Proliferation of human lung cancer in an orthotopic transplantation mouse model

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Abstract. The objective of this study was to clarify the growth and proliferation style of human lung cancer grown in an orthotopic transplantation model. The human lung squamous cell carcinoma SQ5 and adenocarcinoma A549 cell lines were used. Tumor cells suspended in serum-free medium were directly injected into the main bronchi of anesthetized female Balb/c athymic nude mice with simultaneous administration of 0.01 M ethylenediaminetetraacetic acid. Bromodeoxyuridine was injected into mice 20 min before sacrifice. Lung tissue with tumor nodules and subcutaneous tumors were fixed and confirmed by histological examinations. Bromodeoxyuridine-labeled cells in the tumor area were counted, and the proliferation index was calculated. Lung tumor colonies of various sizes were obtained in the SQ5- and A549-cell orthotopically transplanted mice. Orthotopic SQ5 tumors whose minor diameter was 40-700 μm and major diameter was 80-830 μm showed no definite necrosis. Orthotopic SQ5 tumors whose minor diameter was 540-5,200 μm and major diameter was 600-6,100 μm showed definite necrosis in the tumor center. Similar results were also found in the orthotopic A549 tumors. The proliferation index was 7.38 (3.03)/10.63 (3.10) in the orthotopic SQ5 tumors with/without necrosis and 6.99 (2.10) in the subcutaneous SQ5 tumors with necrosis, respectively. The proliferation index was 2.70 (0.88)/3.53 (1.70) in the orthotopic A549 tumors with/without necrosis and 3.91 (0.63) in the subcutaneous A549 tumors with necrosis, respectively. The data suggest that this orthotopic transplantation model may provide the proper organ microenvironment for lung cancer growth and may be suitable for the target therapy research of human lung cancer.

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Introduction

Lung cancer is the most common malignancy in the world (1,2), and no more than 14.9% of patients survive after treatment for lung cancer. These data show that a new therapeutic approach for lung cancer is crucial. Recently, investigations aimed at developing anticancer agents have begun to shift away from general cytotoxic drugs to those that target specific molecules or processes selectively involved in tumor cell survival (3,4). However, the relevance of subcutaneous (s.c.) tumor growth in animal models to that in human studies must be carefully ascertained, since experimental preclinical results frequently cannot be translated to the clinic. The limitation of animal models is, in part, related to tumor transplantation. The use of ectopic tumors does not adequately take into account the interaction between the specific organ microenvironment and tumor cells, and may therefore alter the tumor response to therapy (4-7). Previously, we established an orthotopic transplantation model which simulated the clinical and pathological features of human lung cancer (8). In this study, we further analyzed the growth style and the proliferation profile of human lung cancer in an orthotopic transplantation model, comparing it with an s.c. transplantation model.

Materials and methods

Cell culture and preparation for delivery. The human squamous lung cancer cell line SQ5 was donated by Dr Kubota from the Ibaraki Prefectural University of Health Sciences (9). The human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection cell line repository. Both cell lines were maintained in αMEM medium (Sigma, St. Louis, MO) with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Equitech-Bio Inc., Kerrville, TX), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were maintained in exponential growth in humidified incubators at 37˚C in 5% CO_2. Adherent tumor cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA). Trypsinization was stopped with medium containing 10% serum, and the cells were washed once and resuspended in serum-free medium. Trypan blue staining was used to assess cell viability, and only single-cell suspensions

of >95% viability were used for injections. EDTA (0.01 M) (Sigma) was supplemented in the tumor cell suspensions before intratracheal delivery (8).

Experimental animals. Pathogen-free female BALB/c nu/nu mice (Charles River Laboratories, Tokyo, Japan) were transplanted at the age of 7-9 weeks either intratracheally or subcutaneously with the SQ5 or A549 cell line. Mice were maintained in specifically pathogen-free conditions, fed autoclaved food and water, and handled under stringent sterile conditions. Mice were acclimated for 1 week before the start of the study. All animal experiments were performed in accordance with the Guidelines of Animal Experiments of Yokohama City University, and the protocols were approved by the Animal Care Committee of the Yokohama City University.

