VEGF₁₆₂, A New Heparin-binding Vascular Endothelial Growth Factor Splice Form That Is Expressed in Transformed Human Cells*

Tali Lange‡, Noga Gottmann-Raviv‡, Limor Baruch‡, Marcelle Machluf¶, and Gera Neufeld‡¶

From the Departments of Cell Biology and Anatomy, The Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, P. O. Box 9697, 1 Efron Street, Haifa 31096, Israel and the Faculty of Biotechnology and Food Engineering, Technion-Institute of Technology, Haifa 32000, Israel

The splice forms of vascular endothelial growth factor (VEGF) differ in biological properties such as the receptor types that they recognize and their interaction with heparan sulfate proteoglycans. We have identified a new VEGF mRNA splice form encoding a VEGF species containing 162 amino acids (VEGF₁₆₂) in human A431 ovarian carcinoma cells. This novel mRNA contains the peptides encoded by exons 1–5, 6A, 6B, and 8, and of the VEGF gene. Recombinant VEGF₁₆₂ is biologically active. It induces proliferation of endothelial cells in vitro and angiogenesis in vivo as determined by the alginate bead assay. VEGF₁₆₂ binds less efficiently than VEGF₁₄₅ but more efficiently than VEGF₁₆₅ to a natural basement membrane produced by corneal endothelial cells. VEGF₁₆₂, an artificial VEGF form that contains exon 6B but lacks exons 6A and 7, did not bind to this basement membrane at all, indicating that exon 6B probably interferes with the interaction of exon 6A with heparin and heparan sulfate proteoglycans.

The various forms of vascular endothelial growth factor (VEGF) are generated by alternative splicing from a single gene (1–4). The domains encoded by exons 1–5 of the VEGF gene contain information required for the recognition of the tyrosine kinase VEGF receptors 1 (flt-1) and 2 (KDR/flk-1) (5) and are present in all the VEGF splice forms. Most VEGF splice forms are distinguished by the presence or absence of the peptides encoded by exons 6 and 7 of the VEGF gene that code for two independent heparin-binding domains. VEGF₁₂₁ lacks both exons and does not bind to heparin. VEGF₁₆₅ contains the exon 7-encoded peptide, VEGF₁₄₅ contains the peptide encoded by exon 6A, and VEGF₁₆₂ contains both exon 6A and exon 7 (3, 6–8). VEGF₂₀₆ has a structure similar to that of VEGF₁₆₂, except that it contains both exons 6A and 6B of the VEGF gene. However, the contribution of exon 6B to the biological properties of VEGF₂₀₆ has not been studied. The amino acids encoded by exon 8 are present in most of the VEGF splice forms. However, it was recently found that in the novel VEGF form VEGF₁₆₂, the 6 amino acids encoded by exon 8 are replaced by 6 amino acids derived from a putative ninth exon to yield a VEGF form that probably inhibits angiogenesis (9). In another recently reported VEGF splice form, exon 8 is completely truncated to generate a 148-amino acid form (10). In addition, it was recently found that VEGF forms possessing an extended N termini of unknown function also exist (11).

Most VEGF isoforms induce proliferation of vascular endothelial cells, induce angiogenesis, and cause permeabilization of blood vessels (8). Recently, certain differences between some common VEGF splice forms have been reported. The neuropilin-1 and neuropilin-2 receptors function as receptors for axon guidance factors belonging to the semaphorin family (12, 13). It was found that both neuropilins also function as VEGF receptors that differentiate between various forms of VEGF (14, 15). Thus, VEGF₁₂₁ cannot bind to either of the neuropilins, whereas VEGF₁₆₅ binds efficiently to both receptors, and VEGF₁₄₅ binds well to neuropilin-2 but not to neuropilin-1 (16).

Such differences are also reflected in the functional properties of the VEGF splice forms. It was found that mice expressing only VEGF₁₂₁ do not develop properly (17, 18) because the heparan sulfate-binding VEGF forms are required for correct branching of blood vessels during development (19). Likewise, mice expressing only VEGF₁₄₅ display impaired arterial development (17), suggesting that each VEGF form possesses specific characteristics and that the various VEGF forms complement each other to achieve a balanced angiogenic response.

