NOTE *Immunology*

Nucleotide Sequence and Expression of the Feline Vascular Endothelial Growth Factor

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ABSTRACT. Vascular endothelial growth factor (VEGF) is an angiogenic factor which targets vascular endothelial cells. In this study, cDNA encoding a feline VEGF (fVEGF) isoform was cloned from a feline lymphoid tumor cell line and sequenced. The fVEGF cDNA contained an open reading frame of 567 nucleotides coding for a polypeptide of 163 amino acids with a putative signal peptide of 26 amino acids. The predicted fVEGF amino acid sequence shared 98.4, 94.2 and 94.2% homology with the sequences of canine, bovine and human VEGF, respectively. Though predicted fVEGF polypeptide was two amino acid residues shorter than human $VEGF_{165}$, a potential glycosylation site and regions critical for receptor binding were conserved in all the species examined. Transient expression of fVEGF in mammalian cells resulted in secretion of VEGF which could be detected by antibodies against human $VEGF_{165}$. Furthermore, wide expression of fVEGF mRNA was observed in various feline tissues using RT-PCR methods.
KEY WORDS: cloning, feline, VEGF.

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Vascular endothelial growth factor (VEGF) is a specific key activator of vascular endothelial cells. VEGF induces proliferation, differentiation and migration of endothelial cells *in vitro* [9, 26], and promotes angiogenesis and vascular permeability *in vivo* [17, 20]. VEGF is widely expressed in normal tissues [2] and also in numerous human and rodent tumor cells [4, 11, 24], and its expression is upregulated by hypoxia and by transforming growth factor β (TGF-β, tumor necrosis factor α (TNF-α) and other stimuli [25, 27, 30, 32]. In pathological conditions such as wound healing [12] and tumor angiogenesis [2], and in ischemic diseases [1, 15], the association of VEGF with the disease state and the clinical importance of VEGF have become increasingly apparent. Investigation of the human VEGF gene revealed that the VEGF mRNA is alternatively spliced, resulting in translation at least 5 isoforms composed of 121, 145, 165, 189, 206 amino acids [10, 14, 33]. $VEGF_{165}$ is the predominent isoform in humans, and has been shown to be a heparin-binding basic homodimer of 45 kDa [9]. To investigate the pathophysiological role of VEGF in feline disease, we cloned and sequenced the feline VEGF cDNA from a feline lymphoma cell line, and examined the expression of the corresponding mRNA in normal tissues. Furthermore, we expressed feline VEGF in mammalian cells and confirmed the cross-reactivities of anti-human VEGF antibodies to develop a measurement assay for feline VEGF.

Total RNA was extracted from the feline lymphoid tumor cell line FT-1 [22, 23], and was reverse transcribed using a cDNA cycle kit (Invitrogen, San Diego, CA). Oligonucleotide primers were designed based on the sequences of the 5' and 3' untranslated regions of human and canine VEGF gene sequences, respectively [6]. The 5' end primer sequence was 5'-GCGCCCCGGTCGGGCCTCCGAAA-3', corresponding to human VEGF cDNA position numbers 677–699 (GenBank accession number AF022375) and the 3' end primer was 5'-AGGGAGCCTCCTTCTTCCAG-3', corresponding to canine VEGF cDNA nucleotide positions 671–652 (GenBank accession number AF133250). Polymerase chain reaction (PCR) was performed by using the gene-specific primers and resulted in three bands of about 650 bp, 600 bp and 450 bp as shown by electrophoresis on 2% agarose gels (data not shown). The major band of about 600 bp was purified and directly cloned into the pCR2.1-TOPO cloning vector (Invitrogen). Sequence analysis was performed by using a BigDye Primer Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, CA) and the sequence was read using an Applied Biosystems 377 DNA sequencer.

The nucleotide sequence and predicted amino acid sequence of the feline VEGF cDNA clone are shown in Fig. 1 (GenBank accession number AB071947). The feline VEGF cDNA clone had an open reading frame of 567 bp, coding for a polypeptide of 189 amino acids. The deduced amino acid sequence encoded by the feline VEGF clone was aligned with the amino acid sequences deduced from the canine [31], equine (GenBank accession number AB053350), bovine [20], mouse [7] $VEGF_{164}$ and human $VEGF_{165}$ [20] cDNAs (Fig. 2), and found to share 98.4, 96.3, 94.2, 90.0 and 94.2% homology, respectively, with these sequences. It contained 26 residues of N-terminal signal sequence that were highly conserved among the other species. Without the leader peptide, the predicted feline VEGF contains 163 amino acid residues, which is two amino acids shorter than human VEGF165. Two deletions were observed at positions 5 and 113 in human sequence, a glutamic acid residue and a glutamine residue, respectively. There is one potential Asn-linked glycosylation site, which

