Widespread Skeletal Metastatic Potential of Human Lung Cancer Revealed by Green Fluorescent Protein Expression

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Abstract
To understand the skeletal metastatic pattern of non-small cell lung cancer, we developed a stable high-expression green fluorescent protein (GFP) transductant of human lung cancer cell line H460 (H460-GFP). The GFP-expressing lung cancer was visualized to metastasize widely throughout the skeleton when implanted orthotopically in nude mice. H460 was transduced with the pLEIN retroviral expression vector containing the enhanced GFP and the neomycin (G418) resistance gene. A stable high GFP-expressing clone was selected in vitro using 800 µg/ml G418. Stable high-level expression of GFP was maintained in s.c.-growing tumors formed after injecting H460-GFP cells in nude mice. To use H460-GFP for visualization of metastasis, fragments of s.c.-growing H460-GFP tumors were implanted by surgical orthotopic implantation in the left lung of nude mice. Subsequent micrometastases were visualized by GFP fluorescence in the contralateral lung, plural membrane, and widely throughout the skeletal system including the skull, vertebra, femur, tibia, pelvis, and bone marrow of the femur and tibia. The use of GFP-expressing H460 cells transplanted by surgical orthotopic implantation revealed the extensive metastatic potential of lung cancer in particular to widely disseminated sites throughout the skeleton. This new metastatic model could play a critical role in the study of the mechanism of skeletal and other metastasis in lung cancer and in screening of therapeutics that prevent or reverse this process.

Introduction
Lung cancer is the leading cause of cancer death in the United States with metastasis being the principal cause (1–3). The skeleton is one of the most common sites of metastasis in lung cancer. However, the biology of bone metastasis is poorly understood because of a lack of a bone-metastasis animal model of lung cancer (4).

Models including s.c.-implant models (5, 6) and renal capsule-implant models (7–9) have been developed for human lung cancer, which have been useful. However, these models are not sufficiently representative of the clinical situation (10). In the past 10 years, orthotopic-implant models, such as the intrapulmonary-injection model and the SOI model (10–13), have been established. The SOI model allows extensive lung cancer metastasis due to the maintenance of tissue architecture during the orthotopic implant process. The SOI model of lung cancer required the development of an open thoracotomy procedure in the mouse to suture histologically intact tumor tissue onto the lung (12, 13).

However, the early stages of tumor progression and micrometastasis formation have been difficult to analyze because of the inability to identify small numbers of tumor cells against a background of host tissue. Bone involvement of lung cancer has been observed when cells were injected into the cardiac ventricle of nude mice (14). However, this model does not represent the clinical metastatic process.

To develop a representative experimental model of human lung cancer that could closely represent the clinical situation, we designed an SOI model of GFP-expressing H460 human lung cancer. These models involved the stable transduction of tumor cells in vitro with the jellyfish Aequorea victoria GFP gene that was stably and highly expressed in vivo (15, 16). Our previous studies had shown that high GFP expression in the tumor cells allowed the visualization of tumor cell emboli, micrometastases, and their progression during the course of the disease (15, 16). In the present investigation, GFP expression in the SOI model has revealed the very extensive and widespread skeletal metastatic potential of lung cancer.

Materials and Methods
DNA Expression Vector. The RetroXpress vector pLEIN was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The pLEIN vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an IRES site.

Cell Culture, Vector Production, Transduction, and Subcloning. PT57, an NIH3T3-derived packaging cell line expressing the 10 A virus envelope, was purchased from Clontech Laboratories, Inc. PT57 cells were cultured in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, Calabasas, CA). For vector production, packaging cells (PT57), at 70% confluence, were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim) and saturating amounts of pLEIN plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy after 48 h. For selection of GFP transductants, the cells were cultured in the presence of 500-2000 µg/ml of G418 (Life Technologies, Inc., Grand Island, NY) for 7 days.

Retroviral Transduction of H460 Cells. For GFP gene transduction, 20% confluent H460 cells were incubated with a 1:1 precipitated mixture of retroviral supernatant of PT57 cells and RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA) for 72 h. Fresh medium was replenished at this time. H460 cells were harvested by trypsin/EDTA 72 h after infection and subcultured at a ratio of 1:15 into selective medium that contained 200 µg/ml of G418. The level of G418 was increased to 800 µg/ml gradually. H460 clones expressing GFP (H460-GFP) were isolated with cloning cylinders (Bel- Art Products, Pequannock, NJ) by trypsin/EDTA and were amplified and transferred by conventional culture methods.

