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● *Original Contribution*

TARGETED CONTRAST-ENHANCED ULTRASOUND IMAGING OF ANGIOGENESIS IN AN ORTHOTOPIC MOUSE TUMOR MODEL OF RENAL CARCINOMA

SHUPING WEI,* NINGHUA FU,* YU SUN,† ZHIJIAN YANG,† LI LEI,* PENGFEI HUANG,* and BIN YANG*
*Department of Ultrasound Diagnostics, Jinling Hospital, Nanjing University School of Medicine, Nanjing, Jiangsu, China; and
†Department of Pharmacological Study, Origin Biosciences, Nanjing, Jiangsu, China

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Abstract—Previous studies have reported that microbubbles bearing targeting ligands to molecular markers of angiogenesis can be successfully detected by ultrasound imaging in various animal models of solid cancer. In the present study, we sought to investigate the activity of microbubbles targeted to vascular endothelial growth factor receptor 2 (VEGFR2) in an orthotopic model of renal cell carcinoma (RCC). Microbubbles conjugated to an anti-VEGFR2 antibody (MB_V) were compared with microbubbles conjugated to an isotype control antibody (MB_C) or naked microbubbles (MB_N). An orthotopic mouse model of human RCC was established by surgically implanting an established tumor within the renal capsule in mice. Tumor growth and blood flow were verified by B-mode and color Doppler ultrasound imaging. VEGFR2 expression within the tumor and renal parenchyma was detected by immunohistochemistry. The duration of contrast enhancement of MB_V was much longer than those of MB_N and MB_C when assessed over 10 min. The baseline-subtracted contrast intensity within the tumor was higher for MB_V than for MB_C and MB_N ($p < 0.01$). Additionally, the contrast intensity for MB_V was significantly higher in the tumor region than in normal parenchyma ($p < 0.01$). Microbubbles targeting VEGFR2 exhibit suitable properties for imaging angiogenesis in orthotopic models of renal cell carcinoma, with potential applications in life science research and clinical medicine. (E-mail: yb12yx@hotmail.com) © **2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Targeted ultrasound, Microbubbles, Contrast agent, Molecular imaging, Contrast pulse sequence, Orthotopic, Renal clear cell carcinoma, Nude mouse, Angiogenesis, Vascular endothelial growth factor receptor 2.**

INTRODUCTION

It is widely accepted that angiogenesis, the process of new blood vessel formation and recruitment, is critically involved in the early progression and metastatic potential of many types of cancer (Carmeliet 2005; Hicklin and Ellis 2005). Various specific molecular markers of angiogenesis have been identified that are overexpressed on tumor endothelial cells, including vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR2 is a receptor tyrosine kinase that mediates most of the pro-angiogenic activity of VEGF (Hicklin and Ellis 2005; Shibuya 2006; Vajkoczy et al. 2002). VEGFR2 expression within the tumor vasculature has been linked to the progression and aggressiveness of many types of tumors, including renal cell carcinoma (RCC) (Hicklin and Ellis 2005; Rini et al. 2011).

Renal cell carcinoma accounts for 2%–3% of all adult malignancies and is the third most frequent urologic malignancy after prostate and bladder cancer (American Cancer Society 2008; Rini et al. 2009). Anti-angiogenic drugs targeting the VEGF/VEGFR2 pathway have found particular utility in advanced RCC; these include inhibitors of VEGF receptor such as sorafenib and sunitinib (Escudier et al. 2007; Motzer et al. 2007; Terakawa et al. 2011). However, individual response rates vary among patients, and patient selection to identify responders before implementing treatment is a critical unmet need. Additionally, non-invasive and reproducible methods to assess response to therapy are needed. Finally, quantitative tools for assessing tumor function and phenotype are becoming increasingly important in the context of basic science and the drug discovery process. We

Address correspondence to: Bin Yang, Department of Ultrasound Diagnostics, Jinling Hospital, Nanjing University School of Medicine, 305 East Zhongshan Road, Nanjing, Jiangsu 210002, China. E-mail: yb12yx@hotmail.com

hypothesize that ultrasound-based molecular imaging may be useful for each of these applications.

