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Close association between tumour cells and vascular basement membrane in gastric cancers with liver metastasis

An immunohistochemical and electron microscopic study with special attention to extracellular matrices

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Summary. To investigate morphological features valuable in estimating the propensity of gastric cancer to metastasize to the liver, we examined the primary tumours from 49 surgically resected advanced gastric cancers (24 with liver metastasis) and 45 autopsy cases, 19 with liver metastasis. We paid special attention to extracellular matrices – connective tissue stroma and basement membrane (BM) – using immunohistochemistry and electron microscopy. Type IV collagen staining showed that in differentiated carcinomas neoplastic glands were occasionally located in close proximity to the BM of thin-walled tumour blood vessels in back-to-back fashion. In poorly differentiated lesions, tumour cells were also oriented toward the vascular BM in pseudorosette-like pattern. Type III collagen staining and electron microscopy showed that in such regions tumour cells, with continuous or discontinuous BM, were immediately adjacent to vascular BM with no connective tissue stroma in between. On occasion tumour cells were in direct contact with vascular BM. These close associations were often found in carcinomas with a medullary growth pattern, irrespective of the degree of histological differentiation. However, they were virtually never seen in their benign counterpart. Of the resected cases, all 24 with liver metastasis showed this association, whereas only 10 of 25 (40%) without liver metastasis did so ($P < 0.001$). In the autopsied cases, a similar positive correlation was observed between liver metastasis and this association. Furthermore, the tumor cells showing this juxtaposition showed evidence of vascular invasion. These results suggest that the close association between tumour cells and vascular BM is specific to the malignant neoplasm, and may be related to liver metastasis. Immunohistochemistry can be a great help in estimating the probability of liver metastasis.

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Introduction

Gastric cancers show various patterns of metastatic spread, including haematogenous and lymphatic metastasis and peritoneal dissemination. The pattern of metastasis is one of the most important factors affecting the prognosis in this neoplasm. There are many factors used for the prediction of metastasis, such as depth of intramural invasion (Serlin et al. 1977; Pagnini and Rugge 1985), histological type (Rhomberg and Gruber 1989), degree of lymphatic and venous invasion (Koga et al. 1987; Makino et al. 1989), DNA ploidy (de Aretxabala et al. 1988) and oncogene expression (Tahara et al. 1986). Among them, the histological type of the cancer has considerable prognostic value. Liver metastasis, most of which occurs haematogenously via the portal vein, is frequently found in well-differentiated adenocarcinomas, whereas poorly differentiated adenocarcinomas are less frequently metastatic to liver (Duarte and Llanos 1981; Sugano et al. 1982). However, gastric cancers of medullary type which contain little connective tissue stroma have a high tendency to liver metastasis, irrespective of their degree of histological differentiation (Kaibara et al. 1985; Koga et al. 1987). Even among carcinomas of medullary type, the incidence of liver metastasis varies with the histological subtypes (Maruyama et al. 1989). These results suggest that histological types based on the degree of differentiation and the amount of connective tissue stroma are useful indicators for estimating the metastatic propensity of gastric cancers, but they are not always crucial. The common morphological features shared by the carcinomas with liver metastasis

remain unclear and should be studied with techniques other than those used in conventional histological classification.

Extracellular matrices (ECM) are one of the important factors involved in tumour morphogenesis. There are two different types of ECM in tumour tissue (Liotta et al. 1983; Martinez-Hernandez 1988); the connective tissue stroma which contains type I and type III collagens, fibronectin and hyaluronic acid and basement membrane (BM), which is mainly composed of laminin, type IV collagen and heparan sulfate proteoglycan (Hay 1982). Vascular BM are the major component in neoplastic tissue together with tumour BM produced by tumour cells themselves. Many in vitro studies suggest that interaction between tumour cells and these ECM, especially vascular BM, is of great importance in tumour metastasis (Kramer et al. 1980; Terranova et al. 1986; Nakajima et al. 1987). However little is known of the in situ features of the tumour cell/vascular BM relationships in the primary sites of gastric cancers with liver metastasis.

In the present immunohistochemical and electron microscopic study, we investigated the morphological features associated with gastric cancers with liver metastasis, focusing on the tumour cell/vascular BM interface.

