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Establishment and Characterization of a New Spontaneous Metastasis Model of Human Gastric Carcinoma in Nude Mice

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A poorly differentiated medullary carcinoma of human stomach, designated HY-1, was successfully transplanted to nude mice by either the subcutaneous or intramuscular route for five generations. The transplanted tumor showed spontaneous lung metastases in nearly 100% of KSN and Balb/c female nude mice. There were over 20 visible lung metastatic nodules in KSN and Balb/c nude mice bearing tumors for over 80 days. Immunostaining of type IV collagen and electron microscopy revealed that tumor cells were often in direct contact with basement membrane (BM) of tumor blood vessels in the primary tumor tissue. At the site of contact between tumor cells and vascular BM, focal disappearance of the BM, disruption of endothelial cells and entry of tumor cell clusters into vascular lumen were observed. Immunostaining of 72 kDa gelatinase/type IV collagenase demonstrated that tumor cells expressed this enzyme in their cytoplasm. These results suggest that spontaneous metastasis of this tumor may be partly due to a marked tendency to vascular invasion involving the following sequential events: tumor cell contact with vascular BM, BM degradation possibly by 72 kDa gelatinases and endothelial disruption. This model could be a useful tool for understanding the mechanism of hematogenous metastasis of human gastric cancer.

Key words: Metastasis — Nude mouse — Human gastric carcinoma

Metastasis is one of the most important biological behaviors of cancer cells. Much information on the mechanism of cancer metastasis has been obtained using rodent systems,[1–3] but little comparable experimental data are available on the metastatic process of human cancers. Recently, models for metastasis of human cancers have been developed in nude mice, many of them concerning malignant melanoma and breast carcinoma.[4–7] In contrast, models of hematogenous metastasis of human gastrointestinal tract carcinomas are few and have not been fully delineated. In most metastasis models for carcinomas of colon and pancreas, intravenous or intrasplenic injection of tumor cells was necessary to generate metastasis.[8–10] There are several reports of spontaneous metastasis in xenografted gastric carcinomas in nude mice. Li and co-workers reported a transplantable papillary adenocarcinoma of stomach that formed metastases to the lung and lymph nodes,[11] and a moderately differentiated tubular adenocarcinoma that was metastatic to the lung was reported by Takao *et al.*[12] To date, however, no experimental model has been reported of a poorly differentiated carcinoma of medullary type, which is known to be highly metastatic to the liver and which results in a poor prognosis in various gastric cancers.[13, 14]

In the present study, we established a new spontaneously metastasizing line (HY-1) of poorly differentiated gastric adenocarcinoma of medullary type in nude mice. The process of intravasation by tumor cells at the implanted site, a critical step in spontaneous hematogenous metastasis, was investigated *in situ* using immunohistochemical and electron microscopic techniques.

MATERIALS AND METHODS

Animals Four- to 5-week-old female athymic nude mice with different genetic backgrounds (KSN and Balb/c) were obtained from Shizuoka Laboratory Animal Center, Hamamatsu. They were maintained under SPF (specific-pathogen-free) conditions.

Transplantation of human gastric carcinoma Tumor tissue used was obtained from a liver metastatic lesion which was surgically resected from a 72-year-old Japanese man suffering from a Borrmann II gastric cancer. The tumor tissue was cut aseptically into small pieces and fragments less than 1 mm in diameter were transplanted with a trocar into the subcutaneous tissue on the back and/or intramuscularly at the hind thigh. When the animals were killed, serial transplantations of primary tumor or lung metastatic lesions were performed.

Spontaneous metastasis assay Mice with growing transplant were killed and autopsied when the tumor size

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reached 2.5–3.5 cm in diameter. Autopsy was performed about 90 days after implantation. Lung and all other organs suspected of containing metastases were removed and fixed overnight in Bouin's solution. The number of macroscopic metastases was determined by counting visible parietal nodules. Micrometastases were detected by histological examination of sections stained with hematoxylin and eosin.

