ARTICLE

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Osteosarcoma Cells Enhance Angiogenesis Visualized by Color-Coded Imaging in the In Vivo Gelfoam® Assay

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ABSTRACT

We previously described a color-coded imaging model that can quantify the length of nascent blood vessels using Gelfoam® implanted in nestin-driven green fluorescent protein (ND-GFP) nude mice. In ND-GFP mice, nascent blood vessels are labeled with GFP. We report here that osteosarcoma cells promote angiogenesis in the Gelfoam® angiogenesis assay in ND-GFP mice. Gelfoam® was initially transplanted subcutaneously in the flank of transgenic ND-GFP nude mice. Seven days after transplantation of Gelfoam®, skin flaps were made and human 143B osteosarcoma cells expressing green fluorescent protein (GFP) in the nucleus and red fluorescent protein (RFP) in cytoplasm were injected into the transplanted Gelfoam®. The control-group mice had only implanted Gelfoam®. Skin flaps were made at days 14, 21, and 28 after transplantation of the Gelfoam® to allow imaging of vascularization in the Gelfoam® using a variable-magnification small animal imaging system and confocal fluorescence microscopy. ND-GFP expressing nascent blood vessels penetrated and spread into the Gelfoam® in a time-dependent manner in both control and osteosarcoma-implanted mice. ND-GFP expressing blood vessels in the Gelfoam® of the osteosarcoma-implanted mice were associated with the cancer cells and larger and longer than in the Gelfoam®-only implanted mice ($P < 0.01$). The results presented in this report demonstrate strong angiogenesis induction by osteosarcoma cells and suggest this process is a potential therapeutic target for this disease. J. Cell. Biochem. 115: 1490–1494, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: GREEN FLUORESCENT PROTEIN; RED FLUORESCENT PROTEIN; OSTEOSARCOMA; ANGIOGENESIS; GELFOAM®; NESTIN; TRANSGENIC NUDE MOUSE; CONFOCAL MICROSCOPY

McCarty et al. [2002] described in vivo angiogenesis of Gelfoam® sponges impregnated with 0.4% agarose and different pro-angiogenic factors such as basic fibroblast growth factor (βFGF), vascular epidermal growth factor (VEGF), tumor growth factor-alpha (TGF-α), and endothelial growth factor. In order to quantitate in vivo angiogenesis in this assay, histological and immunohistochemical procedures are required.

In transgenic mice with nestin-driven green fluorescent protein (ND-GFP), ND-GFP is highly expressed in proliferating endothelial cells and nascent blood vessels [Amoh et al., 2005a]. Doxorubicin inhibited the nascent tumor angiogenesis as well as tumor growth in ND-GFP mice transplanted with B16F10-RFP murine melanoma cells [Amoh et al., 2005b, 2007].

Primary-tumor angiogenesis in the ND-GFP transgenic nude mice with orthotopically-transplanted MiaPaCa-2 human pancreatic cancer expressing RFP was visualized by dual-color imaging. Gemcitabine significantly decreased the mean nascent blood vessel density in the tumor as well as decreased tumor volume. These results demonstrated for the first time that gemcitabine is an inhibitor of angiogenesis as well as tumor growth in pancreatic cancer [Amoh et al., 2006a, 2007].

Angiogenesis of liver metastasis of the XPA-1-RFP human pancreatic cancer in the ND-GFP transgenic nude mice was

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visualized by dual-color fluorescence imaging. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing liver metastasis. The density of nascent blood vessels in the liver metastasis was readily quantitated by ND-GFP expression. Gemcitabine significantly decreased the mean nascent blood vessel density in the liver metastases [Amoh et al., 2006b, 2007].

We have also developed of a color-coded imaging model that can visualize the anastomosis between blood vessels of RFP-expressing vessels in vascularized Gelfoam® previously transplanted into RFP transgenic mice and then re-transplanted into ND-GFP mice. Anastomosis between the GFP-expressing nascent blood vessels of ND-GFP transgenic nude mice and RFP blood vessels in the Gelfoam® was imaged 14 and 21 days after re-transplantation [Uehara et al., 2013a].

