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Cell Adhesion Molecule Expression by Vascular Endothelial Cells as an Immune/Inflammatory Reaction in Human Colon Carcinoma

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The cell adhesion of inflammatory cells to vascular endothelial cells is an important process in the recruitment of inflammatory cells to the site. In cancer tissue, infiltration of inflammatory cells has been suggested to be a mechanism of host resistance. To clarify this infiltration mechanism, we investigated cell adhesion molecule expression (E-selectin, P-selectin, and ICAM-1) in vascular endothelial cells by immunohistochemistry in colon carcinoma. Venules distributed along the invasive margin expressed E- and P-selectins and ICAM-1. These phenotypical features are identical to those of endothelial cells observed in active inflammatory lesions, and the vessels can, therefore, be designated as immunologically activated vessels. Nevertheless, the majority of blood vessels within the tumor lacked immunoreactivity for all these adhesion molecules and, therefore, could be designated as immunologically inactive vessels. Granulocytes, lymphocytes and macrophages, bearing the counter-receptors of these adhesion molecules, were more densely distributed along the invasive margin. In contrast, few inflammatory cells were present within the tumor. In conclusion, the present study has demonstrated the phenotypical heterogeneity of tumor vessels; those for inflammatory cell infiltration to the tumor and those for the nutrient supply to the tumor.

Key words: Cell adhesion molecule — Inflammatory infiltrate — Selectin — Colon cancer — Immunohistochemistry

In 1863 Virchow first identified infiltration of inflammatory cells within and at the edge of cancer tissue. The infiltration of inflammatory cells has been considered to be a manifestation of the host reaction, associated with a better prognosis in carcinomas of the breast,[1,2] stomach[3] and colon.[4–7] Lymphocytes isolated from human colon cancer were cytotoxic to cancer cells from the same patient *in vitro*.[8] On the other hand, lymphoreticular infiltration in malignant melanoma was correlated with a poor survival rate.[9] These conflicting results suggest pleiotropic functions of the inflammatory cells to cancer tissue. In colorectal carcinoma, inflammatory cells are particularly dense along the invasive margin.[7,10] However, the exact mechanism responsible for this phenomenon has not been elucidated.

Recently, it has been demonstrated that the recruitment of leukocytes to the inflammatory sites is mediated by the cell adhesion molecules expressed by vascular endothelial cells.[11–14] E-selectin (ELAM-1)[15] and P-selectin (CD62, GMP140)[16,17] contain the N-terminal lectin domain that mediates the adhesion to the carbohydrate ligands on leukocytes. These two selectins are expressed on activated endothelial cells (E- and P-selectins) and platelets (P-selectin). They are involved in the recruitment of granulocytes, cells of a monocyte/macrophage lineage, helper memory T cells and natural killer cells to the sites of inflammation.[18–21] Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin superfamily, is also expressed on endothelial cells and inflammatory cells, and binds to the leukocyte function-associated antigen-1 (LFA-1) expressed in leukocytes.[22] In inflammatory lesions, ICAM-1 is expressed in vascular endothelial cells, macrophages and lymphocytes and squamous epithelial cells.[23]

We have already demonstrated a close association between the expression of the cell adhesion molecules in venules and the degree of inflammatory cell infiltration *in situ* in inflammatory bowel disease.[23,24] These venules are considered to be "activated" from the immune/inflammatory viewpoint. In cancer tissue, most investigations on the tumor vessels have been focused on the mechanism of neovascularization and the relation between angiogenesis and tumor growth.[25,26] Blood vessels have been regarded merely as "ducts" to supply the nutrients to cancer tissue.

In the present study, we investigated the relationship between the expression of the cell adhesion molecules in blood vessels and the distribution of inflammatory cells bearing their counter-receptors. E- and P-selectins were mainly analyzed since they are the best markers of activated venules.[23] Vascular cell adhesion molecule-1 (VCAM-1) was not studied because it was not expressed along the luminal plasma membrane of activated venules

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either in inflammatory bowel disease[23] or in cancer tissue (our preliminary data). We will show that there is a phenotypical heterogeneity among blood vessels in the tumor tissue; i.e., blood vessels along the invasive margin functioning as conduits of an immune/inflammatory reaction, and blood vessels within the tumor which function as nutrient vessels.