Immunohistochemistry Transplanted subcutaneous tumors were fixed in cold ethanol/acetic acid (98/2, v/v) and embedded in paraffin. Immunohistochemical staining of type IV collagen and 72 kDa gelatinase was performed on deparaffinized sections by the indirect immunoperoxidase method. In the former, sections were pretreated with trypsin. Staining of type III collagen was performed by the avidin-biotin-peroxidase complex (ABC) method (Kit, Vectastain Vector Labs, Burlingame, CA). The following antibodies were used as the primary antibody: rabbit antiserum to bovine kidney type IV collagen (Advance Co., Tokyo), goat antiserum to human type III collagen (Iatron Labs, Tokyo). Rabbit antiserum to a synthetic peptide corresponding to an internal domain near the C-terminus (residues 472–490) of human 72 kDa gelatinase was raised in our laboratories. The specificity of antiserum to 72 kDa gelatinase was examined by western blotting. For the negative control, the primary antisera were replaced by nonimmune serum.

Electronmicroscopy Tumor tissue was processed conventionally following glutaraldehyde-paraformaldehyde-osmium tetroxide fixation and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with the electron microscope (Nihon Denshi, JEM-1200 EX).

RESULTS

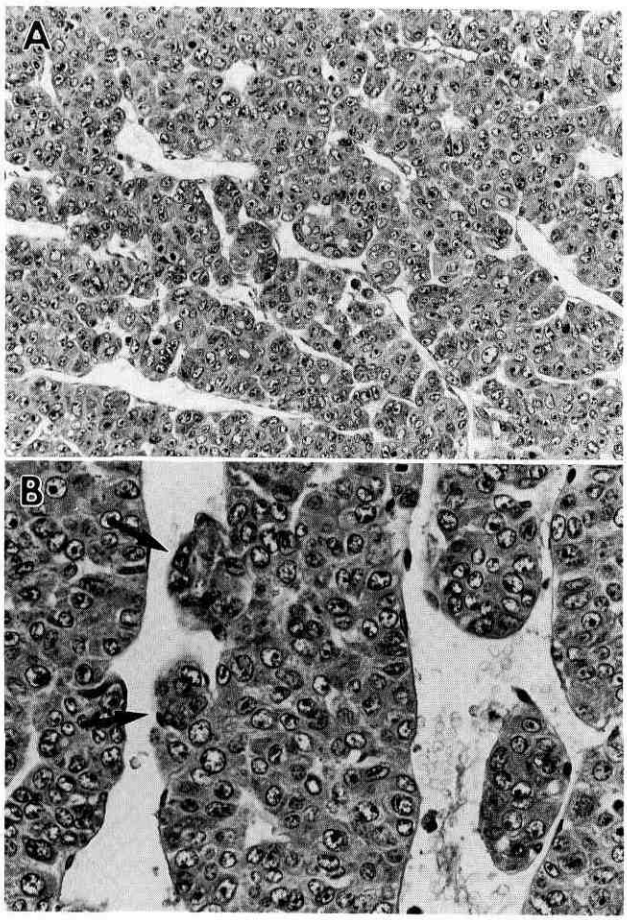
Transplantability and growth characteristics Tumor tissues were transplanted to both subcutaneous and intramuscular sites for the first 3 generations. Total tumor transplantability for 3 generations was 87% (20/23). Thereafter, primary tumor tissues were implanted to subcutis or muscle alone. Transplantability of the implanted tumors was 83% and 69% in KSN and Balb/c nude mice, respectively (Table I). In KSN female nude mice, the mean latent interval of the subcutaneously transplanted tumors was 20 days and tumor size averaged 1.0 cm in diameter at 45 days. The largest tumor was 4.0 cm in maximal diameter at 93 days. Similar growth characteristics were observed in Balb/c female nude mice.

