Jpn. J. Cancer Res. 87, 78–85, January 1996

Establishment and Characterization of High- and Low-lung-metastatic Cell Lines Derived from Murine Colon Adenocarcinoma 26 Tumor Line

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We established and characterized high- (LuM1) and low-lung-metastatic (NM11) cell lines derived from murine colon adenocarcinoma 26 tumor line. LuM1 cell line was established as a clonal cell line from a cultured cell mixture derived from a lung-metastatic nodule after 7 sequential subcutaneous transplantations of lung-metastatic tumors in the abdominal wall of BALB/c mice. NM11 cell line was established from a cultured cell mixture derived from a subcutaneous transplant of murine colon adenocarcinoma 26 tumor cells. LuM1 cells showed marked spontaneous lung metastases, but NM11 cells rarely did. High invasive potential of LuM1 cells was revealed by *in vitro* invasion assay using Matrigel reconstituted membranes. Rapid retraction was observed in monolayers of human umbilical vein endothelial cells and bovine aortic endothelial cells when LuM1 cells were added on the monolayers. Gelatin zymography and immunochemical examinations with monoclonal antibodies against gelatinase B (M_r 95,000 type IV collagenase) showed secretion of large amounts of the gelatinase by LuM1 cells.

Key words: High-lung-metastatic cell line — LuM1 — NM11 — Gelatinase B (M_r 95,000 type IV collagenase) — Basement membrane

Tumor metastasis involves a series of complex processes: detachment of tumor cells from the primary tumor mass, microinvasion into stromal tissues, intravasation into and extravasation from the circulatory system and growth in secondary sites. These processes involve various interactions of tumor cells with host cells or extracellular matrix, and tumor cell properties such as adhesiveness, motility and secretion of proteinases could have crucial roles.[1,2] In order to analyze these processes, it is important to have a suitable model that mimics them, and many metastasis models in the mouse have been reported, including spontaneous metastasis models for the overall process and experimental metastasis models suitable for the analysis of the steps after intravasation.[3–10] Cell lines with differing metastatic potentials were selected by various methods. Fidler established an *in vitro* selection method for metastatic variants from B16 melanoma cell line.[11] A similar method was applied to select cell lines with spontaneous metastatic potential from murine carcinomas such as lung carcinoma, colon adenocarcinoma and bladder cell carcinoma.[12–16] In this study, we describe the establishment and characterization of a new lung-metastatic cell line, LuM1, from murine colon adenocarcinoma 26. LuM1 cells showed marked vascular invasion and high-lung-metastatic potential *in vivo*, as well as highly invasive properties *in vitro*, in terms of penetration through a Matrigel matrix and of retraction of endothelial cell monolayers.

MATERIALS AND METHODS

Tumor line and cell lines Murine colon adenocarcinoma 26 tumor line[17] was obtained from Dr. T. Kato, Laboratory of Chemotherapy, Aichi Cancer Center Research Institute. A subcutaneous tumor of colon adenocarcinoma 26 transplanted in a BALB/c mouse was minced into small fragments and cultured in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and the resultant cultured cells have been maintained as a cultured cell mixture. A clonal cell line, NM11, was established from the cultured cell mixture by the limiting dilution method using culture plates of 96 wells. Then an attempt was made to establish a high-lung-metastatic tumor line, following the method described by Fidler.[11] Half a million cells of the cultured cell mixture were transplanted in the subcutaneous tissue of the abdominal wall of 6-week-old female BALB/c mice. Subcutaneous tumors developed in all transplanted mice, but no macroscopic metastatic nodule was found

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after 5 weeks following the transplantation. Lung tissues of a tumor-bearing mouse were excised, minced and transplanted in the abdominal wall in other BALB/c mice. After 7 sequential transplantations, lung tumor nodules became visible. The lung tumor nodules were minced and cultured. A clonal cell line, LuM1, was established from the resultant cultured cells. These LuM1 and NM11 cells were maintained in RPMI 1640 medium with 10% FBS. They were transferred from Aichi Cancer Center Research Institute to the Department of Pathology, Nagoya University School of Medicine in 1990.