Orthotopic and subcutaneous transplantation of tumor cells. The mice were anesthetized with isoflurane (Merck Hoei Ltd., Osaka, Japan), placed in a supine position and gently immobilized with tape. A 1-cm long ventral incision was made through the skin of the neck to expose the trachea. A 27 3/4-gauge needle (Terumo, Tokyo, Japan), bent to approximately a 135° angle, was used to inject the tumor cell suspensions ($1x10^7$ cells) directly into the main bronchi. The incision was closed with a single surgical clip, and the mice were allowed recovery time under a warm lamp until fully awake (8,10). For subcutaneous transplantation, unanesthetized mice were injected with a 100-μl tumor suspension ($1x10^7$ cells) directly into the flank. The mice were observed daily after tumor cell injection and monitored for signs of wound healing disturbance, evidence of tumor development and decreased physical activity. The weights of the mice were determined twice a week. Necropsies were performed on all animals.

To determine the proliferation of tumor cells, the mice were pulse-labeled with 60 mg/kg of body weight bromodeoxyuridine (BrdUrd) (Sigma) in phosphate-buffered saline (PBS) as an intraperitoneal injection 20 min before sacrifice. After death, the lungs were exposed and inflated with neutral-buffered formalin. Excised lungs and s.c. tumors were collected in 10% neutral-buffered formalin for immunohistochemistry.

Histology and immunofluorescence staining for microscopic evaluation of BrdUrd incorporation in tumor cells. In brief, after fixation in 10% neutral-buffered formalin, 4-μm tissue sections were cut and mounted on silane-coated slides. The sections were stained with H&E and analyzed by microscopy (Olympus CKX41, Tokyo, Japan).

For immunofluorescence staining, the sections were deparaffinized in xylene and rehydrated through decreasing ethanol concentrations to water. The sections were then washed twice for 5 min in PBS. The sections were treated with 2 N HCl for 50 min and neutralized with 0.1 M sodium borate for 2 min. The sections were rinsed twice for 5 min in PBS and then incubated in blocking normal horse serum for 30 min at 37˚C to decrease nonspecific antibody binding. Sections were incubated for 1 h at 37˚C with the antibody IU-4 (1:100 vol/vol in PBS plus 0.5% Triton X-20; PBST) (Sigma). The sections were again washed twice for 5 min in PBS and then incubated for 1 h at 37˚C with a FITC-conjugated goat anti-mouse secondary antibody (GAMFITC) (Sigma) together with normal goat serum (1:100 vol/vol in PBST). The slides were again washed twice for 5 min in PBS, the nuclei counterstained with 1 μg/ml propidium iodide (PI) in PBS, and mounted using Vectashield anti-fade mounting medium (Vector Laboratories, Burlingame, CA) (10). AxioCam MRm microscopy (Zeiss, Tokyo, Japan) was used to detect BrdUrd- and PI-labeled nuclei. Sections were observed under x200 magnification. Both BrdUrd- and PI-labeled tumor cells were counted as proliferating cells (10). The proliferation index was calculated as proliferating cells/10^5 tumor area using NIH ImageJ software. At least 3 mice per group and 3 slides per mouse sample were stained for counting. Proliferating cells were counted in the area of tumors without necrosis.