MATERIALS AND METHODS

Material Twenty surgically resected colon carcinoma samples were obtained at Tohoku University Hospital and Tohoku Rosai Hospital. All the patients were Japanese. No patient had received pre-operative adjuvant chemotherapy. Immediately after resection, small slices of specimens, 5×5×2 mm in size, were fixed in periodate-lysine-4% paraformaldehyde (4% PLP) for 6–8 h. After having been washed in phosphate-buffered saline (PBS) containing 10%, 15% and 20% sucrose, the specimens were embedded in OCT compound (Miles, Elkhart, IN) and rapidly frozen in acetone-dry ice. For the control, normal-appearing colon tissues obtained from regions remote from the carcinoma were used. Colon carcinomas were either well or moderately differentiated adenocarcinoma, including 10 cases of Dukes B (cancer invading beyond the muscularis propria without metastasis), two cases of Dukes C (cancer with lymph node metastasis) and eight cases of Dukes D (cancer with liver metastasis).

Immunohistochemistry Frozen sections, 6 μm in thickness, were cut and mounted on ovalbumin-coated glass slides. They were immersed in non-immunized sheep serum, and the indirect immunoperoxidase method was applied. The primary antibodies (listed in Table I) were applied to the sections for 24 h. Endogenous peroxidase activity was blocked by treating the slides with 0.3% hydrogen peroxidase in methanol for 12 min after the incubation with the primary antibodies. The secondary antibodies were sheep peroxidase-conjugated $F(ab')_2$ fragment of anti-mouse and anti-rat immunoglobulins (Amersham, UK). These were diluted 1:150 with PBS containing 5% human serum and applied overnight. The enzymatic reaction was performed in 0.03% 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.006% H_2O_2 and 0.065% sodium azide. For endogeneous peroxidase staining, frozen sections were directly immersed in DAB/H_2O_2 solution for 5 min.

Morphometrical analysis of immune/inflammatory cells The numbers of $CD3^+$ and $CD4^+$ lymphocytes and granulocytes as revealed by the endogenous peroxidase activity were counted along the invasive margin using frozen sections of 20 cases of colon carcinomas. Three representative fields were chosen in each case where apparently average numbers of inflammatory infiltrates were present. Immunoreactive cells bearing a nucleus were counted in microgrids using a ×400 field. One grid covered an area of 0.065 mm^2. $CD68^+$ macrophages were counted by the same method using formalin-fixed, paraffin-embedded blocks in 63 colorectal carcinomas including 40 cases without liver metastasis and 23 cases with liver metastasis. The average numbers were compared between the groups with and without liver metastasis and analyzed by use of the *t* test.

Immunoelectron microscopy The staining procedure has already been described.[24, 27] Immersion in 0.3% H_2O_2/methanol was replaced by immersion in 0.3% H_2O_2/0.1% sodium azide/PBS for 10 min. After DAB reaction, the specimens were fixed in 1% osmium tetroxide

Table I. List of Monoclonal Antibodies Used

Monoclonal antibody	Specificity	Sources	Working dilution
E-selectin	Activated endothelial cells	British Biotechnology (BBA8)	1:200
		Becton-Dickinson (clone H18/7)	1:500
P-selectin	Activated endothelial cells and platelets	Takara (clone WGA1)	1:500
		Becton-Dickinson (clone AC1. 2)	1:500
E-+P-selectin	Activated endothelial cells and platelets	British Biotechnology (BBA1)	1:500
ICAM-1	Endothelial cells, activated macrophages, etc.	Immunotech (clone 84H10)	1:300
Sialyl Lewis^x	Granulocytes, macrophages, etc.	Dr. Y. Fukushi (clone FH6)	1:20
CD31	Endothelial cells and some lymphocytes	DAKO (clone JC/70A)	1:100
Laminin	Basement membrane	Immunotech (clone 4C12.8)	1:200
CD3	Mature T lymphocytes	Becton-Dickinson (clone SK7)	1:100
CD4	Helper/inducer T lymphocytes	Nichirei (clone NU-$T_{H/4}$)	1:32
CD68	Monocytes/macrophages	DAKO (clones EBM11 and PGM1)	1:500

for 1 h, dehydrated with ethanol and embedded in Epon. Ultrathin sections were stained with lead citrate for 2 min and observed with a JEM-100B electron microscope.