Materials and methods

Forty-nine surgically resected cases of advanced gastric cancer (National Nagoya Hospital, 1985–1987) and 45 autopsied cases (Aichi Cancer Center Hospital, 1983–1988) were studied. Of the 49 resected cases, 24 had liver metastasis (19 synchronous and 5 metachronous) and 25 were non-metastatic controls. These two groups were matched by age, sex and depth of invasion. Of the 45 autopsy cases, liver metastasis was found in 19 cases. In 26 cases without liver metastasis, the organs most frequently involved were peritoneum, followed by lymph node, lung, ovary, bone and pancreas. The surgically resected materials were fixed in cold ethanol/acetic acid (98/2, v/v) for 24 h and embedded in paraffin. Autopsied materials were routinely fixed in 10% formalin for up to 7 days and embedded in paraffin. Paraffin sections at 4 μm were made for routine histological examination with haematoxylin and eosin and for immunohistochemical staining. Ten hyperplastic and adenomatous lesions in the stomach were examined as controls. Fresh specimens removed by biopsy or surgical resection were embedded in OCT compound (Miles Scientific, Naperville, Ill.) without fixation and stored at −80° C until immunohistochemical study. Frozen sections of 10 μm thickness were cut and fixed in cold ethanol for 10 min.

For immunohistochemistry of type IV collagen, deparaffinized sections fixed in ethanol/acetic acid were pretreated with 2 μg/ml trypsin (Sigma, St. Louis, Mo.) in 50 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$ or with 126 units/ml crude collagenase (Worthington, Freehold, N.J.) in the same buffer solution for 15 min at room temperature. In autopsied specimens fixed in 10% formalin, the sections were pre-treated with 0.4% pepsin (Sigma) in 0.01 N hydrochloric acid at 37° C for 2 h as described previously (Kirkpatrick and D'Ardenne 1984). In frozen sections, no enzymatic digestion was performed. Immunostaining of ethanol/acetic acid-fixed, paraffin-embedded and frozen sections was performed by the indirect immunoperoxidase method using peroxidase-conjugated swine antiserum against rabbit immunoglobulin (Dakopatts, Copenhagen, Denmark). In formalin-fixed specimens, the detection system used was the avidin-biotin peroxidase complex (ABC) method (Kit Vectastain, Vector Labs, Burlingame, Calif.). For staining of type III collagen, enzymatic pre-treatment was done in only formalin-fixed specimens and detection was performed by the ABC method. Colour was developed using 3,3′-diaminobenzidine as substrate, followed by methyl green counter-staining. For negative controls, the primary antisera were replaced by non-immune serum. Detection of vascular BM was carried out by type IV collagen immunostaining, but in cases where it was difficult to distinguish vein from lymphatic channel, we added immunostaining of factor-VIII-related antigen using the Dako Kits (Bettelheim et al. 1984). The following monospecific antisera were used as primary antibody: goat antiserum against human type III collagen (Iatron Labs, Tokyo, Japan), rabbit antiserum against bovine kidney type IV collagen (Advance Co., Tokyo, Japan), and rabbit antiserum against human factor VIII-related antigen (Dakopatts).

For electron microscopy, tumour tissues were fixed in 2.5% glutaraldehyde/2% paraformaldehyde/50 mM phosphate buffer, pH 7.3, for 2 h, post-fixed in 2% osmium tetraoxide, and then

Table 1. Histological types and liver metastasis, association between tumour cells and vascular basement membrane (BM) in 49 resected and 45 autopsied cases of gastric cancers

Histological type	Surgery			Autopsy		
	No. of cases	Liver metastasis	Association	No. of cases	Liver metastasis	Association
Differentiated	25	15	20	14	9	9
Pap	8	7	8	5	5	5
Tub	17	8	12	9	4	4
medullary	5	4	5	1	1	1
intermediate	7	4	6	3	2	2
scirrhous	6	0	1	5	1	1
Poorly differentiated[a]	24	9	14	31	10(5)	5
medullary	13	7	11	9	8(5)	4
intermediate	3	2	3	3	2	1
scirrhous	8	0	0	19	0	0
Total	49	24	34	45	19(5)	14

Pap, Papillary adenocarcinoma, most of which are classified as medullary in terms of stroma; Tub, Tubular adenocarcinoma

[a] Signet-ring cell carcinoma is included in poorly differentiated adenocarcinoma. Numbers in parentheses indicate cases with liver metastasis via periportal lymphatics

