Dual-Color Imaging of Nascent Blood Vessels Vascularizing Pancreatic Cancer in an Orthotopic Model Demonstrates Antiangiogenesis Efficacy of Gemcitabine

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Background. The stem cell marker nestin recently has been shown to be expressed in nascent blood vessels in nestin-driven green fluorescent protein (ND-GFP) transgenic nude mice.

Materials and methods. In the present study, we visualized by dual-color fluorescence imaging tumor angiogenesis in the ND-GFP transgenic nude mice after orthotopic transplantation of the MIA PaCa-2 human pancreatic cancer line expressing red fluorescent protein. Mice were treated with gemcitabine at 150 mg/kg dose on days 3, 6, 10, and 13 after tumor implantation. At day 14, mice were sacrificed and mean nascent blood vessel density and tumor volume were calculated and compared to control mice.

Results. Nestin was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumor. Results of immunohistochemical staining showed that CD31 co-localized in ND-GFP-expressing nascent blood vessels. The density of nascent blood vessels in the tumor was readily quantitated. Gemcitabine significantly decreased the mean nascent blood vessel density in the tumor as well as decreased tumor volume.

Conclusion. The dual-color model of the ND-GFP nude mouse orthotopically implanted with RFP-expressing pancreatic tumor cells enabled the simultaneous visualization and quantitation of tumor angiogenesis and tumor volume. These results demonstrated for the first time that gemcitabine is an inhibitor of angiogenesis as well as tumor growth in pancreatic cancer. The results have important implications for the clinical application of gemcitabine in this disease. © 2006 Elsevier Inc. All rights reserved.

Key Words: pancreatic cancer; angiogenesis; in vivo imaging; mouse models.

INTRODUCTION

Nestin-driven green fluorescent protein (ND-GFP), transgenic mice have nascent blood vessels that are labeled with GFP. GFP-labeled blood vessels were first observed in the skin, where they form a network of interconnecting hair follicles. When ND-GFP–labeled vibrissa (whisker) hair follicles are transplanted to unlabeled nude mice, new vessels grow from the transplanted follicle, and these vessels increase when the local recipient skin is wounded. These results indicate that, in the skin, the ND-labeled blood vessels originated from hair follicles. The ND-GFP–expressing structures were confirmed to be blood vessels because they displayed the characteristic endothelial-cell–specific markers CD31 and von Willebrand factor. This model visualizes very early events in skin angiogenesis and can serve for rapid antiangiogenesis drug screening [1].

We also have visualized tumor angiogenesis by dual-color fluorescence imaging in ND-GFP transgenic mice after transplantation of the murine melanoma cell line B16F10 expressing red fluorescent protein (RFP). ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumor. Progressive angiogenesis during tumor growth was
readily visualized during tumor growth by GFP expression. Doxorubicin inhibited the nascent tumor angiogenesis as well as tumor growth in the ND-GFP mice transplanted with B16F10-RFP. This model was thus shown to be useful for direct visualization of tumor angiogenesis and evaluation of angiogenic inhibitors [2].

We also developed a novel transgenic ND-GFP nude mouse for the visualization of human tumor angiogenesis. The nestin ND-GFP gene was crossed into nude mice on the C57/B6 background to obtain ND-GFP nude mice. ND-GFP was expressed in the brain, spinal cord, pancreas, stomach, esophagus, heart, lung, blood vessels of glomeruli, blood vessels of skeletal muscle, testes, hair follicles, and blood vessel network in the skin. Human lung cancer, pancreatic cancer, and colon cancer cell lines as well as a murine melanoma cell line and breast cancer tumor cell line expressing RFP were implanted orthotopically, and an RFP-expressing human fibrosarcoma was implanted subcutaneously in the ND-GFP nude mice. These tumors grew extensively in the ND-GFP nude mice. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumors, visualized by dual-color fluorescence imaging [3]. These results demonstrated that, in the ND-GFP mice, nascent blood vessels were visualized in visceral tumors as well as in the skin.