RESULTS

Identification of tumor vessels Vascular structure was confirmed on the basis of immunoreactivity for laminin and CD31. Laminin staining revealed the basal lamina of blood vessels, nerve fibers and epithelial cells. CD31 was positive in vascular endothelial cells and some lymphocytes and macrophages. In cancer tissue, laminin staining was useful for the identification of blood vessels.[28] Tumor vessels were classified into two groups based on the location; i.e., blood vessels in the stroma in close proximity to tumor cells described as "vessels within the tumor" and blood vessels along the invasive margin (Fig. 1A). The vascular reaction described below was more typically observed along the invasive margin in the submucosa and subserosa than in the muscularis propria.

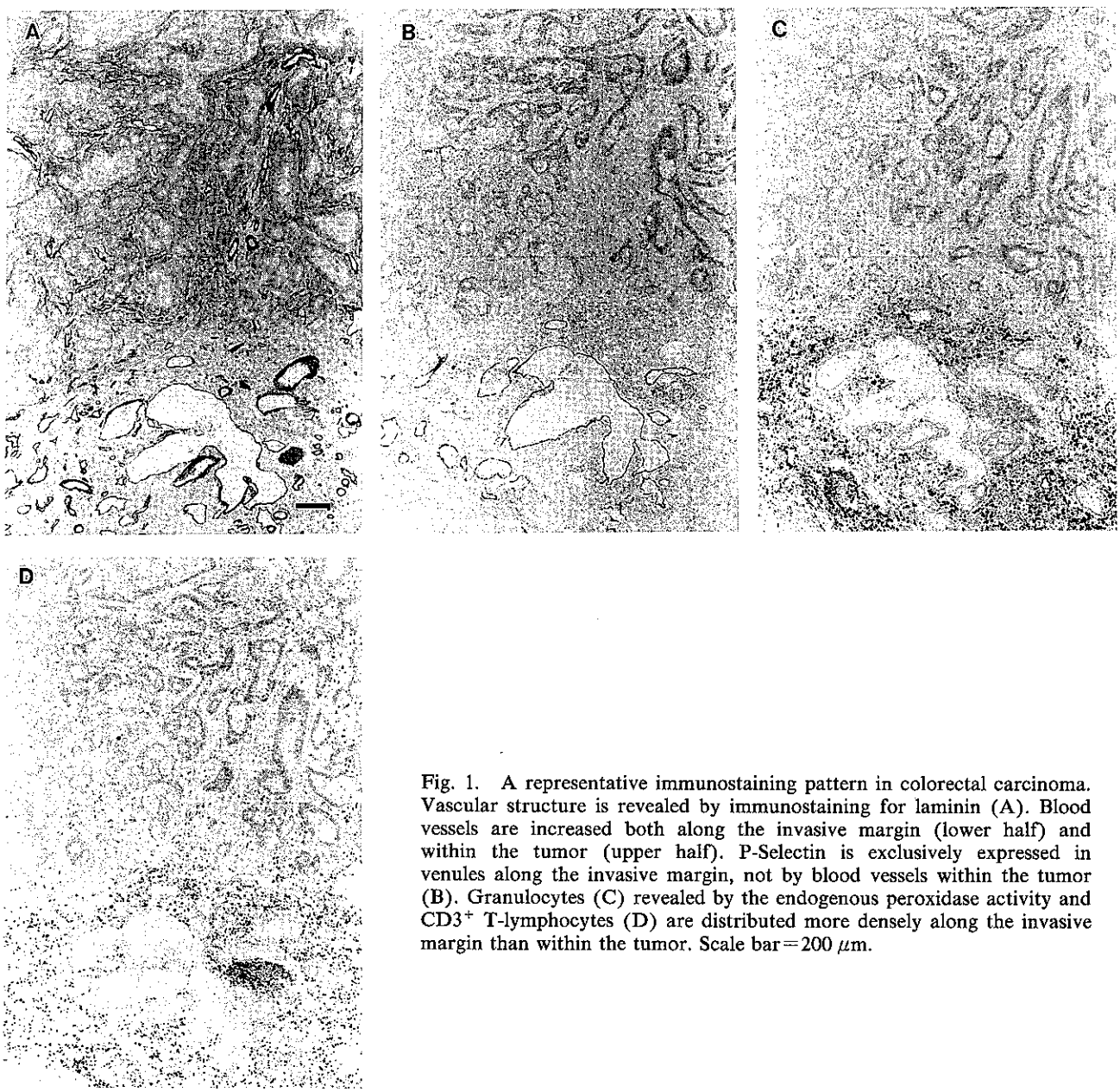

Fig. 1. A representative immunostaining pattern in colorectal carcinoma. Vascular structure is revealed by immunostaining for laminin (A). Blood vessels are increased both along the invasive margin (lower half) and within the tumor (upper half). P-Selectin is exclusively expressed in venules along the invasive margin, not by blood vessels within the tumor (B). Granulocytes (C) revealed by the endogenous peroxidase activity and $CD3^+$ T-lymphocytes (D) are distributed more densely along the invasive margin than within the tumor. Scale bar = 200 μm.

