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Differing microvasculature in the two major types of gastric carcinoma: a conventional, ultrastructural and ultrastructural immunolocalization study of von Willebrand factor

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Summary. The microvasculature of the stroma of human gastric carcinoma was studied by immuno-electron microscopy for factor VIII/von Willebrand factor (vWF) and conventional electron microscopy. In differentiated type (intestinal) gastric carcinoma (9 cases), capillaries were distributed more densely around carcinoma cell nests. vWF was localized in endothelial cells and neighbouring stroma. Ultrastructurally, capillary endothelial cells showed considerable hypertrophic changes with well-developed rough endoplasmic reticulum (rER). vWF was localized in well-developed rER, granules, Weibel-Palade bodies (WPB), in the vascular lumen as clusters, and diffusely deposited in the subendothelium. This indicates that endothelial cells in this group are transformed into a state of active protein production. In undifferentiated type (diffuse) gastric carcinoma (12 cases), capillaries were uniformly distributed and endothelial hypertrophic changes were less remarkable. vWF was localized in WPB, scanty rER and subendothelial matrix. Solid capillary buds were observed in both types; they were composed of a solid strand of endothelial cells without a visible lumen. Our results reveal that the microvasculature in tumour stroma differs significantly according to its histological type.

Key words: Gastric carcinoma – Capillaries – von Willebrand factor – Ultrastructure – Immuno-electron microscopy

Introduction

Carcinomas are composed of tumour cells and a stroma. In recent years, much attention has been paid to the stroma to elucidate carcinoma-stromal interactions including the ability of proteases to initiate carcinoma cell invasion (Liotta et al. 1983; Pauli and Knudson 1988), the mechanism of stromal fibrosis (Roberts et al. 1988) and neovascularization (Folkman 1984). Through these studies, the concept of growth factors has become one of the keys in elucidating stromal reactions (Folkman and Klagsbrun 1987). The deposition of fibrin is also postulated as an important initial step in activation of the stromal reaction (Dvorak 1986; Dvorak et al. 1987). The similarity between the stromal reactions in cancer and the tissue repair process has been stressed (Dvorak 1986).

Our previous work has revealed that stromal fibroblasts/myofibroblasts were morphologically activated by cancerization of epithelial cells (Ohtani and Sasano 1983). This indicates that a certain biological activity of carcinoma cells is closely related to the production of matrix proteins such as collagens and glycosaminoglycans by fibroblasts. Vascular endothelial cells in colonic carcinoma also show morphological features suggestive of activity (Wang and Campiche 1982; Ohtani and Sasano 1987). This implies that tumour-induced neovascularization in human carcinomas does not simply mean proliferation of capillaries, but it is also associated with certain functional changes in the vascular endothelial cells.

Endothelial cells also play crucial roles in the immune responses (Beilke 1989). They express class II immune response genes and the interleukin-1 gene during infection. Morphological studies have elucidated that capillaries also carry the surface antigens common to cells of a macrophages/monocytes lineage and that there is the phenotypical heterogeneity among capillaries in various tissues and lesions (Yamamoto et al. 1988; Takeuchi et al. 1988a; Kinjo et al. 1989; Komatsu et al. 1989). These suggest that the micro-environment is a determinant factor of capillary phenotypic expression. Considering these, it is probable that endothelial cells in cancer stroma may also be heterogeneous according to the difference of micro-environment. In the present investigation we have extended our study to gastric carcinoma to elucidate how histological types of carcinoma determine the morphological changes of microvasculature in the stroma.