Spontaneous lung metastasis The incidence and the number of macroscopic lung metastases varied depending upon the tumor-bearing time. In Balb/c female nude mice bearing tumors for up to 60 days, the incidence of visible lung metastases was 67% and the average number of metastatic nodules was only 1 (data not shown). On the other hand, in Balb/c female nude mice bearing tumors for over 80 days the incidence of macroscopic lung metastases was over 90% and the average number of visible metastases was 24. The incidence and the mean number of metastases in KSN female nude mice were almost the same as those in Balb/c nude mice, but the number of, metastases showed a small variation (Table I). The site of implantation had no significant effect on the visible lung metastases. No metastasis to other organs such as lymph node and liver was observed until the 5th passage. After serial passages of lung metastatic nodules, no significant increase in the number of lung metastases was observed in either KSN or Balb/c female nude mice (data not shown).

Table I. Growth, Transplantability and Spontaneous Metastasis of Serially Transplanted HY-1 Tumor in Nude Mice

No. of generations	Strain /Sex	Time of autopsy (days)	Implantation site	Tumor take rate	Mean tumor size (cm)	Lung metastasis		
						Incidence		No. of nodules mean (range)
						micro	macro	
4	Balb/F	93	sc	1/3	2.8	1/1	1/1	100
	Balb/F	85	im	1/3	3.0	1/1	1/1	9
	KSN/F	90–166	sc	3/5	3.2	3/3	3/3	21(17–25)
	KSN/F	86–103	im	3/3	3.6	3/3	3/3	19 (9–29)
5	Balb/F	81–100	sc	6/6	3.5	6/6	5/6	18 (5–34)
	Balb/F	72–90	im	3/4	3.2	3/3	3/3	16 (2–25)
	KSN/F	85–102	sc	6/6	3.3	6/6	6/6	22(13–31)
	KSN/F	78–91	im	3/4	3.4	3/3	3/3	31(10–100)

F, female mice; sc, subcutis; im, intramuscular; micro, microscopic metastasis; macro, macroscopic metastasis.



Histological analysis The histology of the original tumor of human stomach and the metastatic liver tumor was poorly differentiated adenocarcinoma of medullary type. Tumor cells grew in a trabecular and microglandular pattern separated by sinusoid-like blood vessels. Connective tissue was not conspicuous in the tumor tissue (Fig. 1A). The transplanted tumors in subcutis or muscle of nude mice showed essentially the same histological appearance. In transplanted tumor, penetration of blood vessels by tumor cells was regularly seen. In this region, tumor cell clusters disrupted the vascular endothelium to enter the vascular lumen. No migration of individual tumor cells through the vascular wall was observed (Fig. 1B). Fig. 2A shows the number of visible lung metastatic nodules ranging from 1 to 5 mm in diameter. At the light microscopic level, tumor embolization in the pulmonary arteries or arterioles and expansive growth of tumor cells within the vessels were observed in the lung metastases. In places, breaching of the vessel wall and subsequent extravasation to the lung parenchyma by tumor cells were also observed (Fig. 2B).

Fig. 1. (A) Histology of original human gastric carcinoma metastasized to the liver. The tumor shows poorly differentiated histological appearance with trabecular and microglandular structures separated by sinusoid-like blood vessels. (H-E, ×125). (B) Subcutaneously transplanted tumor in a nude mouse. The histological appearance is the same as that of the original tumor, as shown in A. Vascular invasion by tumor cells is seen (arrows). (H-E, ×250).