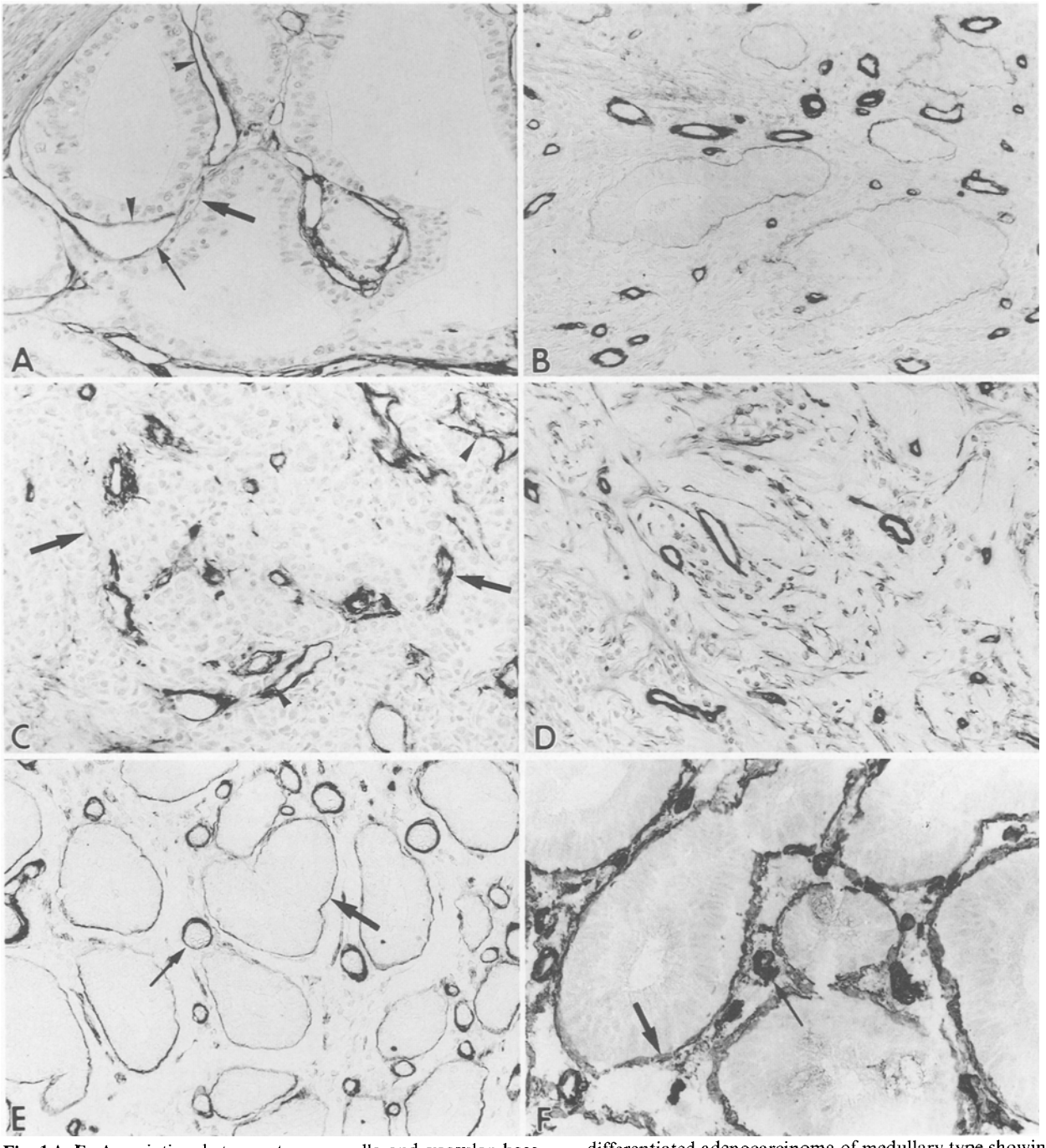

Fig. 1 A–F. Association between tumour cells and vascular basement membrane (BM) in various gastric cancers demonstrated by immunostaining of type IV collagen. **A** Tubular adenocarcinoma of medullary type showing the close association (*arrowheads*). Tumour BM (*thick arrow*) and vascular BM (*thin arrow*) positively stained face each other and meet in places. **B** Tubular adenocarcinoma of scirrhous type. No close association is found. Abundant connective tissue stroma is seen in between the BM. **C** Poorly differentiated adenocarcinoma of medullary type showing the close association (*arrowheads*). Weakly positive discontinuous tumour BM is noted (*arrows*). **D** Poorly differentiated adenocarcinoma of scirrhous type. None of tumour cells are associated with vascular BM. **E** Normal gastric glands. **F** Benign adenomatous lesion of stomach. In these tissues, epithelial BM (*thick arrow*) are separated from vascular BM (*thin arrow*) by the stroma. Indirect immunoperoxidase method; **A–E** ×170, **F** ×340

embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate followed by lead citrate and examined in an electron microscope (Nihondenshi, JEM-1200EX).