The interaction between α_v integrin linked to GFP in osteosarcoma cells and blood vessels in Gelfoam®, vascularized after implantation in RFP transgenic nude mice, was previously observed. The 143B α_v integrin-GFP-expressing cells proliferated into the Gelfoam®, which contained RFP-expressing blood vessels. Strong expression of α_v integrin-GFP in 143B cells was observed near RFP vessels in the Gelfoam®, suggesting that α_v integrin was involved in this interaction [Uehara et al., 2013b].

In the present study, we implanted Gelfoam® into the ND-GFP transgenic nude mouse and generated vascularized Gelfoam®. Human 143B osteosarcoma cells, expressing RFP in the cytoplasm and GFP in the nucleus, were then injected into the vascularized Gelfoam® via a skin flap in the ND-GFP nude mice in order to observe the angiogenesis-stimulatory effect of osteosarcoma cells.

MATERIALS AND METHODS

CELLS

High lung-metastatic variant human osteosarcoma cells, 143B LM4, expressing GFP in the nucleus and RFP in the cytoplasm, were previously established [Tome et al., 2013]. The 143B cells were maintained in RPMI-1640 medium (Cellgro, Herndon, VA) with 10% fetal bovine serum (FBS) (Omega Scientific, San Diego, CA) and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2.

MICE

Female ND-GFP transgenic nude mice (AntiCancer, Inc., San Diego, CA) were used in this study. Mice were fed with an autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, CA). All animal studies were conducted in accordance with principles and procedures outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals under PHS Assurance Number A3873-01. Totally, 12 ND-GFP transgenic nude mice were used in this study.

IMPLANTATION OF GELFOAM®

Four-week-old ND-GFP-transgenic nude mice [Amoh et al., 2005a] were anesthetized with a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 20 μl H_2O) *via s.c.* injection. Gelfoam® (5 mm × 5 mm) (Pharmacia & Upjohn Company, Kalamazoo, MI) was treated with 300 ng basic fibroblast growth factor (βFGF; Millipore, Billerica, MA) in 75 μl RPMI-1640 medium. The treated Gelfoam® was then transplanted into the subcutis on the flank of ND-GFP transgenic nude mice [Amoh et al., 2007].

IMAGING GELFOAM® VASCULARIZATION

ND-GFP transgenic nude mice were anesthetized with the ketamine mixture *via s.c.* injection. An arc-shaped incision was made in the abdominal skin from the axillary to the inguinal region. The subcutaneous connective tissue was separated to free the skin flap without injuring the vessels [Yang et al., 2002]. Mice were laid flat and the skin flap was spread and fixed on the flat stand. Vascularized Gelfoam® was directly imaged with the OV100 Small Animal Imaging system (Olympus Corp., Tokyo, Japan) [Yamauchi et al., 2006] and the FV 1000 confocal microscope (Olympus) [Uchugonova et al., 2011] (please see below). The skin was closed with a 6-0 nylon suture after observation.

IMAGING OF ND-GFP VESSELS IN GELFOAM® WITH IMPLANTED DUAL-COLOR 143B CELLS

Seven days after Gelfoam® implantation, dual-color coded human 143B osteosarcoma cells (5×10^5) were injected with a 0.5 ml 28 G latex-free insulin syringe (TYCO Health Group LP, Mansfield, MA) into the Gelfoam® previously implanted in ND-GFP transgenic nude mice (n = 6). The control group mice had only implanted Gelfoam® (n = 6). Skin flaps were made at 7, 14, 21, and 28 days after Gelfoam® implantation, and the inside surface of the skin flap and Gelfoam® were directly imaged. The skin was closed with a 6-0 suture after each observation.

IMAGING

The Olympus OV100 Small-Animal Imaging System, containing an MT-20 light source (Olympus Biosystems) and DP70 charge-coupled device camera (Olympus), was used for imaging in live mice [Yamauchi et al., 2006]. High-resolution images were captured directly on a PC (Fujitsu Siemens). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and CellR (Olympus Biosystems).

An FV 1000 laser scanning confocal microscope (Olympus) with a XLUMPLFLN 20 × W (0.95 numerical aperture [NA]) water immersion objective was used for imaging [Uchugonova et al., 2011]. GFP was excited at 488 nm, and RFP was excited at 559 nm with an Argon laser. Images were produced with FV10-ASW Fluoview software (Olympus) and ImageJ (NIH, Bethesda, MD) and were not modified beyond the standard adjustment of intensity levels.

