Original Article

VEGF silencing inhibits human osteosarcoma angiogenesis and promotes cell apoptosis via PI3K/AKT signaling pathway

Jian Zhao¹, Zi-Ru Zhang¹, Na Zhao², Bao-An Ma¹, Qing-Yu Fan¹

¹Department of Orthopedic Surgery, Orthopedics Oncology Institute of Chinese PLA, Tangdu Hospital, Fourth Military Medical University, Xi’an 710038, Shaanxi Province, P. R. China; ²Department of Outpatient, Tangdu Hospital, Fourth Military Medical University, Xi’an 710038, Shaanxi Province, P. R. China

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Abstract: Vascular endothelial growth factor (VEGF) is one of the most potently angiogenic factors which promotes generation of tumor vasculature. VEGF is usually up-regulated in multiple cancers include osteosarcoma and gliomas. To further explore the potential molecular mechanism that inhibits tumor growth induced by interference of VEGF expression, we constructed an Lv-shVEGF vector and assessed the efficiency of VEGF silencing and its influence on U2OS cells. Our data demonstrated that Lv-shVEGF has high inhibition efficiency on VEGF expression, which inhibits proliferation and promotes apoptosis of U2OS cells in vitro. Our results also indicated that inhibition of VEGF expression suppresses osteosarcoma tumor growth in vivo, VEGF inhibition reduces osteosarcoma angiogenesis.

We also found that the phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) activation was considerably reduced after osteosarcoma cells were treated with Lv-shVEGF. Taken together, our data demonstrated that VEGF silencing suppresses cells proliferation, promotes cells apoptosis and reduces osteosarcoma angiogenesis through inactivation of PI3K/AKT signaling pathway.

Keywords: Cell apoptosis, angiogenesis, lentivirus-mediated short hairpin RNA (Lv-shRNA), U2OS cells, vascular endothelial growth factor (VEGF)

Introduction

Osteosarcoma is one of the most common malignant bone tumor in adolescents, and tumor angiogenesis is closely related to the cancer cells growth, invasion and metastasis [1-3]. In this process, tumor cells release proteolytic enzymes to degrade the basement of membrane and directly contact with the blood circulation [4]. Tumor growth depends on angiogenesis, which is regulated by the interactions between numerous regulatory factors, such as VEGF, Tie and Eph [5, 6]. Particularly, VEGF has a key role in the tumor angiogenesis [4, 7].

As an important regulator of tumor angiogenesis, VEGF directly promotes angiogenesis, vascular remodeling and increases vascular permeability, which lead to the formation of the tumor stroma [8]. VEGF also promotes tumor growth and metastasis via interaction with metal protease [9]. Human VEGF gene is located on chromosome 6p21.3, with the size of 14 kb, and contains eight exons and seven introns [10]. VEGF can stimulate vascular endothelial cells to produce and release nitric oxide (NO) in a dose-dependent manner in human and rabbit, which plays an important role in blood vessels [11, 12].

Previous studies have shown that VEGF is up-regulated in multiple cancers. Expression of VEGF indirectly provides the necessary oxygen and nutrients for tumor growth, which promotes tumor invasion and metastasis [13]. Many studies revealed that VEGF protein is up-regulated in liver cancer, pancreatic cancer, osteosarcoma, lymphoma and other malignancies, and is positively correlated with poor prognosis [14-17]. In this study, we explored the effect of VEGF inhibition on osteosarcoma angiogenesis in a Wistar rat osteosarcoma model. We inhibited the expression of VEGF in U2OS cells and
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measured the growth of osteosarcoma cells. We found phosphorylation of PI3K and AKT was significantly reduced in U2OS cells after silencing of VEGF. Our data revealed the relationship between VEGF inhibition, osteosarcoma angiogenesis and tumor growth.

Materials and methods

Cell culture

Human osteosarcoma cell line U2OS was purchased from American Type Culture Collection (ATCC). U2OS cells were cultured in RPMI-1640 medium supplemented with 10% heated-inactivation FBS (Life Technologies), 100 mg/ml of streptomycin sulfate and 100 U/ml of penicillin sodium (Life Technologies).

