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Growth Characteristics in the Initial Stage of Micrometastasis Formation by Bacterial *LacZ* Gene-tagged Rat Prostatic Adenocarcinoma Cells

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A micrometastasis model was established using a rat differentiated prostatic adenocarcinoma, designated PLS30lZ, transfected with the *lac*Z gene encoding a bacterial β-galactosidase. The morphology, tumorigenicity and metastatic ability of PLS30lZ were comparable to those of the parental cells. Micrometastatic foci could be specifically detected at the single cell level after X-Gal staining with a dissecting microscope. After intravenous injection, the number of X-Gal positive foci in the lung decreased progressively to a steady-state level (less than 1% of injected cells) by 4–7 days, while the size of persisting positive foci started to increase from 4 days after inoculation, as demonstrated by image analysis. X-Gal and BrdU double staining revealed that BrdU labeling indices of X-Gal-positive cells decreased transiently at the 2-day time point and increased again from 4 days after inoculation. Type IV collagen immunostaining showed the tumor cells to be surrounded by a basement membrane intravascularly at the time point when they started new growth. Electron microscopy confirmed that, 2 days post injection, most tumor cells were degenerative or dead, but on day 4, persisting tumor cells formed multicellular clumps in contact with the vascular basement membrane inside vessels. These results indicate that PLS30lZ cells begin to grow intravascularly depending upon the presence of a basement membrane before extravasation at the initial stage of micrometastasis formation.

Key words: Micrometastasis — Prostatic carcinoma — *LacZ* gene — Cell growth — Basement membrane

Micrometastasis may already be present when primary neoplasms are diagnosed. To improve survival rates of cancer patients, diagnostic and therapeutic approaches to deal with micrometastasis are essential. To date, however, studies on the biology of micrometastases, as well as on the mechanisms permitting their stabilization over months or years and their expansion into overt nodules have been hampered by the inability to identify small numbers of tumor cells in the host tissues. Several methods including fluorescence[1] and radioisotopic labeling,[2] as well as production of drug-resistant mutants,[3] have been used to tag tumor cells to study their fate *in vivo*, their interactions with host cells and the clonal origin of metastatic sub-populations. These approaches, however, all have limitations for long-term studies *in vivo*.

LacZ gene encodes *Escherichia coli* (*E. coli*) β-galactosidase, whose enzymatic activity can be simply detected by staining with the chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal), which generates an intense blue product. Lin *et al.* first demonstrated that i.v.-injected ras-transformed 3T3 cells supertransfected with the *lac*Z gene could be easily detected in mouse tissues at the single cell level following X-Gal staining.[4] Several *lac*Z transfectants of metastatic tumor cells of different origin, including breast cancer,[5] melanoma,[6] glioma,[7] and lymphoma,[8] have subsequently been developed and used to detect micrometastases *in vivo*. However, the growth kinetics of the earliest stage of micrometastasis formation by carcinoma cells, especially by differentiated cells, remain essentially unknown. We previously established well-differentiated, metastatic adenocarcinoma cell lines from prostatic carcinoma induced by DMAB in F344 rats.[9–11] In this study, we transfected a prostatic carcinoma cell line with the *lac*Z gene and established a micrometastasis model. This cell line, which stably expresses the *lac*Z gene and has a metastatic potential comparable to that of the parental cells, was here used to study the initial growth associated with micrometastasis formation both qualitatively and quantitatively.

MATERIALS AND METHODS

Animals Athymic male nude mice of the CD-1 strain were purchased from Charles River Japan, Inc.

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(Kanagawa). They were kept in semisterile laminar flow conditions at 25°C and given sterile food and water.

Cell line The PLS30 cell line was established in our laboratory from a 3,2′-dimethyl-4-aminobiphenyl (DMAB)-induced prostatic carcinoma in a Fisher 344 rat.[9] It was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY) with 100 units/ml penicillin and 100 units/ml streptomycin sulfate (GIBCO) and cultured in a humidified 5% CO_2 incubator at 37°C.

DNA transfection The PLS30 cell line was transfected with the pCMV*lac*Z plasmid using Lipofectin (GIBCO). The pCMV*lac*Z expression vector (kindly provided by Dr. Lloyd Culp) consists of the bacterial *lac*Z gene under the control of the cytomegalovirus immediate early gene promoter (CMV) and the neo resistance gene (neo^R), as described previously.[4] Transfectants were isolated in selection medium supplemented with 0.5 mg/ml G418. G418-resistant colonies were further screened for β-galactosidase activity by X-Gal staining and one particular colony with elevated enzyme activity was isolated, followed by single cell cloning. One clone (PLS30lZ), whose growth rate and metastatic ability proved comparable to those of the parental cell, was chosen for this study.