Chromosome analysis LuM1 and NM11 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Colcemid was added for 2 h at the concentration of 0.02 mg/ml. Then, the cells were collected after simple trypsinization, treated with 0.05 *M* KCl for 20 min at room temperature and fixed with a methanol:acetic acid mixture (3:1). Chromosome preparations were made by the conventional air-drying technique. The chromosomes were stained by a G-banding technique. The G-banded metaphases were obtained by trypsin treatment. Mouse chromosomes were identified according to the standard karyotype.[18]

***In vivo* metastasis assay** LuM1 or NM11 cells (2×10^5) were transplanted subcutaneously in the abdominal wall of BALB/c mice. After 25 days, the mice were killed and internal organs including lungs, liver, kidneys, spleen and subcutaneous tumors were resected. The resected organs and tumors were weighed and parts of them were fixed with 4% paraformaldehyde, and processed for light microscopic examinations.

***In vitro* invasion assay** Double-chamber culture systems were used for *in vitro* invasion assay. Transwell chambers with filters of 8 μm pore size (Costar, Alewife Center, Cambridge, MA) were reconstituted with 20 or 100 μl of Matrigel (Collaborative Research Inc., Bedford, MA) by incubating for 1 h at 37°C. The Transwell chambers with Matrigel reconstituted membranes were filled with 0.5 ml of serum-free ITS+ medium (Collaborative Research Inc.) and set on culture plates with 24 wells filled with 1 ml of the medium. A cover glass was placed at the bottom of each lower well. LuM1 or NM11 cells (2×10^4/well) were added to the upper chambers and incubated in 5% CO_2-air at 37°C. The number of tumor cells that passed through the thin Matrigel membranes (20 μl/chamber) was counted. The cover glasses were changed every day and tumor cells recovered on them were stained with Coomassie brilliant blue and counted. The thicker Matrigel membranes (100 μl/chamber) were used for the morphological examination of penetrating cells. The membranes penetrated by tumor cells were fixed with 4% paraformaldehyde or 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.05 *M* phosphate buffer, and processed for light or electron microscopic examination, respectively.

***In vitro* retraction assay** LuM1 or NM11 cells were dispersed with 0.02% EDTA and 0.05% trypsin in phosphate-buffered saline at 37°C for 2 min. These cells ($0.5–5\times10^5$/well) were seeded on monolayers of human umbilical vein endothelial cells (HUVEC) (Kurabo Co., Osaka)[19,20] or bovine aortic endothelial cells (BAE), provided by Dr. N. Ohmiya, Department of Internal Medicine, Nagoya University School of Medicine, in RPMI 1640 containing 10% FBS. Sequential morphological changes of co-cultured cells were examined under a phase-contrast microscope after the tumor cell seeding.

Detection of gelatinases and production of monoclonal antibody against gelatinase B Gelatinases (M_r 95,000 and M_r 60,000 type IV collagenases) secreted into the serum-free RPMI 1640 culture medium conditioned with LuM1 or NM11 cells were detected by zymography with gelatin as a substrate, as described previously.[21,22] Gelatinases were purified from a total of 20 liters of serum-free medium containing 100 ng/ml phorbol myristate acetate (Sigma, St. Louis, MO) conditioned with LuM1 cells for 24 h by gelatin-Sepharose affinity chromatography. One liter of the serum-free conditioned medium, adjusted to 0.5 *M* NaCl, 5 m*M* $CaCl_2$, 0.01% Brij 35, 20 m*M* Tris-HCl, pH 7.5 and 0.5 m*M* phenylmethylsulfonyl fluoride (Sigma), was applied to 1.0×20 cm gelatin-Sepharose column. The gelatinases were eluted from the column with the same buffer containing 7.5% dimethyl sulfoxide. Then, the fraction containing the gelatinases was passed through an anti-fibronectin antibody column for removal of fibronectin. The purified gelatinases were concentrated and stored at −80°C.

