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Original article

Tumor angiogenesis of SCLC inhibited by decreased expression of FMOD via downregulating angiogenic factors of endothelial cells

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ABSTRACT

Fibromodulin (FMOD), an ECM small leucine-rich proteoglycan (SLRP), was reported to promote angiogenesis not only during wound healing, but also in optical and cutaneous angiogenesis-dependent diseases. However, whether it plays important roles in tumor angiogenesis remains unclear. To explore the role of FMOD in tumor angiogenesis of human small cell lung cancer (SCLC), initially the study analyzed the relationship of FMOD expression in cancer tissues of SCLC with clinical characteristics. The analysis revealed that the positive FMOD expression was significantly associated with extensive stage of SCLC and higher vascular density. In mouse models, xenograft tumors developed with FMOD-silenced H446 cells (H446-shFMOD) exhibited slowed growth rate, decreased microvessel density, and reduced blood perfusion related to that of controls (H446-shCON). Additionally, compared with that of controls, the decreased secretion of FMOD in conditioned medium (CM) from H446-shFMOD inhibited proliferation, migration, and invasion of human umbilical vessel endothelial cells (HUVECs). Moreover, the decreased secretion of FMOD downregulated the expression of VEGF, TGF-β1, FGF-2, and PDGF-B in HUVECs. The findings strongly suggested that the autocrine FMOD of cancer cells may promote tumor angiogenesis of SCLC by upregulating the expression of angiogenic factors that act in concert to facilitate the angiogenic phenotype of endothelial cells as a proangiogenic factor. Therefore, silencing FMOD may be a potentially clinical therapy for repressing tumor angiogenesis.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths with high incidence and mortality worldwide [1]. Small cell lung cancer (SCLC) accounts for approximately 15% of all lung cancer cases [2]. The prognosis of SCLC is highly poor, largely due to its striking aggressiveness and limited effectiveness of current therapeutic regimens [3]. Hence, it is urgent to identify novel therapeutic targets for improvement of therapeutic efficacy and prognosis. Since Judah Folkman reported that tumor growth was angiogenesis-dependent [4] and tumor angiogenesis, the growth of new capillaries from pre-existing pool of blood vessels, plays a pivotal role in tumor growth and metastasis, targeting the tumor vasculature has become a promising clinical application for anticancer therapy [4]. However, currently although a number of antiangiogenic agents, like Bevacizumab which acts on the vascular endothelial growth factor (VEGF) axis, were proved to be effective in treating mouse tumors by arresting tumor growth [5,6], the clinical practices have not been translated successfully [7]. Furthermore, therapeutic resistance and escape, caused by a selection pressure of VEGF therapy to tumor cell population and upregulation of alternative angiogenic pathways such as bFGF and IL-8, have become another conundrum in the clinic [8]. The formation and maintenance of tumor blood vessels involve intercellular signaling between malignant and endothelial cells (ECs). Cancer cells can aberrantly express bioactive molecules, which drive ECs to secrete cytokines, growth factors, i.e., angiopoietin (ANGPT), VEGF, and fibroblast growth factor (FGF) that act in an autocrine, paracrine or juxtacrine manner to facilitate angiogenesis [9]. Therefore, to find the other potential upstream

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regulator of tumor angiogenesis which is endogenous of tumor cells may provide us new targets to overcome the conundrum.

Mounting evidences revealed that interactions between the extracellular matrix (ECM) and ECs contribute to tumor angiogenesis. In addition to providing structural support for blood vessels, the ECM serves as a reservoir for angiogenic growth factors and proteases, transduces signals via integrins in ECs, and regulates the angiogenic phenotype of ECs [10]. Fibromodulin (FMOD), an ECM protein, is a member of the small leucine-rich proteoglycan (SLRP) family. The SLRPs encompass 18 members which are subdivided into five distinct classes based on N-terminal cysteine-rich clusters with specific spacing, C-terminal cysteine-rich capping motif, and chromosomal organization [11]. FMOD belongs to the class II of the SLRPs and is mainly present in connective tissues such as skin, tendon, cartilage, and sclera [12]. Initially, FMOD was reported to play important roles in modulating ECM organization by interacting with collagen I and II [13,14]. The following studies demonstrated that FMOD not only contributed to regulate fluid balance and interstitial pressure [15], acted as a novel tumor-associated antigen (TAA) in CLL [16], but also was able to reprogramme human fibroblasts into multipotent cells [17]. Recently FMOD has been implicated to function in blood vessel regeneration in granulation tissues during wound healing, and in optical and cutaneous angiogenesis-dependent diseases [18,19]. Interestingly, FMOD was not detected in normal lung tissues and mammary tissues [20–22], while its aberrant expression was examined in lung, breast, and prostate carcinomas at the mRNA levels [21,23,24]. Regretfully, these studies did not explore the role of the abnormally expressed FMOD in these carcinomas.

