Genetically Fluorescent Melanoma Bone and Organ Metastasis Models

Meng Yang, Ping Jiang, Zili An, Eugene Baranov, Lingna Li, Satoshi Hasegawa, Maraya Al-Tuwaijri, Takashi Chishima, Hiroshi Shimada, A. R. Moossa, and Robert M. Hoffman


ABSTRACT

We report here the establishment and metastatic properties of bright, highly stable, green fluorescent protein (GFP) expression transductants of the B16 mouse malignant melanoma cell line and the LOX human melanoma line. The highly fluorescent malignant melanoma cell lines allowed the visualization of skeletal and multiorgan metastases after i.v. injection of B16 cells in C57BL/6 mice and intradermal injection of LOX cells in nude mice. The melanoma cell lines were transduced with the pLEIN retroviral vector containing the GFP and neomycin resistance genes. Stable B16F0 and LOX clones expressing high levels of GFP were selected stepwise in vitro in levels of G418 up to 800 μg/ml. Extensive bone and bone marrow metastases of B16F0 were visualized by GFP expression when the animals were sacrificed 3 weeks after cell implantation. Metastases for both cell lines were visualized in many organs, including the brain, lung, pleural membrane, liver, kidney, adrenal gland, lymph nodes, skeleton, muscle, and skin by GFP fluorescence. This is the first observation of experimental skeletal metastases of melanoma, which was made possible by GFP expression. These models should facilitate future studies of the mechanism and therapy of bone and multiorgan metastasis of melanoma.

INTRODUCTION

Cutaneous melanoma is increasing in incidence. This disease is largely confined to whites, in whom the age-adjusted incidence rate in the United States is about 12 per 100,000 persons and is 3-fold higher (30 per 100,000) in some geographic areas (1–3). In 1935, only 1 in 1,500 persons developed melanoma. The incidence has increased dramatically to 1 in 250 persons in 1980 and to 1 in 135 persons in 1987. Assuming present trends, the incidence will be 1 in 90 persons by the year 2000 (4). Therefore, the development of more effective methods for the prevention and treatment of this disease is urgent and would be highly facilitated by a useful animal model that closely mimics the clinical situation.

During the past 15 years, experimental models of the human malignant melanoma LOX have been established in athymic nude mice and rats (5–8). Studies with these models have shown that the LOX cells have a preference for growth in the lungs of the host.

In man, the lymph nodes, lung, brain, liver, and bone (9) are the most common sites of melanoma metastasis (10).

To develop effective therapeutics for this pathological process, a simple, highly reproducible, and easily used animal model is needed.

Although injection of B16 melanoma cells into the left cardiac ventricle resulted in tumor colonies in the skeletal system and most organs of the mouse (11), this procedure is complicated and is not convenient enough to be used in large-scale investigation. There have not been any reports demonstrating bone metastasis from B16 melanoma cells injected into the tail vein. In previous studies, i.v. injection of 10⁵ melanoma cells resulted in observable lung tumor colonies (11).

We have developed a new model of cancer by highly stable transfer and expression of the Aequorea victoria jellyfish GFP gene in tumor cells. The GFP-expressing tumor cells enable visualization of tumor growth, invasion, micrometastasis, and metastasis in fresh viable tissue after transplantation to mice (12–14). In the present investigation, GFP expression in an orthotopic intradermal injection model of LOX cells in nude mice and in an experimental metastasis model of B16 in C57BL/6 mice has revealed the multiorgan and very extensive bone metastatic potential of melanoma. These new metastasis models should be very useful for studying the biology of metastatic melanoma and for the development of therapy of this disease.

MATERIALS AND METHODS

GFP Expression Vector. The pLEIN retroviral vector (CLONTECH, Palo Alto, CA) expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an internal ribosome entry site (IRES) (14).

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2 To whom requests for reprints should be addressed, at AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111. Phone: (858) 654-2555; Fax: (858) 268-4175; E-mail: all@anticancer.com.