The purified gelatinases (50 μg) emulsified with an equal volume of complete Freund's adjuvant were injected into the splenic subcapsular space of a Fischer rat. The same amount of the gelatinases was boosted into the same site 3 weeks after the first injection and again subcutaneously 1 week later. Splenic cells of the immunized rat were fused with Y3 rat melanoma cells by using polyethylene glycol. Screening and cloning of hybridomas producing monoclonal antibodies against gelatinase were performed with enzyme-linked immunosorbent assay using multiwell plates coated with the purified gelatinases. Specificity of monoclonal antibody against the gelatinases was examined by immunostaining with POD Immunostain Set (Wako, Osaka) after separation of gelatinases A and B by polyacrylamide gel electrophoresis and transfer to a nitrocellulose filter membrane by Western blotting. Only antibodies against gelatinase B were obtained.

To detect gelatinase B in LuM1 or NM11 cells, they were cultured on cover glasses with or without 5×10^{-7} *M* monensin (Sigma) for 5 h. The cells were fixed with 4%

paraformaldehyde for 30 min and treated with 0.2% Triton X-100 (Sigma) for 2 min. The cells were examined under a fluorescence microscope after overnight treatment with a rat monoclonal antibody against gelatinase B (12-5F) and then with fluorescence isothiocyanate-conjugated anti-rat IgG (Tago Inc., Burlingame, CA).

RESULTS

LuM1 and NM11 cell lines Under a phase-contrast microscope, LuM1 cells were spindle-shaped, showing a tendency of rounding (Fig. 1A), whereas NM11 cells were an admixture of spindle and polygonal cells, showing a tendency to adhere to the culture dish (Fig. 1B).

Chromosome analysis of LuM1 and NM11 cell lines Thirty-two LuM1 and 33 NM11 metaphases were analyzed for karyotype and chromosomal rearrangements. LuM1 cells were hypotetraploid, and the modal number of the chromosome was 69 (Fig. 2A). NM11 cells were also hypotetraploid, having the modal number of 65 (Fig. 2B). The difference in number between these cell lines might mainly depend on the numerical aberration of autosomes. The following chromosomes were commonly found in both cell lines: isochromosomes of 5, deleted 7, 14 and 18, and Robertsonian translocation between 5 and 15. NM11 cells had more markers: isochromosomes of 3 and 7. There were many other isochromosomes, Robertsonian translocations and deleted chromosomes, but they were sporadic. The Y chromosome was not found in these two lines.

***In vivo* characteristics of LuM1 and NM11 cells** Histological examinations of subcutaneous tumors in the abdominal wall formed by transplantation of LuM1 or NM11 cells revealed that LuM1 cells were more invasive