MEASUREMENT OF VESSEL LENGTH

The lengths of ND-GFP vessels were imaged with the Fluoview FV 1000 laser scanning confocal microscope. Vessel lengths were measured with FV10-ASW Fluoview software (Olympus). A random 10 fields were quantified in each group. The experimental data are expressed as the mean ± SD. Statistical analysis was done using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

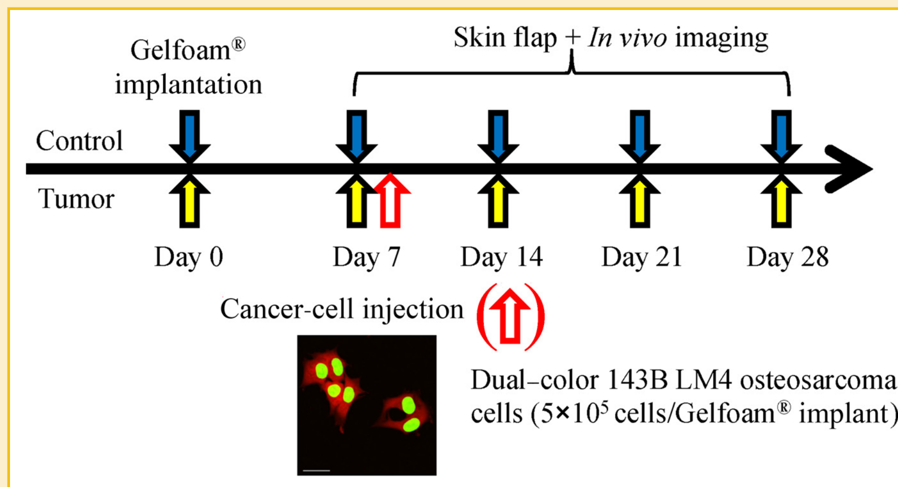

Fig. 1. Experimental schema for Gelfoam® implantation and cancer-cell injection. Gelfoam® (5 mm × 5 mm), impregnated with 300 ng β-fibroblast growth factor in 75 μl RPMI-1640 medium, was transplanted to the subcutis on the flank of nestin-driven green fluorescent protein (ND-GFP) transgenic nude mice. Skin flaps were made at day 7 after transplantation of Gelfoam® under ketamine anesthesia. Angiogenesis was observed by fluorescence in the Gelfoam® in the skin flap using the OV100 Small Animal Imaging System (Olympus Corp.) and confocal fluorescence microscopy with the FV1000 (Olympus Corp.). Dual-color coded 143B osteosarcoma cells were injected into the previously transplanted Gelfoam® in the ND-GFP nude mice (n = 6) on day 7 after Gelfoam® implantation. The control mice (n = 6) had only implanted Gelfoam®. Skin flaps were made days 14, 21, and 28, to observe tumor angiogenesis in the implanted Gelfoam®.