In the present study, we visualized by dual-color fluorescence imaging tumor angiogenesis in the ND-GFP transgenic nude mice after orthotopic transplantation of the MiaPaCa-2 human pancreatic cancer line expressing RFP. Nestin was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumor. We observed for the first time that gemcitab ine has antiangiogenesis as well as antitumor activity in pancreatic cancer. These results suggest that the dual-color model with the ND-GFP nude mouse orthotopically implanted with RFP tumor cells is useful for the visualization and quantitation of tumor angiogenesis and evaluation of angiogenesis inhibitors in pancreatic cancer.

**MATERIALS AND METHODS**

**ND-GFP Transgenic Nude Mice**

ND-GFP transgenic C57/B6 mice [3] carry GFP under the control of the nestin second-intron enhancer. In the present study, the ND-GFP gene was crossed into nude mice on the C57/B6 background to obtain ND-GFP nude mice.

**Red Fluorescent Protein (RFP) Vector Production**

The RFP (DsRed-2) gene (BD Biosciences Clontech, Palo Alto, CA) [3] was inserted in the retroviral-based mammalian expression vector pLNCX (Clontech) to form the pLNCX DsRed-2 vector. Production of retrovirus resulted from transfection of pLNCX DsRed-2 in PT67 packaging cells, which produced retroviral supernatants containing the DSRed-2 gene. Briefly, PT67 cells were grown as mono-layers in DMEM supplemented with 10% fetal calf serum (Gemini Biological Products, Calabasas, CA). Exponentially growing cells (in 10-cm dishes) were transfected with 10 μg of expression vector using a Lipofectamine Plus (GIBCO-BRL, Grand Island, NY) protocol. Transfected cells were replated 48 h after transfection and 100 μg/ml G418 was added 7 h after transfection. Two days later, the amount of G418 was increased to 200 μg/ml. After 25 days of drug selection, surviving colonies were visualized under fluorescence microscopy, and RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titering on the 3T3 cell line.

**RFP Gene Transduction of Tumor Cell Lines**

For RFP gene transduction, 70% confluent MiaPaCa-2 human pancreatic cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 or other culture media (GIBCO) containing 10% fetal bovine serum (Gemini Biological Products) for 72 h [3]. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/ethylene diamine tetraacetic acid and subcultured at a ratio of 1:15 in selective medium, which contained 50 μg/ml G418. To select brightly fluorescent cells, the level of G418 was increased to 800 μg/ml in a stepwise manner. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products) by trypsin/ethylene diamine tetraacetic acid and were amplified and transferred by conventional culture methods in the absence of selective agent.

**Measurement of Length and Density of Nestin-Positive Nascent Blood Vessels**

Angiogenesis was quantified in the tumor tissue by measuring the length of ND-GFP nascent blood vessels in all fields under fluorescence microscopy [3]. All fields at ×40 or ×100 magnification were measured to calculate the total length of ND-GFP–positive nascent blood vessels. The vessel density was calculated by the total length of ND-GFP nascent blood vessels divided by tumor area.

**Immunohistochemical Staining**

Colocalization of ND-GFP fluorescence, the endothelial cell marker CD31, and nestin was visualized in frozen skin sections [3]. Detection was with the anti-rat immunoglobulin horseradish peroxidase (HRP) detection kit (BD PharMingen, San Diego, CA; CD31) and the anti-mouse immunoglobulin HRP detection kit (BD PharMingen; nestin) following the instructions of the manufacturer. The primary antibodies used were CD31 monoclonal antibody (mAb; 1:50) and nestin mAb (1:80). Staining was with 3,3′-diaminobenzidine. Anti-CD31 mAb (CBLI337) was purchased from Chemicon (Temecula, CA). Anti-nestin mAb (rat 40) was purchased from BD PharMingen.

