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VEGF111b, a new member of VEGFxxxb isoforms and induced by mitomycin C, inhibits angiogenesis

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ABSTRACT

Vascular endothelial growth factor (VEGF-A) stimulating angiogenesis is required for tumor growth and progression. The conventional VEGF-A isoforms have been considered as pro-angiogenic factors. Another family of VEGF-A isoforms generated by alternative splicing, termed VEGFxxxb isoforms, has anti-angiogenic property, exemplified by VEGF165b. Here, we identify a new number of VEGFxxx family-VEGF111b induced by mitomycin C, although not detected in mitomycin C-unexposed ovarian cancer cells. SKOV3 cells were transfected with pcDNA$_{3.1}$ empty vector, pcDNA$_{3.1}$-VEGF111b or pcDNA$_{3.1}$-VEGF165b to collect conditioned mediums respectively. VEGF111b overexpression inhibits proliferation, migration and tube formation of endothelial cell by inhibiting VEGF-R2 phosphorylation and its downstream signaling, similar to VEGF165b but slightly lower than VEGF165b. The anti-angiogenic property depends on the six amino acids of exon 8b of the VEGFxxxb isoforms. Our results show that VEGF111b is a novel potent anti-angiogenic agent that can target the VEGF-R2 and its signaling pathway to inhibit ovarian tumor growth.

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1. Introduction

Angiogenesis plays a key role in tumor growth and progression [1]. A principal angiogenic promoter that stimulates the migration of endothelial cells, sprouting of blood vessels, and generation of new vessels from existing vascular endothelium in tumors is the vascular endothelial growth factor (VEGF-A) [2,3]. Anti-angiogenic therapy targeting VEGF-A is becoming an additional therapeutic strategy to surgery, chemotherapy and radiotherapy, which has attracted more attention.

The human VEGF-A gene has been assigned to chromosome 6p21.3. It contains 8 exons, separated by seven introns, and its coding region spans approximately 14 kb [4]. Alternative splicing of full-length VEGF pre-mRNA gives rise to two known families of protein isoforms that differ by only six amino acids at their C-terminal end (Fig. 1). The conventional VEGFxxx isoforms, where xxx refers to the number of amino acids, are formed by the proximal splice site (PSS) selection in exon 8 (termed exon 8a) and differentially splicing in exons 5, 6 or 7 [5]. The six amino acids encoded by exon 8a are CDKPRR (Fig. 1). The major isoforms of VEGFxxx family, demonstrated to be pro-angiogenic, are VEGF165, VEGF189, VEGF121, VEGF145, VEGF183, VEGF206 and VEGF111 [6]. However, another sister family of VEGF isoforms, generically referred to as VEGFxxxb isoforms, are formed by distal splice site (DSS) selection 66 bp downstream of the PSS site in exon 8 (termed exon 8b) [7–9]. Exon 8b encodes a unique amino acids sequence SLTRKD (Fig. 1). In VEGFxxxb family, VEGF165b, VEGF121b, VEGF145b and VEGF183b have been identified in succession and demonstrated to be anti-angiogenic [5]. The first verified and widely reported VEGFxxxb family member is VEGF165b, which has been clearly shown to inhibit endothelial cell growth and migration in vitro and angiogenesis in tumor and non-tumor-related angiogenesis [7,10,11].

Abbreviations: FBS, fetal bovine serum; VEGF, vascular endothelial growth factor; PSS, proximal splice site; DSS, istal splice site; VEGF-R, VEGF receptor; HRP, horseradish peroxidase; HUVECs, human umbilical vein endothelial cells; RT-PCR, reverse transcriptase-PCR.

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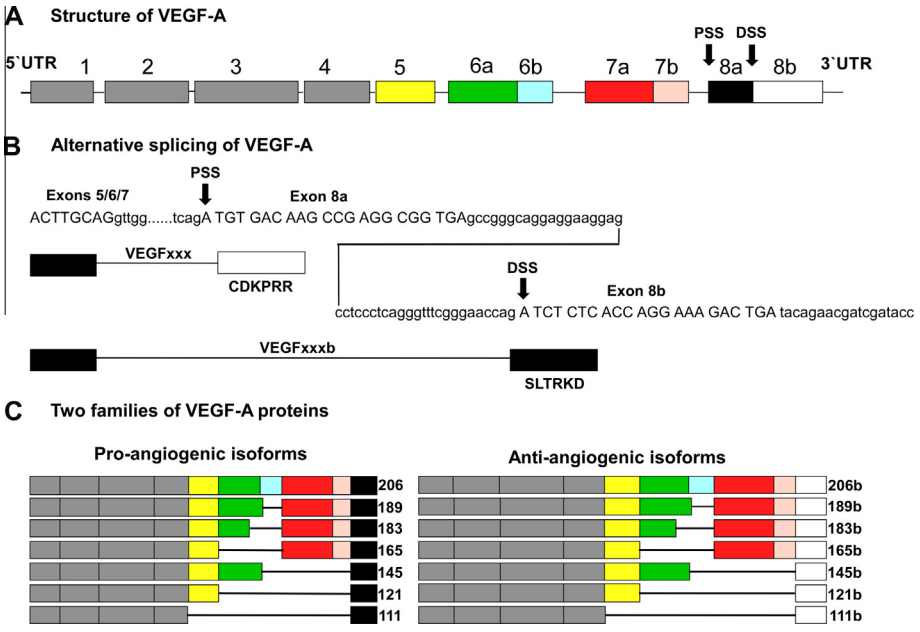