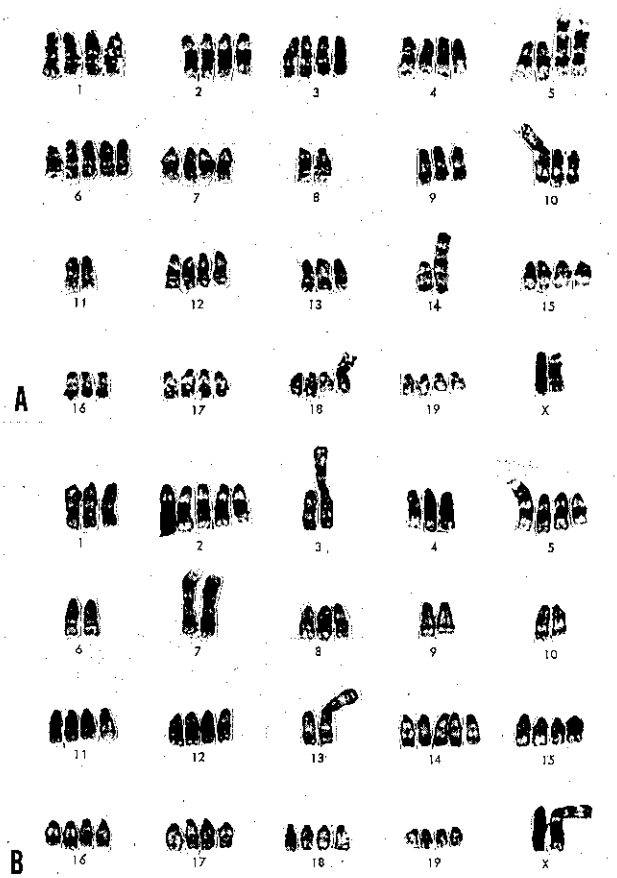

Fig. 2. Karyotypes of LuM1 (A) and NM11 (B) cells, showing hypotetraploidy, isochromosomes 3, 5, 7, 10, 13, 14 and 18, and translocations, (5q15q) and (Xq10q). For details, see the text.

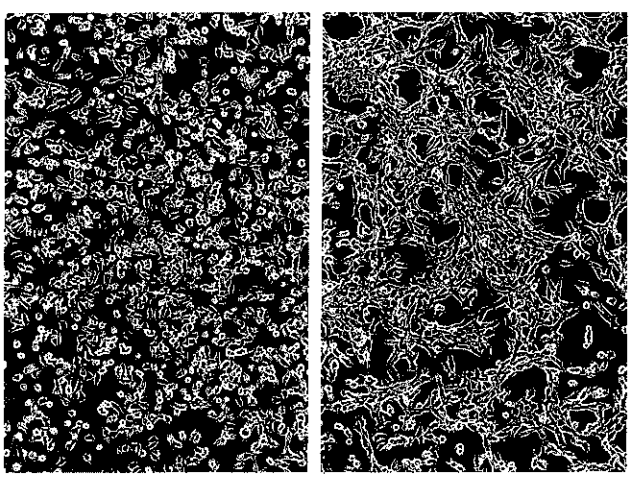

Fig. 1. Phase-contrast micrographs of LuM1 (A) and NM11 cells (B) (×92).

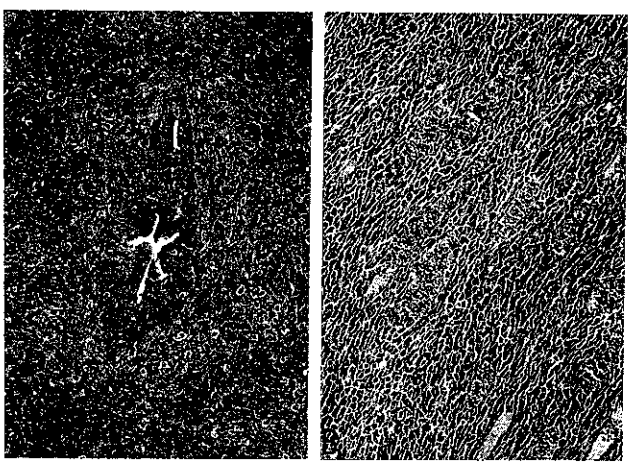

Fig. 3. Micrographs of tumors in abdominal walls of BALB/c mice. (A) LuM1 cells frequently invaded blood vessels and obstructed the lumens (HE, ×50). (B) NM11 cells rarely invaded blood vessels (HE, ×50).