Molecular imaging technology has the potential to detect molecular transformations in various lesions non-invasively and in advance of traditional biopsy-based methods. With the development of molecular imaging, contrast-enhanced ultrasound (CEUS) with microbubbles targeting specific molecular markers is emerging as a uniquely effective imaging modality because of its tolerability, low cost, portability and ability to perform real-time anatomic imaging (Klibanov 2006). By conjugation of targeting ligands (such as antibodies, peptides or small molecules) to its surface, the microbubble can be made to target a desired molecular marker of disease. Subsequent ultrasound imaging enables quantitative detection of the target-bound microbubbles. This is inherently a "late-phase" technique, in which scanning occurs 5–15 min after microbubble administration to ensure that circulating (non-target bound) microbubbles have cleared the imaged tissue. Microbubbles are purely intravascular agents, which makes them uniquely suited for detecting and tracking biological processes on vascular endothelial cells, such as tumor angiogenesis. As a key player in the physiology of angiogenesis and as a drug target, VEGFR2 is a potentially powerful target for molecular imaging.

This feasibility of targeting VEGFR2 with targeted microbubbles has been reported in numerous preclinical studies in mice and rats (Anderson et al. 2010, 2011; Rychak et al. 2007; Willmann et al. 2008, 2010). However, to translate this technology into routine use, further information regarding its behavior must be obtained. In the present study, we have chosen to evaluate a commercially available VEGFR2-targeted agent in an orthotopic model of renal cell carcinoma. This allowed us to evaluate whether VEGFR2 is a suitable imaging target in a model that closely mimics naturally occurring RCC. The expression patterns, growth characteristics, vascular arrangement and geometry of orthotopically grown tumors are known to differ substantially from those of subcutaneous models, and it is important to establish that VEGFR2- targeted microbubbles can bind to these tumors in quantities sufficient for imaging. Additionally, locating a tumor mass within a highly vascularized organ such as the kidney presents some degree of technical challenge. Finally, the time course of microbubble wash-in and uptake is unknown. Our goal in this study was to evaluate the time course and specificity of VEGFR2-targeted microbubbles in an orthotopic model of RCC using non-invasive ultrasound molecular imaging.

METHODS

Animal and cancer cell line

Fifteen 6-wk-old male BALB/C nude mice weighing 18–25g were purchased from the Beijing Kelihua laboratory animal center (Beijing, China). Animals were maintained in a HEPA-filtered environment, where the room temperature was 24°–25°C and the humidity was 50%–60%. The animals were fed an autoclaved laboratory rodent diet. The protocol for animal experiments was approved by the institutional animal care and use committee at our institution. Human renal clear cell carcinoma cells (line 786-0) were purchased from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The cells were maintained in RPMI 1640 (GIBCO Life Technologies, New York, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) and incubated at 37°C in 5% CO_2.

Orthotopic mouse tumor model

We harvested the 786-0 cells at 80% confluence by digestion with 0.25% trypsin solution, washed them twice with phosphate buffered saline and resuspended them at a concentration of 5×10^7/mL Approximately 5×10^6 cells (0.1 mL) of the cell suspension were injected subcutaneously into the right flank region of each nude mouse. Tumors grown in nude mice were harvested at the exponential growth phase and resected under aseptic conditions. Necrotic tissues were removed and viable tissues were cut with scissors and minced into 1-mm^3 pieces. For surgical orthotopic implantation, animals were anesthetized by injection of 0.02 mL of solution of 50% ketamine, 38% xylazine and 12% acepromazine maleate. A 1-cm vertical incision was made in the skin above the right kidney region using surgical scissors. The kidney was exposed and a small cut made on the renal subcapsule. One piece of tumor fragment was inserted into the capsule, and the cut was then covered with surrounding soft tissue using an 8-O sutures. The kidney was then returned to the peritoneal cavity, and the incision was closed in layers using sterile 5-O surgical sutures. All surgical procedures and animal manipulations were conducted under aseptic conditions in an HEPA-filtered laminar-flow hood with a × 8 surgical microscope (YZ20P5, Shanghai, China).

Targeted contrast agent preparation

Streptavidin-coated microbubbles (Targestar-SA) were obtained from Targeson (San Diego, CA, USA; distributed by Origin Biosciences in China). Biotinylated rat anti-mouse VEGFR2 monoclonal antibody (clone Avas12a1, 13-5821, eBioscience, San Diego, CA, USA) was used as the targeting ligand (Rychak et al. 2007). Biotinylated isotype-matched rat control immunoglobulin G (IgG) antibody (13-4321, eBioscience) was used as a specificity control. Targestar-SA agents are streptavidin-coated microbubbles composed of a perfluorocarbon gas core encapsulated by a lipid shell. The outer