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Microvascular Changes in the Stroma of Human Colorectal Carcinomas: Ultrastructural Histochemical Study

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Ultrastructural histochemical studies were performed using *Ulex europaeus* agglutinin-I lectin (UEA-I) and anti-endothelial monoclonal antibody BMA 120 in order to morphologically characterize the microvasculature of human colorectal carcinomas. In the normal mucosa, UEA-I and BMA 120 were bound to luminal plasma membrane of endothelial cells, usually continuously. Capillaries in the stroma of invasive adenocarcinomas showed remarkable structural changes such as swelling of endothelial cells with well-developed cell organelles and narrowing of the lumen. Reaction products for UEA-I and BMA 120 were both observed along the luminal plasma membrane of endothelial cells, usually discontinuously, partially retaining the features of normal capillaries. Furthermore, we have confirmed an occurrence of solid capillary buds composed of a strand of large endothelial cells with a trace of lumen. They showed almost no reactivity to UEA-I and BMA 120. Conventional electron microscopy revealed that they were present frequently in invasive carcinomas, but infrequently in intramucosal carcinomas and inflammatory lesions. Our results suggest that the stroma of invasive colorectal carcinomas abounds in immature capillaries and their precursors, which may indicate active tumor-induced angiogenesis.

Key words: Capillaries — Carcinoma — Histocytochemistry — Ultrastructure — Lectin

Tumor cells induce angiogenesis, a process mandatory for solid tumor growth, by secreting endothelial growth factors.[1,2] Tumor-induced vessels are characterized by morphological changes such as deformity, looping, proliferation and dilatations.[3,4] They are also hyperpermeable to plasma proteins.[5] Few ultrastructural studies on the microvasculature of human carcinomas have been reported,[6,7] however, no ultrastructural histochemical study has appeared except for our study on factor VIII/von Willebrand factor (vWF).[8] *Ulex europaeus* agglutinin I lectin (UEA-I) and mouse monoclonal antibody BMA-120 are known to be markers of vascular endothelial cells.[9,10] Ultrastructural lectin histochemical studies have been done on blood vessels of experimental animals,[11,12] but not so far on human materials except for one case of Kaposi's sarcoma.[13] In the present study, we first morphologically characterized the microvasculature of colorectal carcinoma by ultrastructural histochemistry using UEA-I and BMA 120. Next, we focused our attention on the occurrence of solid capillary buds, which represent capillaries in the earliest stage of development. We describe their distribution in various lesions to show their specificity to invasive carcinomas.

MATERIALS AND METHODS

Ultrastructural histochemistry of UEA-I Surgically resected specimens were obtained from Japanese patients with invasive colorectal carcinomas (11 cases = 5 well and 6 moderately differentiated adenocarcinomas[14]), chronic gastric ulcers (three cases) and Crohn's disease of the ileum (one case). The total number of cases examined by this method was 15. In three cases, normal-appearing mucosa remote from carcinoma was also observed. Fresh specimens, $5 \times 5 \times 2$ mm size, were fixed in 4% paraformaldehyde for 6–8 h and frozen after being washed in phosphate-buffered saline (PBS) containing sucrose. Frozen sections (6-μm thick) mounted on ovalbumin-coated glass slides were immersed in PBS containing 0.5% bovine serum albumin (BSA) for 10 min and incubated with biotinylated UEA-I (20 μg/ml in PBS with 0.5% BSA, Vector Laboratories, Burlingame, CA) for 30 min, followed by incubation with avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Lab.) for 30 min. After fixation in 1% glutaraldehyde for 10 min, the enzymatic reaction was done in 0.03% 3,3′-diaminobenzidine tetrahydrochloride (DAB) with 0.005% hydrogen peroxide for 5–10 min. The specimens were postfixed in 1% osmium tetroxide for 15 min, dehydrated and embedded in Epon 812. Ultrathin sections were stained with lead citrate for 1 min and observed with a JEOL 100B electron microscope. For the specificity control, UEA-I solution was adsorbed with α-L-fucose (20 n*M*) prior to the incubation. This procedure completely blocked the specific reactions. For the study of cancer stroma, areas containing carcinoma cells with desmoplastic stroma were selected. Areas with massive neutrophilic infiltration or necrosis were carefully excluded.