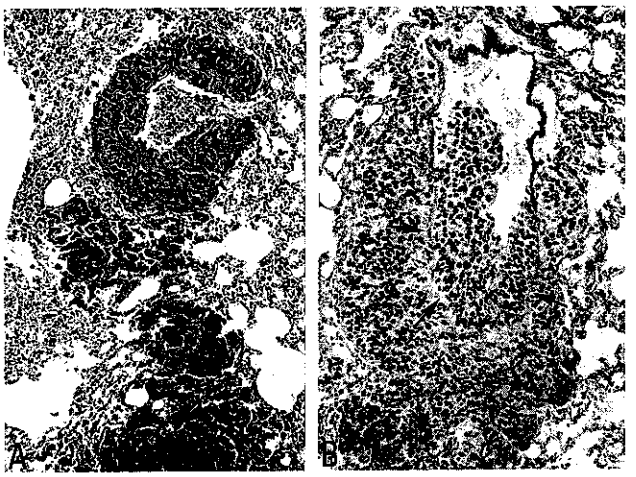

Fig. 4. Micrographs of lung metastases of LuM1 cells. (A) Many metastatic nodules are seen (HE, ×50). (B) Immunostaining of type IV collagen with anti-type IV collagen polyclonal antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) (avidin-biotin-peroxidase complex method). Degradation of type IV collagen of vascular basement membranes invaded by LuM1 cells can be seen (arrows) (×50).

Table I. Metastatic Potentials of LuM1 and NM11 Cells in Lungs of BALB/c Mice

Cell[a)]	Subcutaneous tumor weight (mg)	Lung weight (mg)	Lung metastasis		
			No. of nodules		Incidence
			Mean	Range	
LuM1	1626±220	205±50	135	55–166	4/4
NM11	1047±360	118±5	0		0/3

a) Cells (2×10^5) were transplanted subcutaneously into 6-week-old female BALB/c mice, and lung metastatic nodules were examined after 25 days.

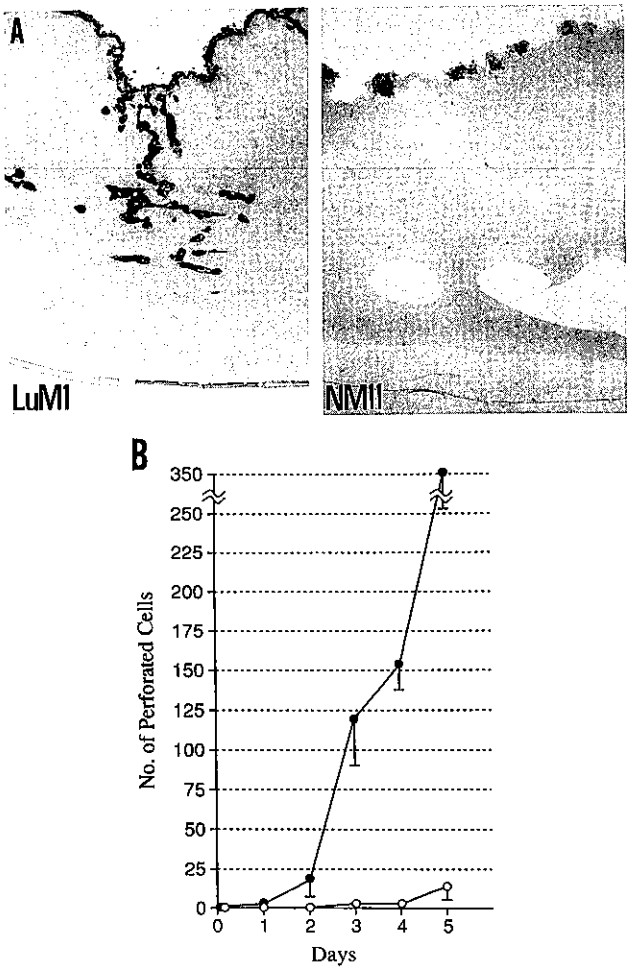

Fig. 5. *In vitro* invasion assay of LuM1 and NM11 cells. (A) Micrographs of reconstituted membranes of 100 μl of Matrigel on Transwell chambers fixed 7 days after culture with LuM1 or NM11 cells. LuM1 cells were more invasive than NM11 cells (HE, ×50). (B) Time sequence of number of penetrated LuM1 (●) and NM11 (○) cells through reconstituted membranes of 20 μl of Matrigel on 8 μm pore-size filters of Transwell chambers.

to vascular vessels (Fig. 3A) than NM11 cells (Fig. 3B). Mice transplanted with LuM1 cells had heavier lungs with many metastatic lung nodules (Fig. 4A), whereas mice transplanted with NM11 cells had lighter lungs without visible nodules (Table I). LuM1 cells maintained in culture for 2 years still had similar metastatic potential to the lungs, inducing many tumor emboli in blood vessels of the lungs. Immunostaining of type IV collagen of the metastatic nodules in the lungs showed degradation of the vascular basement membrane (Fig. 4B). Lymph node metastases were rarely formed.