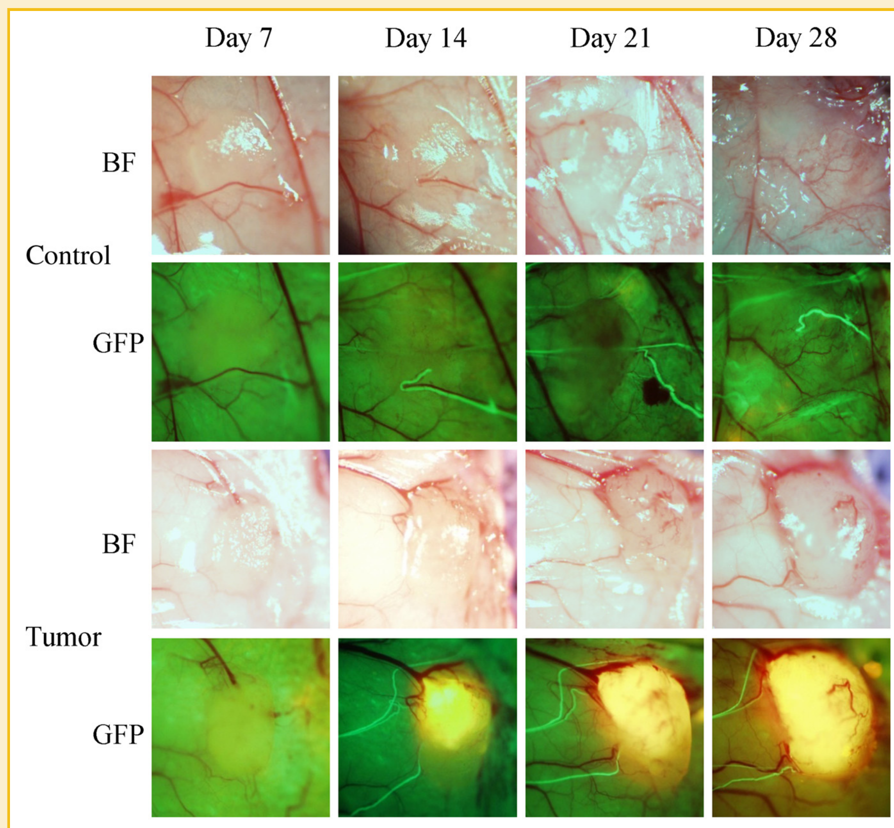

Fig. 2. Real-time color-coded imaging of nestin-driven GFP-expressing blood vessels and dual-color coded osteosarcoma cells implanted in Gelfoam® in ND-GFP mice. Skin flaps were made and observed with the OV100 in bright field (BF) and with GFP excitation (GFP) at days 7, 14, 21, and 28. In the "tumor" group, dual-color osteosarcoma cells were implanted on day-7 after Gelfoam® implantation. Fourteen days after Gelfoam® transplantation, ND-GFP expressing nascent blood vessels were growing in the transplanted Gelfoam® in each group. Osteosarcoma cells formed tumors in the Gelfoam® in a time-dependent manner. Nascent vessels express ND-GFP, which is extinguished as the vessels mature, such that mature vessels are no longer fluorescent.

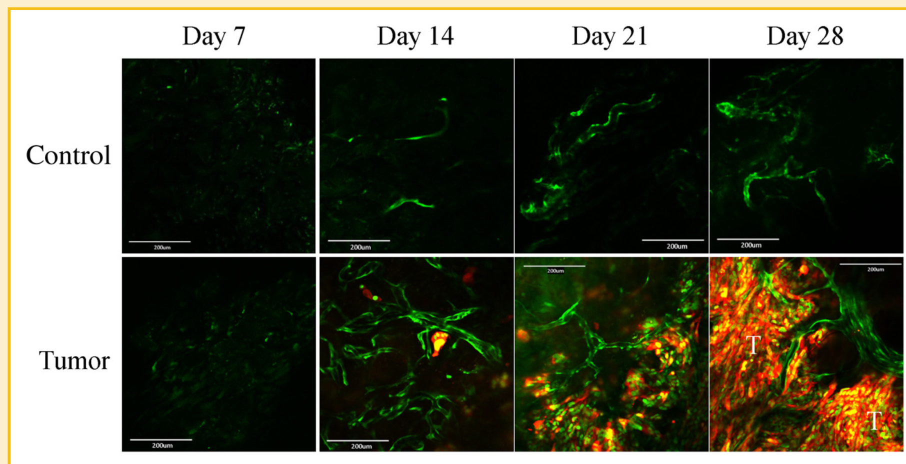

Fig. 3. Subcellular real-time confocal imaging of nestin-driven GFP-expressing blood vessels and dual-color osteosarcoma cells in Gelfoam® in ND-GFP mice. Skin flaps were made and observed by FV1000 confocal microscopy. Control and osteosarcoma implanted mice (Tumor) were observed at days 7, 14, 21, and 28. The dual-color osteosarcoma cells were implanted at day-7 after Gelfoam® implantation. ND-GFP expressing nascent blood vessels grew in the implanted Gelfoam® in a time-dependent manner. The osteosarcoma cells stimulated nascent vessel angiogenesis in the implanted Gelfoam®. ND-GFP expressing blood vessels appeared to be attracted by the osteosarcoma tumor and were more enlarged and longer than in the control Gelfoam®. Bars = 200 μm.