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Immunoelectron microscopy using BMA 120 Methods of fixation and processing were essentially the same as in UEA-I histochemistry. Monoclonal antibody BMA 120 (Behring Diagnostics, Marburg, West Germany, diluted at 1:3) was used as the primary antibody, and a Vectastain ABC Kit was applied in order to raise the sensitivity of immunoreaction. In some cases, HRP-conjugated $F(ab')_2$ fragments of anti-mouse IgG (Cappel Worthington, Malvern, PA; 1:20) were applied overnight as the secondary antibody. Seven cases of colorectal carcinomas and one case of normal mucosa were selected from the cases used in UEA-I histochemistry and observed by this method.

Conventional electron microscopy Ultrathin sections used in our previous study on stromal cells of human colorectal tumors[15] were reviewed for microvasculature in the stroma with some cases added (Table I, total of 37 cases, different from those examined for ultrastructural histochemistry).

RESULTS

Ultrastructural UEA-I histochemistry In the normal mucosa, the luminal plasma membrane of capillaries was labeled with UEA-I, usually continuously and occasionally discontinuously (Fig. 1). Besides this, endothelial abluminal plasma membrane and plasma membrane of pericytes were focally positive (Fig. 1).

In invasive colorectal carcinomas (11 cases), endothelial cells showed hypertrophy with well developed

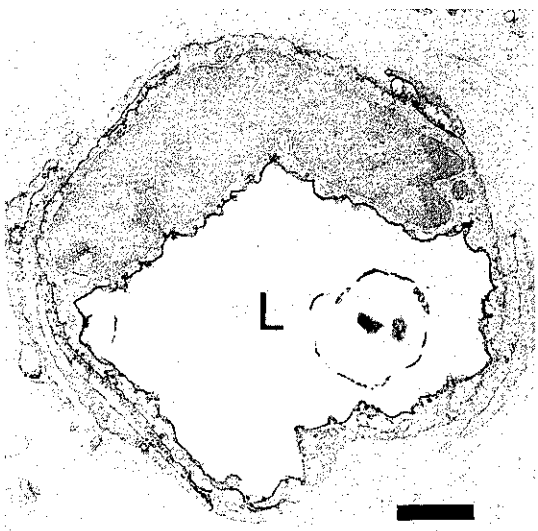

Fig. 1. A capillary in the normal mucosa of ascending colon (UEA-I histochemistry). ×9,900. L=lumen. Scale bar=1 μm.