Lentivirus infection

U2OS cells were seeded onto 6-well plates with 2x10^5 cells per well. After 12 h, U2OS cells cultured in RPMI-1640 medium containing 2% FBS and 8 μg/ml polybrene were infected with Lv-shscramble or Lv-shVEGF at 10 MOI, respectively. Virus-containing culture medium was changed with fresh RPMI-1640 medium 12 h postinfection.

Establishment of rat model

Wistar rats were purchased from the Animal Center of Chinese Academy of Medical Sciences. The animals manipulate was performed under the approved protocol. The Wistar rats were randomly divided into three groups with 6 rats in each group. Osteosarcoma U2OS cells were infected with Lv-shscramble and Lv-shVEGF, respectively. Different amount (1000, 10,000 and 100,000) of untreated, Lv-shscramble treated and Lv-shVEGF treated U2OS cells were suspended in 100 μl of PBS and subcutaneously injected into the left hind legs of rats. Rats injected with untreated U2OS cells were used as control. Tumor volume and weight were measured. Tumor volume was calculated according to the formula ab²/2, where ‘a’ is the length and ‘b’ is the width of a tumor [25].

Quantification of intratumoral MVD

10% formalin-fixed and paraffin-embedded tumor tissues were cut into 4 μM sections. Sections were de-paraffinized in xylene and rehydrated in ethanol. Antigen retrieval was then performed and slides were incubated with anti-CD31 primary antibody (Abcam) overnight at 4°C in a humidified chamber. DAB was used for staining. The slides were also stained with haematoxylin. Tumor angiogenesis was evaluated by a blinded operator.

Cell proliferation assay

The cell proliferative potential was evaluated with Cell Counting Kit-8 assay (Tongren) according to the manufacturer’s instruction. Briefly, 1x10^5 cells were infected with lentivirus Lv-shscramble or Lv-shVEGF respectively. After 1-7 days postinfection, 10 μl of CCK-8 per well was added to each plate and the cells were cultured 2 h. The absorbance at 450 nm was read with multi-well spectrophotometer (Bio-Rad).

RT-PCR and real-time PCR

Total RNA was extracted using RNAiso kit (TaKaRa Bio,) according to the manufacturer’s instruction. RT-PCR and real-time PCR were performed for detection of VEGF expression.
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β-actin was used as a control for normalization.

**Western blotting**

Total proteins were extracted from the U2OS cells with RIPA buffer (Thermo Fisher Sci.). Proteins were separated by SDS-PAGE on 10% gel. Primary antibodies for western blotting were rabbit anti-VEGF (Cell Signaling), anti-p-PI3K and anti-p-AKT (Santa Cruz Biotech.), anti-PI3K (Abcam), anti-AKT (Cell Signaling) and anti-GAPDH (Santa Cruz Biotech.) antibodies.

In order to investigate the role of VEGF in osteosarcoma tumorigenesis, we firstly studied the biological effect of VEGF silencing in osteosarcoma cell line U2OS. We constructed a lentiviral shRNA vector Lv-VEGF shRNA (abbreviated as Lv-shVEGF) to inhibit expression of VEGF. The knockdown efficiency was measured by RT-PCR and western blotting. The data indicate that expression of VEGF mRNA (Figure 1A) and protein (Figure 1B) were markedly reduced in the Lv-shVEGF treated group, compared with the control group or the Lv-shscramble treated group.

**Results**

In order to investigate the role of VEGF in osteosarcoma tumorigenesis, we firstly studied the biological effect of VEGF silencing in osteosarcoma cell line U2OS. We constructed a lentiviral shRNA vector Lv-VEGF shRNA (abbreviated as Lv-shVEGF) to inhibit expression of VEGF. The knockdown efficiency was measured by RT-PCR and western blotting. The data indicate that expression of VEGF mRNA (Figure 1A) and protein (Figure 1B) were markedly reduced in the Lv-shVEGF treated group, compared with the control group or the Lv-shscramble treated group.

