Abstract. Background: Recently we developed a method to observe pulmonary micrometastasis by labeling cancer cells with green fluorescent protein (GFP). We applied the method for observation of micro-dissemination on the visceral pleura. Materials and Methods: RCN9 rat colon cancer cells labeled with GFP were injected into the pleural cavity of Fischer F344 rats. Six weeks after injection, the chest wall was resected under general anesthesia and the lung surface was observed by real-time confocal laser-scanning microscopy. Blood flow was visualized by intravenous injection of fluorescein isothiocyanate-labeled red blood cells, by which blood flow velocity was measured. Results: Dissemination was created in 4 out of 5 rats. Fifteen sites of micro-dissemination were observed (mean diameter, 35.8±13.3 μm). Blood flow velocity was 114.1±26.1 μm/s in the tumor tissue and 183.4±35.0 μm/s out of the tumor tissue. Conclusion: We were able to observe pleural micro-dissemination. Blood flow velocity was significantly lower in the tumor tissue.

Cancer dissemination and metastasis have a significant effect on prognosis, but few experimental models are available and the mechanism of spreading is not well understood. Recently, we developed a method to observe pulmonary metastasis at an early phase (1). In this approach, a site of metastasis comprising only a few cancer cells can be visualized by labeling with green fluorescent protein (GFP). Here, we apply this method to visualization of pleural dissemination in an early phase of spreading.

In a clinical situation, some cancer cells that are present in the pleural space may attach to the surface of the pleura, while others are eliminated by phagocytosis or lymphatic drainage. Only a few attached cells will grow and disseminate as tumor masses, and an understanding of how cancer cells obtain oxygen and nutrition at an early stage and establish a dissemination might lead to strategies to reduce cancer spreading. To address some aspects of this process, we developed a double-fluorescence technique to observe red blood cell (RBC) flow (2) associated with early dissemination tumors.

Materials and Methods

Animals, cancer cells and implantation of cancer cells. Fischer F344 male rats (Charles River, Atsugi, Japan) were used in the study (n=5). Colon cancer cells of the rat RCN-9 cell line (3) (RCN-9; Riken Cell Bank, Tsukuba, Japan) were labeled with GFP [enhanced GFP (EGFP)-N1 cDNA; Takara Bio Inc., Otsu, Japan]. The EGFP-N1 cDNA contains a neomycin-resistance gene. RCN-9 cells were transfected with a GFP vector using calcium phosphate-DNA co-precipitation (4). Following transfection, the RCN-9 cells (GFP-RCN-9) were cultured in RPMI-1640 culture medium (Sigma R8758; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum with antibiotics (G-418, Sigma-Aldrich; antibiotic-antimycotic, Invitrogen, Carlsbad, CA, USA). G-418 blocks polypeptide synthesis, thereby selecting and maintaining cells that were stably transfected with the neomycin-resistance gene and causing non-transfected cells to undergo apoptosis; therefore, we were able to select GFP-transfected RCN-9 cells using this method.

GFP-RCN-9 cells (2.5×10⁶) suspended in the culture medium (0.2 ml) were injected into the pleural space. The chest cavity was approached through a small skin incision and a muscle division on the right side of the chest wall, under general anesthesia induced by intraperitoneal injection of pentobarbital sodium (5 mg/rat). Rats underwent in vivo observation 6 weeks after the cancer cell injection. The experimental protocols were approved by the Animal Experimentation Committee of the University of Tsukuba.
Surgical preparation. To observe the lung surface in vivo, the rats were anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/rat). After cannulation of the external jugular vein, a tracheostomy was performed and the rats were ventilated through a respirator (Model 683; Harvard Apparatus, South Natick, MA, USA). Following intraperitoneal injection of pancuronium bromide (0.2 mg/rat), the anterior chest wall was removed to expose the bilateral lungs. A metal ring of 3 mm in diameter was placed on the lung surface (2); the ring has a tiny round groove connected to tubing that allows suction at low pressure, such that the lung surface can be lifted up slightly to avoid the effects of movement of the respirator and cardiac pulsation. Using this approach, the observation area was fixed under the microscope. To examine the entire surface of the bilateral lungs, wide resection of the anterior chest wall was performed to expose the lungs completely, and the rat was tilted to fit the suction ring on the mediastinal and diaphragm sides of the lungs. Using this approach, we were able to survey the whole area of the bilateral lungs. For detailed observation and video-recording, the respirator was stopped for 15 seconds.