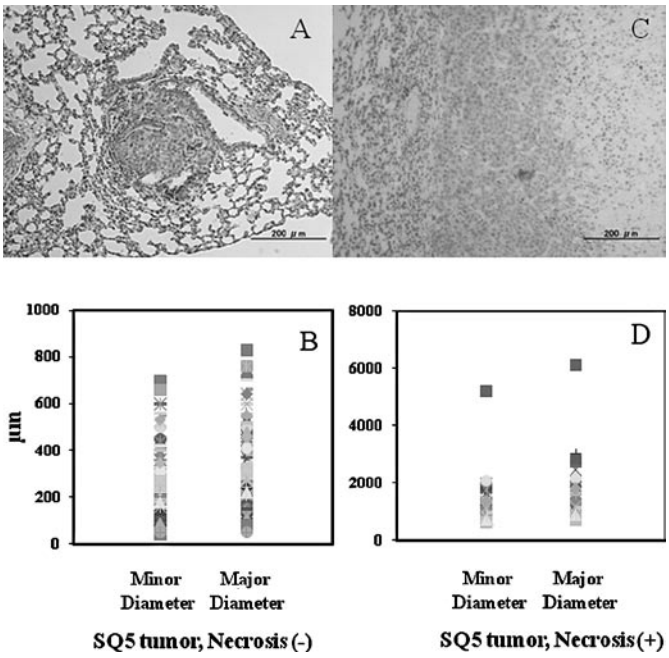

Figure 1. SQ5 tumor nodules in an orthotopic transplantation model (n=20). A total of 86 tumor nodules without necrosis was analyzed. Minor and major diameters were 40-700 and 80-830 μm, respectively (A and B). A total of 50 tumor nodules with necrosis was analyzed. Minor and major diameters were 540-5,200 and 600-6,100 μm, respectively (C and D).

Statistical analysis. Data were presented as the mean (SD), and the statistical significance of differences in mean values was assessed by the Student's t-test. The differences in the proliferation index in orthotopic tumors with/without necrosis and s.c. tumors were considered significant at values of $P<0.05$.

Results

Histology of SQ5 orthotopic tumor colonies. A total of 20 mice with orthotopically transplanted SQ5 tumors were collected. A total of 136 tumor colonies were analyzed. Histological examination showed that the diameter of the tumor colonies was 40-6,100 μm. Tumor colonies whose minor diameter was 40-700 μm and major diameter was 80-830 μm showed no definite necrosis. The average of the

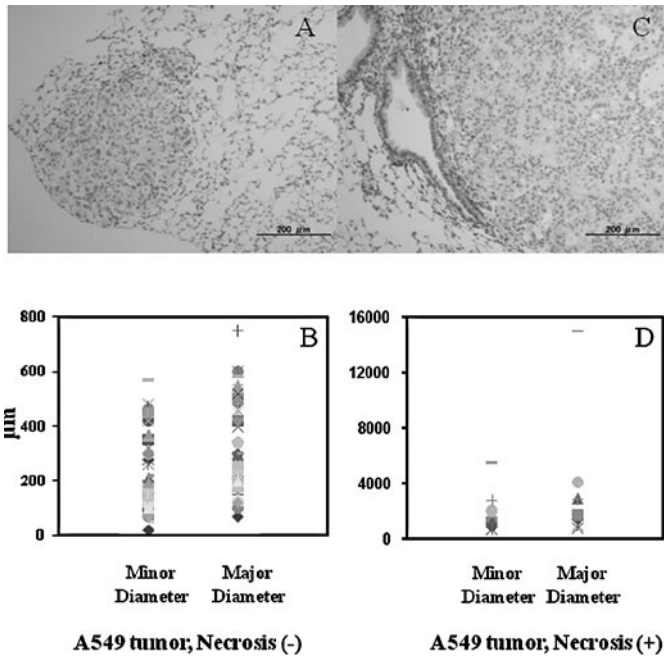

Figure 2. A549 tumor nodules in an orthotopic implantation model (n=24). A total of 51 tumor nodules without necrosis was analyzed. Minor and major diameters were 20-570 and 70-750 μm, respectively (A and B). A total of 9 tumor nodules with necrosis was analyzed. Minor and major diameters were 680-5,500 and 800-15,000 μm, respectively (C and D).