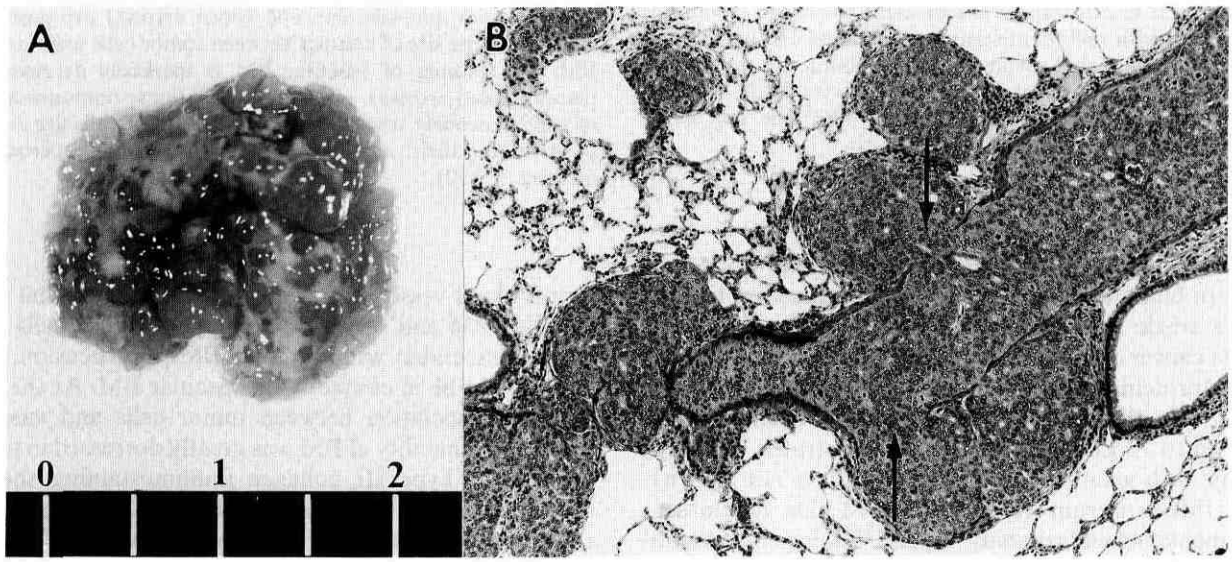


Fig. 2. Metastatic lung tumors in a nude mouse. (A) Macroscopic appearance of the lung with a number of metastatic nodules. (B) Microscopic appearance of the lung metastasis. Extensive intravascular tumor growth and occasional extravasation of tumor cells into lung parenchyma (arrows) are seen. (H-E, ×62).

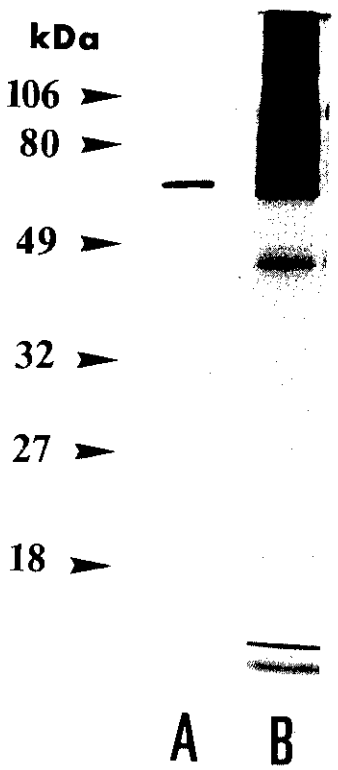


Fig. 3. Western blotting of gelatinases using antiserum to human 72 kDa gelatinase synthetic peptide. (A) Partially purified gelatinases were prepared from serum-free conditioned medium of human gastric cancer cells by gelatin affinity chromatography. Samples were run on a SDS polyarylamide gel (10% acrylamide) under non-reducing conditions. Proteins in the gel were electrotransferred to a nitrocellulose membrane and stained with rabbit antiserum to gelatinase and horseradish peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins. Peroxidase reaction was visualized by using 3,3′-diaminobenzidine as the substrate. (B) The gel before blotting was silver-stained.