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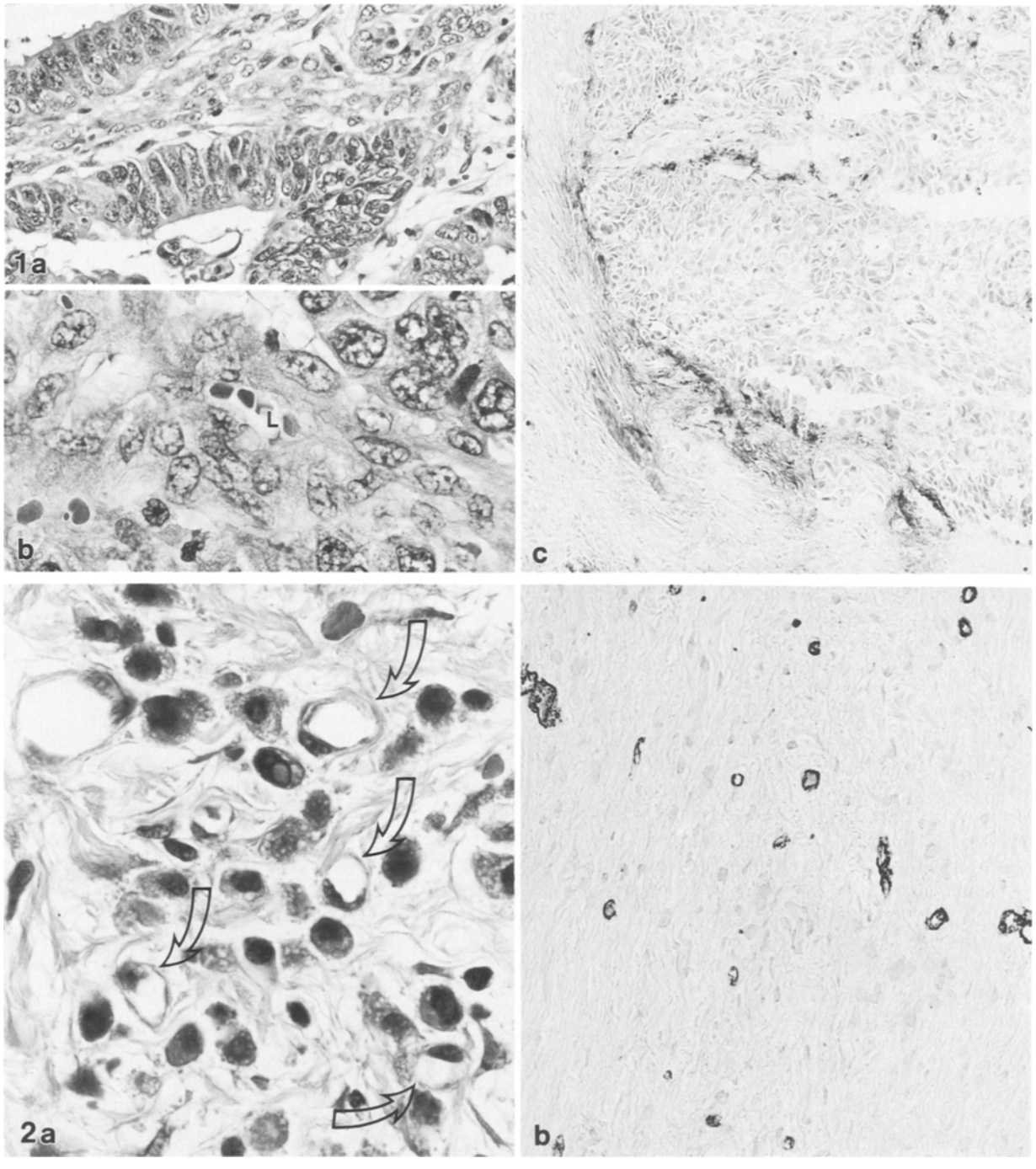
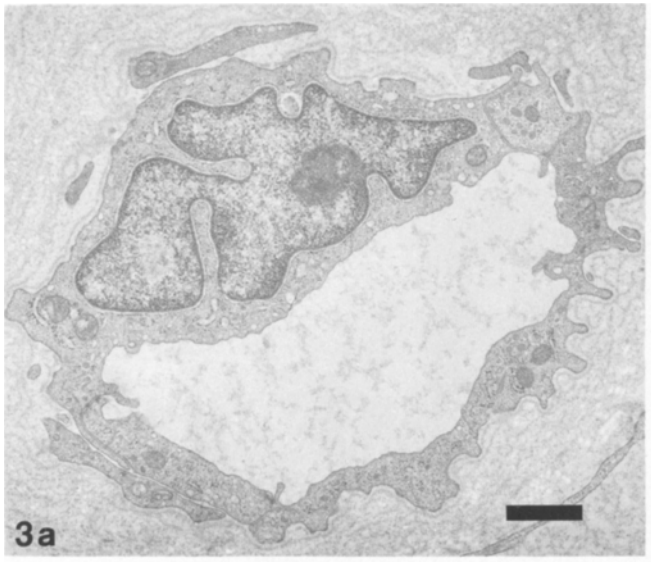


