelial cells are emerging as targets of cancer therapy. The present study compares the expression of CD105 with that of other endothelial markers in all tissue layers during the development of colon cancer. We immunohistochemically analyzed the expression of the colon adenoma–carcinoma sequence by endothelial cells using a panel of eight endothelial markers. We examined sections from endoscopic mucosal resection and surgical resection of tubular adenoma (n=31), carcinoma in adenoma (n=11), and adenocarcinoma (n=34). Cylindrical cores were punched out from donor paraffin blocks of normal mucosa adjacent to tumors, from tumor lesions of mucosa, submucosa, muscularis propria, subserosa, and serosa, and from lymph node metastases. CD31 (PECAM-1) was universally expressed in the blood vessels of adenoma–carcinoma lesions as well as in normal mucosal vessels (80–95%), with no significant differences. In contrast, cancer-associated blood vessels (up to 80%) and cancer cells themselves expressed high levels of CD105. In normal mucosa, CD105 was weakly expressed in endothelial cells of capillaries (≦21%), and significant differences in its expression in endothelial cells between the normal mucosa and adenoma, carcinoma in adenoma, and adenocarcinoma were found. Flt-1, Flk-1, transforming growth factor-β1, transforming growth factor-β receptor II, and CD44 were strongly expressed in the cancer cells but were not expressed in the blood vessels. Vascular endothelial growth factor was expressed at <30% in the blood vessels of adenoma, carcinoma in adenoma, and carcinoma. Moreover, this study provided evidence that CD105 was expressed exclusively in endothelial blood vessels by double immunostaining of CD105 and D2-40. The present study shows that de novo blood vessels of colon cancer specifically express CD105. These findings provide the basis for novel antiangiogenic cancer therapies.

Keywords Colon tumor · CD105 · Tissue microarray · Angiogenesis · Endothelial markers

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Introduction

Colon cancer is a widespread human disease that is one of the major causes of morbidity and mortality worldwide [19]. Most colorectal carcinomas are considered to originate from precursor lesions (adenomas) [18]. Adenoma is thought to progress to carcinoma over many years because of multiple accumulated molecular alterations [25].

Angiogenesis is an important step in the process of cancer growth. It promotes metastatic spread by providing the means for cells to detach from the primary tumor and to travel in the bloodstream to distant metastatic sites. Moreover, the most important prognostic factor for colon cancer is the depth of cancer invasion. Cancer is always accompanied by angiogenesis as it grows and invades the surrounding tissues, and angiogenesis is a complex of endothelial cell growth and stem cell differentiation brought about by interactions of growth factors and their ligands.

Vascular endothelial growth factor (VEGF) is a potent mitogen and cytoprotective factor for vascular endothelial cells, and its receptors (VEGFR-1/Flt-1 and VEGFR-2/Flk-1), which are expressed exclusively by endothelial and neoplastic cells, have been documented [6, 11, 26].

CD105 is a receptor for transforming growth factor (TGF)-β1 and TGF-β3, and it modulates TGF-β signaling by interacting with TGF-β receptor I (TGF-βRI) and/or TGF-β receptor II (TGF-βRII) [22]. CD105 is predominantly expressed on cellular lineages within the vascular system and is overexpressed on proliferating endothelial cells. Several studies indicate that CD105 is involved in the development of blood vessels and that it represents a

powerful marker of neovascularization of various types of tumors, including colon cancer [1, 4, 8, 9, 17, 28]. CD105 is emerging as a prime vascular target of antiangiogenetic cancer therapy [10]. We reported that the predominantly CD105-positive microvessel count of polyploid-type early colon cancer is higher than that of nonpolyploid-type early cancer [20]. However, to our knowledge, the expression of CD105 in vessels of lesions invading all the various tissue layers during the colon adenoma–carcinoma sequence (adenoma, carcinoma in adenoma, and adenocarcinoma) has not been compared with that of other endothelial markers.