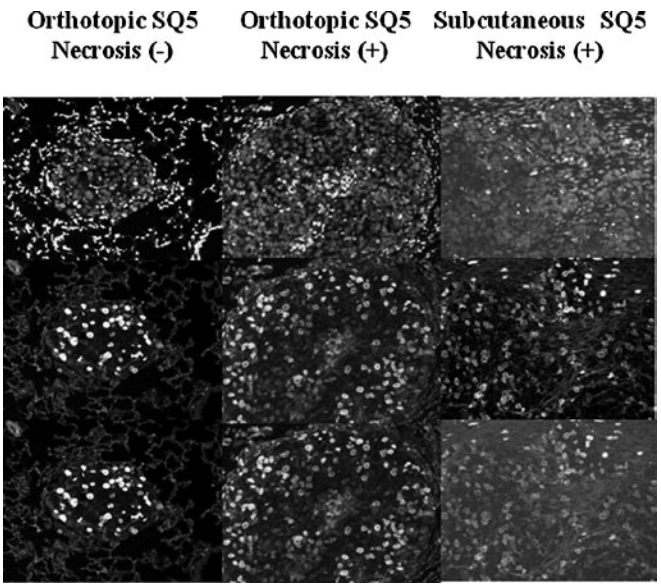

Figure 3. Immunofluorescence examination of SQ5 tumors in the orthotopic and s.c. transplantation models. PI-labeled nuclei indicate the nuclei of the total cells (top row). BrdUrd-labeled nuclei indicate the nuclei of proliferating cells (middle row). Both BrdUrd- and PI-labeled cells were counted as proliferating cells (bottom row). Left column: proliferation of the orthotopic SQ5 tumor colony without necrosis. Middle column: proliferation of the orthotopic SQ5 tumor colony with necrosis. Right column: proliferation of the s.c. SQ5 tumor colony.

minor and major diameters of the SQ5 tumors without necrosis was 283 and 297 μm, respectively (Fig. 1A and B). SQ5 tumor colonies whose minor diameter was 540-5,200 μm and major diameter was 600-6,100 μm showed definite necrosis. The average of the minor and major diameters of the SQ5 tumors with necrosis was 1,182 and 1,507 μm, respectively (Fig. 1C and D).

Histology of A549 orthotopic tumor colonies. A total of 24 mice with orthotopically transplanted A549 tumors were collected. A total of 59 tumor colonies was analyzed. Histological examination showed that the diameter of the tumor colonies was 20-15,000 μm. Tumor colonies whose minor diameter was 20-570 μm and major diameter was 70-750 μm showed no definite necrosis. The average of the minor and major diameters of the A549 tumors without necrosis was 220 and 304 μm, respectively (Fig. 2A and B). Tumor colonies whose minor diameter was 680-5,500 μm and major diameter was 800-15,000 μm showed definite necrosis. The average of the minor and major diameter of the A549 tumors with necrosis was 1,890 and 3,749 μm, respectively (Fig. 2C and D).

Proliferation of SQ5 tumor cells in the orthotopic and s.c. transplantation models. Fig. 3 shows the BrdUrd- and PI-labeled tumor cells in the orthotopic SQ5 tumors with/without necrosis and the s.c. tumors. In the orthotopic SQ5 tumors without necrosis, proliferating tumor cells were distributed in all areas of the tumor colonies (Fig. 3, left column). The proliferation index was 10.63 (3.10) (Table I). In the orthotopic SQ5 tumors where necrosis was found in the center, proliferating tumor cells were mainly distributed in the peripheral of the tumor colonies (Fig. 3, middle column). The proliferating tumor cells were counted in the part of the tumor area without necrosis. The proliferation index of the orthotopic SQ5 tumors with necrosis was 7.38 (3.03) (Table I). In the s.c. transplantation model, the SQ5 tumors grew as a large block which showed several necrotic areas in the tumors (Fig. 3, right column). Proliferating tumor cells were counted in the area of the tumors without necrosis. The proliferation index of the s.c. SQ5 tumors was 6.99 (2.10) (Table I).