Micrometastasis study PLS30lZ cells (1×10^6 cells) were inoculated intravenously into mice, which were killed after 2 h, 2 days, 4 days, 7 days, or 14 days. The lungs were removed and stained as whole organs with X-Gal. Parts of some of the samples were embedded in methacrylate and sectioned as described below for microscopic examination.

X-Gal staining X-Gal staining was performed according to the method of Lin *et al.*[4] Briefly, the lung tissues were removed and fixed in 2% formaldehyde plus 0.2% glutaraldehyde in phosphate-buffered saline (PBS) at 4°C for 60 min, and then treated with X-Gal (Wako, Osaka). Micrometastatic foci appearing as blue-stained spots were enumerated under a dissecting microscope. In some cases, the lungs stained in this way were sliced at 200 μm with a mechanical tissue chopper and quantitatively evaluated using a video image-analyzing system (Mitani-Shoji Co., Chiba).

Methacryrate embedding and BrdU staining Whole lungs from mice given an intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU, Sigma Chemical Co., Ltd., St. Louis, MO) 1 h before death were stained with X-Gal and rinsed three times in PBS. Then they were infiltrated overnight at 4°C with glycol methacrylate monomer (JB-4 A; Polyscience, Inc., Warrington, PA), transferred to embedding molds filled with embedding medium (25 ml of JB-4 A, 1 ml of JB-4 B, and 0.7% catalyst), and kept anaerobic at −18°C for polymerization.[12] Before being mounted, 4 μm sections were cut with a rotary microtome and exposed to biotin-labeled mouse monoclonal antibody to BrdU (Zymed Lab. Co., South San Francisco, CA). The avidin-biotin complex method was applied to identify BrdU-positive cells, labeling indices being obtained by counting the numbers of labeled cells among at least 300 X-Gal positive cells and expressed as percentage values.

Cell growth analysis and spontaneous metastasis To examine the growth rate of PLS30 cells and PLS30lZ cells *in vitro*, 1×10^5 cells were seeded into 35-mm culture dishes, and cell numbers were counted with a hemocytometer after 3 days. For the study of tumor growth in nude mice, cells (5×10^6) were injected s.c. into the abdominal flanks of mice. Tumor size was measured at regular intervals in two dimensions and expressed as the average diameter. For the spontaneous metastasis study, tumor-bearing mice were killed 8 weeks after s.c. injection of the cells, and the numbers of lung metastases were assessed macroscopically. The histology of subcutaneous primary tumor tissues was examined with hematoxylin and eosin staining.

Type IV collagen immunohistochemistry and electron microscopy Parts of the lung tissues were fixed in cold ethanol/acetic acid (98/2, v/v) and exposed to rabbit polyclonal antibody to bovine type IV collagen (Advance Co., Tokyo), then subjected to the avidin-biotin complex method to demonstrate the basement membrane of blood vessels. Some tissue samples were fixed in 2% paraformaldehyde plus 2.5% glutaraldehyde in 50 m*M* phosphate buffer (pH 7.4) for 2 h, postfixed in 1% osmium tetroxide, and then embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate followed by lead citrate and examined under a Nihon Denshi JEM-1200EX electron microscope.

RESULTS

Phenotype of *lacZ* gene transfectants PLS30lZ cells in culture stained positive with X-Gal in their cytoplasm, even after 10 passages (Fig. 1). The histological features of PLS30 and PLS30lZ tumors were essentially the same, both being well differentiated adenocarcinomas with abundant fibrous stroma (data not shown). Cell growth in culture (Fig. 2A), the growth rate of subcutaneous tumors (Fig. 2B), and the spontaneous metastasis rates (Fig. 2C) in nude mice were also comparable.