**Cell apoptosis measurement with flow cytometry**

U2OS cells were treated with Lv-shscramble or Lv-shVEGF. After 24 h treatment, 1×10⁶ cells were harvested and double-stained with fluorescein APC-labeled annexin V and propidium iodide (PI, Becton Dickinson). The percentage of apoptotic cells was then measured with flow cytometry (Becton Dickinson).

**Statistical analysis**

All results were presented as mean ± SD. Statistical significance was determined using t-test or analysis of variance (ANOVA) with SPSS15.0 program. *P* < 0.05 was considered as statistically significant.

**Effect of silencing of VEGF by lentiviral vector-mediated shRNA knockdown in U2OS cells**

![Figure 1](image1.png)

**Figure 1.** VEGF silencing inhibits proliferation of U2OS cells in Wistar rats. (A) Tumor volume during tumor formation, **P** < 0.01, ***P** < 0.001. (B and C) Quantification of tumor weight (B) and volume (C) in each group. (D) Tumorigenicity in Wistar rats.

![Figure 2](image2.png)

**Figure 2.** VEGF silencing inhibits proliferation of U2OS cells in Wistar rats. (A) Tumor volume during tumor formation. **P** < 0.01, ***P** < 0.001. (B and C) Quantification of tumor weight (B) and volume (C) in each group. (D) Tumorigenicity in Wistar rats.

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To further investigate the effect of VEGF silencing in osteosarcoma cells in vivo, U2OS cells were used to generate a xenograft osteosarcoma model in Wistar rats. U2OS were infected with Lv-shscramble and Lv-shVEGF, respectively, then subcutaneously injected into the left hind legs of the rats. When the subcutaneous tumors reached approximately 0.5 cm in length, the tumor volume and tumor weight were measured. As shown in Figure 2A, silencing of VEGF significantly inhibited the growth of tumor in the rats. The tumor volume and weight were significantly lower in the Lv-shVEGF group at day 15 compared with the untreated group and the Lv-shscramble group (Figure 2B and 2C). The results also indicated that tumorigenesis of U2OS cells infected with Lv-shVEGF was decreased compared with the untreated group and the Lv-shscramble group (Figure 2D).

Knockdown of VEGF effectively inhibits angiogenesis in xenograft osteosarcoma model

We next studied the effect of VEGF knockdown on osteosarcoma angiogenesis. As shown in Figure 3A, Lv-shVEGF treatment decreased the counts of intratumoral MVD compared with the Lv-shscramble group and the untreated group ($P < 0.001$), which confirmed that angiogenesis was significantly inhibited in the Lv-shVEGF group. Since VEGF is one of the most effective pro-angiogenic growth factors, we measured expression of VEGF mRNA and protein in the rats. The data showed that expression of VEGF mRNA and protein was markedly decreased in the Lv-shVEGF group (Figure 3B and 3C). In summary, these results indicate that knockdown of VEGF in U2OS cells reduce angiogenesis and generate anti-tumor effects.

Knockdown of VEGF inhibits U2OS cells proliferation

To verify that VEGF has similar anti-tumor effects on U2OS cells in vitro, we treated U2OS cells with Lv-shVEGF and applied CCK-8 assay to detect U2OS cells proliferation. As shown in Figure 4, the proliferative potential of U2OS cells was inhibited markedly in the Lv-shVEGF group compared with the untreated group and the Lv-shscramble group. The in vitro data are consistent with our in vivo results, and supports the hypothesis that VEGF plays an important role in human osteosarcoma cells through inhibition of cell proliferation.

Knockdown of VEGF promotes U2OS cells apoptosis

To further investigate whether VEGF plays a crucial role in human osteosarcoma cells through induction of cell apoptosis, we stained the cells with APC-AnnexinV/PI and measured cell apoptotic rates by flow cytometry. The
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results showed that the apoptotic rate of U2OS cells after infected with Lv-shVEGF was markedly increased compared with the untreated group and the Lv-shScramble group (Figure 5). The data suggest that VEGF silencing can suppress osteosarcoma cell survival by induction of cell apoptosis.

