Real-Time Whole-Body Imaging of an Orthotopic Metastatic Prostate Cancer Model Expressing Red Fluorescent Protein

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BACKGROUND. We describe here, a whole-body imageable spontaneous metastatic model of human prostate cancer developed by surgical orthotopic implantation (SOI) and visualized by red fluorescent protein (RFP) expression.

METHODS. Human prostate cancer PC-3 cells were transduced with the pLNCX2-DsRed-2-RFP retroviral vector containing the RFP and neomycin-resistance genes. A stable RFP-expressing PC-3 clone was selected in 800 μg/ml G418 in vitro and injected subcutaneously in nude mice. Stable high-level expression of RFP was maintained in the subcutaneously-growing tumors. To utilize RFP expression for metastasis studies, fragments of the subcutaneously-growing tumor, which were comprised of RFP-expressing cells, were implanted by SOI in the prostate of nude mice.

RESULTS. Primary tumor growth, progression, and subsequent lymphatic metastases were visualized in live, intact animals in real time by whole-body RFP fluorescence imaging. In total, 100% of the experimental animals developed lymphatic metastasis, the growth of which was monitored in real time by whole-body imaging. The aggressive lymphatic metastasis in this model reflects one of the major metastatic routes of prostate cancer in human patients. Intravital RFP imaging visualized single cancer cells in the lung and bladder. Open RFP imaging at autopsy visualized extensive primary growth and highly disseminated lymph-node metastases.


KEY WORDS: red fluorescent protein; orthotopic; metastatic; fluorescence imaging; real-time

INTRODUCTION

In order to evaluate and rapidly screen new therapeutics for human prostate cancer progression, an imageable in vivo model that reflects the natural history of clinical prostate cancer would be very useful [1].

Early experimental models involving implanting human prostate cancer cells subcutaneously in athymic nude mice failed to yield metastatic disease [2]. With the androgen-independent PC-3 human prostate cancer cell line as a model, which originated from a bone metastasis [3], osseous metastasis were induced in nude mice by injecting tumor cells intravenously with concomitant occlusion of the inferior vena cava or by intracardiac implantation [4–6].

In orthotopic transplant models of human prostate cancer, Fu et al. [7], Stephenson et al. [8], Pettaway et al.

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[5], Saito et al. [9], Rembrink et al. [10], An et al. [11], and Wang et al. [12] have observed prostate cancer metastasis but only in the lymph nodes and the lung. Thalmann et al. [13] reported a spontaneous bone metastasis model of androgen-dependent human prostate cancer LNCaP-derived sublines.

The early stages of tumor progression and micrometastasis formation have been difficult to visualize in current models due to the inability to identify small numbers of tumor cells against a background of many host tissues. We have developed new models of human and animal cancer by transfer of the green fluorescent protein (GFP) gene to tumor cells, which enables visualization of fluorescent tumors and metastases [14]. The PC-3 human prostate carcinoma expressing GFP was implanted by surgical orthotopic implantation (SOI) in the prostate of a series of nude mice. Subsequent micrometastases and metastases were directly visualized by GFP fluorescence throughout the skeleton, including the skull, rib, pelvis, femur, and tibia. The central nervous system, including the brain and spinal cord, were also involved with tumor, as visualized by GFP fluorescence. Systemic organs, including the lung, plural membrane, liver, kidney, and adrenal gland, also had fluorescent metastases [1].

Human androgen-sensitive LNCaP-GFP cells were orthotopically inoculated to the SCID mouse, and metastases to distant organs were examined in the open animal by intravital fluorescence microscopy [15]. There was no difference in growth rates and androgen-responsiveness in vitro between LNCaP-GFP and LNCaP cells. LNCaP-GFP cells inoculated in SCID mice produced prostate specific antigen. Colonies consisting of a few LNCaP-GFP cells were directly visualized in the lung under fluorescence microscopy as early as 4 weeks after inoculation. Numerous lymph node metastases were detected at various time-points.

