A probable novel splicing isoform of human vascular endothelial growth factor

ZHOU Zhongjiang1, YE Haiyan1, CUI Kai1, CHEN Xianghui1, LIU Yili1

1Department of Cardiology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China; 2Department of Gynecology, Guangdong Provisional People's Hospital, Guangzhou 510080, China

Abstract: Objective To characterize a new alternative splicing isoform of human vascular endothelial growth factor (VEGF) gene. Methods The total RNA was extracted from the lung tissue of a legally aborted 4-month-old fetus and amplified by RT-PCR. The amplified product was cloned into the plasmid pMD18-T and plasmid pcDNA3.1 for sequence analysis. Results Electrophoresis of the RT-PCR products displayed one short band for VEGF1a (487 bp) and a long band. The latter was characterized to contain two fragments: one was normal VEGF1a (619 bp), and the other (639 bp) had an identical nucleotide sequence to VEGF1a with a 20 bp fragment inserted between exons 3 and 4. Sequence analysis showed that this 20-bp nucleotide was inserted from the 3’ end of the third intron containing a splicing signal, thus causing shift mutation in the reading frame of VEGF gene and early appearance of the stop codon UAG in the middle of exon 4. Conclusion A new alternative splicing isoform of VEGF probably exists in the lung tissue of a legally aborted human fetus, and its biological significance remains to be further investigated.

Key words: vascular endothelial growth factor; alternatively spliced form; exons; introns; gene mutation

INTRODUCTION

Human vascular endothelial growth factor (VEGF) has diverse alternative splicing variants in different organs and tissues during many physiological and pathological processes such as embryogenesis, angiogenesis, tumor growth, and endothelial cell proliferation4,5,6. So far at least 6 splicing variants of VEGF have been identified, namely VEGF121, VEGF165, VEGF183, VEGF189, and VEGF206, consisting of 121, 145, 165, 183, 189 and 206 amino acids, respectively4,6, as shown in Fig.1.

In this paper, we report our finding of a probable novel alternative splicing variant of VEGF gene in the lung tissue of a legally aborted female fetus.

MATERIALS AND METHODS

Fetal lung tissue sample source

A healthy 4-month-old female fetus was aborted due to serious medical maternal conditions that required immediate termination of pregnancy. The fetus weighed about 200 g at abortion, and the lung tissue was collected immediately with written informed consent from the parents. The sample was collected in a −20 °C ice box on the gene and enzyme-free super-clean bench, and was then stored in liquid nitrogen immediately.

Reagents

The total RNA isolation mini kit, RT-PCR mini kit, gel extraction mini kit, and plasmid mini kit were purchased from QIAGEN Company. T4 DNA ligase, EcoR V and Kpn I DNA endonucleases were purchased from New England Biolabs Company. pMD18-T vector and DNA markers were supplied by Takara Company. Taq DNA polymerase was purchased from the Academy of Military Medical Sciences. DH5α and JM109 engineering strains were obtained from the Laboratory of Molecular Immunology, Southern Medical University (Guangzhou, China). pcDNA3.1 expression plasmid was a product of Invitrogen. The primer synthesis and DNA sequencing were completed by Takara Company. The primer sequences for VEGF1a were 5’-GGGATAATCGAATTTCCGGCTCCGAAACCATGAACT-3’ (sense), and 5’-AAGGTACCTGCAGTCACCGGCTGGCTGTAC-3’ (antisense).
Total RNA isolation

The total RNA was isolated from the lung tissue homogenate and underwent electrophoresis by a standard technique according to the manufacturer’s instructions of the kits. All the operations were carried out in RNAase-free environment treated by a high temperature (180 °C for 6 h) and coke diethyl (DEPC).

RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a one-step technique. The reverse transcription was performed under 50 °C for 15 min, followed by PCR amplification with a pre-denaturation at 95 °C for 15 min and 30 thermal cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 70 °C for 1 min. The PCR products then underwent DNA gel electrophoresis.