Since lung cancer is one of angiogenesis-dependent diseases, the detection of FMOD in the tissues of lung cancer gave us clue that FMOD may contribute to the tumor angiogenesis. To test the hypothesis, the relationship of FMOD expression in cancer tissues of SCLC and xenograft tumors of SCID mice with the microvessel density was analyzed. The comparison displayed that higher FMOD expression was significantly associated with increased microvessel density, blood perfusion, and tumor growth. In addition, compared with controls, the decreased secretion of FMOD in H446-shFMOD cells inhibited proliferation, migration, and invasion of HUVECs via downregulating several key angiogenic factors including VEGF, TGF-β1, FGF-2, and PDGF-B, strongly suggesting that FMOD acted as a proangiogenic factor in the tumor angiogenesis.

2. Materials and methods

2.1. Clinical materials

Tumor tissues (n = 20) from SCLC patients who underwent transthoracic needle biopsy were collected between February 2013 and August 2014 at 324th Hospital of the People's Liberation Army. All diagnoses were based on pathological and/or cytological evidence according to 2004 World Health Organization (WHO) classification. Pathologically normal lung tissues (n = 12) were obtained from Department of Pathology of Xinqiao Hospital as control. Clinicopathological information was obtained from patient records. Five-micrometer sections of paraffin-embedded, formalin-fixed specimens were prepared for immunohistochemical staining. After explaining the purpose and procedures of the study, all of the participants signed a written informed consent form. The study was approved by the Ethics Committee of Xinqiao Hospital, the Third Military Medical University.

2.2. Immunohistochemistry and quantification of microvessel density

Immunohistochemical (IHC) staining for FMOD and CD31 was carried out on sections of SCLC specimens from patients and xenograft tumors from SCID mice. IHC was performed as described as Mehta et al. [25]. The primary antibodies and dilutions were as follows: polyclonal rabbit anti-FMOD antibody (1:50, Abcam, USA), polyclonal goat anti-mouse CD31 antibody (1:100, R&D Systems, USA), and monoclonal mouse anti-human CD31 antibody (Maxim, China). Negative controls were incubated with a non-specific mouse, goat, or rabbit IgG. Microvessel density (MVD) was evaluated by counting CD31-positive immunostained ECs and that were in clusters. Three highly vascular regions were identified as "hot spots" and counted at 200× or 400× magnification. The average number of microvessels was recorded and the mean value was used for statistical analysis. Negative controls were incubated with non-immune sera.

2.3. Cell culture, conditioned medium(CM), and transfection

The human SCLC cell line H446 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA, https://www.atcc.org/Products/All/HTB-171.aspx) and cultured in RPMI1640 (Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, USA). The human umbilical vascular endothelial cells (HUVECs) were kindly gifted by Cardiovascular Laboratory of Xinqiao Hospital, and cultured in DMEM (Hyclone, Thermo Scientific, USA) supplied with 10% FBS. All cells were incubated in 5% CO_2 at 37 °C. The lentivirus-mediated short hairpin RNA (shRNA) was purchased from GenePharma Co. Ltd. (Shanghai, China). The pGLV/H1/GFP + Puro lentivirus vector, constructed with green fluorescent protein (GFP) for detection, was used as the expression system. The recombinant vector (LV3-shFMOD) contained a FMOD-targeting shRNA with the sequence 5′-CCGCATGAAGTACGTCTACTT-3′ [26]. The negative control vector (LV3-shCON) contained a nonsense shRNA insert with the sequence 5′-TTCTCCGAACGTGTCACGT-3′. The viral titre was 1×10^8 TU/mL for both LV3-shFMOD and LV3-shCON. Stable transfected cells with shRNA against FMOD (shFMOD) or control (shCON) were selected using 1 μg/μL puromycin. For conditional medium (CM) preparation, equal numbers of non-transfected H446 cells (H446), LV3-shFMOD-transfected H446 cells (H446-shFMOD), LV3-shCON-transfected H446 cells (H446-shCON) were cultured in RPMI-1640 medium with 10% FBS. After 24 h, the culture medium was replaced with DMEM. After 48 h, CM was collected, centrifuged, concentrated (20 μg/μL), filtered through a 0.22 μm filter (Millipore, Billerica, USA), and stored at −80 °C for future use.

2.4. Mouse models and contrast-enhanced ultrasound

Four to five-week-old female SCID mice (n = 12) were divided into two groups which were inoculated subcutaneously with 1×10^7 H446-shFMOD cells and H446-shCON cells into the right back, respectively. Tumors were monitored on the day of tumor formation and at six to seven day intervals for a total of 28 days. Tumor size was recorded and tumor volume (mm^3) was calculated according to the formula $a \times b^2/2$ (a = largest diameter; b = smallest diameter) [27]. After 28 days, the contrast-enhanced ultrasound (CEUS) imaging using lipid-coated microbubbles was performed as previously described with alterations [28]. Briefly, the survival animals were anesthetized by an intraperitoneal injection of 2% pentobarbital sodium at 25 mg/kg 0.01 mL lipid-coated microbubbles (a gift from Department of Ultrasound, Xinqiao Hospital) were injected into the caudal vein of each mouse followed by a 500 μL saline flush. Vevo®2100 high-resolution ultrasound imaging system (VisualSonics, Canada) equipped with a MS-250 probe (18 MHz) was used for CEUS. The CEUS images were analyzed in the form of internal time-intensity curve by GE Logiq 9 system to record the peak time. Then the CEUS images at