Time-course of micrometastasis formation in the lung Micrometastases in the lungs, 2 h, 2 days, 4 days and 7 days after intravenous injection of PLS30lZ cells into nude mice, could be clearly visualized after X-Gal staining under a dissecting microscope (Fig. 3, A–D), allowing quantitative analysis of their numbers and the sizes with a video image-analyzing system. The numbers of X-Gal-positive foci progressively decreased to a steady-state level (about two foci/mm^2) by 7 days after inoculation

(Fig. 4A). In contrast, the sizes of individual foci substantially increased from the 4-day time point (Fig. 4B). On histological examination of methacryrate-embedded tissues, most micrometastases at 2 h and 2 days postinjection were composed of single and several blue-staining cells, respectively, while the foci on day 4 mostly consisted of more than ten tumor cells (data not shown), indicating that tumor cell proliferation had already occurred.

Cell proliferation kinetics X-Gal and BrdU double staining made it possible to detect easily and specifically tumor cell entry into the S-phase of the cell cycle in the lung (Fig. 5A–C). BrdU labeling indices of tumor cells immediately after inoculation were relatively high at 23%, then decreased transiently to 8% at 2 days post inoculation before increasing again to 23% at 4 days (Fig. 5D).

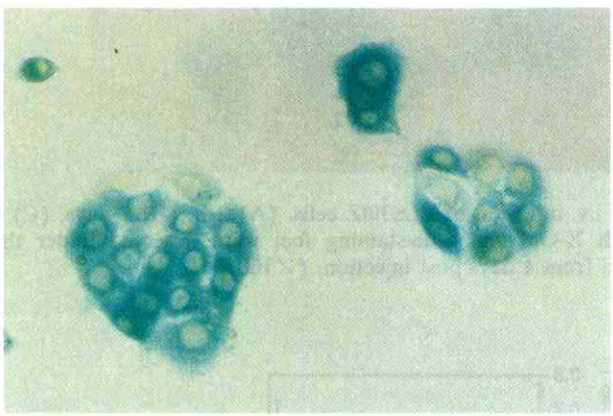

Fig. 1. X-Gal staining of cultured PLS30lZ cells. Note the intense blue colour of tumor cells after incubation with X-Gal solution (×150).

Type IV collagen immunohistochemistry and electron microscopy Since the acetic acid-ethanol fixation used for type IV collagen immunohistochemistry is inappropriate for X-Gal staining, double staining was unsuccessful. However, relatively large metastatic foci composed of more than 10 tumor cells could be identified with hematoxylin counter-staining. Type IV collagen immunostaining revealed that most metastasized tumor cells were surrounded by a vascular basement membrane at 4 days after injection (Fig. 6). Electron microscopic examination further demonstrated that while most tumor cells showed degenerative changes at 2 days post injection (data not shown), on day 4 they formed viable multicellular clumps inside vessels, in direct contact with the basement membrane without interruption by endothelial cells (Fig. 7).

DISCUSSION

The present growth-kinetic study of micrometastasis formation using X-Gal and BrdU double staining demonstrated that the BrdU labeling index of tumor cells immediately after injection decreases transiently before increasing again by day 4. The relatively high labeling index observed at 2 h post injection may reflect persistence of DNA synthesis of tumor cells after harvesting from *in vitro* culture. The transient decrease in the labeling index at two days post injection reflected tumor cell damage or cell death in the capillary bed of the lung, as demonstrated by electron microscopy. Thus more than 99% of the X-Gal-positive foci were cleared from the lungs within 2–3 days post injection. This clearance of tumor cells may be caused by hemodynamic destruction,[13] or by elements of the host surveillance system, such as macrophages and natural killer cells.[14] Alternatively, tumor cell death may be related to difficulty in

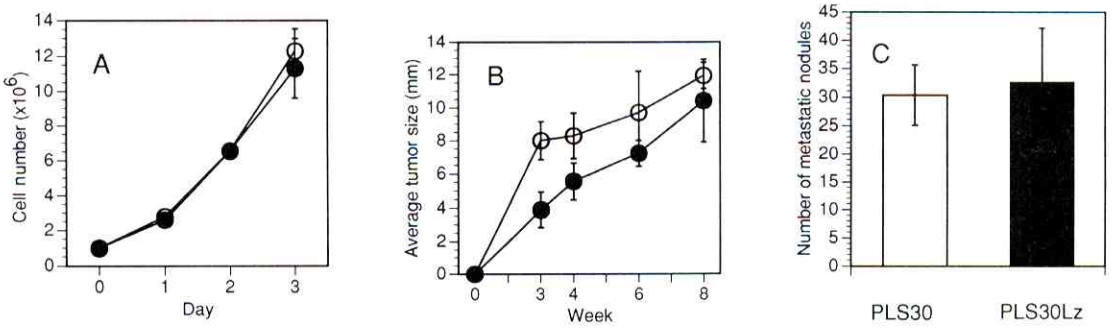

Fig. 2. Comparison of cell growth in culture (A), primary tumor growth (B) and spontaneous metastasis (C) in nude mice between PLS30 and PLS30lZ cells. PLS30 (○) and PLS30lZ (●) cells were seeded onto plastic dishes or injected s.c. into nude mice and cell numbers, tumor sizes and the numbers of lung metastatic nodules were assessed as described in "Materials and Methods." Data presented are means±SD values.