The present study, taking advantage of the strong signal and long-wavelength fluorescence of red fluorescent protein (RFP), demonstrates that metastatic prostate cancer progression, in this case in the PC-3 model, can be visualized in nude mice by real-time whole-body fluorescence imaging.

**MATERIALS AND METHODS**

**Expression Vectors**

The pLNCX2 vector was purchased from CLONTECH Laboratories, Inc., (Palo Alto, CA). The pLNCX2 vector contains the neomycin resistance gene for antibiotic selection in eukaryotic cells. The RFP gene, DsRed2 (CLONTECH Laboratories, Inc.) was inserted in the pLNCX2 vector at the Egl II and Not I sites [16].

**RFP Vector Production**

For retroviral transduction, PT67, an NIH3T3-derived packaging cell line, expressing the 10 A1 viral envelope, was purchased from CLONTECH Laboratories, Inc. PT67 cells were cultured in DME (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-products, Calabasas, CA). For vector production, packaging cells (PT67), at 70% confluency, were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim), and saturating amounts of pLNCX2-DsRed2 plasmid for 18 hr. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 hr post-transfection. For selection, the cells were cultured in the presence of 200-1,000 µg/ml of G418 increased in a step-wise manner (Life Technologies, Grand Island, NY) [16].

**RFP Gene Transduction of PC-3 Cell Line**

For RFP gene transduction, ~60% confluent PC-3 human prostate cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and F12K medium containing 7% FBS (Gemini Bio-products, Calabasas, CA) for 72 hr. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium which contained 200 µg/ml of G418. The level of G418 was increased to 1,000 µg/ml in a step-wise manner. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent.

**Subcutaneous Tumor Stock**

In order to have growing tumor tissue stock for subsequent SOI, 6-week-old nu/nu male mice were injected subcutaneously with a single dose of 10^6 RFP-expressing PC-3-RFP cells. Cells were first harvested by trypsinization and washed three times with cold serum-containing medium, then kept on ice. Cells were injected in the subcutaneous space of the flank of the animal in a total volume of 0.4 ml within 40 min of harvesting. The nude mice were sacrificed to harvest tumor tissue 4 weeks after tumor cell injection for SOI of tumor fragments (see below).

**Surgical Orthotopic Implantation (SOI)**

Tumor fragments (1 mm³) were prepared from the nude mouse PC-3-RFP subcutaneous tumor. Two tumor fragments were implanted by SOI in the lateral
lobe of the prostate, which was exposed following a lower midline abdominal incision. After proper exposure of the bladder and prostate, the capsule of the prostate was opened and the two tumor fragments (1 mm³) were inserted into the capsule. The capsule was then closed with an 8-0 surgical suture. The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery. All procedures of the operation described above were performed with a 7x magnification microscope (Olympus) [1].

**Analysis of Metastases**

The performance status in the mice began to decrease after tumor progression, 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 approximately 1 mm thickness and observed directly under fluorescence microscopy without any processing.

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**Fig. 1.** Stable high-level RFP expressing PC-3 cells. The RFP- and neomycin-containing expression-vector transduced cells were able to grow in levels of G418 up to 1,000 mg/ml. The selected G418 resistant PC-3 cells had a striking bright RFP fluorescence.

**Fig. 2.** Whole-body real-time imaging of orthotopically-growing and metastasizing PC-3-RFP human prostate cancer in nude mice. A: Whole-body image of orthotopically-growing PC 3-RFP primary tumor (arrowhead) and metastases (arrows). Image was acquired 2 weeks after tumor implantation. B: Same as (A), except 4 weeks after tumor implantation. Image shows progression of the primary tumor (arrowhead) and extensive tumor metastases (arrows). C: Quantitative imaging data of primary tumor growth and metastases.
Fig. 3. Intra-vital imaging of micrometastasis. **A:** Single cell metastasis in the lung. **B:** Single cell metastasis in the bladder. **C:** Micrometastasis in mesenteric lymph node.