3 The abbreviation used is: GFP, green fluorescent protein.
Stable high-level GFP expression LOX human melanoma transductants in vitro and in vivo. a, the human malignant melanoma cell line LOX was transduced with retroviral vector pLEIN that expresses GFP and the neomycin resistance gene on the same bicistronic message. Stable clones expressing high levels of GFP were selected in 800 µg/ml G418. Bar, 80 µm. b, LOX-GFP orthotopic tumor with intense expression of GFP in the nude mouse. The tumor is growing through the whole layer of the skin. The epidermis (EP) and dermis (D) are indicated. Bar, 400 µm.

Table 1  Multi-organ, brain and skeletal metastases of GFP-expressing LOX human melanoma cells after intradermal implantation in nude mice

Ten nude mice were each injected intradermally with $1 \times 10^6$ cells. The mice were sacrificed at 6–8 weeks, at the time of significant decline in performance status.

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Packaging Cell Culture, Vector Production, Transfection, and Subcloning. PT67, an NIH 3T3-derived packaging cell line expressing the 10 Al viral envelope, was purchased from CLONTECH. PT67 cells were cultured in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA). For vector production, packaging cells (PT67) at 70% confluence were incubated with a precipitated mixture of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate 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 48 h after transfection. For selection, the cells were cultured in the presence of 500-2000 μg/ml G418 (Life Technologies, Inc., Grand Island, NY) for 7 days.

Retroviral GFP Transduction of LOX and B16 Cells. For GFP gene transduction, LOX cells (National Cancer Institute, Bethesda, MD) and B16 cells (American Type Culture Collection, Manassas, VA) at ~25% confluence were incubated with a 1:1 precipitated mixture of retroviral supernatants of

Fig 2 Lung metastasis of LOX cells visualized by GFP. a, the surface of the lung of a nude mouse. No metastasis was detected under bright-field microscopy. Bar, 1280 μm. b, same field as in a. Numerous micrometastases and metastases are visualized by GFP expression in the lung under fluorescence microscopy. Bar, 1280 μm.
PT67 cells and RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (Gemini Bioproducts) for 72 h. Fresh medium was replenished at this time. Cells were harvested by trypsin-EDTA 72 h after transduction and subcultured at a ratio of 1:15 into selective medium that contained 200 \( \mu \text{g/ml} \) G418. The level of G418 was increased stepwise to 800 \( \mu \text{g/ml} \) for both LOX and B16 cells. Clones expressing GFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) using trypsin-EDTA and then amplified and transferred by conventional culture methods.

**Doubling Time of Stable GFP Clones.** GFP or non-transduced cells were seeded at 1.5 \( \times \) 10^4 in 35-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.

**Intradermal Injection of LOX.** Ten 6-week-old BALB/c nu/nu male mice were injected intradermally with a single dose of 1 \( \times \) 10^6 LOX-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 0.1 ml within 30 min of harvesting. Cells were inoculated into dorsal skin using a 30 G1/2 precision glide needle (Becton Dickinson) and a 1-ml latex-free syringe (Becton Dickinson).

**Tail Vein Injection of B16.** Female 6-week-old C57BL/6 mice (Harlan, Indianapolis, IN) were injected with a single dose of 5 \( \times \) 10^6 B16F0-GFP C1 cells in the lateral tail vein. 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 30 min of harvesting.

**Analysis of Metastases.** Tumor progression occurred in the tumor-bearing animals along with decreased performance status. When performance status was poor, as defined by the onset of cachexia, the animals were sacrificed and autopsied. The primary tumor and all major organs as well as the whole skeleton were explored. The fresh samples were sliced at approximately 1 mm in thickness by using disposable microtome blades (Model 818; Leica Instruments GmbH, Nussloch, Germany) and observed directly under a fluorescence microscope.
Microscopy. Light microscopy 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).

Animal Care. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

RESULTS AND DISCUSSION

Isolation of Stable Transductants of LOX Cells Expressing High Levels of GFP. The GFP-transduced cells were able to grow in levels of G418 of up to 800 μg/ml. The selected G418-resistant LOX cells had a strikingly bright GFP fluorescence (Fig. 1a). There was no difference in the cell proliferation rates of parental cells and selected transductants determined by comparing their doubling times in monolayer culture.