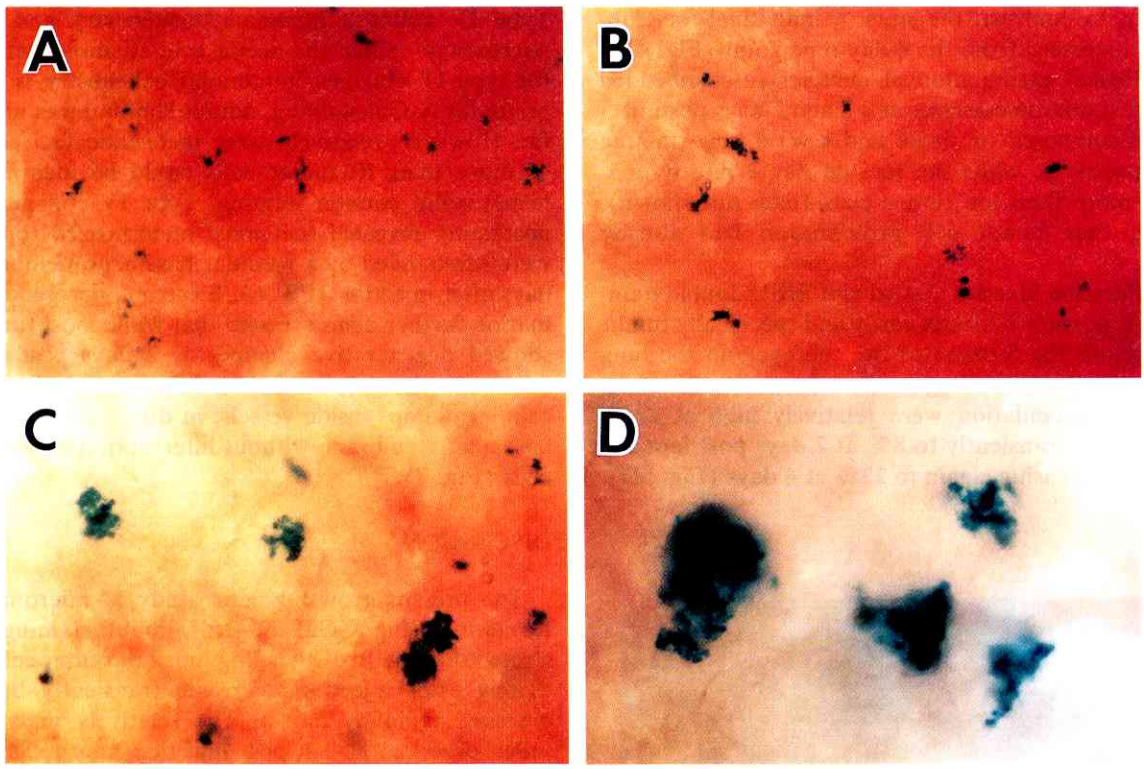

Fig. 3. Time course of micrometastasis formation in the lung after i.v.-injection of PLS30lZ cells. (A) 2 h, (B) 2 days, (C) 4 days, (D) 14 days post injection. Whole lungs were stained with X-Gal and blue-staining foci were observed under the dissecting microscope. Note the increase in size of X-Gal-positive foci from 4 days post injection. (×160).