Doubling Time of Stable GFP Clones. H460-GFP or nontransduced cells were seeded at 5.5 × 10^4 in 60-mm culture dishes. The cells were harvested and counted every 24 h using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY). The doubling time was calculated from the cell growth curve over 6 days.

s.c. Tumor Growth. Three BALB/c nu/nu female mice, 6 weeks of age, were injected s.c. with a single dose of 5 × 10^6 H460-GFP cells. Cells were first harvested by trypsinization and washed three times with cold serum-free medium and then injected in a total volume of 0.2 ml within 40 min of harvesting.

SOI Tumor. Tumor fragments (1 mm³) derived from H460-GFP s.c. tumor growing in the nude mouse were implanted by SOI on the left lung in eight nude mice.
mice (12, 13). The mice were anesthetized by isoflurane inhalation. The animals were put in a position of right lateral decubitus, with four limbs restrained. A 0.8-cm transverse incision of skin was made in the left chest wall. Chest muscles were separated by sharp dissection, and central and intercostal muscles were exposed. A 0.4-0.5-cm intercostal incision between the third and fourth rib on the chest wall was made, and the chest wall was opened. The left lung was then taken up by a forceps, and tumor fragments were sewn promptly into the upper lung by one suture. The lung was then returned into the chest cavity. The incision in the chest wall was closed with a 6-0 surgical suture. The closed condition of the chest wall was examined immediately and, if a leak existed, was closed by additional sutures. After closing the chest wall, an intrathoracic puncture was made by using a 3-ml syringe and 25-gauge 1/2 needle to withdraw the remaining air in the chest cavity. After the withdrawal of air, a completely inflated lung could be seen through the thin chest wall of the mouse. Then the skin and chest muscle were closed with a 6-0 surgical suture in one layer. All procedures of the operation described above were performed with a ×7 microscope (Olympus).

Analysis of Metastases. After tumor progression in the SOI animals, the performance status of the mice began to decrease, at which time the animals were sacrificed and autopsied. The orthotopic primary tumor and all major organs as well as the whole skeleton were explored. The fresh samples were sliced at a 1 mm thickness and observed directly under fluorescence microscopy.

Microscopy. Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply. A Leica stereo fluorescence microscope model LZ12 equipped with a mercury lamp power supply was also used. Both microscopes had a GFP filter set (Chroma Technology, Brattleboro, VT).

Results and Discussion

Isolation of Stable High-Level Expression GFP Transductants of H460 Cells. The retroviral-vector transduced cells were able to grow in vitro at levels of G418 up to 800 µg/ml. The selected G418-resistant H460-GFP cells had bright GFP fluorescence (Fig. 1). There was no difference in the cell proliferation rates of parental cells and the GFP transductants as determined by comparing their doubling times in vitro (data not shown).

Stable High-Level Expression of GFP in H460 Tumors in Nude Mice. Three weeks after s.c. injection of H460-GFP cells, the mice were sacrificed. All three mice had a s.c. tumor, which ranged in diameter from 1.5 to 2.1 cm (mean, 1.82 ± 0.3). The tumor tissue was strongly GFP fluorescent, thereby demonstrating stable high-level GFP expression in vivo during s.c. tumor growth. Lung metastases were found, but no metastases were found in systemic organs in the s.c. tumor model of H460-GFP (data not shown).

GFP-expressing Lung and Bone Metastases in Nude Mice. Eight nude mice were implanted in the left lung by SOI with 1-mm³ cubes of H460-GFP tumor tissue derived from the H460-GFP s.c. tumor. The implanted mice were sacrificed at 3–4 weeks at the time of significant decline in performance status. All mice had tumors in the left lung weighing from 0.985 to 2.105 g (mean, 1.84 ± 0.4; Fig 2A, a). All tumors (eight of eight) metastasized to the contralateral lung and chest wall (Fig. 2B, C and c; Table 1). Seven of eight tumors metastasized to the skeletal system (Fig. 3; Table 1).

In the present investigation, it was determined that the vertebral bone was the most involved skeletal site of metastasis, because seven of eight mice had vertebral metastasis. Figs. 3, 4 and 5, are examples of tumor metastasis in the lumbar vertebral visualized by GFP. Fig. 3A shows −7.13 mm² of a vertebral bone involved with tumor. Fig. 3C shows a tumor metastasis in the pelvis. Approximately 0.32 mm² of the pelvis was involved with tumor.

Three of seven mice had skull metastases visualized by GFP. Fig. 3D shows very strong GFP fluorescence in the skull of one mouse, which was involved with tumor of −0.46 mm².