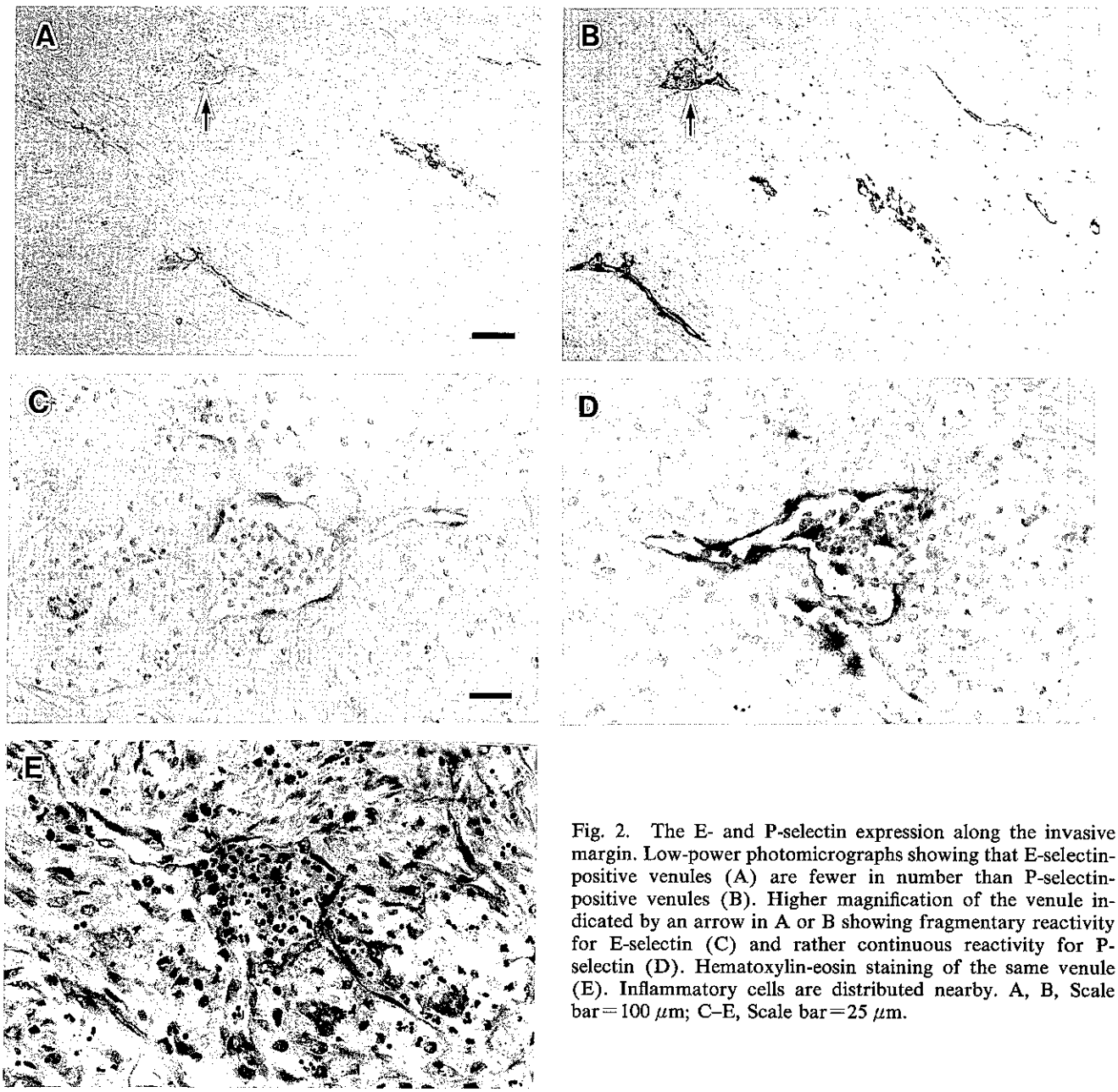

Fig. 2. The E- and P-selectin expression along the invasive margin. Low-power photomicrographs showing that E-selectin-positive venules (A) are fewer in number than P-selectin-positive venules (B). Higher magnification of the venule indicated by an arrow in A or B showing fragmentary reactivity for E-selectin (C) and rather continuous reactivity for P-selectin (D). Hematoxylin-eosin staining of the same venule (E). Inflammatory cells are distributed nearby. A, B, Scale bar = 100 μm; C–E, Scale bar = 25 μm.

Immunohistochemical study

1) Colorectal carcinoma: In colorectal carcinoma, blood vessels increased within the tumor and along the invasive margin as revealed by laminin (Fig. 1A) or CD31 staining. Venules with a relatively wide lumen, 10–100 μm in diameter, were observed along the host side of the invasive margin. Nearly all venules