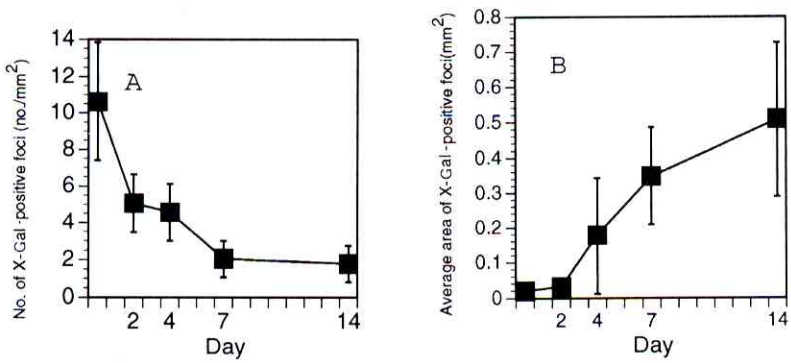

Fig. 4. Sequential changes in the numbers (A) and average areas (B) of X-Gal-positive foci in the lung after i.v.-injection of PLS30lZ cells. Lung tissues were sliced with a tissue chopper after X-Gal staining and examined with a video image-analyzing system. Data presented are means ($n=4$) ± SD values.

becoming securely attached to the endothelium and subsequently to the subendothelial matrix. Recently, Frish *et al.* demonstrated that interruption of cell adhesion to the extracellular matrix by suspension culture or RGD peptides induces apoptotic cell death in epithelial cells.[15, 16] In the present model, however, tumor cell death appeared to be necrotic rather than apoptotic, based on the ultrastructural features. Therefore, programmed cell death seems to be a minor factor, if it does occur. The high rate of BrdU labeling index of tumor cells at 4 days