Histological types of carcinomas identified were: differentiated adenocarcinomas, which were subdivided into papillary and tubular adenocarcinomas, and poorly differentiated adenocarcinomas according to the classification of the Japanese Research Society for Gastric Cancer (1981). In addition, we divided each type into three groups based on the degree of stromal deposition: the medullary type contains absent or slight stroma, the intermediate type a moderate stroma, the scirrhous type abundant stroma. The incidence of venous invasion in resected cases was determined in accordance with the pathological report written by the clinical pathologist in compliance with the general rules of the Japanese Research

Society for Gastric Cancer. Statistical correlations between parameters in cross-tabulations were analysed by the chi-square test. A value of $P<0.05$ was considered to be significant.

Results

Histological types of gastric cancers resected and autopsied are presented in Table 1. In autopsy cases, the most frequent histological type manifesting liver metastasis was papillary adenocarcinoma (36%), followed by poorly differentiated adenocarcinoma of medullary type (21%). A similar result was obtained in resected cases. However, there was a minor difference between these two groups. In autopsy cases, 5 of 8 poorly differentiated carcinomas of medullary type with liver metastasis, most of which contained signet-ring cells, appeared to metastasize to the liver via periportal lymphatics. This was suggested by the fact that they involved the liver in a lymphangitic distribution rather than in a multinodular pattern as is usual in haematogenous liver metastasis. They were also often accompanied by systemic lymph node metastasis and lymphangitis carcinomatosa of the lung.

In normal gastric mucosa, type IV collagen was detected in the BM of gastric glands, smooth muscles, peripheral nerves and blood vessels. In gastric cancers, continuity of tumour BM varied greatly from nearly intact BM in well-differentiated carcinomas to almost complete absence in some poorly differentiated ones. In normal mucosa and benign adenomatous lesions, epithelial cells were clearly separated from blood vessels by the epithelial BM and connective tissue stroma (Fig. 1 E, F). In carcinomas, however, tumour cells often showed close proximity to vascular BM and these two distinctive tissue elements were occasionally contiguous. In differentiated adenocarcinoma, neoplastic glands lay adjacent to and occasionally met the BM of dilatated blood vessels in a back-to-back fashion (Fig. 1 A). This association between tumour cells and vascular BM was most frequently seen in papillary adenocarcinoma and tubular adenocarcinoma with medullary growth pattern in both resected and autopsy cases, but was rarely seen in tubular adenocarcinoma of scirrhous type (Table 1). In the latter, abundant connective tissue stroma intervened between tumour cells and vascular BM (Fig. 1 B). In poorly differentiated adenocarcinomas, both resected and autopsied, tumour cells with discontinuous BM were oriented toward vascular BM. This association was often found in carcinoma of medullary type (Fig. 1 C). In carcinoma of scirrhous type, however, this association was absent and tumour cells proliferated in disorderly fashion (Fig. 1 D).

We looked for the presence or absence of a connective tissue stroma at the site of the association between tumour cells and vascular BM. Type III collagen staining showed that in differentiated carcinomas, both resected and autopsied, tumour BM and vascular BM occasionally faced each other, where few collagen fibres could be seen (Fig. 2 A). In poorly differentiated adenocarcinoma, a similar result was obtained; tumour cells appeared to