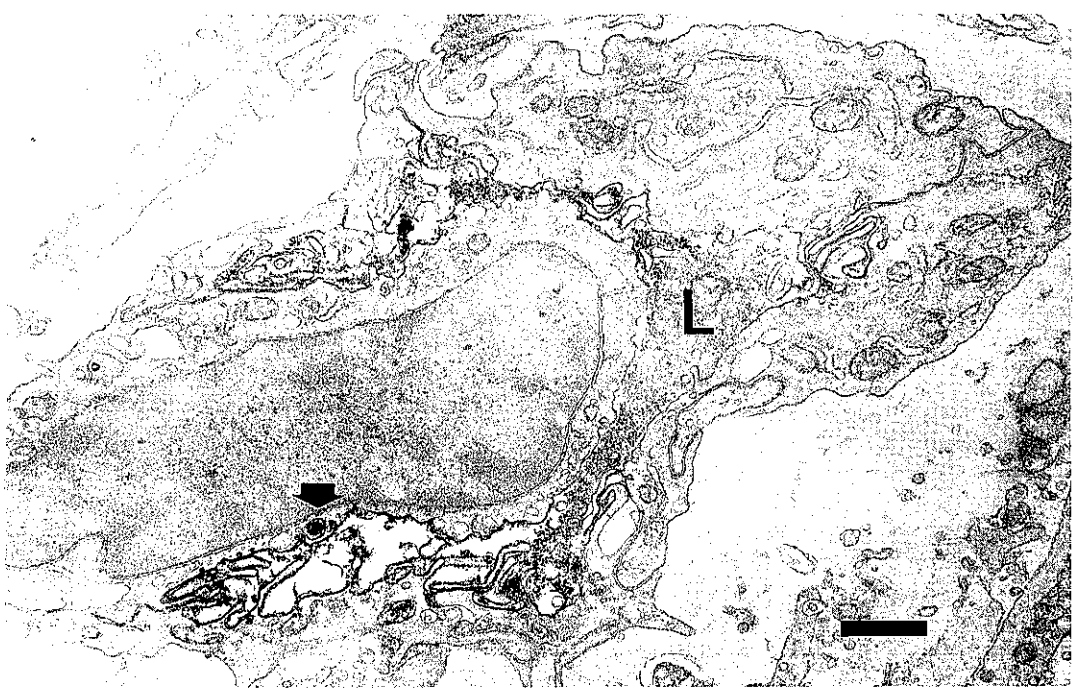

Fig. 2. A typical capillary in the stroma of colonic carcinoma (UEA-I histochemistry). The luminal plasma membrane is discontinuously positive. An arrow indicates a granule with a positive reaction. ×11,500. L=lumen. Scale bar=1 μm.

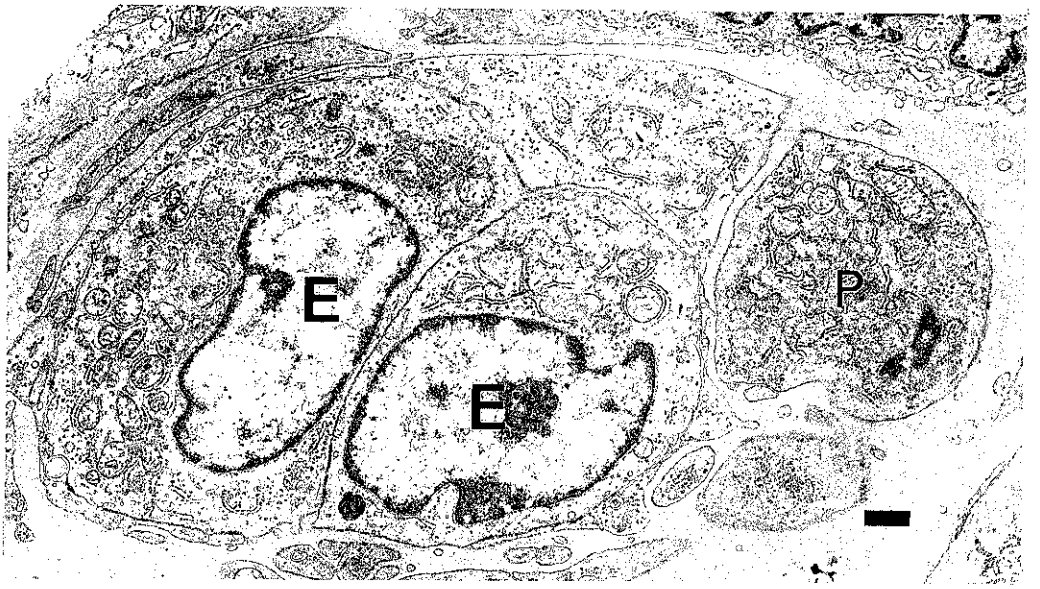

Fig. 3. A soild capillary bud in the stroma of carcinoma (conventional electron microscopy). Luminal differentiation is nearly absent and no pinocytotic vesicles are observed. P=pericyte, E=endothelial cell. ×6,000. Scale bar=1 μm.

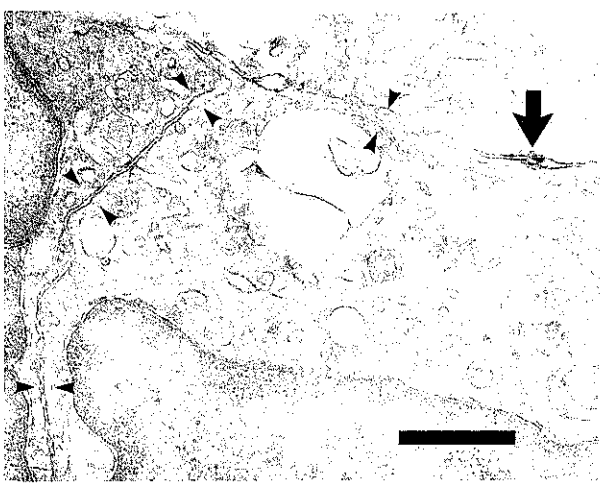

Fig. 4. A part of a solid capillary bud in the stroma of carcinoma (UEA-I histochemistry). Note that most of the plasma membrane is devoid of reactivity (arrowheads) except for focal positive reactivity (arrow). ×15,300. Scale bar=1 μm.