Knockdown of VEGF inhibits PI3K/AKT signaling pathway in U2OS cells

PI3K/AKT signaling pathway is one of the main downstream of VEGF, which is involved in many cellular biological processes. In addition, many studies support that VEGF/PI3K/AKT signaling pathway plays a critical role in tumor development and progression. We therefore determined expression of VEGF in U2OS cells after Lv-shVEGF treated. A representative data is shown in Figure 1B. Then the total PI3K, total AKT, the activated PI3K and AKT were analyzed with western blotting in U2OS cells. The data suggest that Lv-shVEGF treatment did not influence expression of PI3K or AKT, in contrast, the activated PI3K and AKT were dramatically reduced in the U2OS cells (Figure 6). Taken together, our data suggest that the effects of VEGF silencing on tumor angiogenesis and growth is correlated with down-regulation of PI3K/AKT signaling pathway.

Discussion

Osteosarcoma is one of highly malignant solid tumors, which is derived from mesenchymal tissues [18]. It is common in children and adolescents and 50% osteosarcoma patients have lung metastasis within a few months [19]. At present, the most effective clinical treatment strategy is still operation resection. Sometimes, it The operation sometimes is combined with chemotherapy, but patients are prone to relapse and have drug resistance [20].

RNA interference (RNAi) is one of the most commonly used gene therapy methods with high specificity and efficiency [21]. VEGF is a crucial factor that promotes tumor angiogenesis. Since it had been reported that angiogenesis is an efficient target for cancer therapy, many studies focused on treating malignant tumors by down-regulation of VEGF using RNAi [2, 21-23]. In order to identify potential therapy for osteosarcoma, we constructed Lv-shVEGF vector which reduces the mRNA and protein expression of VEGF both in vitro and in vivo. Our data showed that osteosarcoma tumor was significantly inhibited in the rat model when treated with Lv-shVEGF. Moreover, MVD of the osteo-

Figure 4. VEGF silencing inhibits proliferation of U2OS cells. It was shown that the proliferation of U2OS cells was measured with CCK-8 assay. Untreated group cells were used for normalization. ***P < 0.001.

Figure 5. VEGF silencing promotes U2OS cells apoptosis. The data showed that the apoptotic rates of U2OS cells are shown. Untreated group cells were used for normalization. **P < 0.01.
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sarcoma in the Lv-shVEGF group was remarkably reduced compared with the control group, which suggest that malignancy of osteosarcoma can be reduced by Lv-shVEGF treatment. Our data also showed that tumor angiogenesis was obviously inhibited by down-regulation of VEGF, which may lead to tumor hunger, suppressed tumor growth, reduced tumor invasion and metastasis, and eventually leads to complete treatment of osteosarcoma.

It has been reported that malignantly solid tumor cells secrete VEGF to promote formation of vascular system [24]. Our results suggest that the MVD of osteosarcoma had a positive correlation with expression of VEGF in the untreated group and the Lv-shScramble group, which consistent with prior research. In contrast, the MVD of osteosarcoma was significantly decreased in the Lv-shVEGF group. The data implied that VEGF is a key factor in the process of tumor angiogenesis and indicates that VEGF expression may be negatively related to the prognosis of osteosarcoma. Therefore, specific inhibition of VEGF may provide us an appropriate method to improve the prognosis of osteosarcoma.

In order to explore the molecular mechanism of suppressed osteosarcoma through down-regulation of VEGF, we studied the effect of VEGF silencing on PI3K/AKT signaling pathway. The results suggest that along with clearly down-regulation of VEGF, the levels of p-PI3K and p-AKT was subsequently decreased in the U2OS cells infected with Lv-shVEGF, which suggest that VEGF may affect occurrence and development of osteosarcoma by regulating PI3K/AKT signaling pathway [25, 26]. Taken together, these results may provide a basis for developing a therapeutic strategy that targets osteosarcoma angiogenesis.

Disclosure of conflict of interest
None.

Address correspondence to: Dr. Qing-Yu Fan, Department of Orthopedic Surgery, Orthopedics Oncology Institute of Chinese PLA, Tangdu Hospital, Fourth Military Medical University, 569 Xinsi Road, Xi’an 710038, Shaanxi Province, P. R. China. Tel: +86 29-84777639; Fax: +86 29-84777639; E-mail: bonetm@fmmu.edu.cn

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