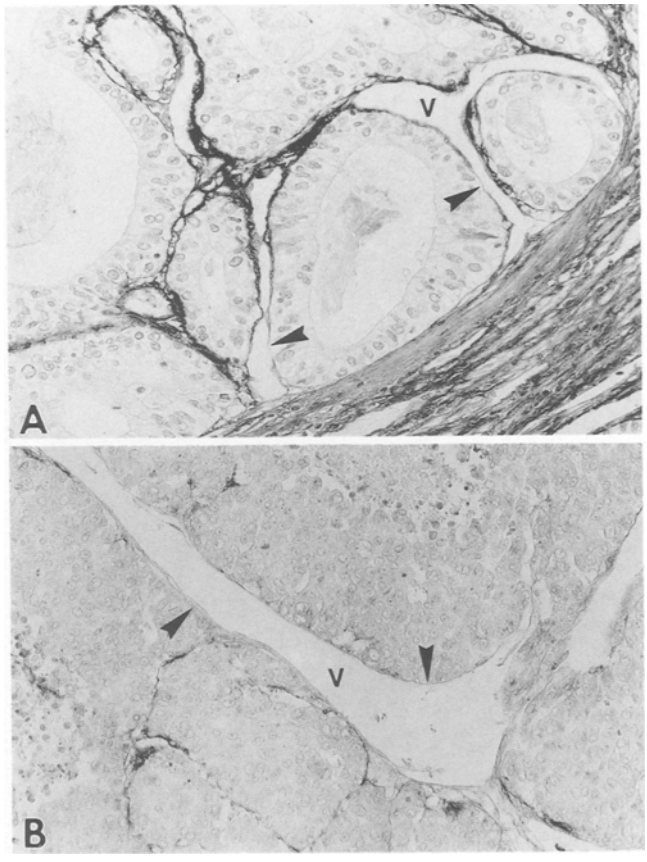

Fig. 2 A, B. Distribution of type III collagen at the site of association between tumour cells and vascular BM in gastric cancers. **A** Tubular adenocarcinoma of medullary type. **B** Poorly differentiated adenocarcinoma of medullary type. Little or no positively stained stroma is observed at the site of the association between tumour cells and vascular BM (*arrowheads*). *V*, Lumen of blood vessel. ABC method, ×170

Table 2. Relationship between liver metastasis and association of tumour cells with vascular BM or venous invasion in gastric cancers

Metastasis to liver parenchyma	Incidence of association (%)	Incidence of venous invasion (%)
<Resected cases>		
Pap+Tub	20/25(80)	7/25(28)
present	15/15(100)*	5/15(33)[a]
absent	5/10(50)	2/10(20)
Por	14/24(58)	12/24(50)
present	9/ 9(100)*	4/ 9(44)[a]
absent	5/15(33)	8/15(53)
<Autopsy cases>		
Pap+Tub	9/14(64)	9/14(64)
present	8/ 9(89)*	8/ 9(89)*
absent	1/ 5(20)	1/ 5(20)
Por	5/31(16)	6/31(19)
present	5/10(50)**	5/10(50)**
via portal vein	4/ 5(80)	4/ 5(80)
via lymphatics	1/ 5(20)	1/ 5(20)
absent	0/21(0)	1/21(5)