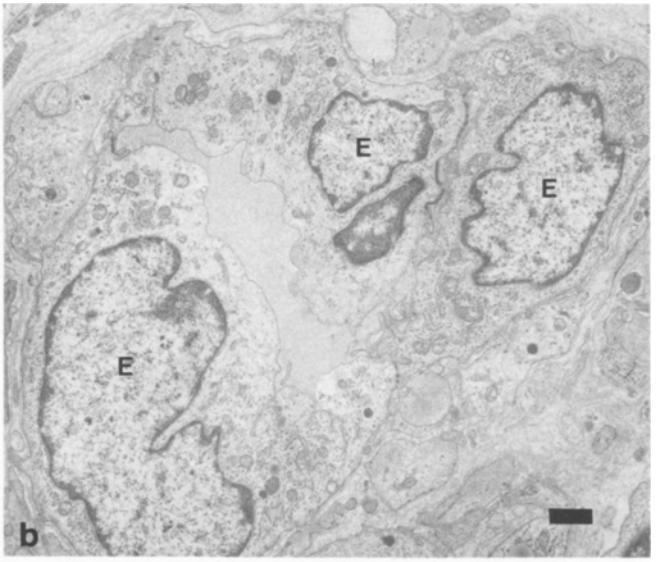
Fig. 1a–c. Light microscopy of differentiated type (intestinal) carcinoma. Capillaries in the stroma of this group are difficult to detect **a**, ×250. Higher magnification reveals endothelial hypertrophic change of a capillary **b**, ×625; *L*, lumen. Immunostain for von Willebrand factor (vWF) on cryostat section **c**, ×175 reveals denser distribution of capillaries near carcinoma cell nest with diffuse stromal reactivity

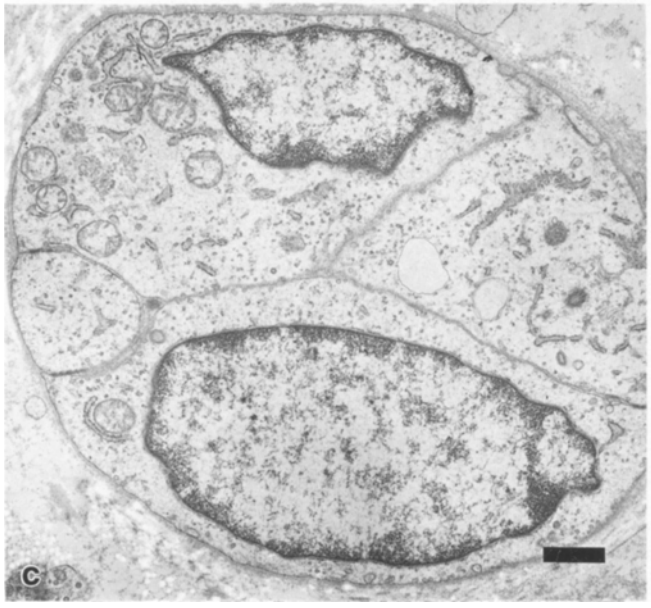
Fig. 2a, b. Light microscopy of undifferentiated type (diffuse) carcinoma. Capillaries in the stroma (*arrows*) have a wide lumen which are easily identified **a**, ×875. Immunostaining for vWF on cryostat section **b**, ×230 reveals uniform distribution of capillaries in the stroma

Materials and methods

Cases of surgically resected gastric carcinoma (21) were obtained at Tohoku Rosai Hospital and Tohoku University Hospital. All the patients were Japanese, and the male to female ratio was 11:10. Histologically they were classified into differentiated type (intestinal) (9 cases, average age 64.4 years) and undifferentiated type (diffuse) (12 cases, average age 59.4 years) according to Sugano et al. (1982) and Lauren (1965). All the cases showed invasive growth beyond the muscularis mucosae. Immediately after resection, tissue specimens (5 × 5 × 2 mm in size) taken from areas of invasive growth were fixed in periodate-lysine-paraformaldehyde fixative (McLean and Nakane 1974) or 4% paraformaldehyde for 6–8 h at 4° C. They were washed in phosphate-buffered saline (PBS) containing 10% sucrose and 20% sucrose. The specimens were rapidly frozen after embedding in O.C.T. compound. In the study of the