VEGF cDNA cloning and sequence analysis

The PCR products were analyzed with 1.2% agarose electrophoresis, recovered by cutting the gel under ultraviolet light, and purified with the gel extraction kit. The target fragments were subsequently cloned into plasmid pMD18-T Vector to construct the recombinant eukaryotic expression vector, and was identified by sequence analysis.

Construction and identification of VEGF eukaryotic expression plasmid

The recombinant plasmid was digested with the endonucleases EcoR V and Kpn I. The full-length VEGF cDNA was separated with 1.2% agarose electrophoresis and purified with the gel extraction kit. VEGF cDNA obtained was then connected using T<sub>4</sub>DNA ligase to pcDNA3.1<sup>−</sup> eukaryotic expression plasmid to construct the recombinant eukaryotic expression vector pcDNA3.1<sup>−</sup>/VEGF.

RESULTS

Electrophoresis of the RT-PCR products displayed two distinct bands of about 564 bp (Fig.2). The lower band was cloned into pMD18-T (T vector) and pcDNA3.1<sup>−</sup> expression vector, and was identified by sequencing as the 487-bp VEGF<sub>121</sub> (the full-length VEGF<sub>165</sub> cDNA was 444 bp). The upper band was cloned into T vector and identified to contain two fragments: one was 619-bp natural VEGF<sub>165</sub> (the full length VEGF<sub>165</sub> cDNA was 576 bp), and the other the 639-bp VEGF<sub>165</sub> nucleotide sequence with a 20-bp fragment inserted between exon 3 and exon 4 (Fig.3). The terminal base of the inserting sequence was AG, which was consistent with the typical GT---AG gene splicing rule. The variant splicing isofoms of VEGF was clearly shown by gene splicing. Sequence analysis showed that the 20-bp inserting sequence was the retention terminal fragment of the third intron, which induced a frame shift mutation of the full sequence (Fig.4). The presence of the new insertion not only produced a shift of the open reading frame, but also introduced a stop codon (TAG) in the middle of the fourth exon. At the protein level, the peptide encoded by the fourth exon was shortened, and that encoded by the fifth and the subsequent exons were lost. The results were confirmed by repeated sequencing (Fig.5).

DISCUSSION

In this study, we successfully cloned VEGF<sub>121</sub> and VEGF<sub>165</sub> into the eukaryotic expression vector pcDNA3.1<sup>−</sup> plasmid, which provides a basis for further study. From the fetal lung tissue sample, we found a novel splicing variant containing an intron coding for 20 amino acids with frameshift mutations in the sequences because of the retention of the third intron. The mRNA of this novel variant consists of exons 1-3 and partial exon 4 sequences, and might encode amino acids with frameshift mutations in the sequences. Sequence analysis showed that the 20-bp inserting sequence was the retention terminal fragment of the third intron, which induced a frame shift mutation of the full sequence with the typical GT---AG gene splicing rule. The variant splicing isofoms of VEGF was clearly shown by gene splicing. Sequence analysis showed that the 20-bp inserting sequence was the retention terminal fragment of the third intron, which induced a frame shift mutation of the full sequence (Fig.4). The presence of the new insertion not only produced a shift of the open reading frame, but also introduced a stop codon (TAG) in the middle of the fourth exon. At the protein level, the peptide encoded by the fourth exon was shortened, and that encoded by the fifth and the subsequent exons were lost. The results were confirmed by repeated sequencing (Fig.5).
splicing of VEGF mRNA results in the isoforms of varying mitogenicity and solubility. This probable novel variant of VEGF was found in the lung tissue of a 4-month-old fetus, possibly indicating its important roles in such physiological processes as embryonic development, lung angiogenesis, and lung development. Variable splicing is an important mechanism of gene expression regulation in eukaryotes, but the difference and significance of variable splicing remain to be investigated.