Stable High-Level Expression of GFP in LOX Tumors Growing Intradermally in Nude Mice. The mice were sacrificed 6–8 weeks after intradermal injection of LOX-GFP cells. Ten of 10 mice had very large intradermal and s.c. tumors. The tumor tissue was strongly fluorescent, thereby demonstrating stable high-level GFP expression in vivo during tumor growth (Fig. 1b).

Organ and Bone Metastases of GFP LOX Tumors. Tumor-bearing nude mice were sacrificed at 6–8 weeks when performance status was poor. As summarized in Table 1, tumors metastasized to the lung (10 of 10 mice; Fig. 2, a and b); pleural membrane (5 of 10 mice; Fig. 3a); liver (3 of 10 mice; Fig. 3b); kidney (1 of 10 mice; Fig. 3c, large arrows); adrenal gland (3 of 10 mice; Fig. 3c, small arrows); lymph nodes (7 of 10 mice; Fig. 3d), and skeletal system (1 of 10 mice; Fig. 4a). In five mice, single cancer cells or small colonies could be visualized in the brain by GFP fluorescence (Fig. 4, b–d).

Although previous studies have already demonstrated that pulmonary metastases have been produced with high efficiency after i.v., i.p., and s.c. injection of LOX cells, other distant metastases were not observed (5–8). This situation is similar in other human melanoma models. For example, the s.c. implantation MeWo melanoma model can result in numerous large lung metastasis nodules. Transfer of the metastatic lung nodules to new recipients also led to the appearance of lung metastases. However, other distant metastases were not observed (15). Subdermal injection of the MeWo cells resulted in the development of highly melanotic and nonencapsulated primary tumors, which grew quickly into the dermis and epidermis and metastasized at high frequency to the draining lymph nodes (16). Although these spontaneous metastasis models have provided useful tools for basic and preclinical studies, they still have limitations because they do not fully match the clinical situation of highly...
malignant melanoma, perhaps the most aggressively metastatic cancer (17).

The LOX-GFP model revealed by GFP fluorescence the extensive multiorgan metastasizing potential of human malignant melanoma, which also includes the brain and skeletal system. These data demonstrate the far-reaching malignancy of this tumor (Table 1). Such a high incidence of multiorgan and skeletal metastases of human melanoma could not have been visualized before the development of the intradermal GFP model described here, which provided the necessary tools.

Isolation of Stable Transductants of B16F0-GFP Cells Expressing High Levels of GFP. The GFP- and neomycin-containing expression vector-transduced cells were able to grow in increasing levels of G418 (up to 800 μg/ml). The B16F0-GFP C1 clone resistant to 800 μg/ml G418 had a strikingly bright GFP fluorescence (Fig. 5). There was no difference in the cell proliferation rates of parental cells and selected transductants as determined by a comparison of proliferation rates in monolayer culture. Both cell lines had a doubling time of approximately 24 h (data not shown). The GFP-transduced B16F0 and parental cells still produced melanin in vitro and in vivo. There are no obvious changes in morphology of the GFP-transduced cells compared with the parental cells. In vitro properties of transformation such as the serum requirement and anchorage dependence of GFP-transformed and parental cells will be compared in future studies.

Stable High-Level Expression of GFP in B16F0 Tumors Growing in C57BL/6 Mice. Mice were sacrificed 3 weeks after i.v. injection of B16F0-GFP C1 cells. Five of five mice had extensive skeletal and visceral metastases (Table 2). The tumor tissue was strongly fluorescent, thereby demonstrating stable high-level GFP expression in vivo during tumor growth (Figs. 6 and 7).

**Table 2** Skeletal and multi-organ metastases of GFP-expressing B16F0 cells after i.v. injection in C57BL/6 mice

Five C57BL/6 mice were each injected with $5 \times 10^6$ B16F0-GFP C1 cells. Three weeks after the injection of the cells, the mice were sacrificed and examined.