consistently expressed P-selectin and ICAM-1 in all cases (Figs. 1B, 2A, 2B and 3), and approximately half of them expressed E-selectin (Fig. 2, A–D). This difference was not an artifact because dilutions of the two antibodies were determined consistently to label venules in the ulcer base of inflammatory bowel disease.[23] CD41 staining revealed immunoreactive platelets in the vascular lumen and no staining was observed in endothelial cells (data not shown, see ref. 23). The phenotypical features of these venules were the same as those of "immunologically activated venules" in inflammatory lesions.[23] In contrast, most of the blood

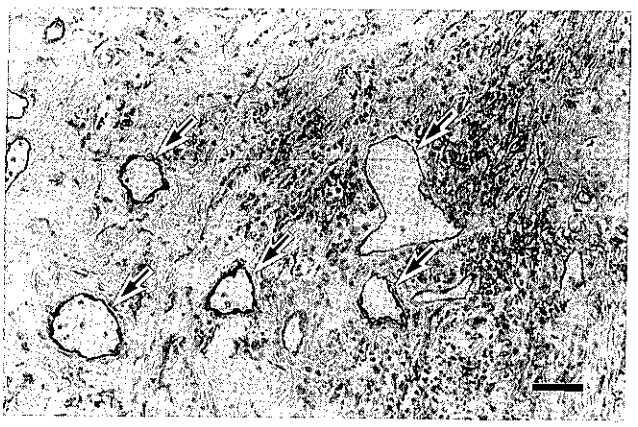

Fig. 3. ICAM-1 staining along the invasive margin. Venules are positive (arrows), as well as inflammatory cells. Scale bar = 50 μm.

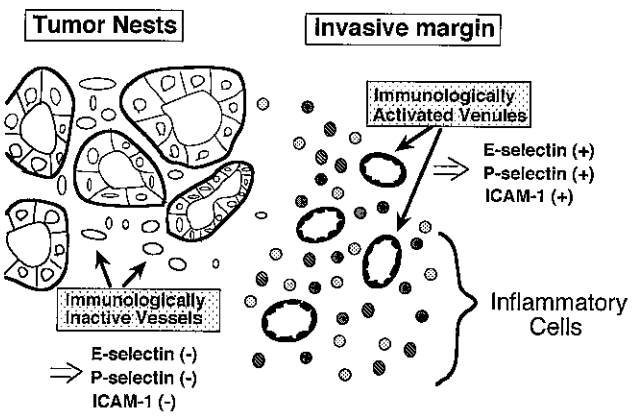

Fig. 4. Schema of the results.