**Fluorescence Imaging**

A Leica fluorescence stereo microscope model LZ12 equipped with a mercury 50W lamp power supply was used [14–16]. To visualize both GFP and RFP fluorescence at the same time, excitation was produced through a D425y60 band pass filter, 470 DCXR dichroic mirror, and emitted fluorescence was collected through
a long pass filter GG475 (Chroma Technology, Brattleboro, VT). Macroimaging was carried out in a light box (Lightools Research, Encinitas, CA). Fluorescence excitation of RFP was produced through an interference filter 4401/220 nm using slit fiber optics for animal illumination. Fluorescence was observed through a 520-nm long pass filter. Images from the microscope and light box were captured on a Hamamatsu C5810-3-chip cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) [11].

Images were processed for contrast and brightness and analyzed with the use of Image Pro Plus v.4.0 software (Media Cybernetics, Silver Spring, MD). High resolution images of 1024 × 724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR model SLV-R1000 (Sony, Tokyo; [16]).

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under assurance number A3873–1. Animals were kept in a barrier facility [1]. Mice were fed with autoclaved laboratory rodent diet Teklad LM-485 (Western Research Products, Orange, CA).

RESULTS AND DISCUSSION

Isolation of Stable High-Level Expressing RFP Transductants of PC-3 Cells

The RFP- and neomycin-containing expression-vector–transduced cells were able to grow in levels of G418 up to 800 mg/ml. The selected G418 resistant PC-3 cells had a striking bright RFP fluorescence (Fig. 1). There was no difference in the cell proliferation rates of parental cells and selected RFP transductants determined by comparing their growth rate in monolayer culture (data not shown).

Stable High-Level Expression of RFP in PC-3 Tumors Growing Subcutaneously in Nude Mice

Six weeks after subcutaneous injection of PC-3 cells, the mice were sacrificed. The tumor tissue was strongly fluorescent, thereby demonstrating stable high-level RFP expression in vivo during tumor growth (data not shown).

Whole-Body Imaging of Primary Tumor Growth and Metastasis in the Orthotopic PC-3-RFP Model

Whole-body imaging visualized primary tumor growth and lymph node metastasis by day 10 after SOI. By 4 weeks, the primary tumor had greatly expanded and more metastatic lymph nodes were visible. The primary tumor growth and metastasis were readily quantified at various imaging time-points (Fig. 2).

Intravital Micro-Imaging of Metastasis

Intravital imaging visualized micrometastases of PC-3-RFP in various internal organs. Figure 3a visualizes a single PC-3-RFP cell in the lung. Figure 3b shows two PC-3-RFP cells in the bladder (white arrow). Figure 3c visualizes a micrometastasis in a mesenteric lymph node.

Open Imaging of Lymph Node Metastasis at Autopsy

Five of five tumors metastasized to the lymphatic system. The lymphatic metastasis included the inguinal lymph nodes, mesenterial lymph nodes, and mediastinal lymph nodes. Orthotopically-growing primary PC-3-RFP and widespread lymph node metastases were visualized only 3 weeks after SOI (Fig. 4).

In conclusion, in the present study, we established an imageable spontaneous orthotopic metastasis mouse model of human prostate cancer. RFP expression of PC-3 enabled metastases to be visualized throughout the lymphatic system and to other organs as well by whole-body imaging in real-time. With fluorescence imaging, the growth and metastasis of PC-3-RFP could be whole-body imaged as early as 10 days after SOI, an important advantage for studying the early events of metastasis and for the discovery of drugs active against this process. Intravital imaging visualized micro-metastases on the internal organs including lung, bladder, and lymph nodes at the single-cell level. This model of PC-3 is highly aggressive with

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**Fig. 4.** Open imaging at autopsy of orthotopic PC-3-RFP human prostate cancer and extensive lymphatic metastasis. Image shows orthotopically-growing primary PC-3-RFP human prostate cancer and widespread lymph node metastases 3 weeks after surgical orthotopic implantation of tumor.
the animal succumbing to local-regional disease. Future studies will emphasize resection of the primary tumor such that there will be a longer time period to develop more distant metastasis, in particular to the bone, which will be followed by real-time whole-body imaging.

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