The use of tissue microarrays in colorectal carcinoma has been validated by analyzing the immunohistochemical expression of some markers [12]. We therefore compared the expression of CD105 in several hundred target spots with that of other endothelial markers in all tissue layers during the development of colon cancer using tissue microarrays and immunohistochemistry. To specifically differentiate the vascular expression of CD105 and CD31 as pan-endothelial markers, we compared them using double immunofluorescence. Additionally, to exclude the idea that the CD105-stained vessels represent lymphatic vessels, we performed a double immunohistochemistry of CD105 and D2-40, which has been reported as a new selective marker for lymphatic endothelial markers [13]. We also compared the vascular expression of the antibody between the normal mucosa adjacent to tumors and the neoplastic lesions.

Materials and methods

Tumor samples

Between 1998 and 2003, 36 consecutive resected colon samples and 40 endoscopic mucosal resection (EMR) samples from 76 patients (42 men, 34 women; age range 39–95 years) with colonic tumors were obtained from the files of the Department of Pathology, Faculty of Medicine, Saga University and its affiliated hospital. The specimens were formalin-fixed and paraffin-embedded, and the tumor was diagnosed from observations of sections stained with hematoxylin and eosin (H&E). Histological subtypes were classified according to the Japanese classification of colorectal carcinoma [29]: 31 tubular adenomas (10 mild, 10 moderate, and 11 severe atypias), 11 carcinomas in adenoma (focally expressed cancer cells in adenoma), and 34 adenocarcinomas (16 well, 16 moderate, and 2 poor differentiations). Table 1 shows the details of the patients and their diseases.

Tissue microarray

To identify the targets of core samples, we examined the microscopic features of each layer of invasion on slide sections and marked the locations to be punched out (Fig. 1).

Table 1 Clinicopathological data

Variable	Number
Number of patient	76
Age (years)	
Range (median)	39–95 (67)
Gender	
Male	42
Female	34
Histopathology	
Tubular adenoma	31
Mild atypia (low-grade dysplasia)	10
Moderate atypia	10
Severe atypia (high-grade dysplasia)	11
Carcinoma in adenoma	11
Adenocarcinoma	34
Well differentiated	16
Moderately differentiated	16
Poorly differentiated	2
Tissue cores	
Normal adjacent tumor	76
Depth of invasion	
Mucosa	76
Submucosa	33
Muscular propria	31
Subserosa	25
Serosa	6
Lymph nodes	11

Under a light microscope, we selected and marked areas of normal mucosa and representative tumor from each layer on H&E slides and corresponding paraffin blocks. The tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA) was used to prepare cylindrical cores of 1.5 mm in diameter. Cores were punched out from donor blocks and placed in recipient blocks.

Two array blocks of 258 cores were derived from the normal mucosa adjacent to tumors (n=76), from tumor lesions from mucosa (n=76), submucosa (n=33), muscular propria (n=31), subserosa (n=25), and serosa (n=6), and from lymph node metastases (n=11). The depth of each core depended on the extent of tumor cell invasion (Table 1). The array blocks were then incubated for 30 min at 37°C to improve adhesion between the cores and the paraffin of the recipient block.

Double immunostaining

For double immunofluorescence, the tissue array slides were deparaffinized and soaked in 0.01 M citrate buffer (pH 6.0) at 90°C for 40 min for antigen retrieval. The samples were treated with 10 mg/ml bovine serum albumin (BSA) to inhibit nonspecific antibody binding and then were incubated with the primary murine monoclonal antibody CD105 for 1 h at 37°C. After washing thrice