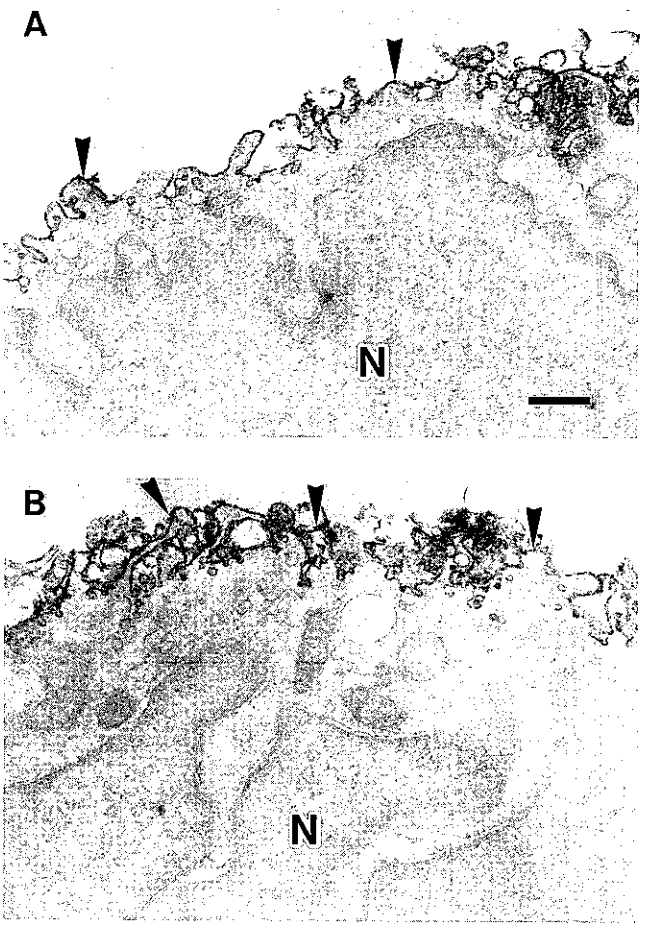

Fig. 5. Immunoelectron microscopy for E- (A) and P-selectins (B) in colon carcinoma. Note continuous reactivity (revealed by black deposits) along the luminal cell membrane of endothelial cells (arrowheads). N; nucleus. Scale bar = 0.5 μm.

vessels within the tumor identified by laminin staining lacked immunoreactivity for E- and P-selectins and ICAM-1 in 19 of 20 cases (Fig. 1, A and B). We have defined these vessels as "immunologically inactive vessels" (see "Discussion" for details). In the area between clusters of tumor cell nests (i.e., the intervening stroma), a part of the blood vessels was positive for E- and P-selectins and ICAM-1 (data not shown). In one case, venules positive for E- and P-selectins appeared in the intervening stroma to the same degree as along the invasive margin.

Distribution patterns of inflammatory cells are shown in Fig. 1, C and D. Granulocytes (revealed by the endogenous peroxidase activity), $CD3^+$ and $CD4^+$ cells, and macrophages (revealed by CD68) were all distributed more densely along the invasive margin. In contrast, these inflammatory cells were fewer within the tumor. The area densely populated by inflammatory cells corresponded to the area where venules expressed the cell adhesion molecules. This area extended along the invasive margin, being 1–2 mm in width. This relationship was confirmed in 15 of 20 cases for $CD3^+$ and $CD4^+$ cells, in 13 of 20 cases for granulocytes and in 15 of 20 cases for macrophages. Other cases showed inflammatory cells also within the tumor (mainly in the intervening stroma) to nearly the same degree as in the invasive margin.

There were many sialyl $Lewis^x$-positive inflammatory cells (granulocytes, macrophages and some lymphocytes) along the invasive margin (data not shown), and carcinoma cells were occasionally positive.

The results are schematically summarized in Fig. 4.

2) Changes at the ulcer base: Carcinoma tissues were frequently associated with ulceration. Ulcer bases in car-