In vivo observation and measurement of blood flow velocity. The exposed lungs were observed using a real-time confocal laser scanning microscope (CLSM) fitted with objective lens (×20) optics (CSU10; Yokogawa Electric Corporation, Tokyo, Japan) and image processing devices (Model In-Sight Plus, Meridian Instruments, Okemos, MI, USA). The laser source was an argon ion laser adjusted to a wavelength of 488 nm. The resolution of the system was 0.625 μm. Rubart (5) reported that CLSM imaging depths in a variety of tissues are less than 500 μm with a common mode-locked laser. Fluorescent isothiocyanate-labeled red blood cells (FITC-RBCs) were injected intravenously to detect blood flow. The RBCs were obtained from syngeneic rats just before use, washed with saline twice by centrifugation (2,000 ppm/min for 3 min), and suspended in phosphate-buffered saline (PBS) containing FITC (1 mg/ml). After incubation at 25°C for an hour in the dark, the FITC-RBCs were washed once with saline and twice with a PBS solution containing 1% bovine albumin by centrifugation (1,500 ppm/min for 3 min). The FITC-RBCs were re-suspended in saline and injected through the external jugular vein. For detailed analysis, CLSM images were collected by DVD recorder (Model DVR-7000; Pioneer, Tokyo, Japan) through a silicon intensifier target tube camera (Model C2400-8; Hamamatsu Photonics, Shizuoka, Japan). These recordings were used to measure the size of the tumor mass of the micro-dissemination and velocity of movement of the FITC-RBCs. The velocity was measured by a frame-by-frame method in playback of video-recordings: each bright spot of FITC-RBCs was traced for 1 second and the number of tracts was counted (2, 6). The FITC-RBC velocity is interpreted as the blood flow velocity.

Statistics. Differences between means were determined using a t-test when groups passed both a normality test and a test of equal variance; if this did not occur, a Mann-Whitney rank sum test was used. Statistical significance was taken as \( p < 0.001 \).

Results

Observation of visceral pleural dissemination. Pleural dissemination was created successfully in 4 out of the 5 rats that received an injection of RCN-9 cells, and a total of 15 tumor masses were found on the lung surfaces in the 4 rats. The margins of the tumor masses were obscure, but we were able to measure the diameters of the long and short axes on recorded images. These data are summarized in Table I. The maximum and minimum tumor sizes were 100×60 and 17×17 μm, respectively. We were unable to determine the tumor distribution on the lung surface in a low-power image because each tumor was too small to be seen. Since the smallest size detectable by the naked eye is generally >0.2 mm in diameter, observation of pleural disseminations in a later phase will be necessary to determine the distribution.

Blood flow detected by FITC-RBCs. FITC-RBCs were clearly observable as bright spots (Figure 1) after intravenous injection and they moved in a non-pulsatile manner. The FITC-RBCs flowed through the tumor tissue in 9 out of the 15 tumor masses observed as micro-disseminations in the visceral pleura. The FITC-RBCs flowed rectilinearly to the tumor mass in the normal visceral pleura (Figure 1 a-c and g-h), but then flowed irregularly in the tumor tissue (Figure 1 e-g). The rectilinear flow pattern has not been reported previously. Most of the FITC-RBCs were observed to move in a circular manner. We have observed this flow pattern in previous studies following

### Table I. Size of tumor masses of pleural dissemination (n=15).

<table>
<thead>
<tr>
<th>Diameter axis</th>
<th>Length range (min to max) (μm)</th>
<th>Mean±SD</th>
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<tbody>
<tr>
<td>Long axis</td>
<td>17-100</td>
<td>35.8±13.3</td>
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<tr>
<td>Short axis</td>
<td>13-60</td>
<td>23.1±8.6</td>
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Size: diameters were measured from recorded images in DVD and expressed by the range and mean values with standard deviations (SD). n=15: total number of tumor masses observed on the visceral pleura in 4 rats.