Metastasis could be visualized in the tibia and femur marrow by GFP fluorescence. Fig. 3E shows the bone marrow of the tibia under bright-field microscopy. Fig. 3, F and G, show the same fields as Fig. 3 under fluorescence microscopy. A strong GFP-fluorescing metastasis could be detected in the tibia bone marrow. Fig. 3H shows tumor metastasis in the bone marrow of the femur visualized under fluorescence microscopy. The tumor lodged in the bone marrow and seemed to begin to involve the bone as well.

Fig. 3I shows the surface of the femur. No metastatic lesion was detected on the surface under bright-field microscopy. Fig. 3J shows the same field as Fig. 3I under fluorescence microscopy, where a strongly GFP-fluorescent metastasis could be visualized on the surface of the femur.

Fig. 3K shows the tibia. No metastatic lesion was detected on the bone under bright-field microscopy. Fig. 3L shows the same field as Fig. 3K under fluorescence microscopy, where a strongly GFP-fluorescent metastasis was visualized on the tibia with an area of −0.24 mm².

Table 1 summarizes the metastatic pattern of human lung tumor H460-GFP. All of the experimental animals were found with contralateral lung metastases. Extensive and widespread skeletal metastasis, visualized by GFP expression, were found in 90% of the animals explored. Thus, the H460-GFP SOI model revealed the extensive skeletal metastasizing potential of lung cancer. Such a high incidence of skeletal metastasis could not have been visualized previously before the development of the GFP-SOI model described here, which provided the necessary tools. Although previous studies have suggested that bone is one of the most favored sites of solid tumor metastasis, the present study revealed that the bone microenvironment provided a highly fertile soil for lung cancer. Patients with other common solid tumors, such as breast and prostate cancer, also may have a major portion of the tumor burden present in bone at the time of death (17).

Our previous studies have shown that GFP expression allowed the visualization of tumor cell emboli, micrometastases, and their progression in fresh tissue down to the single-cell level (15, 16). These models provided a powerful and convenient tool for the study of micrometastasis in experimental animal models.

In the present investigation, an extensive metastatic process, involving the bone marrow and bone, were visualized by GFP directly under fluorescence microscopy. This method has higher resolution and is much more facile than the traditional cumbersome pathological examination procedures, such as histology and immunohistochemistry. A major advantage of GFP-expressing tumor cells is that they can be visualized in fresh live tissue. It is possible that when GFP-expressing cells undergo apoptosis, they could be engulfed by macrophages. However, when
Fig. 2. Orthotopic growth and contralateral lung and plural membrane metastases visualized by GFP. Two 1-mm^2 fragments of s.c.-grown H460 GFP tumor were implanted to the left lung of nude mice by SOI. A, the primary tumor formed in the left lung visualized under bright-field microscopy. Bar, 1280 μm. A, same as A, but the tumor is visualized by intense expression of GFP under fluorescence microscopy. Bar, 1280 μm. B, numerous metastases and micrometastases can be visualized by GFP in the contralateral lung of the nude mouse. Bar, 200 μm. C, metastases in the contralateral plural membrane under bright-field microscopy. Bar, 1280 μm. C, same as C, but visualized by GFP under fluorescence microscopy (white arrowheads). Bar, 1280 μm.

Table 1: Metastasis of GFP-expressing H460 cells after SOI in nude mice

Eight nude mice were implanted in the left lung by SOI with 1-mm^3 cubes of H460-GFP tumor tissue derived from the H460-GFP sc. tumor grown previously in a nude mouse. The implanted mice were sacrificed at 3-4 weeks at the time of significant decline in performance status. All transplanted mice (eight of eight) had metastases to the contralateral lung and chest wall. Seven of eight tumors metastasized to the skeletal system including skull, vertebra, femur, tibia, pelvis, and bone marrow of the femur as well as the tibia.

<table>
<thead>
<tr>
<th>Tumor #</th>
<th>Primary Tumor Weight (mg)</th>
<th>Brain</th>
<th>Contralateral Lung</th>
<th>Pleural Membrane</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Adrenal Gland</th>
<th>Bone</th>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>1388</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>+ (vertebra)</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>+ (vertebra, tibia, bone marrow of femur)</td>
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<td>5</td>
<td>1710</td>
<td>-</td>
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<td>+</td>
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GFP expressing cells die, they lose their fluorescence, such as in necrotic areas of tumors, suggesting that these macrophages would not interfere with detection of metastases.

The data presented here reveal the extensive, widespread skeletal metastatic potential of lung cancer. This new metastasis model will be relevant for the study of the mechanism of skeletal and other metastases in lung cancer and for their therapy.

References

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