RESULTS AND DISCUSSION

REAL-TIME IN VIVO IMAGING OF OSTEOSARCOMA FORMATION IN GELFOAM® IMPLANTED IN ND-GFP TRANSGENIC NUDE MICE

To obtain angiogenesis of Gelfoam® in the ND-GFP nude mouse in vivo, Gelfoam® was treated with βFGF, and then transplanted into the subcutis on the flanks. Seven days after transplantation of Gelfoam®, dual-color-coded 143B osteosarcoma cells (Fig. 1) were injected in the transplanted Gelfoam®. The control mice were implanted with Gelfoam® only. Skin flaps were made on days 14, 21, and 28, and tumor angiogenesis was observed with the OV100 Small Animal Imaging System (Fig. 2). ND-GFP expressing nascent blood vessels vascularized the transplanted Gelfoam® in each group. The osteosarcoma cells formed tumors in the implanted Gelfoam® in a time-dependent manner (Fig. 2, lower panels).

OSTEOSARCOMA CELLS PROMOTED ANGIOGENESIS IN GELFOAM® IMPLANTATION IN ND-GFP TRANSGENIC NUDE MICE

Skin flaps were made in the ND-GFP mice and observed with an Olympus FV1000 confocal laser scanning microscope. The control mice and osteosarcoma-implanted mice were observed on days 7, 14, 21, and 28 after transplantation of the Gelfoam®. ND-GFP expressing nascent blood vessels vascularized the Gelfoam® in a time-dependent manner in each group (Fig. 3). ND-GFP expressing blood vessels in the mice with implanted osteosarcoma cells had larger and longer vessels than the control group (Fig. 3, right, lower panel). On day 14 after implantation of osteosarcoma cells (day 21 after implantation of the Gelfoam®), dual-color cancer cells were associated with ND-GFP expressing nascent blood vessels. Furthermore, on day 21 after implantation of osteosarcoma cells, ND-GFP expressing nascent blood vessels were visualized growing into the dual-color tumor.

Vessel length in the Gelfoam® was measured with FV10-ASW Fluoview software (Olympus). Nascent blood vessels grew in the Gelfoam® in a time-dependent manner. A random 10 fields were quantified in each group (Fig. 4). The mean length of ND-GFP expressing blood vessels in the Gelfoam® in mice with implanted osteosarcoma were 43.1, 81.1, and 88.8 mm/mm^2, at days 14, 21, and 28, respectively. The mean length of ND-GFP expressing blood vessels in the Gelfoam® in mice of the control group were 23.6, 43.2,

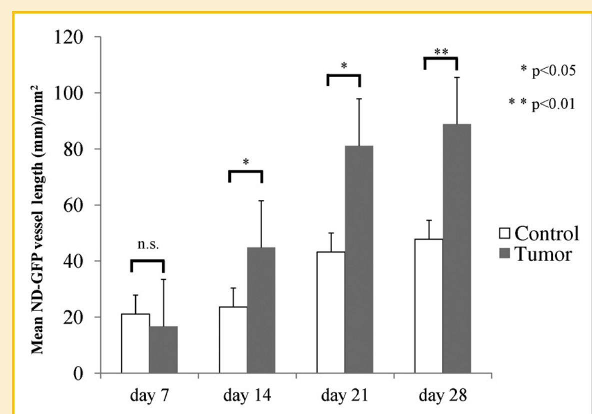

Fig. 4. Comparison of the mean length of ND-GFP expressing vessels in Gelfoam® with and without osteosarcoma cells. Vessel length in the Gelfoam® was measured with FV10-ASW Fluoview software (Olympus). A random 10 fields were quantified in each group in a time-dependent manner. The length of ND-GFP expressing vessels was stimulated by the osteosarcoma cells (at days 14 and 21: $P < 0.05$, at day 28: $P < 0.01$). The experimental data are expressed as the mean ± SD. Statistical analysis was performed using the Student's *t*-test.

and 47.8 mm/mm^2 on day 14, 21, and 28, respectively. Thus, the extent of nascent blood vessel growth was significantly increased by osteosarcoma cells (on days 14 and 21: $P < 0.05$, on day 28: $P < 0.01$).

The results presented in this report demonstrate the strong angiogenesis induction by osteosarcoma cells, readily measured in the in vivo Gelfoam® assay, and suggest this is a potential therapeutic target for this cancer [Folkman, 1971]. The color-coded in vivo Gelfoam® angiogenesis imaging assay is very convenient to study angiogenesis and its stimulation by cancer cells and to screen for novel antiangiogenic agents.

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