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Fig. 5 Stable high-level GFP-expressing B16F0-GFP C1 murine melanoma transductants in vitro. The murine malignant melanoma cell line B16F0 was transduced with the pLEIN vector that expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message. Stable high expression clones were selected in 800 μg/ml G418. Bar, 80 μm.
Fig. 6  Bone metastasis of B16F0 GFP C1 visualized by GFP.  

a, skull; top, no metastasis was detected under bright-field microscopy. Bar, 640 μm. 
Bottom, same area as shown at the top, bone metastasis visualized in the skull under fluorescent microscopy.

b, vertebral body; top, no metastasis was detected under bright-field microscopy. Bar, 1280 μm. 
Bottom, same area as shown at the top, bone metastasis visualized in the vertebral body under fluorescent microscopy.

c, bone metastases visualized by GFP expression in the humerus and scapula. Bar, 1280 μm.
d, bone metastases visualized by GFP expression in the distal end of the femur. Bar, 1280 μm.
e, bone metastases visualized by GFP expression in the head of the femur. Bar, 1280 μm.
f, bone metastases visualized by GFP expression in the pelvis. Bar, 1280 μm.
Fig. 7 Multiorgan metastasis of B16F0-GFP C1 visualized by GFP expression. 

a, brain metastasis visualized by GFP expression. Bar, 1280 μm.
b, extensive metastases visualized in the lung and pericardium by GFP expression (arrows). Bar, 1280 μm.
c, metastases visualized in the pleural
such as the skull (four of five mice) and scapula (three of five mice; Fig. 6, a–f).

**GFP-expressing Experimental Systemic Organ Metastases.** Tumor-bearing C57BL/6 mice were sacrificed 3 weeks after injection of B16F0-GFP C1 cells. Experimental metastases were visualized by GFP in the major systemic organs: (a) brain (three of five mice); (b) lung (five of five mice); (c) pleural membrane (five of five mice); (d) liver (five of five mice); (e) kidney (five of five mice); (f) adrenal gland (five of five mice); (g) lymph node (five of five mice); and (h) skin and skeletal muscle (five of five mice; Fig. 7; Table 2).

Previous studies have shown that injection of melanoma cells into the left cardiac ventricle resulted in tumor colonies in the skeletal system and in most organs of the mouse (11). In contrast, the vast majority of i.v. injected tumor cells arrest rapidly in the lung (11), and arterially injected embryonal carcinoma cells appear to arrest immediately in the capillary beds (18). In spontaneous metastasis to extrapulmonary organs, some cells must pass through the lung capillary bed into the arterial supply and lodge in other organs. A major role of the lung capillaries may be to prevent metastasis by trapping and mechanically destroying almost all of the tumor cells that enter the venous circulation (19). In the present study, we increased the amount of i.v. injected tumor cells up to $5 \times 10^6$ and caused extensive skeletal and visceral metastasis as visualized by GFP, indicating that some of the tumor cells could pass through the lung capillary.

In the present study, all five mice injected with tumor cells had bone metastasis as well as lung metastasis. No mouse had only bone metastasis. Despite the injection of $5 \times 10^6$ cells (100× routine innoculum), the survival of all experimental animals was longer than 3 weeks, which was sufficient for seeding of the bone and colonization. The model described in this report used tail vein injection of tumor cells, which could simultaneously seed all organs of appropriate “soil.” Bone seeding and colonization would not then necessarily be late events.

Bones with fluorescent tumor colonies were those rich in hematopoietic bone marrow, such as vertebral bodies, femur, tibia, and the pelvis. There were no colonies in the bones that did not have bone marrow. The presence of bone marrow also appears to be a prerequisite for the establishment of human bone metastasis (20, 21). Bone marrow appears to support tumor colonization very extensively. Histopathological evidence of bone metastases was demonstrated by H&E staining (Fig. 8) and correlates to GFP fluorescence in the bone (Fig. 6d). The GFP-fluorescent human tumor cell transductants we have produced can be visualized as single cells when seeded in various tissues (12, 13). However, thus far, we have not yet found any single cells seeded in the bone. Future time course experiments will clarify how many GFP-expressing tumor cells must be present in the bone to be detectable. In the present study, the pattern of skeletal distribution of tumor colonies closely mimics the pattern of metastasis to bone in humans.