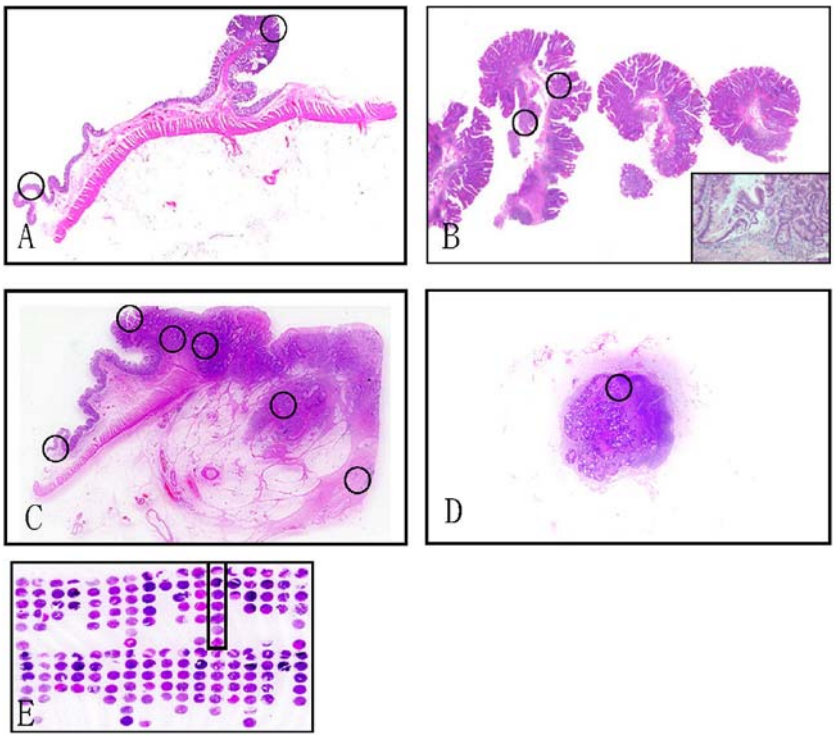

Fig. 1 Sources of punctured cores. Cores were punched out from the normal mucosa and neoplastic layer of adenoma at the mucosal level (**a** and **b**), carcinoma in adenoma (**b**), and normal mucosa, as well as in the mucosa, submucosa, muscular propria, subserosa, and serosa levels (**c**) of cancer tissues, and in lymph node metastasis (**d**). The box shows all layers from one advanced cancer in a representative tissue microarray (**e**). *Inset*: A transition area of adenoma (*left half*) and cancer (*right half*) in the right circle of **b**

with PBS (pH 7.2), the samples were incubated with fluorescein-isothiocyanate (FITC)-labeled secondary rabbit polyclonal antibody against murine immunoglobulin G (IgG) for 30 min at 37°C. To inactivate the primary and secondary antibodies, the samples were heated in the citrate buffer at 90°C for 15 min. This treatment made it possible to carry out the second immunoreaction with the monoclonal antibody. For the second immunoreaction, the same procedure was repeated; the samples were treated with 10 mg/ml BSA, incubated with another primary antibody CD31, and then incubated with rhodamine-labeled secondary antibody. After washing with PBS, FITC-labeled and rhodamine-labeled samples were examined using a fluorescence microscope (Olympus BX60, Tokyo, Japan). To detect nonspecific antibody binding, control sections were incubated with either normal murine or rabbit serum or PBS instead of primary antibody. No staining was observed in these control samples.

For double immunohistochemistry, after incubation with the first primary antibody CD105 and then washing with PBS, the alkaline-phosphatase-labeled secondary rabbit polyclonal antibody against murine IgG was reacted for 30 min at 37°C and visualized with the BCIP/NBT substrate system (Dako, USA), which produces a blue purple color. After microwave heating in ethylenediaminetetraacetic acid (EDTA; pH 8) for 5 min, the specimens were incubated with the second primary antibody (D2-40, mouse monoclonal antibody; Nichirei, Japan) for 3 h. The same procedure with the first step was repeated, and the expression of the second antibody was visualized with the New Fuchsin Substrate Kit (Nichirei), which produces a red color.

Immunohistochemistry

Sections from each array block were cut at a thickness of 4 μm. Briefly, the slides were placed at 60°C for 15 min, deparaffinized in xylene, and rehydrated in a series of graded alcohols. Thereafter, antigen retrieval was performed by microwave heating in EDTA (pH 8) for 6 min (for CD105, mouse monoclonal clone 4G11), then the slides were autoclaved (121°C) in EDTA (pH 8) for 5 min using the appropriate antibodies (for CD44, mouse monoclonal MH114; VEGF, mouse monoclonal clone C-1; Flt-1, rabbit polyclonal C-17; Flk-1, rabbit polyclonal N-931; TGF-β1, rabbit polyclonal V; and TGF-βRII, rabbit polyclonal L-21) and with proteinase K (Dako, Japan) for 10 min (for CD31, mouse monoclonal clone JC70A; Table 2). The sections were incubated with primary antibodies overnight at 4°C, washed, and incubated with goat antimouse or goat antirabbit Igs (EnVision Peroxidase; Dako) for 30 min at room temperature.