### Table II. Blood flow velocity inside and outside of tumor masses

<table>
<thead>
<tr>
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<th>n</th>
<th>Blood flow velocity (μm/s)</th>
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</thead>
<tbody>
<tr>
<td>Inside</td>
<td>11</td>
<td>114.1±26.1*</td>
</tr>
<tr>
<td>Outside</td>
<td>18</td>
<td>183.4±35.0</td>
</tr>
</tbody>
</table>

Blood flow velocity was measured using FITC-RBC tracings in the records with a frame-by-frame method, expressed by mean values±standard deviations. FITC-RBCs moved through 9 out of 15 recognized tumor masses on the visceral pleura by real-time confocal laser scanning microscopy. n: Number of measurements of blood flow velocity; Inside: inside tumor tissue of the dissemination; Outside: normal pleural surface before entering tumor tissue or after flowing out tumor tissue. *Significantly different compared with outside velocity, \( p<0.001 \).

#### Reference

Figure 1. A series of recorded images showing a tumor micro-dissemination with a diameter of approximately 60×40 μm and a flowing fluorescein isothiocyanate (FITC)-labeled red blood cell (RBC) as a dot close to the tumor. The FITC-RBC moved at different velocities. a, The micro-dissemination is dimly fluorescent in the center of the image (delineated by a rectangle). The FITC-RBC is brightly fluorescent (arrow) just before entering the tumor and is accompanied by a comet-like tail since it is moving quickly and in a rectilinear manner. b, The FITC-RBC next to the tumor. c-f, The FITC-RBC showing irregular flow inside the tumor, with a velocity of 91.7 μm/s. Since the FITC-RBC flowed at a low velocity, the fluorescence appears as a bright spot. g, h, The FITC-RBC flowing out of the tumor tissue at a velocity of 177.8 μm/s, with re-establishment of rectilinear motion. The increased velocity blurs the fluorescence and causes it to appear as a comet shape.
intra-venous injection of FITC-RBCs (2, 6) and the circular shape might reflect flow in alveolar capillaries, which we have recently demonstrated to be involved in intracapillary tumor growth (1). Based on these observations, we speculate that the tumor tissues are supplied by the bronchial circulation.

**Comparison of blood flow velocity inside and outside of the disseminated tumor.** The velocity of FITC-RBCs inside the tumor tissue was measured 11 times in 9 disseminated tumors, and the velocity was measured 18 times before or after flowing in the tumor tissue of the 9 disseminated tumors. These data are summarized in Table II. There was a significant difference between the two velocities \( p<0.001 \), with the blood flow velocity decreasing significantly when blood entered the tumor, and then soon returning to the prior level upon flowing out of the tumor.

**Discussion**

Ebright et al. (7) demonstrated a cytotoxic effect of herpes virus NV1020 to pleural dissemination of the human lung cancer cell line A459 in a model of cancer dissemination in the pleural cavity in athymic rats. In contrast, we were able to create pleural dissemination in normal immune rats using a colon cancer cell line from the same rats (3). Furthermore, we were also able to observe blood flow patterns in the pleural dissemination and to measure blood flow velocity. Therefore, we suggest that this model might be appropriate to study the mechanism of pleural dissemination under conditions that are similar to the clinical situation in human disease. Furthermore, the high rate of successful dissemination (4 out of 5 rats) and the similar values for blood flow velocity at each measurement point (Table II) show that reproducible results can be obtained.