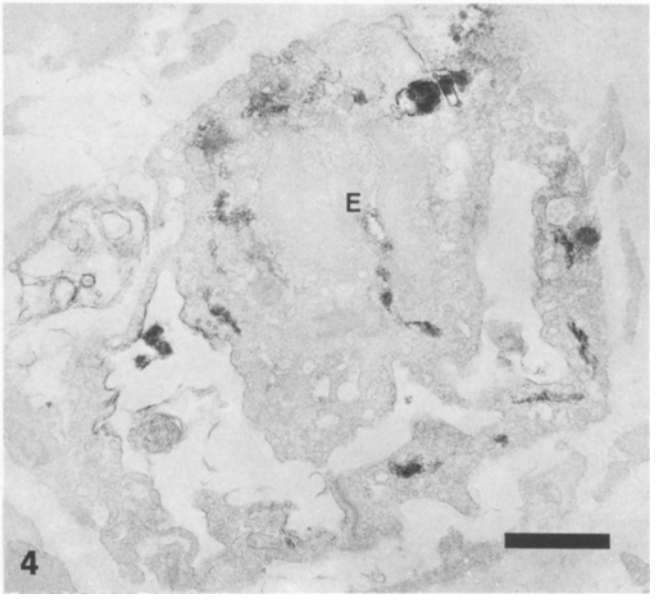


Fig. 3a, b. Conventional electron microscopy of capillaries. **a** A capillary with mild endothelial hypertrophic change (undifferentiated-type carcinoma), × 10000. **b** A capillary with severe endothelial hypertrophic change in differentiated-type carcinoma, × 5600. **c** A solid capillary bud in undifferentiated-type carcinoma, × 8200. *E*, Endothelial cell. *Scale bar* = 1 μm

Fig. 4. Immuno-electron microscopy for vWF in a control capillary in the muscularis propria. Scanty rough endoplasmic reticulum (rER), round granule, and part of luminal plasma membrane are reactive. × 14200. *E*, Endothelial cell. *Scale bar* = 1 μm

cancer stroma, tumour tissue without marked inflammatory cell infiltration or massive necrosis was selected. Areas of ulceration were also eliminated. Histologically an intermediate type between the two described was excluded from the present study. Non-neoplastic tissues were obtained from normal-appearing gastric mucosa and metaplastic gastric mucosa remote from gastric cancer.

Immunostaining for von Willebrand factor (vWF) was performed by the indirect antibody method as previously described (Ohtani and Sasano 1987; Yamamoto et al. 1988). After immersion in 10% non-immunized goat serum, 6-μm-thick frozen sections were incubated with anti-human vWF antibody (Dako, Copenhagen, Denmark; diluted at 1:2000) for 24 h at 4° C and then overnight with the horseradish-peroxidase-labelled Fab fragment of secondary antibody (Cappel Lab., West Chester, Calif., USA; diluted at 1:200). In 4 cases, undiluted mouse monoclonal antibody (Dako) was also used. After fixation in 1% glutaraldehyde, the specimens were reacted with 0.03% 3-3′diaminobenzidine tetrahydrocloride, post-fixed in 1% osmium tetroxide for 1 h, and embedded in Epon. Ultrathin sections were stained with lead citrate for 1 min and observed with a JEOL 100B electron microscope. For the negative control, the primary antibody was pre-absorbed with human factor VIII or replaced by non-immunized rabbit serum or PBS. For light microscopy, parallel sections were processed in the same way and mounted after methylgreen counter-staining. A total of 212 capillaries were identified and analysed by immunoelectron microscopy.

For conventional electron microscopy, specimens, 1 × 1 × 1 mm in size, were fixed in 2.5% glutaraldehyde containing 2% paraformaldehyde for 2 h in 13 of the 21 cases. After osmification, they were dehydrated and embedded in Epon. Blocks containing carcinoma cells without significant necrosis or inflammatory infiltrate were selected. Ultrathin sections were stained with uranyl acetate and lead citrate. Whole areas of each section were observed and all capillaries detected were tentatively classified into four cate-

gories – capillaries with little or mild endothelial hyperplastic change (Fig. 3a), moderate change, severe change (Fig. 3b) and solid capillary buds (Fig. 3c). Capillaries with little or mild hypertrophic change showed no or mild swelling of endothelial cells and had a wide capillary lumen. Capillaries with severe changes showed marked enlargement of endothelial cells with narrowing of the lumen. Capillaries with moderate changes were intermediate between the two. Solid capillary buds were characterized by marked enlargement of endothelial cells without the formation of a lumen (Ohtani and Sasano 1989). The numbers of each category of capillaries were added together (total 384) and the data were statistically analysed by the chi-square method using the 3×4 contingency table.