Histological assessment

Images of an immunostained section (×200) were captured on a computer (Windows XP, 7000 cl; Fujitsu) with a

Table 2 Used antibody

Antibody	Clone	Pretreatment	Dilution	Company
CD31	Mono	Proteinase K	×20	Dako
CD105	Mono	MW—EDTA	×50	Novocastra
CD44	Mono	AC—EDTA	×50	MBL
VEGF	Mono	AC—EDTA	×100	Santa Cruz
Flt-1	Poly	AC—EDTA	×200	Santa Cruz
Flk-1	Poly	AC—EDTA	×200	Santa Cruz
TGF-β1	Poly	AC—EDTA	×100	Santa Cruz
TGF-βRII	Poly	AC—EDTA	×200	Santa Cruz

Antigen retrieval method for immunohistochemistry
For double immunostaining, see "Materials and methods"
MW Microwave, *AC* citrate buffer

Nikon ACT-1 software by using a Nikon digital camera (DXm 1200) attached to an operator light microscope (Nikon Eclipse, E600). Assessment was performed on live images from each core. Endothelial staining of at least one blood vessel from each core was considered as a positive expression of the blood vessels. Staining of ≧10% of the neoplastic or normal epithelial cells was considered positive in neoplastic or normal epithelial cells. Positive immunoreactivity was graded as weak (+), moderate (++), and intense (+++).

Statistical analysis

Differences in the vascular endothelial expression of an antibody between a normal mucosa and neoplastic mucosal adenoma, carcinoma in adenoma, and adenocarcinoma from each layer were analyzed using the *t* test of independent samples. $P<0.05$ was considered significant.

Results

Double immunofluorescence staining revealed that CD31 (PECAM-1) was universally expressed in small blood vessels and capillaries. On the other hand, CD105 was rarely expressed in normal mucosal vessels; however, in the cancer tissue, CD105 was intensely expressed in newly formed numerous capillaries, which reflect angiogenesis (Fig. 2). After the demonstration that CD105 was specifically expressed in angiogenetic blood vessels, the detailed relationship between markers of blood vessels and tumor histology was assessed by immunohistochemistry.

CD31 was universally expressed in the blood vessels of all layers of adenoma, carcinoma in adenoma, and adenocarcinoma lesions (Fig. 3), as well as in those of normal mucosal blood vessels (80–95%) (Fig. 5). Vascular endothelial expression of CD31 in normal and neoplastic