* $P<0.01$; ** $P<0.001$; Por, Poorly differentiated adenocarcinoma

[a] Not significant

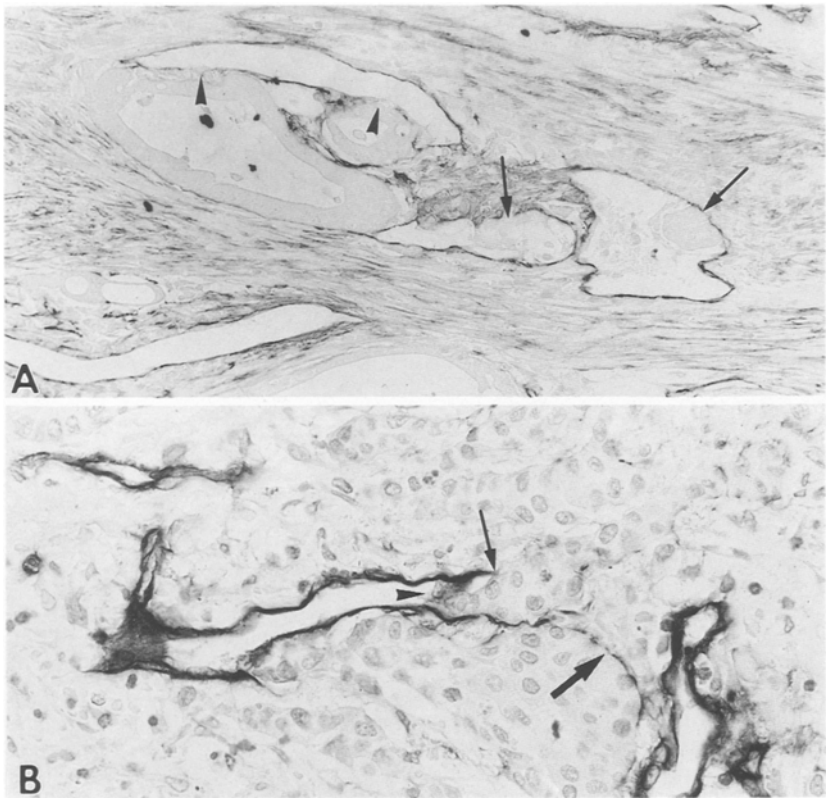

Fig. 3A, B. Vascular invasion by tumour cells at the site of the association between tumour cells and vascular BM demonstrated by immunostaining of type IV collagen. **A** Tubular adenocarcinoma. Neoplastic glands in contact with locally fragmented vascular BM are seen in extravascular space (*arrowheads*). In adjacent area, intravascular tumour cells growing on the vascular BM are visible (*arrows*). **B** Poorly differentiated adenocarcinoma. Tumour cell nests with discontinuous BM (*thick arrow*) disrupt the vascular BM (*thin arrow*) and enter into vascular lumen (*arrowhead*). Indirect immunoperoxidase method; **A** ×170, **B** ×340

be in contact with vascular BM without the connective tissue stroma in between (Fig. 2B).

The relationship between this association or venous invasion and liver metastasis was investigated. As shown in Table 2, there was a significant correlation between liver metastasis and this association in both resected and autopsy cases, irrespective of the degree of differentiation. For example, in differentiated adenocarcinoma cases autopsied, 8 of 9 with liver metastasis exhibited this association, while 1 of 5 without liver metastasis showed this feature ($P<0.01$). There was a similar correlation between liver metastasis and venous invasion in autopsy cases. However, this was not the case with resected cases, probably because of the low incidence of venous invasion detected. A significant correlation between the incidence of this association and that of venous invasion was also observed in autopsy cases only ($P<0.01$).

To investigate the pathological significance of this association in metastasis, we analysed the tumour cell/vascular BM interface in more detail. Although relatively rare, immunostaining of type IV collagen showed that in differentiated adenocarcinomas, at the site of the association between tumour cells and vascular BM, tumour cells appeared to enter the blood vessels (Fig. 3A). In some cases of poorly differentiated adenocarcinoma, tumour cells crossed the vascular wall and intravasated into the vascular lumen (Fig. 3B). Fibrin deposition was occasionally observed in these lesions. Electron microscopic study showed that at the interface between tumour cells and vascular BM, their respective BM ran parallel to or met each other with no connective tissue stroma in between (Fig. 4A, B). On occasion, tumour cells were in direct contact with a single BM shared by the vascular endothelial cells (Fig. 4C). At the site of contact of tumour cells with vascular BM, vascular endothelial cells sometimes showed degenerative changes such as cytoplasmic vacuolation, development of gaps and disappearance of vascular BM (Fig. 4D). Disruption of the endothelial cells was also occasionally seen (data not shown).

Discussion

We have found that carcinoma cells occasionally show a close association between tumour cells and vascular BM. This was very seldom observed in benign proliferative lesions, suggesting that the association is specific to malignant neoplasms and may not simply represent localization as a result of extensive growth of tumour cells. It may be a phenomenon reflecting a distinctive interaction between epithelial tumour cells and vascular BM in malignant tumours. To date, no similar observations have been reported in human cancers. However, it is noteworthy that human prostatic and oesophageal carcinoma cells spread intraductally over pre-existing ductal BM (Kovi et al. 1985; Takubo et al. 1987). Murine mammary carcinoma cells invade lung parenchyma through BM fusion between tumour BM and epithelial BM followed by the replacement of lung epithelial cells on what appears to be the same BM in metastatic foci (Pitelka et al. 1980). Furthermore, in muscular invasion, mouse Lewis lung carcinoma cells come in direct contact