Results

By light microscopy of differentiated-type carcinoma, identification of capillaries was difficult owing to swelling and proliferation of endothelial cells, narrowing of the lumen and proliferation of stromal fibroblasts (Fig. 1a). Higher magnification revealed the vessels (Fig. 1b). Undifferentiated-type carcinomas were generally less populated by fibroblasts, with a diffuse deposition of collagen fibers (Fig. 2a). Included capillaries usually had slender nuclei and a patent lumen which was easily detected on the haematoxylin and eosin stain.

By immunohistochemistry, the intensity of staining for vWF was variable with each type of case. In differentiated-type carcinoma, capillaries were usually more densely located near carcinoma cell nests (Fig. 1c). Diffuse immunoreactivity in the stroma around capillaries was observed in 7 of 9 cases (Fig. 1c). In undifferentiated-type carcinoma, capillaries were more uniformly distributed and stromal immunoreactivity was not discernible at the light microscopic level (Fig. 2b). Negative controls uniformly showed no reactivity.

On electron microscopy, differentiated-type carcinoma showed marked hypertrophic changes of capillary endothelial cells (Fig. 3b) and infrequent occurrence of those with mild change (Fig. 3a, Table 1). The development of rough endoplasmic reticulum (rER) in the endothelial cells was more remarkable in this group (Fig. 3b) than in undifferentiated-type carcinomas (Fig. 3a). The latter type was characterized by more frequent occurrence of capillaries with mild hypertrophic changes (Fig. 3a, Table 1). Solid capillary buds were present in both groups (Fig. 3c).

By immuno-electron microscopy, results with the monoclonal antibody were essentially the same as those obtained using the polyclonal, and the following data were obtained mainly with the polyclonal antibody. vWF antigens were localized along the luminal plasma membrane, in scanty rER, round granules and Weibel-Palade bodies (WPB) in the control capillaries in the non-neoplastic gastric tissue (Fig. 4). All of them belonged to capillaries with little endothelial hypertrophic change. Subendothelial immunoreactivity was observed, but was usually minimal in the control group.

In differentiated-type carcinomas, vWF antigens were localized in the cisternae of well-developed rER (Figs. 5, 8) and in round granules (Fig. 7a) in endothelial cells. Cases showing these features were classified into "rER-type", to which 5 of 9 cases belonged (Table 2). The round granules measured 300–400 nm in diameter and were surrounded by a limiting membrane with thin peripheral halo (Fig. 7b). This is different from WPB (Fig. 6, inset). vWF was also found in the narrow lumen as a cluster, and was apparently diffusely deposited in the subendothelium (Fig. 8). One case showed an increase of immunoreactive WPB without rER, which was classified into "WPB-type". Three cases showed weak reactivity in endothelial cells and were classified into "weak reactivity type".

In undifferentiated-type carcinomas, vWF antigens were detected in WPB and scanty rER (Fig. 6). Increase of immunoreactive WPB was detected n 7 of 13 cases (WPB-type) (Table 2). vWF was also deposited in the subendothelium, but less than in differentiated-type carcinoma (Fig. 9). The luminal plasma membrane of endothelial cells was occasionally positive continuously. Only 1 case showed moderate development of immunoreactive rER (rER-type). The weak reactivity type comprise 4 cases.

Solid capillary buds were negative for vWF in both groups of carcinoma.

Table 1. Occurrence rate of each type of capillary (indicated by %)