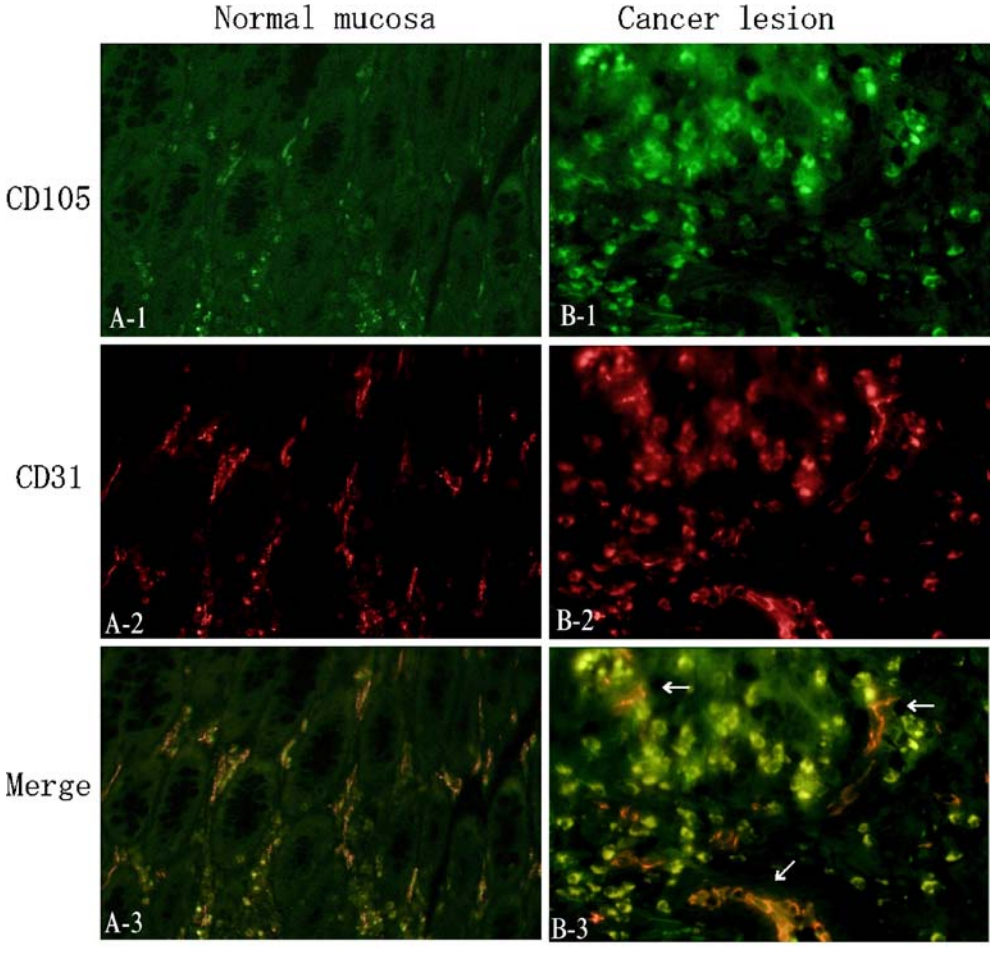

Fig. 2 Double immunofluorescence of CD105 and CD31. Normal mucosa (*upper layer*) and cancer lesion (*lower layer*). CD105 is rarely expressed in the normal mucosa (**a1**) and is intensely expressed in angiogenetic small blood vessels and capillaries (**b1**). An intense vascular expression of CD31 is universally noted in all large and small blood vessels and capillaries (**a2** and **b2**). Their characteristics are demonstrated in the merged images (**a3** and **b3**, *arrows*). Original magnification, ×200