**RFP-Expressing Orthotopic Human Pancreas Cancer Model**

Fifty microliters containing 2 × 10⁶ RFP-expressing MiaPaCa-RFP pancreatic cancer cells per mouse were injected in the subcutis in 6- to 8-week-old nude mice with a 1-mL 27G1/2 latex-free syringe (Becton Dickinson) [4]. Tumor fragments (1 mm³), stably expressing RFP, previously grown subcutaneously in nude mice, were implanted by surgical orthotopic implantation on the pancreas [4] of the ND-GFP nude mice that were 6 to 8 weeks old. After proper exposure of the pancreas, 7-0 surgical sutures were used to penetrate the tumor pieces and attach them to the pancreas. The incision in the abdominal wall was closed with a 6-O surgical suture in one layer. The animals were kept under tribromoethanol anesthesia during surgery. On day 14 after implantation of the tumor, the mice were anesthetized with tribromoethanol. The tumor in the pancreas was directly observed by fluorescence microscopy. Tumor samples were excised. All procedures of the operation described above were
done with a 7X magnification microscope (MZ6, Leica). The tumor samples were divided into two parts, one for fluorescence microscopy and the other for frozen sections.

**Fluorescence Microscopy**

Fluorescence microscopy [3] was performed using an Olympus IMT-2 inverted microscope equipped with a mercury lamp power supply. The microscope had a GFP filter set (Chroma Technology). Tissue samples were directly observed.

**Treatment With Gemcitabine**

The mice were given intraperitoneal injections of 150 mg/kg of gemcitabine or PBS (Cellgro, Herndon, VA; vehicle controls) at day 3, 6, 10, and 13 after implantation of tumor cells [4–6]. Tumor samples were excised under anesthesia at day 14 after implantation of tumor cells. At the end of experiment, the mice were euthanized. The tumors visible with the naked eye were surgically removed. The tumors were measured in three dimensions with calipers. Tumor volume (mm$^3$) was calculated with the formula $V = \frac{1}{2} \times \text{length} \times \text{width} \times \text{height}$. Angiogenesis was quantified in the tumor mass by measuring the total length of ND-GFP-expressing nascent blood vessels under fluorescence microscopy. The vessel density at day 14 was calculated by the total length of nestin-positive nascent blood vessels divided by the tumor volume (mm$^3$). Each experimental group consisted of five mice.

**Statistical Analysis**

The experimental data are expressed as the mean $\pm$ SD. Statistical analysis was performed using the two-tailed Student’s $t$ test.

**RESULTS**

ND-GFP was expressed in the acinar cells of the pancreas in the ND-GFP transgenic nude mice (Fig. 1), which is consistent with previous observations in ND-GFP immunocompetent mice [3].

![FIG. 1. ND-GFP expression in the pancreas. The pancreas in ND-GFP transgenic nude mice: (A1) bright field; (A2) fluorescence. Acinar cells of the pancreas in the ND-GFP transgenic nude mice (arrows): (B1) fluorescence; (B2) bright field.](image)

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Antiangiogenesis Efficacy of Gemcitabine</th>
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<tbody>
<tr>
<td></td>
<td>MIA PaCa-2 PBS</td>
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<tr>
<td>Tumor volume</td>
<td>$704 \pm 381 \text{ mm}^3$</td>
</tr>
<tr>
<td>Total nascent vessel length</td>
<td>$1169 \pm 698 \text{ mm}$</td>
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<tr>
<td>Mean nascent vessel length/mm$^3$</td>
<td>$2.25 \pm 1.42 \text{ mm}$</td>
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* Statistically significant vs PBS treatment ($P < 0.05$).

The RFP-expressing MIAPaCa-2 human pancreas cancer line was orthotopically transplanted to ND-GFP transgenic nude mice. ND-GFP-expressing nascent blood vessels were visualized to grow into the RFP-expressing tumor mass as early as day 7 after surgical orthotopic implantation. The newly formed ND-GFP-expressing blood vessels apparently had blood flow after 14 days (Fig. 2). Immunohistochemical staining showed that CD31 and ND-GFP fluorescence were positive in the newly formed ND-GFP-expressing blood vessels growing into RFP-expressing tumor mass (Fig. 3).

By day 14 after tumor implantation, ND-GFP-expressing nascent blood vessels formed a network in the tumor mass. The ND-GFP–expressing nascent blood vessels had many branches that were connected to each other.