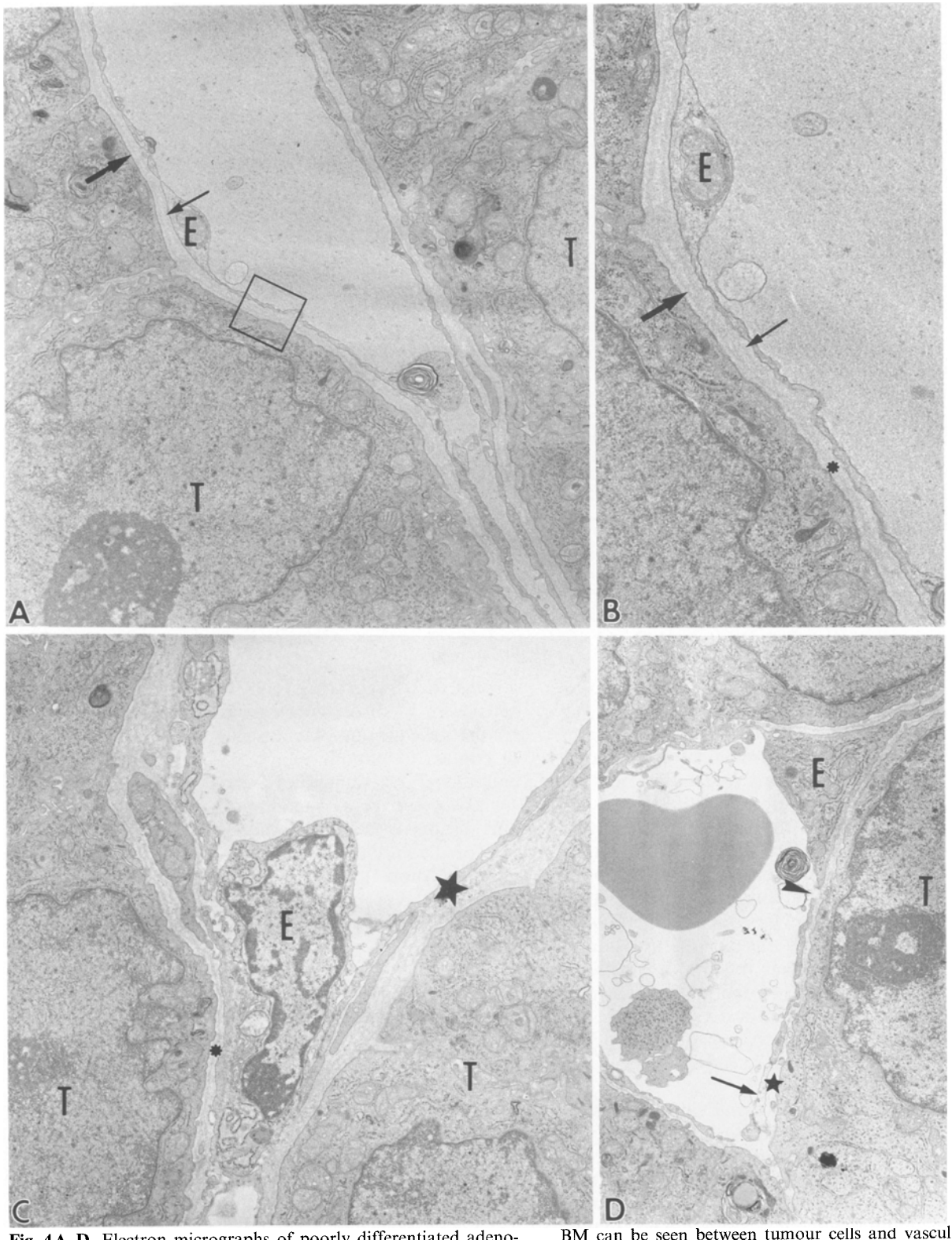

Fig. 4 A–D. Electron micrographs of poorly differentiated adenocarcinoma of medullary type, showing the close association between tumour cells and vascular BM. **A** Tumour cells lie adjacent to thin-walled blood vessel. Tumour BM (*thick arrow*) and vascular BM (*thin arrow*) run parallel or face each other with no collagen fibrils in between. × 8600. **B** Higher magnification of the area indicated by *open square* in **A**. Both BM meet or become indistinct to form a single BM (*asterisk*). × 16000. **C** Multilayered or folded BM can be seen between tumour cells and vascular BM, but no collagen fibrils intervene (*star*). Some tumour cells are in direct contact with vascular BM (*asterisk*). × 8600. **D** At sites of intimate contact between tumour cells and vascular BM, some degenerative changes of vascular endothelial cells such as vacuolation (*arrow*), gap (*arrowhead*) and disappearance of BM (*star*) are observable. *T*, Tumour cell; *E*, Endothelial cell. × 8600