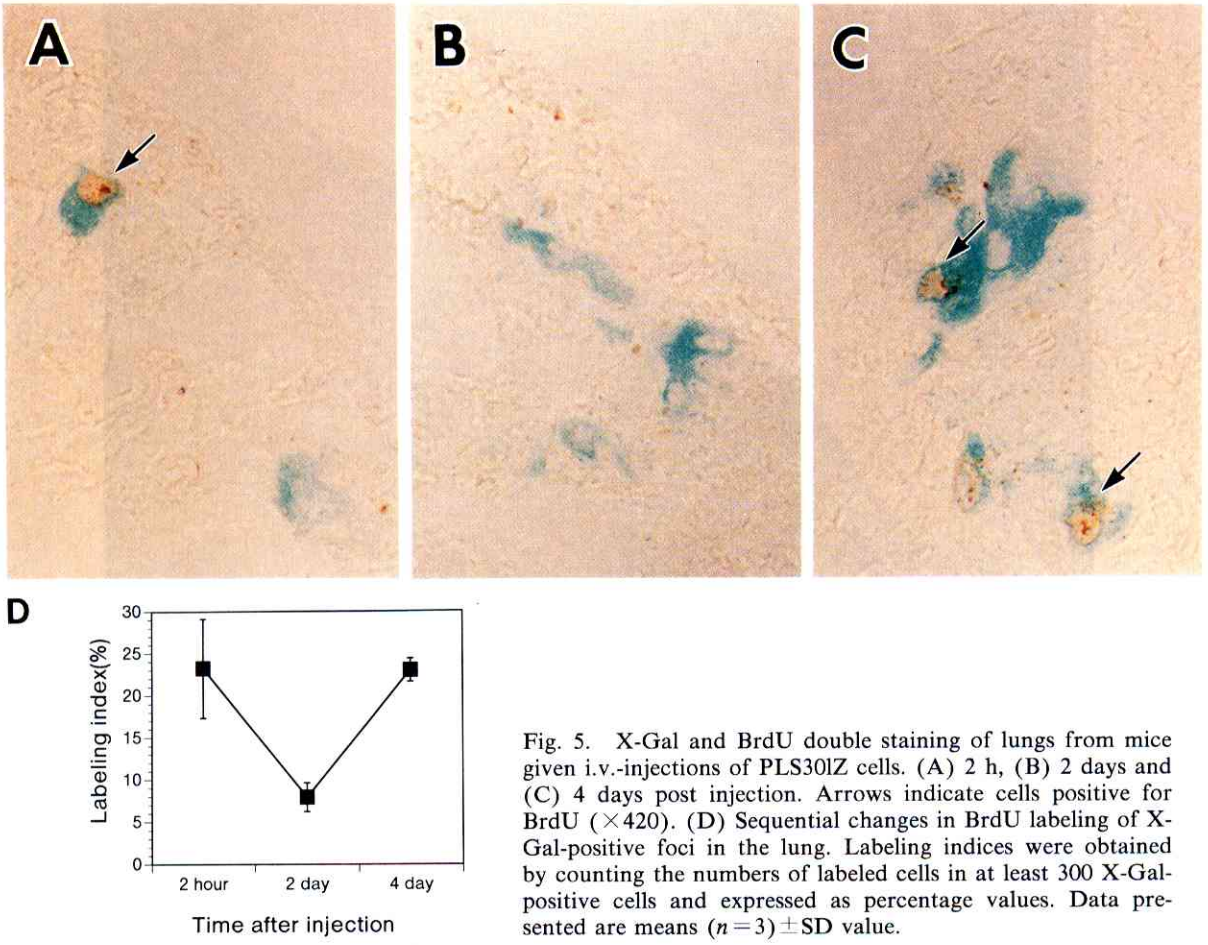

Fig. 5. X-Gal and BrdU double staining of lungs from mice given i.v.-injections of PLS301Z cells. (A) 2 h, (B) 2 days and (C) 4 days post injection. Arrows indicate cells positive for BrdU (×420). (D) Sequential changes in BrdU labeling of X-Gal-positive foci in the lung. Labeling indices were obtained by counting the numbers of labeled cells in at least 300 X-Gal-positive cells and expressed as percentage values. Data presented are means ($n=3$) ± SD value.

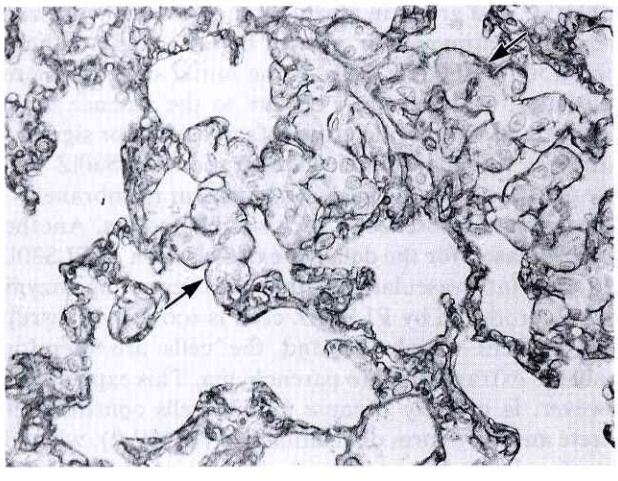

Fig. 6. Type IV collagen immunostaining of lung from mice 5 days after i.v.-injection of PLS301Z cells. Tumor cells with large, atypical nuclei are surrounded by positively stained basement membrane (arrows) inside vessels. (×125).

post injection would appear to represent the commencement of proliferation by the less than 1% of injected cells persisting at this point. These results indicate that multicellular clumps observed in the blood vessels at 4 days post injection are not artificial emboli but established micrometastases.

The most important findings of this study are that such tumor cell growth and formation of multicellular clumps occurs intravascularly, using the subendothelial basement membrane as a scaffold, and is followed by extravasation into lung parenchyma by 7 days post injection. This is in contrast to the previously accepted scenario that micrometastases develop as a result of a sequence of events which includes arrest of tumor cells in the blood vessels, extravasation into the parenchyma and new growth at the secondary site.[17] For example, Lin *et al.* reported that i.v.-injected, *lac*Z-transfected, ras-transformed 3T3 cells extravasate into lung parenchyma within 24 h.[18] The discrepancy between these results presumably reflects differences in the cell types used. Our