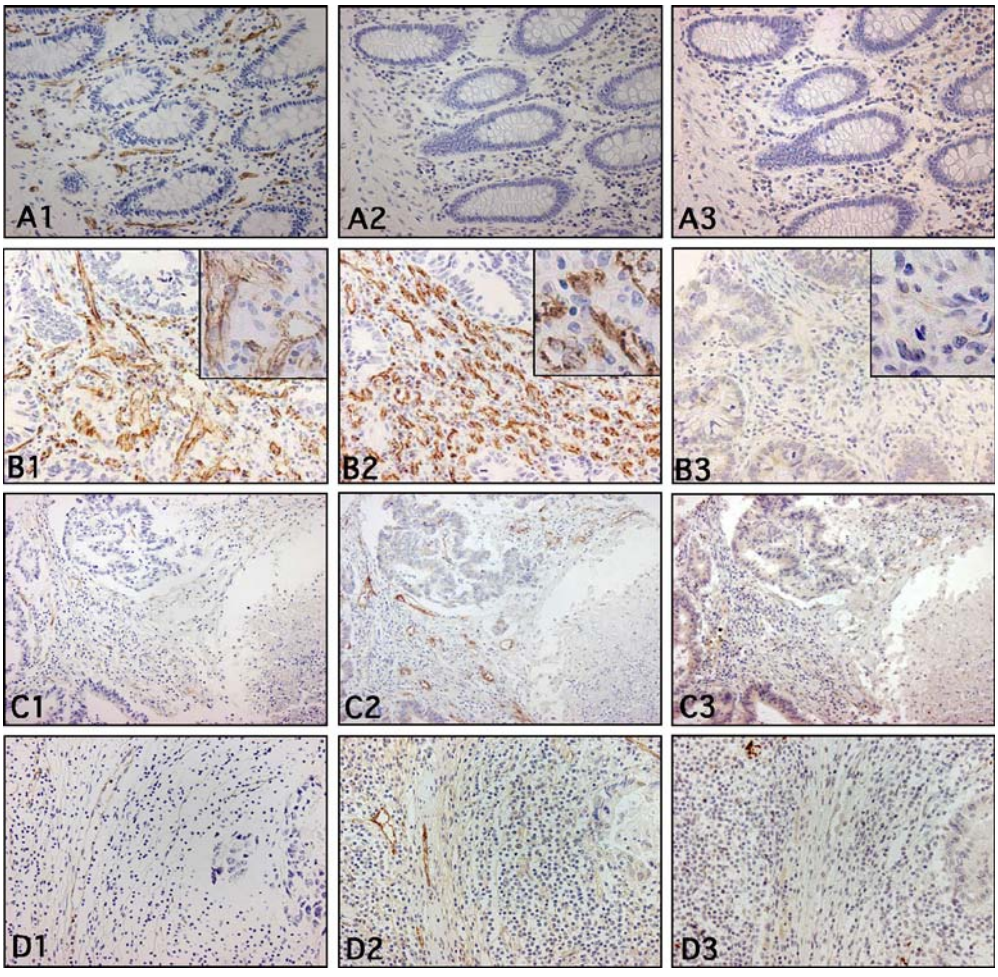

Fig. 3 Expression of CD31 (**a1**–**d1**), CD105 (**a2**–**d2**), and VEGF (**a3**–**d3**). Immunohistochemistry. Original magnification, ×200. Normal mucosa (**a1**–**a3**), neoplastic lesion at mucosal level (**b1**–**b3**), subserosal level (**c1**–**c3**), and lymph node metastasis (**d1**–**d3**) from 1.5-mm tissue cores of the same specimen. Intense vascular expression of CD31 (**a1**); absent expression of CD105 (**a2**) and VEGF (**a3**) in normal mucosa. Intense expression of CD31 (**b1**) in large blood vessels and CD105 (**b2**) in smaller blood vessels from serial sections. Cancer tissues expressed VEGF but not the blood vessels (**b3**). In subserosal layer and lymph node metastasis, only CD105 is expressed (**c2** and **d2**). *Inset*: Original magnification ×400

mucosal layers of adenoma, carcinoma in adenoma, and adenocarcinoma from all layers did not significantly differ (Fig. 5). CD105 was intensely expressed in vascular endothelial cells of neoplastic mucosal adenoma, carcinoma in adenoma, and various layers of adenocarcinoma at a level corresponding to the depth of cancer invasion. The CD105 expression was also seen in the cytoplasm of neoplastic epithelial cells (Fig. 3). CD105 was expressed by 30 and 54% of vascular endothelial cells in the neoplastic mucosa of adenoma and carcinoma in adenoma, respectively. Moreover, CD105 was also expressed in endothelial cells of blood vessels in neoplastic mucosa (65%), submucosa (72%), muscular propria (80%), subserosa (68%), serosa (50%) of adenocarcinoma, and lymph node metastasis (45%). In contrast, only 12, 18, and 21% of blood vessels in normal mucosal layers of adenoma, carcinoma in adenoma, and adenocarcinoma, respectively (Fig. 5), weakly expressed CD105, and its expression in normal epithelial cells was not found. Unlike CD31, vascular endothelial expression of CD105 significantly differed between the normal and neoplastic mucosal layers of adenoma, cancer in adenoma, adenocarcinoma lesions ($p<0.05$, $p<0.05$, and $p<0.0001$, respectively; Fig. 5). Additionally, by using a double immunostaining of CD105 and D2-40, our results demonstrated that CD105 was expressed exclusively in the endothelium of newly formed blood vessels within and around tumors from all layers, and no double-positive staining in the same vessels was found (Fig. 4).

VEGF was expressed on vascular endothelial cells (<30%) and in the cytoplasm of normal and neoplastic cells (<50%) throughout all layers of adenoma, cancer in adenoma, and adenocarcinoma. Vascular expression of VEGF

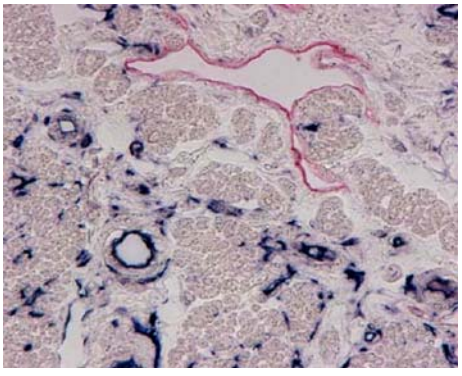

Fig. 4 Double immunostaining of CD105 (*blue purple*) and D2-40 (*red*). No apparent double labeling of CD105 and D2-40 in the vessels. Original magnification, ×200