***In vitro* invasive properties of LuM1 and NM11 cells** To ascertain the invasive character of LuM1 cells observed in histological examinations of primary subcutaneous tumors, the cells were examined *in vitro* using Matrigel reconstituted membranes. LuM1 cells, cultured on the membranes for 7 days, deeply infiltrated into them, in contrast with NM11 cells, which proliferated on the surface of the membranes (Fig. 5A). The number of LuM1 cells perforated through the Matrigel reconstituted membranes was 10 times more than that of NM11 cells, at 2 days following the seeding (Fig. 5B). Electron microscopic examinations revealed that LuM1 cells penetrated into the membranes, showing elongated cytoplasmic processes (Fig. 6).

Retraction of endothelial cells by LuM1 cells Fig. 7 shows a series of micrographs taken at different times after LuM1 cell seeding on HUVEC monolayers. LuM1 cells were recognized as rounded cells on the monolayers

by 1 h after the seeding (Fig. 7A), and adhered to the monolayers 2 h after (Fig. 7B). Cleavage of HUVEC intercellular junctions began around the adherent LuM1 cells, resulting in retraction of HUVEC monolayers. HUVEC formed small round foci containing acellular fenestrations. Thereafter, the margins of the foci expanded, forming many larger acellular fenestrations (Fig. 7, C–E). Seeding of NM11 cells on the HUVEC monolayers resulted in much later retraction, starting 6 h after the seeding. BAE monolayers seeded with LuM1 cells gave similar but slower retraction (data not shown), while co-culture of BAE monolayers with NM11 cells gave no retraction.

Production and secretion of gelatinases by cultured LuM1 and NM11 cells Zymographic examinations and immunostainings of gelatinases in the media showed that LuM1 cells secreted a remarkably larger amount of gelatinase B than NM11 cells, whereas secretion of gelatinase A (M_r 60,000 type IV collagenase) was little different between LuM1 and NM11 cells (data not shown). Gelatinase B in serum-free conditioned media of LuM1 cells was recognized as a single band with a molecular weight of 95 kDa by the monoclonal antibody (12-5F) (Fig. 8). Immunostaining of the monensin-treated cells with 12-5F monoclonal antibody gave positive staining in LuM1 cells, but not in NM11 cells (Fig. 8).

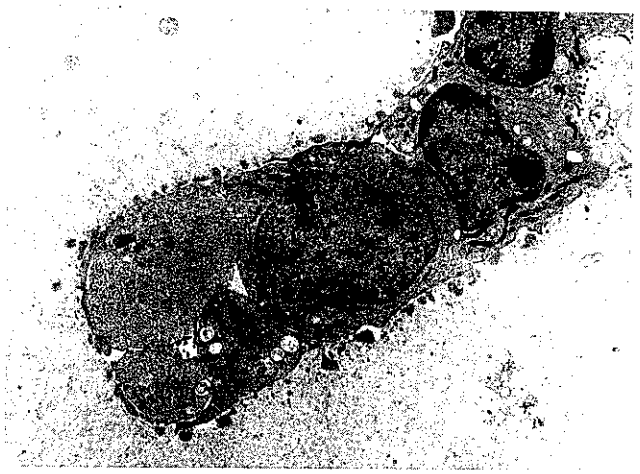

Fig. 6. Electron micrograph of Matrigel reconstituted membrane penetrated by LuM1 cells. LuM1 cells have many slender cytoplasmic processes in compact Matrigel (×3600).