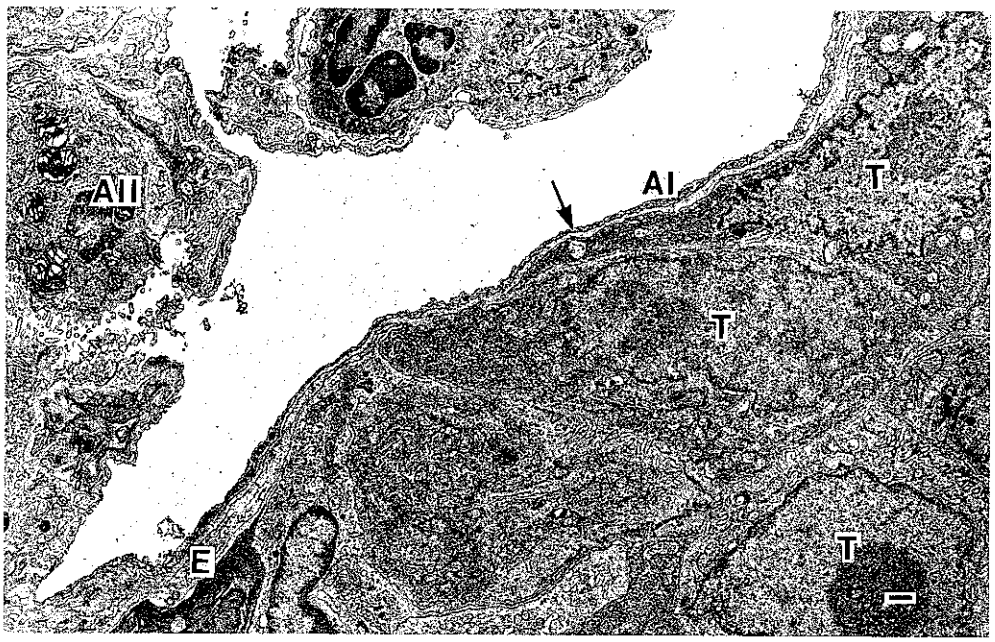

Fig. 7. Electron micrograph of lung from mice 4 days after i.v.-injection of PLS30lZ cells. Note the direct contact of tumor cells with the basement membrane (arrow) of a vessel. A I, Type I alveolar cell; A II, Type II alveolar cell; E, endothelial cell; T, tumor cell. Bar = 1 μm.

model utilizes well differentiated adenocarcinoma, whereas the previously reported models mostly employed non-epithelial cells such as transformed fibroblasts, melanoma cells or lymphoma cells. In fact, the present results are in agreement with the finding that metastatic human gastric adenocarcinoma cells extensively proliferate intravascularly in the lung after subcutaneous implantation.[19] This suggests that patterns of micrometastasis formation vary greatly from "extravascular" growth type to "intravascular" growth type, and this heterogeneity of micrometastasis formation is dependent on the tissue origin and degree of differentiation of tumor cells. With the differentiated, intravascular growth type, co-operation between tumor cells in the blood vessels may be important for their stabilization and/or extravasation.

The reason why our tumor cells in contact with the vascular basement membrane grew intravascularly rather than exhibiting transendothelial migration is unknown. Attachment to the extracellular matrix is known to be critical for growth stimulation by growth factors or serum in anchorage-dependent cells.[20] On the other hand, synergistic interaction between integrins and growth factors or cytokines was found to be necessary for cell migration.[21] Integrin $\alpha V\beta 5$-dependent cell migration requires specific epidermal growth factor (EGF) receptor activation in human pancreatic carcinoma cells.[22] Furthermore, hepatocyte growth factor (HGF) and the heparin-binding EGF-like growth factor (HB-EGF) are considered to be involved in transendothelial migration of human breast and esophageal cancer cells due to their stimulation of integrin $\alpha 2\beta 1$ and $\alpha 3\beta 1$ expression and the concomitant increase of cancer cell adhesion to collagen.[23–25] The PLS30lZ cell line, however, is refractory to various growth factors such as EGF and HGF, and grows in an autonomous fashion *in vitro*.[9] Therefore, intravascular growth before transendothelial migration of PLS30lZ cells at the initial stage of micrometastasis formation may be due to the absence or inactivation of the specific growth factor receptor signaling pathways which lead to cell migration. PLS30lZ cells may proliferate on the vascular basement membrane as a substrate in the absence of such soluble factors. Another possible reason for the delay of extravasation of PLS30lZ cells after intravascular growth is that degrading enzyme activity produced by PLS30lZ cells is too low to disrupt the basement membrane and the cells are therefore unable to extravasate into parenchyma. This explanation, however, is unlikely because PLS30 cells constitutively secrete an active form of gelatinase B (MMP-9), which is considered to play an important role in metastasis, as demonstrated by gelatin zymography (unpublished result). Further cell biological studies *in vitro* are in progress in our laboratory to clarify the relationship between cell growth and transendothelial migration.

PLS30lZ stably expresses β-galactosidase for at least 1–2 weeks after intravenous injection into mice. However, when the inoculation is subcutaneous, the spontaneous metastatic foci which form 8 weeks after injection show definitely reduced β-galactosidase activity (data not shown). This decreased expression may be attributable to selective growth of new subclones with reduced activity under non-selective conditions for drug resistance. Therefore, this micrometastasis model has some limitations. Nevertheless, it is very useful for examination of the earliest events of micrometastasis formation of differentiated adenocarcinomas, as shown here, and could be simply applied for estimation of the anti-metastatic and metastasis-enhancing effects of anti-cancer drugs and cytokines, respectively.

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