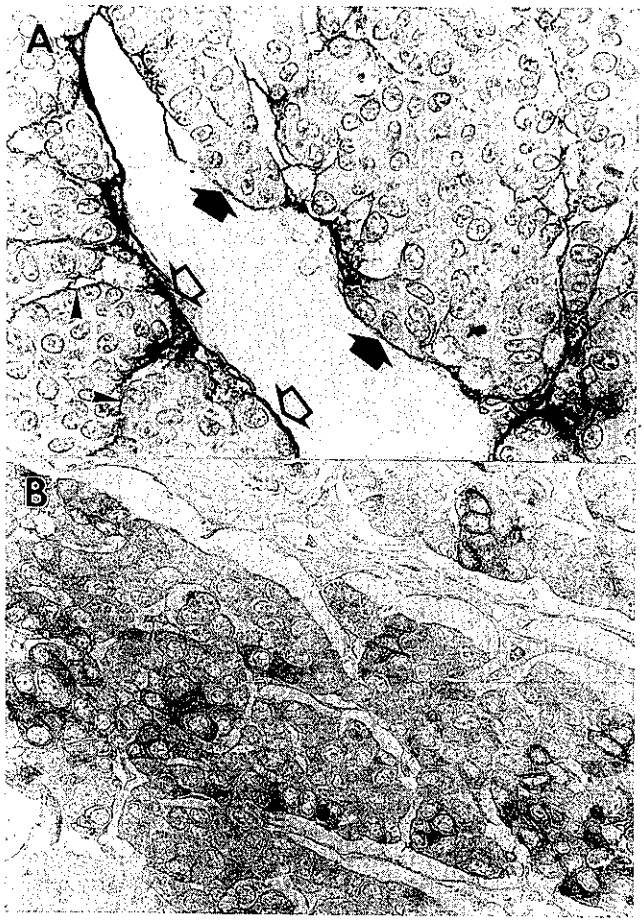


Fig. 4. (A) Type IV collagen immunostaining of subcutaneously transplanted tumor in a nude mouse. Tumor BM (arrowheads) and vascular BM (open arrows) are positively stained. At the site of contact between tumor cells and vascular BM, the staining of vascular BM is markedly decreased in places (closed arrows). (B) 72 kDa gelatinase immunostaining of subcutaneously transplanted tumor. Tumor cells are stained positive in their cytoplasm. (indirect immunoperoxidase method, ×250).

Specificity of anti-human 72 kDa gelatinase antiserum Western blot analysis showed that this antiserum reacted with a single spieces in conditioned medium of human gastric cancer cells (Fig. 3), indicating that other neutral metalloproteinases present in this sample do not cross react with this antiserum. The single species corresponded to 72 kDa gelatinase as demonstrated by zymography with gelatin as the substrate (data not shown). Thus, this antiserum was specific to 72 kDa gelatinase.

Immunohistochemical study Type IV collagen immunostaining of transplanted tumor clearly showed the presence of discontinuous tumor BM surrounding the groups of tumor cells and vascular BM. In the vicinity of the tumor blood vessels which consisted of endothelial cells, vascular BM and occasional pericytes, tumor cells were closely associated with vascular BM. On occasion, they appeared to be in contact with vascular BM. At the sites of close association between tumor cells and vascular BM, the stainability of BM was greatly decreased in places (Fig. 4A). Type III collagen immunostaining showed the absence of interstitial collagens between tumor cells and vascular BM (data not shown). When the localization of 72 kDa gelatinase was investigated, positive staining was principally detected in the cytoplasm of tumor cells, but not in other cell types (Fig. 4B).