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      10        20        30        40        50        60        70        80        90
ATGAACTTTCTGCTCTCTTGGGTGCATTGGAGCCTTGCCTTGCTGCTCTACCTCCACCATGCCAAGTGGTCCCAGGCTGCACCCATGGCA
 M  N  F  L  L  S  W  V  H  W  S  L  A  L  L  L  Y  L  H  H  A  K  W  S  Q  A  A  P  M  A

      100       110       120       130       140       150       160       170       180
GATGGAGAGCACAAACCCCACGAAGTGGTGAAGTTCATGGATGTCTACCAGCGCAGCTACTGCCGTCCCATCGAGACCCTGGTGGACATC
 D  G  E  H  K  P  H  E  V  V  K  F  M  D  V  Y  Q  R  S  Y  C  R  P  I  E  T  L  V  D  I

      190       200       210       220       230       240       250       260       270
TTCCAGGAGTACCCTGATGAGATCGAGTATATCTTCAAGCCATCCTGCGTGCCCCTGATGCGATGCGGGGGCTGTTGCAATGACGAGGGC
 F  Q  E  Y  P  D  E  I  E  Y  I  F  K  P  S  C  V  P  L  M  R  C  G  G  C  C  N  D  E  G

      280       290       300       310       320       330       340       350       360
CTGGAGTGTGTGCCCACGGAGGAGTTCAACATCACCATGCAGATTATGCGGATCAAACCTCATCAAGGCCAGCACATAGGGGAGATGAGC
 L  E  C  V  P  T  E  E  F  N  I  T  M  Q  I  M  R  I  K  P  H  Q  G  Q  H  I  G  E  M  S

      370       380       390       400       410       420       430       440       450
TTCCTACAGCATAGCAAATGTGAATGCAGACCAAAGAAAGATAGAGCGAAAGAAAATCCCTGTGGGCCTTGCTCAGAGCGGAGAAAGCAT
 F  L  Q  H  S  K  C  E  C  R  P  K  K  D  R  A  K  E  N  P  C  G  P  C  S  E  R  R  K  H

      460       470       480       490       500       510       520       530       540
TTGTTTGTACAAGATCCGCAGACGTGTAAATGTTCCTGCAAAAACACAGACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTAAACGAACGT
 L  F  V  Q  D  P  Q  T  C  K  C  S  C  K  N  T  D  S  R  C  K  A  R  Q  L  E  L  N  E  R

      550       560       570       580
ACTTGCAGATGTGACAAGCCCAGGCGGTGAGCCGGGC
 T  C  R  C  D  K  P  R  R  *
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Fig. 1. Nucleotide and deduced amino acid sequence of feline VEGF cDNA (GenBank accession number AB071947). The termination codon is denoted by an asterisk (*).

is conserved in all species. There are 16 cysteine residues at positions completely equivalent to those in canine, equine, bovine, human and mouse VEGF, including 8 cysteine residues within the receptor binding domains (i.e. amino acids 1–109). Several deteminants critical for the binding to two VEGF receptors, kinase domain receptor (KDR/flk-1) and fms-like tyrosine kinase (flt-1) [18], were also completely conserved (Fig. 2).

The feline VEGF cDNA was next used for a protein expression study. The VEGF cDNA was subcloned into the *Bam*HI and *Eco*RV sites of the pcDNA3.1 expression vector (Invitrogen), which contains the cytomegalovirus promoter and other features that result in high-level expression in mammalian cells. Plasmid DNAs containing feline VEGF cDNA or cDNA for the lacZ gene for β-galactosidase, which was used as a control, were prepared using a QIAGEN plasmid kit (Qiagen, Studio City, CA). Transient transfections of these plasmids into the human embryonic kidney cell line 293 were carried out using cationic lipid, LIPOFECTAMINE 2000 Reagent (Invitrogen). More than 90% transfection efficiency was confirmed by X-gal staining in cells transfected with LacZ-containing control vector after 48 hr of incubation (data not shown) and the supernatants from each type of transfected cell were collected. The expression of feline VEGF was detected using a sandwitch enzyme-linked immunosorbent assay (ELISA) kit using monoclonal antibody to recombinant human $VEGF_{165}$ protein (Quantikine, R&D Systems, Minneapolis, MN). The concentration of VEGF was quantitated by interpolation of a standard curve using linear regression analysis.