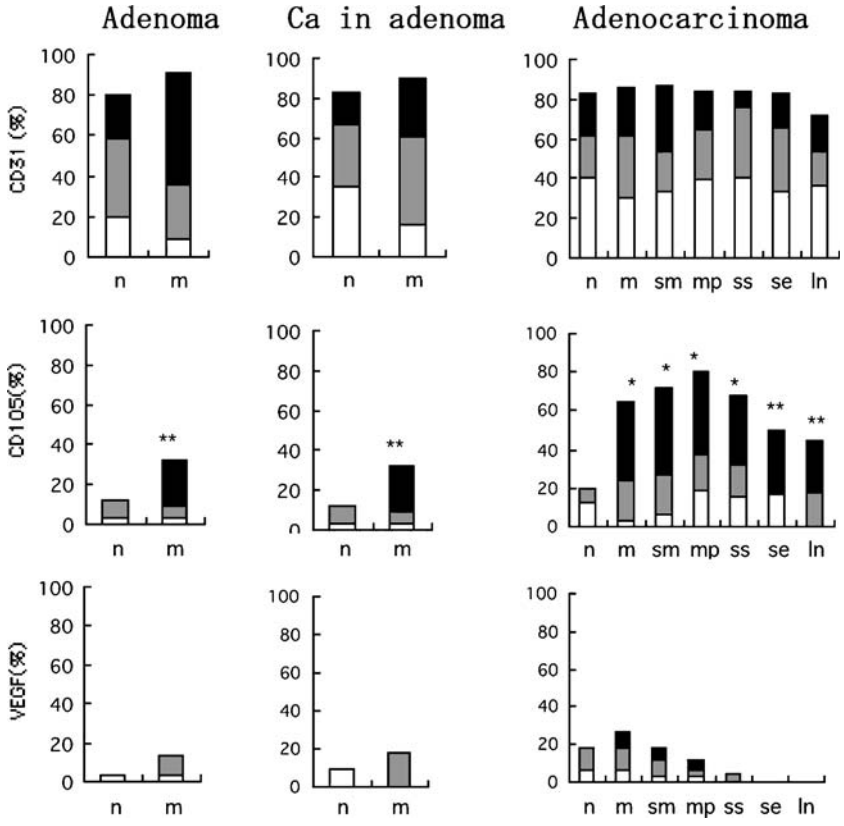

Fig. 5 Expression of CD31, CD105, and VEGF. Vascular endothelial cells express CD31, CD105, and VEGF throughout all tissue levels of colon adenoma, carcinoma in adenoma, and adenocarcinoma. Only CD105 significantly differs between the normal mucosa and adenoma or cancer (*middle three boxes*). CD31 (*upper boxes*) and VEGF (*lower boxes*) are not different between them. *n* Normal mucosa adjacent to the tumors at the level of tumor/cancer cells, *m* mucosal layers, *sm* submucosa, *mp* muscular propria, *ss* subserosa, *se* serosa. Significantly different: **p*<0.001 and ***p*<0.05. Grade of immunoreaction: (*rectangle*) weak, (*dotted rectangle*) moderate, and (*filled rectangle*) intense

was lost in the serosa of adenocarcinoma and lymph node metastasis. The expression of VEGF in the vascular endothelium did not significantly differ between normal and neoplastic mucosal layers of adenoma, cancer in adenoma, or adenocarcinoma (Fig. 5).

CD44 was not expressed in the vascular endothelium in normal tissues and in all layers of adenoma, carcinoma in adenoma, and adenocarcinoma, and was intensely expressed in the neoplastic cell membrane (Table 3). Flt-1 (cytoplasm and cell membrane), Flk-1 (cytoplasm and nuclei), TGF-β1 (cytoplasm), and TGF-βRII (cytoplasm and nuclei) were intensely expressed in neoplastic cells in all layers of adenoma, cancer in adenoma, and adenocarcinoma, but not in endothelial cells (Table 3).