with muscle BM, penetrate into the space between muscle cells and the BM, and spread on the inner surface of the BM (Paku et al. 1990). These observations indicate a possible significance for the intimate relationship between carcinoma cells and the pre-existing BM of host tissue in tumour invasion. In the present study, the incidence of liver metastasis in cases with this association was significantly higher than in cases without it. Therefore, the association between neoplastic cells and vascular BM may have some relation to malignant behaviour of tumour cells as in metastasis. We analysed the association in more detail in relation to metastasis. Even if relatively rarely, it was nevertheless observed that carcinoma cells in association with vascular BM appeared to cross the thin-walled tumour blood vessels. Ultrastructurally, at the sites of association between tumour cells and vascular BM, tumour cells were in direct contact with vascular BM in places and shared the BM with endothelial cells. In this region, vascular endothelial cells sometimes showed degeneration and subsequent disruption. The endothelial degeneration and breakdown of inter-endothelial junction occurred prior to vessel invasion by tumour cells in vivo (Azzarelli et al. 1985; Constantinides et al. 1989). It is possible that the close association of tumour cells with vascular BM presents an advantage, permitting tumour cells to attach to vascular BM and to disrupt the vascular wall, which assumed to be an important steps in vascular invasion by tumour cells (Liotta et al. 1983). In other words, this association may be a morphological sign indicative of the risk of vessel invasion.

Liver metastasis was inversely correlated with the degree of stromal deposition. Seventy-five percent of the resected cases which developed liver metastasis haematogenously were differentiated and poorly differentiated adenocarcinomas of medullary type and the remaining cases were adenocarcinomas with relatively poor connective tissue stroma. Scirrhous carcinoma with abundant stroma of both differentiated and poorly differentiated type was not encountered in cases manifesting liver metastasis. Similar observations have been reported by other authors. Differentiated and poorly differentiated adenocarcinomas with medullary growth pattern showed a high tendency to liver metastasis (Kaibara et al. 1985; Koga et al. 1987). Recently, it was reported that the incidence of venous invasion, especially subserosal vein involvement, was higher in cases of gastric cancers of medullary type than those of scirrhous type and that there was a positive correlation between venous invasion and liver metastasis (Makino et al. 1989). The high incidence of liver metastasis in carcinoma of medullary type may be, at least partially, attributable to their high tendency to venous invasion. However, little is known as to why carcinomas of medullary type tend to invade blood vessels. In the present study, we found that all the differentiated adenocarcinomas of medullary type and 85% of the poorly differentiated adenocarcinomas of medullary type often exhibited the above-mentioned association between tumour cells and vascular BM, whereas differentiated and poorly differentiated carcinoma of scirrhous type showed this association in only 17% and 0%, respectively, of the cases ($P<0.001$). Thus, it seems likely that carcinoma of the medullary type might readily gain access to vascular BM and disrupt vessel walls. Although we cannot exclude other possibilities, such as increased degradation enzymatic activity for BM (Nakajima et al. 1987), increased motility (Liotta et al. 1986) and altered microvasculature (Ohtani and Nagura 1990) in carcinoma of the medullary type, the close association of tumour cells with vascular BM may be responsible for high tendency to venous invasion, leading in turn to a high incidence of liver metastasis of carcinoma of medullary type.

In differentiated carcinomas showing this association, tumour BM, in fact, interposed between tumour cells and vascular BM. However, both BM occasionally fused to form a single BM and tumour cells were sometimes in direct contact with the BM. Similar ultrastructural findings were observed in poorly differentiated carcinoma with tumour BM which is more or less discontinuous, as previously reported by Grigioni et al. (1986). Tumour BM has long been considered to be a barrier to tumour cell locomotion and invasion (Barsky et al. 1983; Forster et al. 1986); however, present results suggest that tumour BM does not serve as a barrier that isolate tumour cells from vascular BM; rather, it may mediate the formation of this association, leading to intimate contact between tumour cells and vascular BM.

In lymph node metastasis, the so-called budding or microtubular cancer nests and "mucin leakage" into the stroma are histological features for estimating the probability of lymph node metastasis in rectal carcinoma and gastric, cervical adenocarcinoma, respectively (Morodomi et al. 1989; Rhomberg and Gruber 1989; Konishi et al. 1990). However, to our knowledge, a particular morphological feature associated with haematogenous metastasis other than histological types and venous invasion has not been reported. We have demonstrated that the close association between carcinoma cells and vascular BM is a morphological feature with prognostic value for liver metastasis in gastric cancer. Immunohistochemical detection of the association together with the detection of the venous invasion using anti-type IV collagen antibody may be a useful adjunct in predicting the metastatic propensity of gastric cancers.

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