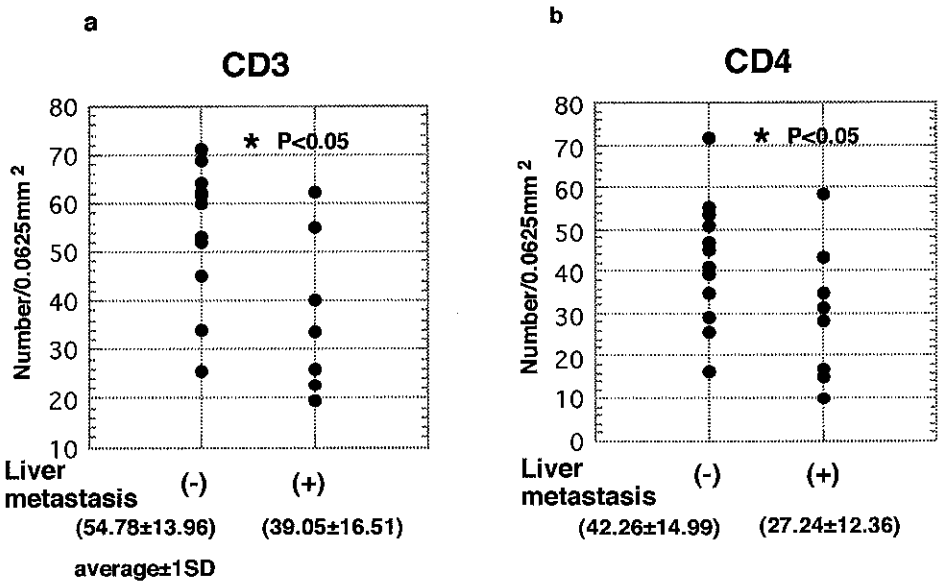

Fig. 6. Cell counting of $CD3^+$ and $CD4^+$ lymphocytes along the invasive margin in colon carcinoma. The number is statistically significantly larger in cases without liver metastasis than in cases with liver metastasis ($P<0.05$).

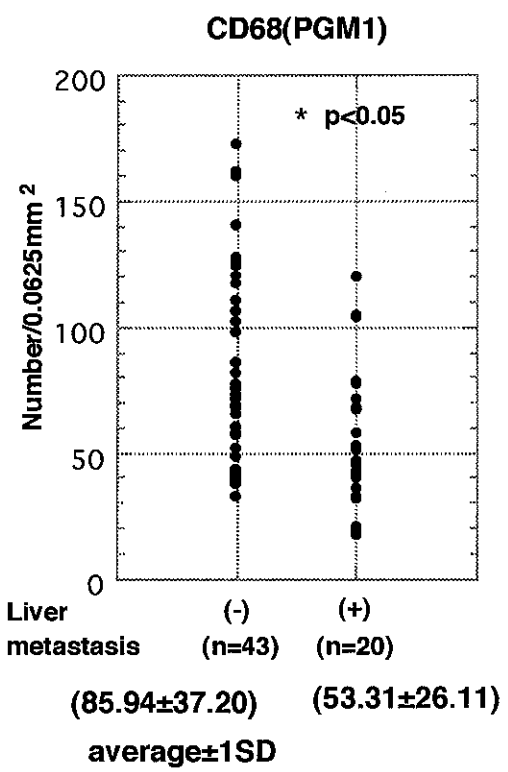

Fig. 7. Cell counting of $CD68^+$ macrophages along the invasive margin in colon carcinomas using paraffin blocks. The number is larger in cases without liver metastasis than in cases with liver metastasis ($P<0.05$).

cinoma abounded with venules expressing E- and P-selectins and ICAM-1 (data not shown). These vascular reactions were apparently secondary to inflammatory changes at the ulcer bases, as in the ulceration area in inflammatory bowel disease.

3) Normal tissue: Endothelial cells of venules were only sporadically positive for E- and P-selectins, as stated in our previous paper.[23, 24] While total vessels were identified by laminin and CD31 expression, ICAM-1 was consistently expressed in most of the capillaries and venules in the mucosa.[23] Small numbers of granulocytes and lymphocytes were observed in the lamina propria.

Immunoelectron microscopy Immunoreactivity for E- and P-selectins was continuously localized along the luminal plasma membrane of endothelial cells of venules in the invasive margin (Fig. 5, A and B). This subcellular localization is consistent with their transmembrane receptor protein character. This also excluded the possibility that immunoreactivity for P-selectin represents platelets aggregated on endothelial cells. Occasionally, E- and P-selectins were observed in round granules[24, 27] and in the Weibel-Palade bodies in endothelial cells, respectively (data not shown). We did not observe remarkable expression of E-selectin in rough endoplasmic reticulum or frequent occurrence of exocytosis of E-selectin into the vascular lumen, as observed in inflammatory bowel disease.[24] This suggested that endothelial activation in the present study was not as marked as in the active inflammatory area in inflammatory bowel disease.