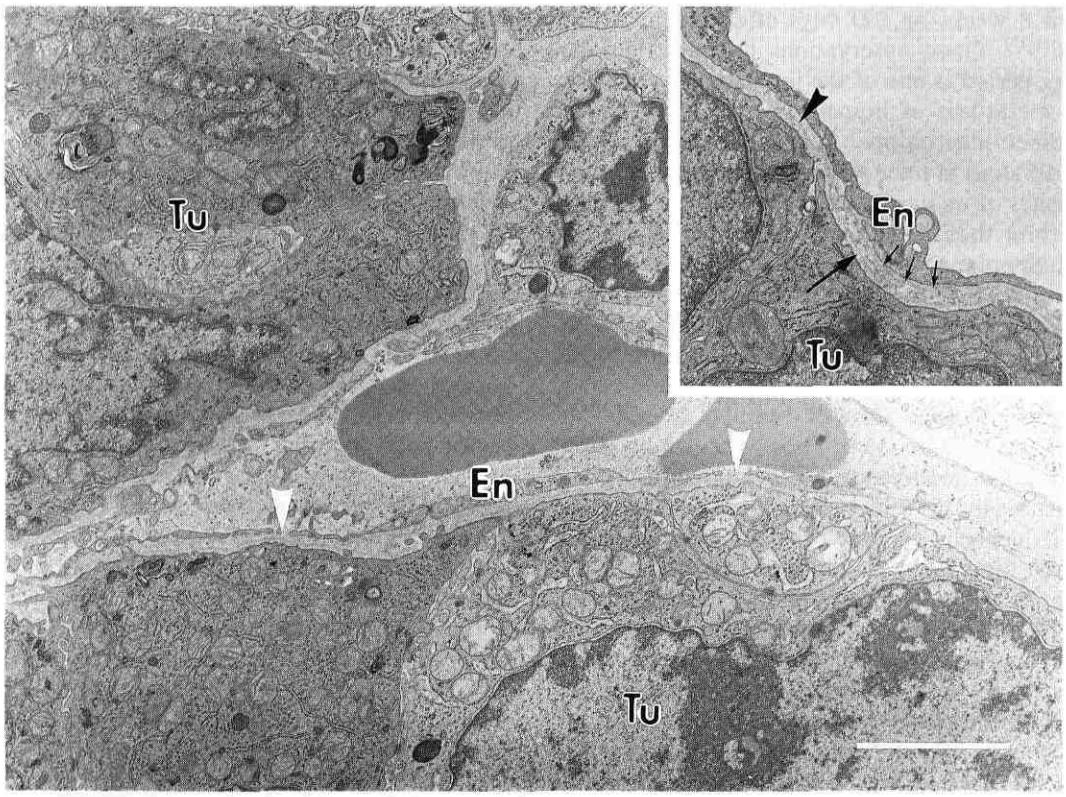


Fig. 5. Electron micrograph of subcutaneously transplanted tumor in a nude mouse, showing the close association between tumor cells and vascular BM. Tumor BM (large arrow, inset) and vascular BM (small arrows, inset) meet to form a single BM, and tumor cells are in direct contact with the BM (arrowhead, inset). Degenerative changes of endothelial cells such as gaps and focal disappearance of vascular BM are evident (white arrowheads). Tu = tumor cell, En = endothelial cell. (Bar = 5 μm).

Electron microscopic study At the site of association between tumor cells and vas-cular BM, tumor BM and vascular BM ran in parallel and met each other. On occasion, tumor cells were in direct contact with the single BM with epithelial cell polarity. No collagen fibrils were found between them. Focal disappearance of vascular BM and degenerative changes of endothelial cells such as vacuolation and gaps were observed (Fig. 5). There was no evidence of definite diapedesis of tumor cells between endothelial cells.

DISCUSSION

In the present study, we established a new spontaneous metastasis model of transplantable human gastric cancer in nude mice. This model is unique in the following two respects. First, this model shows not only micrometastases but also severe and stable macroscopic metastases to the lung. The incidence of macroscopic lung metastases of HY-1 in KSN and Balb/c female nude mice was nearly 100% and the average number of lung nodules was over 20. Second, this is the first model of a poorly differentiated adenocarcinoma of medullary type, which is known to be highly metastatic to the liver among various types of gastric cancers.[13, 14] Though the original tumor was obtained from liver metastatic lesion, this tumor metastasizes to the lung but not to the liver in nude mice. This discrepancy may be attributed to the difficulty of entry of tumor cells into the systemic circulation from the first organ encountered. Intraportal or intraarterial inoculation of tumor cells may result in dissemination to other organs including liver. This animal model provides a useful tool for the quantitative experimental study of hematogenous metastasis of a particular type of gastric carcinoma with clinical and pathological importance.