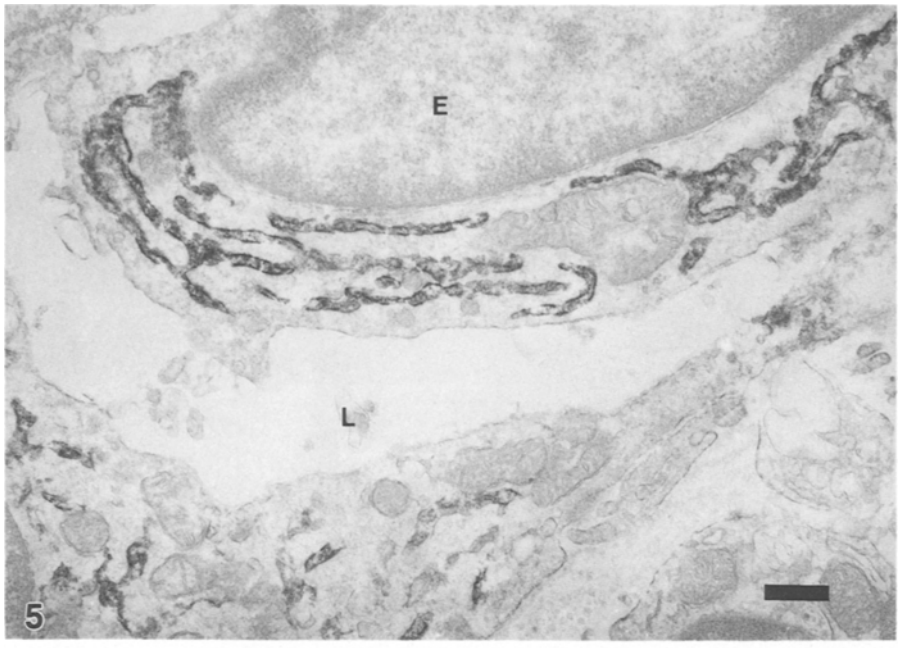
	Severity of endothelial cell hypertrophic change			Solid capillary buds
	Mild	Moderate	Severe	
Differentiated-type gastric carcinoma ($n=186$)	7	45	39	9
Undifferentiated-type gastric carcinoma ($n=158$)	38	30	19	13
Granulation tissue ($n=40$)	75	25	0	0

See the Materials and methods section for the classification of capillaries. n, Total numbers of capillaries examined in each group. Differences of the data among three groups of lesions were statistically significant by the chi-square test ($P<0.1\%$)

Table 2. Summary of the results of immunolocalization of von Willebrand factor (vWF) in endothelial cells in gastric carcinoma

Gastric carcinoma	Localization pattern of vWF in endothelial cells (indicated by numbers of cases)			
	rER-type	WPB-type	Weak reactivity	Total
Differentiated type (intestinal)	5	1	3	9
Undifferentiated type (diffuse)	1	7	4	12

rER, Rough endoplasmic reticulum; WPB, Weibel-Palade body. See the Results section for details of classification



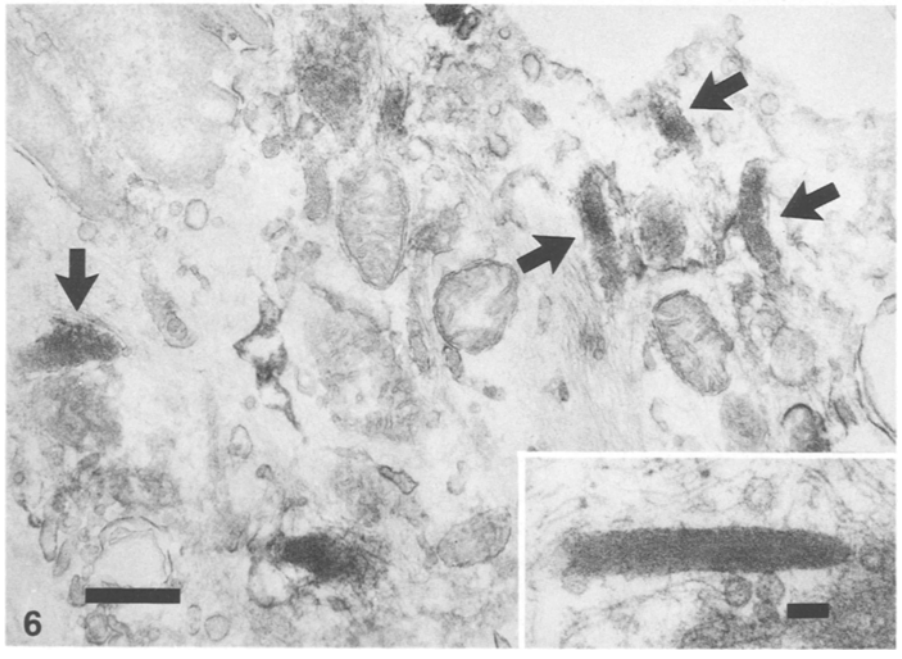


Fig. 5. Immuno-electron microscopy for vWF in a capillary with severe endothelial hypertrophic change in differentiated-type carcinoma. Note well-developed rER with immunolabelled cisternae. *E*, Endothelial cell; *L*, lumen. ×16700. *Scale bar* = 0.5 μm