Morphometrical analysis of inflammatory cells The numbers of $CD3^+$ and $CD4^+$ lymphocytes and macrophages distributed along the invasive margin were significantly smaller in cases with liver metastasis than in cases without liver metastasis (Figs. 6 and 7). $CD8^+$ cells were fewer than $CD4^+$ cells and were not measured, because helper memory T cells have been reported to have a variant of sialyl-Lewis^x antigen.[21]

There was no significant difference in the distribution of E- or P-selectin-positive vessels between the two groups.

DISCUSSION

It is now well documented that cell adhesion molecules play important roles in tumor invasion and metastasis.[29] However, we were interested in the significance of the expression of cell adhesion molecules from a different standpoint, i.e., the immune/inflammatory reaction to tumor growth. We showed that: a) there was a close association between the expression of cell adhesion molecules on venules and the distribution of inflammatory cells, and b) a phenotypical difference was observed between blood vessels along the invasive margin and blood vessels within the tumor.

In colon carcinomas, venules expressing the cell adhesion molecules occurred specifically along the invasive margin, where inflammatory cells were accumulated. In contrast, blood vessels within the tumor were usually negative for these adhesion molecules, and fewer inflammatory cells were present. The situation along the invasive margin is similar to that of inflammatory lesions. Therefore, our data suggest that the infiltration of inflammatory cells in cancer tissue is also regulated by the cell adhesion molecules expressed by vascular endothelial cells. We have already defined vessels in active inflammatory lesions as "immunologically activated vessels."[23] Our recent study further clarified that E-selectin was restricted to more active inflammatory areas than P-selectin in inflammatory disease (Nakamura *et al.*, unpublished data). The present data on E- and P-selectins are consistent with that.

In colorectal carcinoma, increased infiltration of eosinophils,[6] macrophages[4] and lymphocytes[5] is associated with a better survival rate. Furthermore, the inflammatory cells along the invasive margin are a more significant indicator of prognosis than those observed in the central part of the carcinoma.[5, 10] We have also shown that T-lymphocytes and macrophages present along the invasive margin are more abundant in colon cancer without liver metastasis than in that with liver metastasis. Therefore, we speculated that immune/inflammatory cells function as a defensive mechanism against cancer cell metastasis.

The lack of E-selectin has been noted in blood vessels within basal cell carcinoma of the human skin.[30] In an experimental model, the interaction between leukocytes and vascular endothelial cells was diminished in tumor microvessels.[31] Tumor necrosis factor (TNF)-α and interleukin-1 stimulate the expression of E- and P-selectins and ICAM-1.[22] Therefore, markedly diminished expression of the cell adhesion molecules within the tumor may be a result of a lack of stimulation by these cytokines. However, TNF-α mRNA was detected within the tumor in colon carcinoma.[32, 33] Therefore, we speculate that endothelial cells within the tumor lack the capacity to express the cell adhesion molecules owing to the influence of certain microenvironments in the tumor. We named these vessels "immunologically inactive vessels." They probably function solely as nutrient vessels for the tumor cells. We have already described the morphological details of these vessels. Blood vessels in the stroma within the tumor were characterized by swelling of endothelial cells and narrowing of the lumen, a feature of immaturity.[34] These vessels may proliferate in response to a stimulus from the tumor cells, such as vascular endothelial growth factor (VEGF), which is believed to be secreted from cancer cells and to bind to vascular endothelial cells.[35]

Several carbohydrate antigens, previously defined as cancer-associated antigens, are ligands for selectins, including sialyl Lewis^x and sialyl Lewis^a (CA19-9),[18, 36–38] of which the former is also localized in inflammatory cells. Many reports have described the correlation between metastasis and the expression of these carbohydrate antigens in cancer cells.[29, 39] However, the present study revealed that the major cells expressing sialyl Lewis^x antigen are immune/inflammatory cells in human colon carcinomas. This difference may be a consequence of the difference of methodology; we used frozen sections in which glycolipids were well preserved.[40]

Our results may have important implications for cancer therapy; e.g., modulation of the phenotypical characters of blood vessels in the tumor may enhance the access of immuno-competent cells. Further clinicopathological study is needed to clarify the pathophysiological significance of the immune/inflammatory changes in human tumors.

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