Proliferation of A549 tumor cells in the orthotopic and s.c. transplantation models. Fig. 4 shows the BrdUrd- and PI-labeled tumor cells in the orthotopic A549 tumors with/without necrosis and the s.c. tumors. In the orthotopic A549 tumors without necrosis, proliferating tumor cells were distributed in all areas of the tumor colonies (Fig. 4, left column). The proliferation index of the orthotopic A549 tumors without necrosis was 3.53 (1.70) (Table I). In the orthotopic A549 tumors with necrosis, proliferating tumor cells were counted in the region of the tumor area without necrosis (Fig. 4, middle column). The proliferation index of the orthotopic A549 tumors with necrosis was 2.70 (0.88) (Table I). In the s.c. transplantation model, proliferating tumor cells were counted in the part of the tumor area without necrosis (Fig. 4, right column). The proliferation index of the A549 s.c. tumors was 3.91 (0.63) (Table I).

Discussion

We previously established an orthotopic transplantation model of human lung cancer. Tumor growth and distribution in this orthotopic transplantation model simulated the clinical

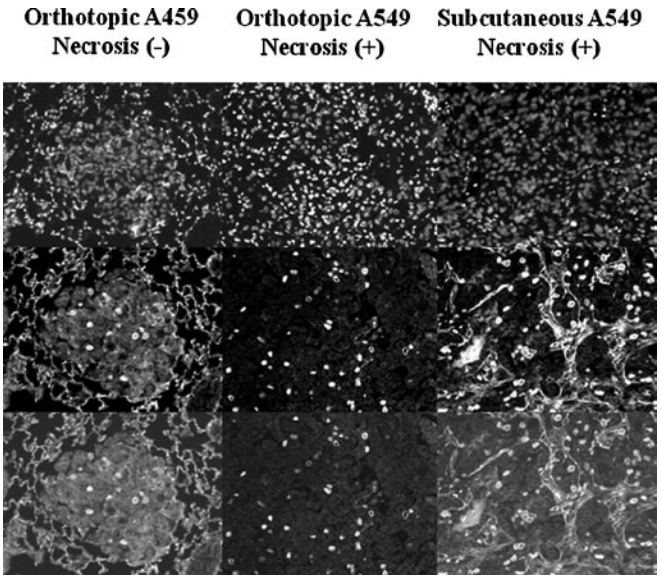

Figure 4. Immunofluorescence examination of A549 tumors in the orthotopic and s.c. transplantation models. PI-labeled nuclei indicate the nuclei of the total cells (top row). BrdUrd-labeled nuclei indicate the nuclei of proliferating cells (middle row). Both BrdUrd- and PI-labeled cells were counted as proliferating cells (bottom row). Left column: proliferation of the orthotopic A549 tumor colony without necrosis. Middle column: proliferation of the orthotopic A549 tumor colony with necrosis. Right column: proliferation of the s.c. A549 tumor.

features of human lung cancer (8). The aim of this study was to determine the influence of the organ microenvironment on the proliferation profiles of human lung cancer cells in orthotopic or s.c. tumor transplantation models.

In the orthotopic transplantation model, tumor colonies of various sizes were found in both the SQ5- and A549-cell transplanted mice. Tumor necrosis was not definitely found in the orthotopic SQ5 tumor colonies when the minor diameter of the tumor colonies was less than 700 μm and the major diameter of the tumor colonies was less than 830 μm (Fig. 1). Similar results were also found in the orthotopic A549 tumor colonies (Fig. 2). These results agree with the fact that tumors less than 1 mm in diameter are considered to have an adequate vascular supply (11,12). It has been reported that the organ microenvironment influences tumor growth. The microenvironmental factors impacting tumor cell growth include interstitial fluid pressure in the tumor tissue, pH, partial pressure of oxygen, focal concentration of cytokines, extracellular matrix and microvessel density (13,14). Without exception, lung cancer cells growing in lung tissue depend on the structure of the host tissue (15). Our present and previous studies found that orthotopic SQ5 tumors expanded locally and invaded the adjacent normal lung fields (Figs. 1 and 3) (8). Orthotopic A549 tumors mainly replaced the normal alveolar epithelial cells and were firmly attached to the alveolar interstitium (Figs. 2 and 4) (8). These growth profiles are similar to those found in human squamous and adenocarcinoma lung tumor growth, respectively (15). This orthotopic transplantation model was considered to provide the proper organ microenvironment for lung cancer growth.