rough endoplasmic reticulum (rER) and narrowing of the lumen, with mature type capillaries being rather rare, as described in our previous study.[8] Reactivity to UEA-I was observed along the luminal plasma membrane of endothelial cells with a discontinuous pattern predominating over a continuous one (Fig. 2). Focal reactivity was occasionally observed along the endothelial abluminal plasma membrane and plasma membrane of pericytes. Cytoplasmic round granules, 200–300 nm in diameter, were rarely positive (Fig. 2). No significant differences were noted among the 11 cases. Solid capillary buds, composed of strands of large endothelial cells (Fig. 3), were detected in 4 cases by this method. They lacked reactivity to UEA-I except for occasional focal reactivity (Fig. 4).

In gastric ulcers and Crohn's disease (4 cases), capillaries were labeled with UEA-I along the luminal plasma membrane, usually continuously (data not shown). Endothelial hypertrophic change was observed but mature-type capillaries were frequently admixed. Solid capillary buds were not detected.

Immunoelectron microscopy using BMA 120 Luminal plasma membranes of capillaries in the normal mucosa were continuously labeled (data not shown). There was no difference in the results by ABC and the indirect methods, except for lower sensitivity in the latter case. In the stroma of carcinomas (seven cases), the immunoreactivity was also observed along endothelial luminal plasma membrane, usually discontinuously (Fig. 5). Solid capillary buds were detected in one of seven cases. They were completely devoid of immunoreactivity (data not shown). Intracytoplasmic immunoreactivity was not observed in normal mucosa or cancer stroma.

Conventional electron microscopy for solid capillary buds Conventional electron microscopy was required for

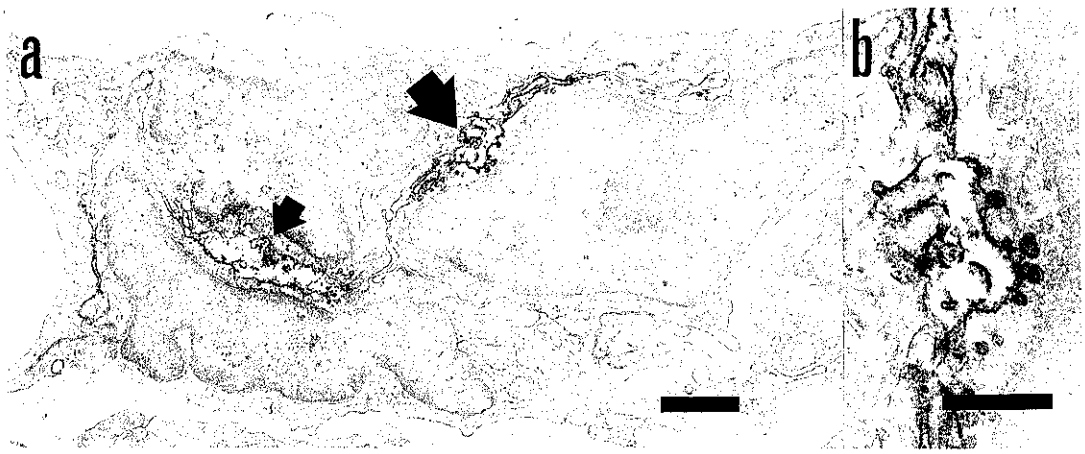

Fig. 5. a. An oblique section of a capillary in the stroma of colonic carcinoma stained with BMA 120. Parts of slit-like lumen are positive (arrows). This vessel is probably in an early stage of differentiation. ×9,900. Scale bar=1 μm. b. Higher magnification of slit-like lumen in the area indicated by the larger black arrow in Fig. 5a. Note pinocytotic vesicles with positive reaction. ×28,000. Scale bar=0.5 μm.