Table 3 Immunohistochemical results

Markers	Vascular endothelial cells	Tumor cells
CD31	+[a]	−
CD105	+[a]	+ (cc)
CD44	−	+ (cm)
VEGF	+	+ (cc)
Flt-1	−	+ (cc, cm)
Flk-1	−	+ (cc, cn)
TGF-β1	−	+ (cc)
TGF-βRII	−	+ (cc, cn)

cc Cell cytoplasm, *cm* cell membrane, *cn* cell nucleus
[a]CD31 is mostly positive in preexisting larger blood vessels, whereas CD105 is positive in newly formed small blood vessels

Discussion

Endoglin (CD105), a cell membrane glycoprotein, was obviously upregulated in endothelial cells in de novo blood vessels of various tumors compared with those in normal tissues [3, 15, 16, 22, 27]. As far as we know, no study has compared the endothelial expression of CD105 with a lymphatic endothelium marker to exclude the idea that the CD105-stained vessels represent lymphatic vessels. The present study provides evidence that CD105 was expressed exclusively in the endothelium of newly formed blood vessels compared with D2-40, which was reported as a selective lymphatic endothelial marker [13]. Our results show no double-positive staining of CD105 and D2-40 in the same vessels (Fig. 4). CD105 is expressed in vascular endothelial cells of polypoid-type early colon cancer [20]. To our knowledge, the present study is the first to identify CD105 expression at levels corresponding to the depth of cancer invasion in the colon adenoma–carcinoma sequence (adenoma, carcinoma in adenoma, adenocarcinoma, and lymph node metastasis).

Angiogenesis always accompanies the development of cancer and metastasis, and the depth of tumor invasion of the colon wall determines the stage of cancer, which is a useful prognostic indicator and basis for the selection of appropriate therapy. We therefore considered that the expression of CD105 in each layer of the colon should be defined and compared with that of other angiogenetic markers. In this respect, we showed that CD105 was expressed at high levels in vascular endothelial cells in de novo blood vessels of the adenoma–carcinoma of the colon

and was weakly expressed (≦21%) in blood vessels of the normal mucosal layer. CD105 expression significantly differed between the normal mucosa and the corresponding layers of adenoma, carcinoma in adenoma, and adenocarcinoma ($p<0.05$, $p<0.05$, and $p<0.0001$, respectively), whereas CD31 and VEGF expression did not differ among them. This finding supports that of Akagi et al. [1], who showed that the expression of CD105 significantly differed from low-grade to high-grade adenoma and to carcinoma, whereas the MVD for a pan-endothelial marker (CD34) did not differ in the colorectal adenoma–carcinoma sequence. Moreover, when compared with CD31 and VEGF, CD105 was more intensely expressed in de novo microvessels (Fig. 3).

A higher expression of both VEGF and CD105 (MVD) correlates with a deeper invasion and lymph node metastasis in gastrointestinal cancer [30]. Our study also shows that CD105 expression is related to the depth of invasion, but not to VEGF expression (Fig. 3).

Flk-1 is expressed in the colon [24], lung [14], and prostate [2], and Flt-1 is expressed in pancreatic cancer [23, 26]. In contrast, we found that Flk-1 and Flt-1 were not expressed in vascular endothelial cells, whereas Flt-1 was intensely expressed in the cytoplasm and membrane of neoplastic cells and Flk-1 was found in the cytoplasm and nucleus of neoplastic cells.

Several studies have shown that CD31 is expressed in blood vessels in normal tissues, as well as in cancer tissues [1, 5, 7]. We found that 80–95% of the normal mucosa adjacent to the tumor and all layers in adenoma, carcinoma in adenoma, and adenocarcinoma expressed CD31.

One report describes that CD44 is expressed in the vascular endothelium [21]. However, we discovered a weak expression in lymphatic endothelial cells (data not shown) and an intense expression in normal and neoplastic cell membranes, but none in vascular endothelial cells.

The cytoplasm and nucleus of neoplastic cells expressed high levels of TGF-β1 and its receptor (TGF-βRII), whereas vascular endothelial cells expressed none.

These results confirm that CD105 was specifically expressed in de novo blood vessels of colon cancer.

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