Fig. 1. Structure of VEGF-A gene and alternative splicing of VEGF-A generate VEGFxxx and VEGFxxxb isoforms. (A) VEGF-A contains eight exons. Proximal splice site (PSS) selection in the terminal exon 8 generates the pro-angiogenic VEGFxxx family, whereas distal splice site (DSS) selection results in the anti-angiogenic VEGFxxxb family. (B) Alternative splicing of the C-terminal end leads to the possibility of two sister families of VEGF-A isoforms: VEGFxxx and VEGFxxxb, differing only in last six amino acids (CDKPRR or SLTRKD).

Although a large number of evidences on the expression and property of VEGF165b have already been published, there is very little evidence on other VEGFxxxb family members, and their existence and property is still unknown. In 2007, Mineur reported a new VEGFxxx family member, VEGF111, and demonstrated that it could only be induced in the condition of genotoxic agents, such as camptothecin, mimosin, mitomycin C and UV-B [12]. The VEGF111 coding sequence consists of exons 1–4 and 8a. DSS in exon 8 has stronger splicing advantages than PSS [13]. Whether VEGF111b exits and plays a role in anti-angiogenic effect has never been demonstrated. Thus we speculate the presence of VEGF111b. Therefore, in this study, we detected and discovered a new member of VEGFxxxb family, VEGF111b, under the induction of mitomycin C. We constructed eukaryotic expression vector of VEGF111b for sequencing, prepared VEGF111b polyclonal antibody, and finally confirmed the hypothesis that VEGF111b also show anti-angiogenic properties.

2. Materials and methods

2.1. Reagents and antibodies

Mitomycin C was obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Anti-VEGF-R1, and anti-VEGF-R2 were purchased from Beyotime (Jiangsu, China). All other primary antibodies were purchased from Abcam (Cambridge, TX, USA). Horseradish peroxidase (HRP)-labeled anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz (Dallas, TX, USA).

2.2. Cell lines

Human umbilical vein endothelial cells (HUVECs) were extracted from umbilical cords from caesarean sections (The General Hospital of the People's Liberation Army, Beijing, China). The study protocol was approved by the local ethics committee. The cells were cultured in Endothelial Cell Medium (ECM, Science) consisting of 5% foetal bovine serum (FBS), 1% endothelial cell growth supplement and 1% penicillin and streptomycin solution. Human ovarian cancer cells SKOV3 were obtained from the Chinese Academy of Medical Sciences and cultured in Roswell Park Memorial Institute-1640 culture (RPMI-1640, HyClone), supplemented with 10% FBS (Invitrogen). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. RT-PCR analysis

SKOV3 cells were treated with 100 μg/ml mitomycin C for 24 h, then total RNA was extracted using Trizol reagent (Invitrogen, USA). Complementary DNA was made using oligo dT primer (TransGen, Beijing) by the manufacturer. According to alternative splicing of VEGF-A, the VEGF111b mRNA is composed of exons 1–4 and 8b. We designed forward primer of VEGF111b in exon 4, and reverse primer in the junction of exon fourth and 8b. GAPDH was amplified as an internal control. Primers sequences are listed as follows: VEGF111b 5′-CCACTGAGGAGTCCAACATCA-3′ (forward); 5′-AATGCAGATGTGACAAGCCGAG-3′ (reverse). VEGF165b 5′-GAGATGAGCTTCCTACAGCAC-3′ (forward); 5′-TTAAGCTTTCAGT CTTTCCTGGTGAGAGATCTGCA-3′ (reverse). GAPDH 5′-CGGAGTCAA CGGATTTGGTCGTAT-3′ (forward); 5′-AGCCTTCTCCATGGTGGTGAA GAC-3′ (reverse). PCR products were separated and visualised using 4% agarose/ethidium bromide gel.

2.4. Production of polyclonal antibody VEGF111b

Synthetic peptide fragments of the 8 amino acids CRSLTRKD in the C-terminal sequence of VEGF111b were coupled to KLH serving as carrier molecules and then used to immunize two male New Zealand long ear rabbits. The animals received subcutaneous injections of 0.5 ml peptide-KLH conjugates in Freund's Complete Adjuvant every 2 weeks. A week after the last immunization ear vein blood was taken for enzyme linked immunosorbent assay (ELISA) titer analysis. When the titers reached to the requirement, serum was collected to purify VEGF111b polyclonal antibody by ammonium sulfate precipitation.

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