Suspended cancer cells were injected directly into the pleural cavity with a 23 G needle, but pneumothorax was not present at the time of surgery 6 weeks after cancer-cell injection. However, although we obtained favorable results for creation of pleural dissemination, future studies will require more careful instillation of cancer cells into the pleural cavity, as described by other investigators (7, 8). Observation of the visceral pleural surface by CLSM is a potential further problem with the approach, since this may be influenced by respiratory movement and cardiac pulsation. However, we were able to avoid the effects of movement by pulling up the site of observation with a metal ring connected to a suction apparatus (2, 6). To avoid the effect of the negative pressure of the suction apparatus, the lung could be isolated and perfused *in situ* without ventilation, but with a diffusion air supply, and Al-Mehdi et al. (9) have shown that pulmonary metastasis can occur under such *in situ* conditions.

Folkman (10) stated that promotion of tumor growth requires neovascularization, with resulting increases in blood perfusion of the tumor tissue and release of paracrine factors from endothelial cells of the neovascularization. The paracrine then stimulates tumor growth further. At an early stage of dissemination, as in our experiments, we cannot determine if the vascularization of the tumor tissue is newly constructed or was originally present, and to clarify this point it will be necessary to observe growth of the tumor dissemination for a longer period. Furthermore, a time-course study is required to reveal the vascularization of the tumor dissemination if the tumor starts from the single-cell stage.

The velocity of blood flow in the tumor tissue of the dissemination was slower than that in normal tissue. Funakoshi et al. (2) have also demonstrated that the velocity of FITC-RBCs in the tumor microcirculation of a pulmonary metastasis was significantly slower than in normal microcirculation. The reduced blood flow velocity in the tumor tissue may be due to the abnormal structure of the tumor vascular wall, as shown by Fukumura and Jain (11). The blood supply to the tumor mass of a pleural dissemination may arise from the pulmonary circulation and the bronchial circulation. The pattern of movement of FITC-RBCs in the tumor tissue differed from that outside the tumor: a rectilinear motion was observed for FITC-RBCs flowing before and after the tumor, whereas inside the tumor, the FITC-RBCs flowed irregularly. However, most FITC-RBCs were not associated with the tumor circulation and showed a circular flow pattern, which might reflect the shape of the alveolus. Collectively, these observations suggest that the tumor circulation arises from the bronchial circulation, and the FITC-RBCs inside the tumor were either moving in neovascularization or in the native bronchial circulation primarily present in the visceral pleura. Dye injection or microsphere studies comparing flow in the pulmonary artery and the aorta are needed to examine these possibilities. Regardless of this, the vasculature provides nutrition and oxygen to the tumor tissue, and at an extremely early phase of tumor growth (at the level of only a single cell or several cancer cells) the tumor tissue of the dissemination may obtain nutrition and oxygen from pleural liquid, which is in a continuous state of turnover.

We attempted to obtain histological evidence of tumor dissemination, but we were unable to prepare histology specimens by hematoxylin and eosin staining, since it was very difficult to slice the disseminated tumors of diameter of about 0.1 mm and only 3 to 4 tumors were found in each animal. An immunohistochemical method to localize cancer cells could be applied to detect very small and infrequent cancer nest in the pleural surface in future experiments. However, we feel that our results confirm that cancer dissemination tissue was present on the pleural surface, since the brightly fluorescent materials used in the study were
limited to GFP-labeled rat colon cancer cells and FITC-RBCs. Therefore, the fluorescent objects observed by CLSM before intravenous injection of FITC-RBCs must have been GFP-labeled cancer cells.

In conclusion, in this study we created a model of pulmonary dissemination of size less than 100 μm in diameter, indicating an extremely early stage of dissemination. We were able to demonstrate microcirculation inside and outside of the dissemination in this model, and we showed that the blood flow velocity was reduced inside the tumor tissue, as demonstrated by the movement of FITC-RBCs. The reduced velocity might be due to a difference in the vascular structure between the tumor tissue and normal tissue. CLSM with fluorescent labeling of cancer cells and RBCs allowed an examination of the pathophysiology of the pulmonary dissemination, and we suggest that this technique can be used to study formation of the early phase of cancer spreading in any kind of malignancy.

Acknowledgements

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References