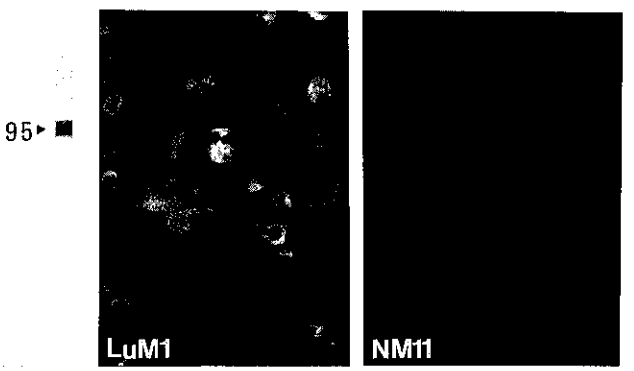

Fig. 8. Immunofluorescence of LuM1 and NM11 cells with monoclonal antibody against gelatinase B, 12-5F. Cells were incubated with 5×10^{-7} *M* monensin overnight. Cytoplasm of LuM1 cells was stained, but NM11 cells were rarely stained (×200). Western blot analysis with monoclonal antibody against gelatinase B, 12-5F, is shown on the left.

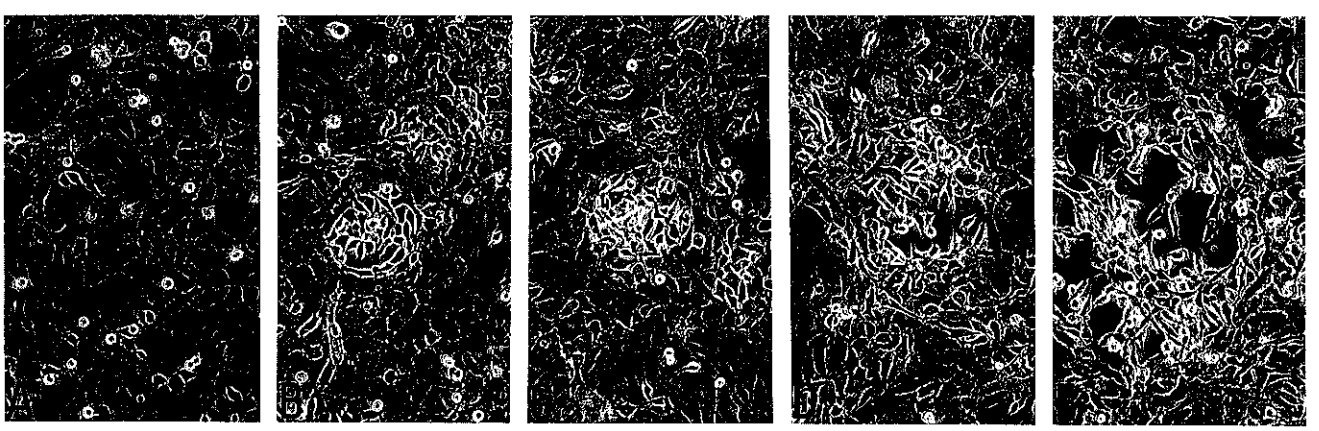

Fig. 7. Phase-contrast micrographs of co-culture of monolayers of HUVEC with LuM1 cells, showing time sequence after LuM1 cell seeding. A: 1 h, B: 2 h, C: 3 h, D: 6 h, E: 9 h after the seeding. Later the margins of foci enlarged and many acellular fenestrations developed (C–E) (×137).

DISCUSSION

We have established two murine colon cancer cell lines, LuM1 and NM11, expressing different potentials of lung metastasis. A clear cytological difference was found between the high-lung-metastatic LuM1 cells and the low-lung-metastatic NM11 cells. However, no obvious difference could be found in karyotypes between these cell lines. Histological observation of subcutaneous tumors often revealed the presence of LuM1 cells in blood vessels. This could be a reflection of the invasive property of LuM1 cell.