Fig. 6. Immuno-electron microscopy for vWF in a capillary with moderate endothelial hypertrophic change in undifferentiated-type carcinoma. Immunolabelled Weibel-Palade bodies (WPB) are observed (*arrows*). Immunolabelled rER are scanty. ×25500. *Scale bar* = 0.5 μm. *Inset* A WPB by conventional electron microscopy in another endothelial cell of the same case. ×56000. *Scale bar* = 0.1 μm

Discussion

The purpose of the study was to shed new light on the morphological and functional changes of microvasculature in the stroma of human carcinoma, in relation to its histological types. The results in differentiated type (intestinal) gastric carcinoma are similar to those of colorectal carcinoma (Ohtani and Sasano 1987) both showing well-developed immunoreactive rER. These common changes suggest that a transformation takes place in endothelial cells into "an actively protein-producing state" in the stroma of differentiated-type adenocarcinomas. An increase of rER positive for vWF was also reported in endothelial cells of glioblastoma (Miyagami et al. 1987). Such changes of vWF may be regarded as one of the host reactions to malignant tumours. However, similar increase of rER positive for vWF was observed in rat aortic endothelial cell after injury and endotoxin treatment (Reidy et al. 1989). The physiological significance of these changes is difficult to clarify at present, but it might be related to the function of vWF on endothelial cell adhesion (Dejana et al. 1989).

In contrast, the microvasculature in undifferentiated type (diffuse) gastric carcinoma is characterized by more frequent occurrence of capillaries with mild endothelial hypertrophic change and vWF antigen in WPB. Transformation into the actively protein-secreting state is rare in this group. This difference of microvasculature is of particular interest because the two major types of gastric carcinomas are different in the biological behaviour and the nature of their background mucosa. The differentiated-type tends to metastasize to the liver haematogen-