Expression of VEGF, ranging from 311.5 to 483.6 *n*g/m*l* in duplicate assays, was demonstrated in the medium of the cells transfected with feline VEGF cDNA-containing vector, whereas less than 0.02 *n*g/m*l* VEGF was detected in the control medium from cells transfected with the control vector. This result indicated that the cDNA clone obtained in this study could produce a soluble protein antigenically related to human $VEGF_{165}$, and that the feline VEGF could be detected by the anti-human $VEGF_{165}$ monoclonal antibody, which did not cross-react with mouse VEGF.

To investigate natural VEGF expression in normal feline tissues, we performed reverse transcription-polymerase chain reaction (RT-PCR) using primers specific for feline VEGF. A sense primer with the sequence 5'-ATGAACTTTCTGCTCTCTTGGG-3' (nucleotide positions 1–22), and an antisense primer with the sequence 5'-

Fig. 2. Alignment of predicted amino acid sequence of feline VEGF with the canine, equine, bovine, human and mouse VEGF sequences. The signal peptide is numbered from -26 to -1, and the mature peptide is numbered from 1 to 163. Amino acids identical with those in feline VEGF are indicated by asterisks (*), and dashes (-) were introduced where the sequence alignment failed. The putative glycosylation site (open box) and receptor binding determinants for KDR (Arg^{81}, Lys^{83}, His^{85}) and Flt-1 (Asp^{62}, Glu^{63}, Glu^{66}) (shaded boxes) are shown.

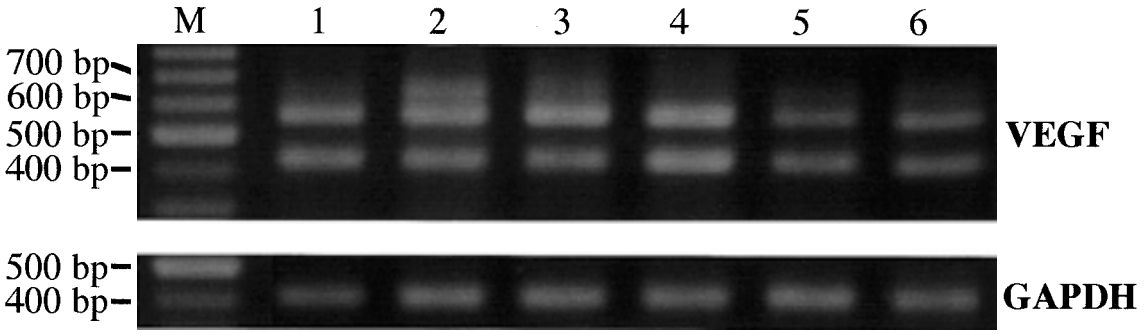

Fig. 3. Expression of feline VEGF mRNA in various tissues. Total RNA samples extracted from normal tissues (lane 1, spleen; lane 2, lung; lane 3, kidney; lane 4, adrenal grand; lane 5, colon; lane 6, skin) were analyzed by RT-PCR using gene-specific primers for feline VEGF.

TCACCGCCTGGGCTTGTCA-3' (nucleotide positions 570–552), which were expected to detect all isoforms of feline VEGF, were used. Total RNA was extracted from various cat tissues, including the spleen, lung, kidney, adrenal grand, colon and skin of healthy adult cats, and RT-PCR was performed using a GeneAmp RNA PCR kit (Applied Biosystems). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in each sample. Agarose gel analysis of PCR products showed at least two major bands in each tissue (Fig. 3). In addition to the expression of $fVEGF_{163}$ (572 bp), bands of about 440 bp were also clearly observed, and these were expected to be homologous to human $VEGF_{121}$. In lung tissue, another faint band of about 650 bp was also detected, and was expected to be homologous to the $VEGF_{189}$ isoform of human VEGF. These results revealed that the feline

VEGF gene is widely expressed in normal feline tissues, as in other species, with predominant expression of mRNAs coding for homologs of the $VEGF_{165}$ and $VEGF_{121}$ isoforms.

The plasma concentration of VEGF is elevated in some malignant diseases, including several solid tumors [19], and in hemangiosarcoma [8] and inflammatory diseases [3, 13] in humans and dogs, emphasizing that the measurement of circulating VEGF is useful for diagnosis or disease monitoring [16]. Furthermore, important advances have been made regarding the therapeutic applications of VEGF. For instance, VEGF gene therapy targeting peripheral or coronary artery diseases [15, 21], or inhibition of VEGF activity using anti-VEGF antibodies [28] or antisense oligonucleotides [5, 29] for a variety of malignancies has been investigated. The feline VEGF cDNA cloned in this study will not only lead to the elucidation of the pathology of feline diseases associated with aberrant angiogenesis or vascular permeability, but should be useful for use in VEGF therapy in serious cats diseases as well.

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