Stable macroscopic lung metastasis could be seen only in mice bearing tumors for more than 80 days in this model. Other investigators reported that spontaneous metastasis of human gastric cancer in nude mice was not

detected until 8 weeks or 100 days after subcutaneous implantation.[11, 12] These observations indicate that the tumor-bearing period is one of the important factors for spontaneous metastasis of human gastric carcinoma in nude mice. Since macroscopic lung metastasis was not significantly different in mice with different genetic backgrounds and sites of implantation, these host factors may be less important than the tumor-bearing period in this tumor model. Spontaneous visible lung metastasis tended to be higher in KSN female mice than KSN male mice, but not in Balb/c nude mice (data not shown). The effect of sex of mice used on metastasis remains to be elucidated.

HY-1 tumors regularly showed vascular invasion at the implanted site in nude mice. Thus, vascular invasion seems to be of great importance in development of spontaneous metastasis in this tumor. Based on *in vitro* studies of invasion, it has been proposed that vascular invasion involves at least three steps, 1) adhesion of tumor cells to vascular BM, 2) degradation of the BM by proteinases such as type IV collagenase or heparitinase, and 3) tumor cell migration through endothelium.[15, 16] To understand the *in vivo* process by which the tumor cells penetrate the blood vessels in this metastasis model, we investigated the above steps *in situ*. We first observed the tumor cell-vascular BM interface at the implanted site, and found that tumor cells often associated with vascular BM and occasionally came in direct contact with the BM with extension of their cytoplasm along the BM. These morphological findings suggest that in this model, tumor cells are not simply contiguous with vascular BM, but virtually adhered to the vascular BM *in vivo*.

Second, we investigated the degradation of vascular BM. As demonstrated by type IV collagen immunostaining and electron microscopy, the vascular BM focally disintegrated and disappeared at sites of close contact between tumor cells and vascular BM. This finding suggests the degradation of vascular BM by tumor cells or endothelial cells, although other possibilities, such as decreased synthesis of BM components or impaired BM assembly by endothelial cell themselves, cannot be excluded. Recently, it has been demonstrated that 72 kDa gelatinase cleaved BM type IV collagen.[17] To examine the enzyme responsible for the degradation of the vascular BM in this tumor, we studied the localization of 72 kDa gelatinase and found intense positive staining only in the cytoplasm of tumor cells. This suggests that tumor cells are the essential source of the enzyme in tumor tissue, as previously demonstrated in human cancers.[18] Seventy-two kDa gelatinase is a latent form required for activation to degrade native type IV collagen.[19] At present, we have no evidence of activation of this enzyme *in vivo*. However, it seems likely that degradation of vascular BM may be at least in part due to 72 kDa gelatinase derived from tumor cells.

For tumor cell entry into blood vessels, the final step of vascular invasion, essentially two mechanisms have been proposed.[20, 21] Tumor cells enter the vessel lumen either by active migration between endothelial cells or by causing endothelial degeneration and subsequent destruction of the vessel wall (endotheliolysis). In the tumor model under study, entry of tumor cells into the vascular lumen seemed to occur in cell clusters after disruption of endothelial cells, and no active migration or diapedesis of tumor cells through interendothelial junctions was observed. Therefore, tumor cell entry into the lumen may be through the latter mechanism. This is supported by the observation that extravasation occurred through the combination of extensive intravascular proliferation of tumor cells and subsequent destruction of vascular wall in the lung. However, the mechanism of endothelial cell destruction is unknown at present. It might involve degradation of the BM scaffold by tumor cells, secretion of some endotheliotoxic factor by tumor cells or mechanical force due to proliferation of tumor cells at the site of contact.[16, 21]

In conclusion, all these results suggest that vascular invasion in this tumor consists of the following sequential events: close contact of tumor cells with the vascular BM, degradation of vascular BM, and degeneration and disruption of endothelial cells, allowing tumor cells to penetrate the blood vessels. Active migration or diapedesis of tumor cells through endothelial cells does not involve intravasation in this model. We previously demonstrated that close contact between tumor cells and vascular BM at the primary site was correlated to liver metastases of human gastric cancers.[22] We propose that contact between tumor cells and vascular BM at the primary site may be an initial step for hematogenous metastasis in human gastric cancers.

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