It is well known that controlling the proliferation of tumors is the key to treating human lung cancer. In this

Table I. Summary of the proliferation indices in SQ5 and A549 tumors.

Tumor model	SQ5		A549	
	Mean	SD	Mean	SD
Orthotopic, necrosis (-)	10.63[a]	3.10	3.53	1.70
Orthotopic, necrosis (+)	7.38	3.03	2.70[a]	0.88
Subcutaneous, necrosis (+)	6.99	2.10	3.91	0.63

n=3 mice per group. The proliferation index was calculated as BrdUrd- and PI-labeled cells/10^5 tumor area using NIH imageJ software. Data were presented as the mean (SD), and the statistical significance of differences in mean values was assessed by the Student's t-test. [a]P<0.05, compared with the s.c. group, respectively. The proliferation index of the SQ5 tumor groups was significantly higher than that of the A549 tumor groups (P<0.01).

study, we analyzed the proliferation style of squamous and adenocarcinoma lung tumors growing in orthotopic and s.c. transplantation models. The proliferation index of the orthotopic squamous SQ5 tumors with/without necrosis and the s.c. tumors was significantly higher than that of the orthotopic adenocarcinoma A549 tumors with/without necrosis and the s.c. tumors, respectively (P<0.01) (Table I). Our previous study showed that in an *in vitro* culture, the doubling time of these two cell lines was almost identical. Tumor cells collected from transplanted tumors did not change the doubling time (8). However, these two cell lines showed a different proliferation style in the transplanted tumors. The different proliferation index of SQ5 and A549 tumors was considered, in part, due to the cell numbers which entered the cell cycle. This finding was supported by Terry *et al* who reported that the proliferation index, but not the cell cycle, was altered by stimulation *in vivo* (10). The proliferation index may reflect the balance of proliferation and dormancy of tumor cells growing in a proper organ microenvironment.

The proliferation style of the orthotopically transplanted tumors was further analyzed and compared with that in the s.c. transplanted tumors. The proliferation index of the orthotopic SQ5 tumors with or without necrosis had no significant difference. The same result was also found in the A549 orthotopic transplanted tumors (Table I). However, the proliferation index of the orthotopic SQ5 tumors without necrosis was significantly higher than that of the s.c. transplanted tumors, while the proliferation index of the orthotopic A549 tumors with necrosis was significantly lower than that of the s.c. transplanted tumors (Table I). These data suggest that tumor proliferation depends on both tumor cell character and host organ microenvironment; blood supply is only one of the factors influencing tumor proliferation. Sun *et al* reported that the microenvironment influences the interstitial fluid pressure in tumor tissue, which may change several proliferation factors (17). Gene expression in tumors is also markedly altered when they are subcutaneously implanted as compared to orthotopically implanted ones (7). In this orthotopic transplantation model, lung tumor cells grew in lung parentima, which has a particular microenvironment such as blood and

oxygen supply and air space in the organ. The balance of proliferation and dormancy in orthotopic transplanted tumors may depend on how the tumor cells respond to the organ microenvironment (5-7,15-17). The proliferation style in this orthotopic transplantation model may support the findings of Onn *et al* who found that paclitaxel had a different effect on human lung cancer cells growing orthotopically compared to those growing subcutaneously (3,18).

In conclusion, lung tumor colonies of various sizes and growth stages were obtained in this orthotopic transplantation model. The growth and proliferation of orthotopic lung tumor colonies were found to depend on both tumor cell character and organ microenvironment. This orthotopic transplantation model may provide a proper organ microenvironment for lung tumor growth and may be a suitable tool for the biological research of human lung cancer.

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