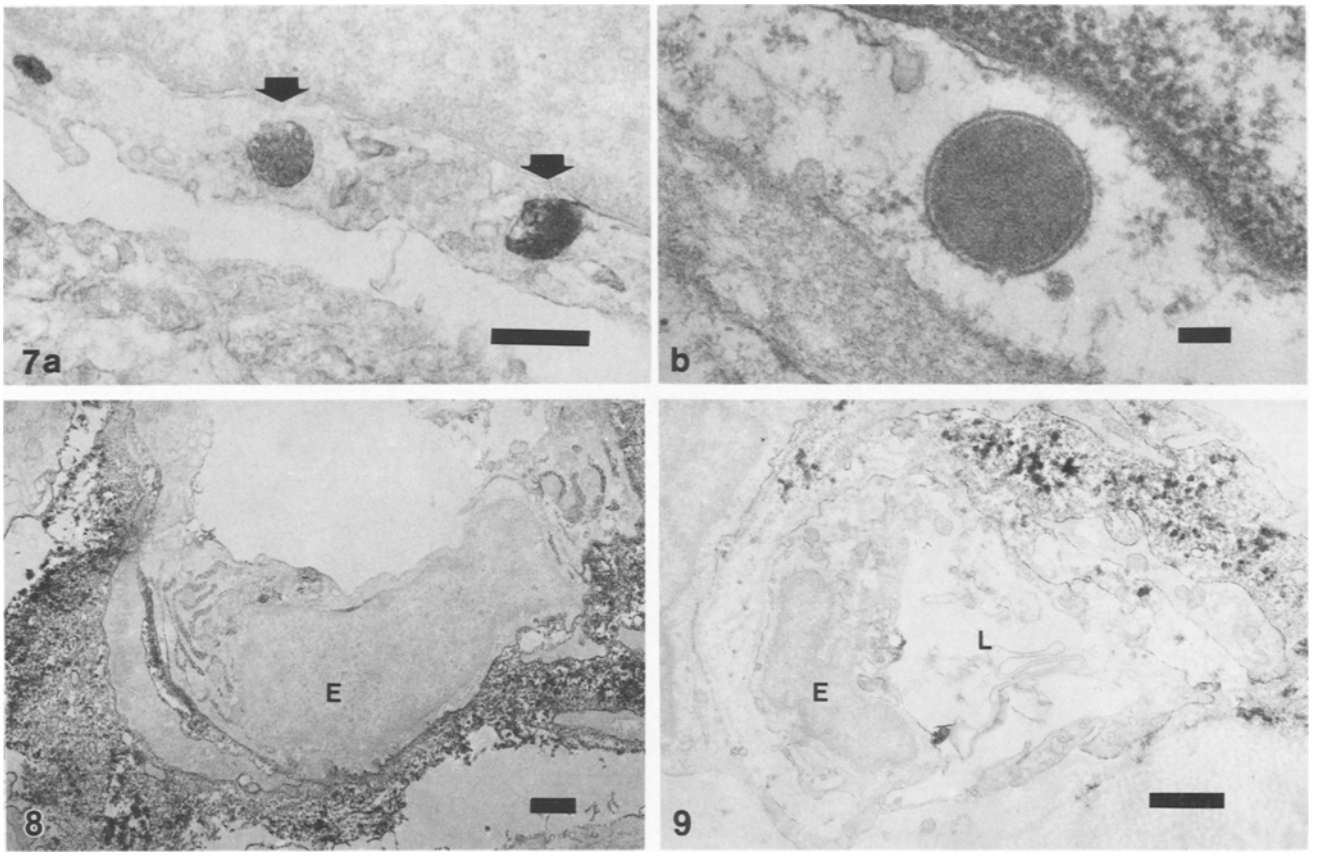


Fig. 7. a Immuno-electron microscopy for vWF in a part of capillary endothelial cells in differentiated-type carcinoma showing immunolabelled round granules (*arrows*). ×26000. Scale bar = 0.5 μm. **b** Conventional electron microscopy of a part of a capillary endothelial cell showing round granules with a limiting membrane containing homogeneously electron-dense material. ×65000. *Scale bar* = 0.1 μm

Fig. 8. Immuno-electron microscopy for vWF showing remarkable subendothelial reactivity in differentiated-type carcinoma. Abundant rER are also positive. *E*, Endothelial cell. ×6100. *Scale bar* = 1 μm

Fig. 9. Immuno-electron microscopy for vWF in undifferentiated-type carcinoma showing diffuse subendothelial reactivity without remarkable cytoplasmic reactivity. *E*, Endothelial cell; *L*, lumen. ×10000. *Scale bar* = 1 μm

eously and to originate from metaplastic mucosa; the undifferentiated-type tends to spread onto the serosal surface and to originate from the fundal mucosa (Nakamura 1982). The rarity of central necrosis in undifferentiated-type carcinoma may also be related to the uniform distribution of more mature capillaries. Difference in the distribution pattern of the microvasculature between the two types has already been demonstrated by infusion of India ink into vessels (Tsuchihashi et al. 1984, 1987).

Diffuse distribution of vWF in the subendothelium may be caused both by increased permeability of capillaries in the cancer stroma (Dvorak et al. 1988) and exocytosis of vWF into subendothelium (Ohtani and Sasano 1987; Takeuchi et al. 1988b). This exocytosis may be important in that aspect of platelet function relating to adherence to the subendothelium, an important step in haemostasis (Fressinaud et al. 1987; Takagi et al. 1989).

Structures similar to solid capillary buds have been seen in experimental studies (Folkman and Haudenschild 1980; Dvorak et al. 1987) but their identification in the stroma of human carcinoma was first described by us (Ohtani and Sasano 1989). These buds are ubiquitous in the stroma of gastrointestinal carcinoma, and they are considered to be immature capillaries because they lack vWF antigen or *ulex europaeus*-I lectin receptors (Ohtani and Sasano 1989).

Fibroblasts also show morphological activation in cancer stroma (Ohtani and Sasano 1983). Paracrine mechanism operating via the following factors is postulated to explain the stromal reactions by capillaries and fibroblasts (Roberts et al. 1988): transforming growth factor-β, platelet-derived growth factor, insulin-like growth factor, acidic and basic fibroblast growth factors and tumour necrosis factor (Folkman and Klagsbrun 1987).

In conclusion, the present study has demonstrated that the microvasculature of the stroma in human carcinomas differs significantly according to its histological types. This is an important fact to be considered both in the study of cancer therapy and in experimental studies of tumour angiogenesis.

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