To examine this property, two experiments were conducted using *in vitro* cultured cells: examinations of cellular invasiveness through Matrigel reconstituted basement membrane and the effect on endothelial cell monolayers in co-culture. Matrigel has been used for *in vitro* invasion assay of tumor cells in several studies, and it was demonstrated that invasive potential of tumor cells into Matrigel reconstituted membranes was well correlated with *in vivo* metastatic ability.[6, 8] The *in vitro* experiments of the present study clearly showed that LuM1 cells had a stronger penetrating ability into Matrigel membranes than NM11 cells, confirming the usefulness of this method to assess the infiltrating potential of tumor cells. Retraction of cultured endothelial cell monolayers caused by tumor cells has been supposed to mimic the process of vascular invasion by tumor cells. Two distinct mechanisms of retraction, one by a soluble factor from tumor cells and the other by the plasminogen activator system on the surface of endothelial cells, have been proposed. In the former, medium conditioned with tumor cells has been reported to contain a factor retracting cultured bovine aortic endothelial cells,[23, 24] and in the latter, aprotinin, an inhibitor of plasmin, completely inhibited the process.[25] Rapid retraction of a HUVEC monolayer caused by LuM1 cells requires cellular attachment of LuM1 cells to the HUVEC monolayer. Medium conditioned with LuM1 or with LuM1 and HUVEC did not cause retraction of a HUVEC monolayer. The retraction caused by LuM1 cells was not inhibited by proteinase inhibitors such as aprotinin, pepstatin, leupeptin, E-64 and phosphoramidon. The addition of cycloheximide to culture medium inhibited the process (data not shown). These results suggest that some unknown mechanism could operate in retraction of HUVEC and BAE monolayers caused by LuM1 cells. The ability of LuM1 cells to induce the retraction of an endothelial monolayer is another manifestation of the invasive property of LuM1 cells, although the underlying mechanism remains to be elucidated.

Degradation of the extracellular matrix by tumor cells is considered to be essential for stromal invasion. In particular, destruction of the basement membrane by tumor cells is important. As the main components of the basement membrane are laminin and type IV collagen, secretion of proteolytic enzymes having specificity for these components, such as gelatinases and stromelysins, could favor successful invasion of tumor cells and formation of metastases. A number of studies have reported overexpression of matrix metalloproteinases by tumor cells and relatively decreased expression level of tissue inhibitors of matrix metalloproteinases.[26–35] The association of the overexpression of gelatinases with high metastatic potential of tumor cell lines has been reported.[26, 28–30, 34, 36–38] We also reported previously that LuM1 cells secreted a larger amount of gelatinase B and smaller amounts of inhibitors than NM11 cells.[21, 22] Thus, the balance of the enzyme and its inhibitors in LuM1 cells is in enzyme excess.[39] A recent study showed that overproduction of gelatinase B by LuM1 cells is due to autocrine factors enhancing the secretion of gelatinase B.[40] The present immunohistochemical and zymographical examinations revealed disruption of the type IV collagen of the vascular basement membranes by LuM1 cells. As the secretion of gelatinase A was similar between LuM1 and NM11 cells, the ability of LuM1 cells to disrupt the basement membranes may be correlated with the expression level of gelatinase B.

An experimental model of a high-metastatic cell line is necessary for the study of tumor metastasis. LuM1 and NM11 cells exhibit different potentials of invasion or metastasis, though they were derived from the same tumor. LuM1 cells show hematogenous lung metastasis and degeneration of type IV collagen in the vascular basement membranes, producing gelatinase B. Therefore, LuM1 cells and the counterpart low-lung-metastatic NM11 cells should prove to be a useful experimental model of lung metastasis of tumor cells.

ACKNOWLEDGMENTS

We are grateful to Dr. N. Ohmiya, Department of Internal Medicine, Nagoya University School of Medicine for providing BAE, and to Mr. J. Aoki, Mr. K. Imaizumi, and Miss M. Kozuka for their technical assistance. This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan, by Special Coordination Funds of the Science and Technology Agency of Japan and by a grant from Imanaga Medical Foundation.

(Received August 23, 1995/Accepted October 12, 1995)

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