Table I. Occurrence Rate of Solid Capillary Buds by Conventional Electron Microscopy

	Numbers of positive cases/ numbers of total cases
Normal mucosa	0/3
Adenoma	0/3
Adenoma with severe atypia	1/3
Intramucosal portion[a)] of invasive carcinoma	0/4
Invasive carcinoma (with desmoplastic stroma)	13/16
Ulcerative colitis (active inflammatory phase)	1/8

a) This indicates carcinoma tissue with the lamina propria as stroma, obtained from the surface area of polypoid early submucosal invasive carcinomas.

the consistent detection of solid capillary buds. The buds were composed of solid strands of large endothelial cells with virtually no luminal differentiation (Fig. 3). Pinocytotic vesicles were sparsely observed between apposing plasma membrane. They were detected in 11 out of 16 cases of invasive adenocarcinomas (Table I). They were particularly frequent in 4 cases. Solid capillary buds were not detected in adenomas or the intramucosal portion of adenocarcinomas. They were detected in one of the 3 adenomas with severe atypia, corresponding to intramucosal carcinomas, and in one of the 8 cases of ulcerative colitis (Table I).

DISCUSSION

An increase of cytoplasmic volume of endothelial cells with an abundance of cell organelles is known to be a common feature of the microvasculature of experimentally induced tumors[16,17] and human tumors.[6,7] The similarity of these vessels to newly formed, immature capillaries has also been pointed out.[17] The present study has revealed that capillaries in cancer stroma with the above-mentioned structural changes partially retained the UEA-I and BMA 120 labeling patterns of normal capillaries; i.e. some of the capillaries lose these phenotypes, probably due to their immaturity, while others show the same pattern as in normal capillaries. These results may indicate that stroma of our cases contains capillaries in various developmental stages.

In the present study we did not detect significant differences in labeling patterns of UEA-I between microvasculature in carcinoma stroma and granulation tissue. This contrasts with our previous results[8]; remarkable labeling of vWF was observed in rough endoplasmic reticulum in carcinoma stroma while it was not observed in capillaries in granulation tissue. These results are intriguing, showing both similarity and difference of microvasculature between carcinoma stroma and granulation tissue.

Next we focused our attention on the occurrence of solid capillary buds. It is already known that solid buds of this type are present in an early stage of angiogenesis in experimental studies[18,19] and in hemangioma.[20] But precise ultrastructural identification has not been reported in cancer stroma of human surgical materials as

far as we know. In the present study we have confirmed that there is very little labeling of UEA-I or BMA 120 in solid capillary buds. This is probably due to immaturity. Since no other ultrastructural histochemical studies dealing with such immature capillaries have been published so far, further studies will be necessary to establish the details. Our observation by conventional electron microscopy revealed frequent occurrence of buds in invasive carcinomas, which suggests that this phenomenon can be regarded as one of the stromal reactions induced by invasive growth of carcinoma cells beyond the muscularis mucosae. Furthermore, the low occurrence rate in inflammatory lesions may suggest that newly-formed capillaries induced by carcinoma differ morphologically from those in inflammatory lesions in human materials. These points require further studies.

Several peptides are known to be angiogenetic at present: acidic and basic fibroblast growth factors (FGF), angiogenin, transforming growth factor-α (TGF-α), and TGF-β, and tumor necrosis factor (TNF)-α.[21, 22] Therefore, it is reasonable to speculate that the microvessels we have observed are induced by such endothelial cell growth factors derived directly or indirectly from carcinoma cells. Peres *et al.*[23] suggested that there is a network of humoral factors among cancer cells, fibroblasts and vascular endothelial cells, and these cells are mutually influenced. This hypothesis is attractive, since we have observed concomitant morphological activation of fibroblasts and vascular endothelial cells in the stroma of human colorectal carcinoma.[8, 15] Roberts *et al.*[24] proposed that the mode of release of growth factors is crucial for the differentiation between the finite process of tissue repair and the continuous growth of carcinoma stroma. It is possible that frequent occurrence of solid capillary buds may be related to the continuous effects of growth factors.

In conclusion, our study suggests the importance of morphologically characterizing tumor vessels of human materials in order to elucidate the tumor-host relationship. Further studies on other carcinomas are needed to deepen our knowledge.

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