ND-GFP mice with orthotopically-transplanted MIAPaCa-2 were given daily intraperitoneal injections of gemcitabine or PBS at day 3, 6, 10, and 13 after tumor implantation. By day 14 after tumor implantation, the number of nascent blood vessels in the gemcitabine-treated mice was significantly less than the PBS-injected mice ($P < 0.05$; Fig. 4). Gemcitabine also significantly decreased the tumor volume, total nascent blood vessel length, as well as the vessel density determined by mean nascent blood vessel length per 1 mm$^3$ of the tumor volume ($P < 0.05$ versus PBS-injected mice; Table 1).

**DISCUSSION**

Angiogenesis usually is determined by immunohistochemical staining of tumor tissue using various antibodies specific for endothelial cells. The process of blood vessel formation is crucial to tumor development as a source of oxygen and nutrients. The blood vessels that form which nourish a small tumor enable the tumor to grow and eventually metastasize [7]. In the present study, proliferating endothelial cells during active angiogenesis were visualized in the growing tumor by ND-GFP expression. Simultaneously, the tumor was visualized by RFP. The dual-color model provides a powerful and specific model to visualize nascent tumor angiogenesis simultaneously with tu-
mor growth. These data suggest that nascent angiogenesis by the ND-GFP-expressing blood vessels is a critical target to prevent tumor spread.

Gemcitabine is a nucleoside analog that inhibits pancreatic tumor growth and is first-line treatment for patients with metastatic pancreatic cancer since 1997 [8]. In the present study, we demonstrated the tumor growth-suppressing and antiangiogenic efficacy of gemcitabine. In a previous study [4], we used the orthotopic RFP MiaPaCa-2 model to demonstrate the time course of gemcitabine activity on growth of this tumor.

The concept of using chemotherapeutic agents as antiangiogenic agents either alone or in combination with other known inhibitors of angiogenesis has recently been proposed by several investigators [8-10]. Therefore, our findings of the antiangiogenic effects of gemcitabine warrant further investigation. Furthermore, these results suggest that the ND-GFP transgenic nude mouse model is uniquely useful for the visualization of nascent tumor angiogenesis and evaluation of angiogenesis inhibitors against this critical target.

There have been several recent clinical studies that suggest that gemcitabine may be a potential antiangiogenesis agent [9, 11–13]. For example, Bellone et al. [11] evaluated the impact of gemcitabine, irinotecan, and oxaliplatin +5-FU on serum vascular endothelial growth factor levels in patients with locally advanced and/or metastatic cancer. Their findings indicated that vascular endothelial growth factor levels were decreased after treatment with this chemotherapeutic regimen in longer survivors. The authors concluded that these preliminary results provided a rationale for exploring whether continuous or frequent administration of some anti-neoplastic agents may elicit a global anti-angiogenic activity and whether different administration schedules of the same drug could have a synergistic or an antagonist effect, which obviously would need to be taken into account in determining combinations with new agents targeting angiogenesis.

Jia et al. [12] reported on antiangiogenic therapy for human pancreatic carcinoma xenografts in nude mice. In this study, a nonfluorescent surgical ortho-
The SOI model was established by suturing small pieces of SW1990 pancreatic carcinoma into the tail of the pancreas of nude male mice [13]. Mice received either gemcitabine, TNP-470, or a combination of both. A significant inhibitory effect on PCNA index and microvessel density was observed in the gemcitabine-treated and TNP-470–treated group, respectively, whereas both microvessel density and PCNA index were significantly inhibited in the combination group. These results suggested that angiocytotoxic therapy may provide a new safe and effective strategy for the treatment of advanced pancreatic cancer.

**CONCLUSION**

The dual-color model of the ND-GFP nude mouse orthotopically transplanted with RFP-expressing pancreatic tumor cells enabled the simultaneous visualization and quantitation of tumor angiogenesis and tumor volume. These results demonstrate for the first time that gemcitabine is an inhibitor of angiogenesis as well as tumor growth in pancreatic cancer. These results have important implications for the clinical use of gemcitabine in this disease. Future studies will concentrate on the
anti-angiogenesis and anti-tumor efficacy of combination chemotherapy of pancreatic cancer in this model.

REFERENCES