VEGF is essential for normal embryonic development and plays a major role in the physiological and pathological events of angiogenesis in adults. As a member of platelet-derived growth factor (PDGF) family,
consisting of about 44 amino acids. The sequences about encode a peptide chain 44 amino acids; Exon sequences. Exon kinase -insert- domain receptor/fetal liver containing 26 and 8 found to contain only the sequences encoded by exons. VEGF was recently sequences encoded by exons 7 and 6, while VEGF completely lacks these sequences. VEGF was recently found to contain only the sequences encoded by exons 1 and 8. As a typical secreted protein, VEGF has an N-terminal signal peptide consisting of 26 hydrophobic amino acids encoded by exon 1. VEGF receptors, known as kinase-insert-domain-containing-receptor/fetal liver kinase 1 (KDR/flk-1), was identified by amino acid sequences. Exon 7 encodes a peptide chain consisting of about 44 amino acids; Exon 6 encodes a peptide chain consisting of about 24 amino acids. VEGF includes VEGF-A (usually referred to as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). Currently, at least 5 different mRNA species encoding VEGF-A have been characterized. These variants result from alternative splicing of the VEGF-A transcript and encode human isoforms of VEGF protein consisting of 121, 145, 165, 189, or 206 amino acids. VEGF protein is a 34 to 42 kD dimeric, disulfide-bound glycoprotein existing in different homodimeric variants. All the variants exist as homologous dimer and share similar spatial structure. The various VEGF forms bind to two tyrosine-kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), which are expressed almost exclusively in endothelial cells. The expression of VEGF is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. VEGF induces angiogenesis as well as permeabilization of blood vessels in vivo, and plays a central role in the regulation of vasculogenesis[1].

VEGF is a glycoprotein consisting of 165 amino acids. The precursor of VEGF contains 191 amino acid residues, 2 disulfides and a glycosylation site: disulfide plays an important role in maintaining the tertiary structure, and glycosylation is related to the secretory functions. The NH-terminal signal peptide of -tertiary structure, and glycosylation is related to the disulfide plays an important role in maintaining the acid residues, disulfides and a glycosylation site: bondings state.

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[Diagram]

Fig.4 Genetic make-up of human VEGF₀ and sequence of the inserting nucleotides. a: The full-length mRNA of VEGF₀ 1 to VII are exons; b: The shortened mRNA of VEGF₀ sequences. Exon kinase -insert- domain receptor/fetal liver containing 26 and 8 found to contain only the sequences encoded by exons. VEGF was recently sequences encoded by exons 7 and 6, while VEGF completely lacks these sequences. VEGF was recently found to contain only the sequences encoded by exons 1 and 8. As a typical secreted protein, VEGF has an N-terminal signal peptide consisting of 26 hydrophobic amino acids encoded by exon 1. VEGF receptors, known as kinase-insert-domain-containing-receptor/fetal liver kinase 1 (KDR/flk-1), was identified by amino acid sequences. Exon 7 encodes a peptide chain consisting of about 44 amino acids; Exon 6 encodes a peptide chain consisting of about 24 amino acids. VEGF includes VEGF-A (usually referred to as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). Currently, at least 5 different mRNA species encoding VEGF-A have been characterized. These variants result from alternative splicing of the VEGF-A transcript and encode human isoforms of VEGF protein consisting of 121, 145, 165, 189, or 206 amino acids. VEGF protein is a 34 to 42 kD dimeric, disulfide-bound glycoprotein existing in different homodimeric variants. All the variants exist as homologous dimer and share similar spatial structure. The various VEGF forms bind to two tyrosine-kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), which are expressed almost exclusively in endothelial cells. The expression of VEGF is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. VEGF induces angiogenesis as well as permeabilization of blood vessels in vivo, and plays a central role in the regulation of vasculogenesis[1].

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Fig.5 Sequencing result of the probable novel VEGF isoform VEGF₀. The sequence in box is the inserting 20-bp intron. ATG is the start codon, and TGA is the stop codon.
Although all the 6 isoforms can induce angiogenesis in vitro, each form offers advantages in different situations, and the kind of cells that produce a specific variant of VEGF still remains to be identified. The limitation of this study lies in the lack of further information about this novel splicing isoform, including its protein characteristics and function, which are the subjects of our ongoing investigation ³. 

References


可变剪切形式, 其生物学意义有待进一步研究。