In the present investigation, GFP expression has revealed membrane by GFP expression (arrow). Bar, 800 μm. d, metastases visualized in the liver by GFP (arrows). Bar, 400 μm. e, kidney (large arrows) and adrenal (small arrow) metastasis visualized by GFP expression. Bar, 1280 μm. f, mesenteric lymph gland metastases visualized by GFP expression. Bar, 1280 μm. g, skin metastases visualized by GFP expression. Bar, 1280 μm. h, skeletal muscle metastases visualized by GFP expression. Bar, 200 μm.
bone and visceral metastasis of human and murine melanoma. The fluorescence models have demonstrated for the first time the very high metastatic potentials of the B16F0 and LOX melanomas.

In the present study, the pLEIN vector was used to transfect cells with the GFP gene along with the neo gene conferring resistance to G418. In previous studies, the bicistronic GFP vector was used, which contains the dihydrofolate reductase gene to confer resistance to methotrexate (12, 13). In recent and present studies, selection was carried out in stepwise increments of the selective agent in vitro. After selection in what was determined to be the maximum concentration of the selective agent, the cells were cultured in the absence of the selective agent. Only the highest concentration of the selective agent, the cells were carried out in stepwise increments of the selective agent trexate (12, 13). In recent and present studies, selection was tested for stability in the absence of the selective agent were then selected and cultured in the absence of the selective agent. Only the maximum concentration of the selective agent, the cells were carried out in stepwise increments of the selective agent trexate (12, 13). In recent and present studies, selection was tested for stability in vitro and in vivo. This procedure has resulted in the selection of tumor cells capable of stable GFP expression in vivo as they form tumors and metastasize. Both the GFP-transduced melanoma cell lines are very stable in vitro and in vivo. B16F0-GFP C1 cells have been passaged for over 20 generations in vitro with no obvious changes observed in fluorescence. In s.c. B16F0 melanoma models, the survival of the mice is longer than 8 weeks. The tumors are fluorescent until the time of the animal’s death. LOX-GFP cells have been passaged for six generations in vitro and grown in vivo for over 3 months, during which time there were no obvious changes in fluorescence seen in vitro or in vivo.

Previous studies transfected tumor cells with the lacZ gene to detect micrometastases (22). However, detection of lacZ requires extensive histological preparation; therefore, it is impossible to detect and visualize tumor cells in viable fresh tissue at the microscopic level. The GFP technique has greatly enhanced the resolution of the visualization of micrometastases in fresh tissue, allowing detection down to the single-cell level in fresh tissue (12, 13).

GFP is more easily visualized than melanin, due to its bright fluorescence. The purpose of the present report was to demonstrate the potential power of visualization of genetically fluorescent models of melanoma. Fig. 8 demonstrates by standard H&E staining that GFP is reporting histologically proven bone metastasis. Future studies will compare the histopathology of GFP B16 and the parental cells in detail.

B16F0-GFP C1 cells produced melanin in vitro and in vivo, similar to the parental cells. GFP-LOX cells did not produce melanin in vitro and in vivo similar to their parental cells. Transduction controls without GFP will be a subject of future studies.

The clonogenicity of GFP-transformed tumor cells has been approached in three different experiments. In a study by Chishima et al. (12), a s.c. growing GFP-expressing Chinese hamster ovary tumor was minced, and the cells were grown in culture with expression of GFP maintained in vitro. In another study by Chisima et al. (23), nude mice were injected in the tail vein with GFP-expressing human lung tumor cells. These cells seeded the lungs, which were removed and grown in three-dimensional histoculture. Progressive colonization of the lung by the GFP-expressing tumor cells over 52 days in histoculture was observed. In the present study, we observed comparable bone metastatic cells by both GFP expression (Fig. 6d) and H&E staining (Fig. 8). The in vitro subcloning of GFP-expressing cells recovered from in vivo metastasis will be undertaken in future studies.

The new metastasis models of LOX and B16F0 should be very useful for the study the biological behavior of metastatic melanoma and for the development of therapy of this disease.


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