Exon 6B was first identified in VEGF₂₀₆ (Fig. 1C), but the effect of exon 6B on the biological properties of VEGF₂₀₆ had not been studied because VEGF₂₀₆, like VEGF₁₈₉, is not secreted into the medium of cells that produce these VEGF forms and is thus difficult to isolate and characterize. We report here the identification of a new exon 6B-containing form of VEGF expressed by A431 ovarian carcinoma cells. This VEGF form is 162 amino acids long (VEGF₁₆₂). In this work, we have characterized the properties of VEGF₁₆₂ and compared them with those of the closely related VEGF₁₆₅ and those of VEGF₁₈₉, an artificial exon 6B-containing VEGF form.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant VEGF₁₆₅ was purified from SF-9 insect cells as described previously (20). The pDCHIP11 plasmid containing the DHFR minigene was kindly provided by Dr. Lawrence Chasin (Columbia University, New York, NY) (21). Rabbit polyclonal antibody directed against VEGF was produced in our laboratory as described previously (20). Monoclonal anti-VEGF antibodies (V4758), peroxidase-conjugated anti-rabbit IgG antibodies, and alkaline phosphatase-conjugated anti-rabbit IgG antibodies were purchased from Sigma. The EZ-ELISA kit was from Biological Industries Inc. (Beth Haemek, Israel). Heparin-Sepharose was purchased from Pharmacia Corporation. Tissue culture plasticware was from Corning. Tissue culture media were obtained from Invitrogen. Other tissue culture reagents were from Biological Industries Inc.

Detection of VEGF₁₆₂ mRNA in Cells—Total mRNA was prepared from A431 human ovarian carcinoma cells. Complementary DNA was synthesized from 5 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). A specific oligonucleotide derived from the 3′-untranslated region of VEGF mRNA (nucleotides...
VEGF₁₆₂, A New Heparin-binding VEGF Splice Form

VEGF₁₆₂ mRNA—The presence of the peptide encoded by exon 6B in VEGF₂₀₆ (2) (Fig. 1C) indicates that this peptide may be present in additional forms of VEGF. We have used a primer derived from the 3′-untranslated region of the VEGF mRNA (nucleotides 827–850) to generate first-strand cDNA species from total RNA prepared from several human tumor-derived cell lines. We subsequently used a 5′ primer corresponding to a hypothetical junction between exon 6B and exon 8 in conjunction with a 5′ primer corresponding to the beginning of the VEGF mRNA to amplify the cDNA in order to detect exon 6B-containing VEGF splice forms differing from VEGF₂₀₆. Using these primers, we have found that A431 squamous carcinoma cells contain a VEGF mRNA of a size corresponding to a VEGF mRNA species that contains exons 1–5, 6A, 6B, and 8 (Fig. 1A, lane 1). No amplified cDNA could be detected in an identical control experiment in which the reverse transcriptase enzyme was omitted (Fig. 1A, lane 2). The structure of this new VEGF cDNA was verified by sequencing, and it did indeed correspond to the expected sequence of a VEGF splice form encoding a 162-amino acid-long form of VEGF (VEGF₁₆₂) (Fig. 1C).

Because the 3′ primer used corresponded to a hypothetical exon 6B/exon 8 junction, it could perhaps have hybridized via the exon 6B matching portion of the primer to an exon 6B-containing cDNA such as that encoding VEGF₂₀₆ during the PCR amplification stage. Such amplification would generate the VEGF₁₆₂ cDNA, even though VEGF₁₆₂ mRNA was not present. This possibility is somewhat unlikely because the annealing temperature we used was 68 °C, making annealing between VEGF₂₀₆ and the 10 exon 6B-derived bases of the primer difficult. Nevertheless, to exclude this possibility, we tried to find out whether the primer pair we used to amplify the VEGF₁₆₂ cDNA was able to amplify cDNAs encoding VEGF splice forms other than VEGF₁₆₂. The pair of primers successfully amplified cDNAs encoding VEGF₁₃₈ (an artificial cDNA containing exons 1–5, 6B, and 8; Fig. 1C; Fig. 1B, lane 2) and VEGF₁₆₂ (Fig. 1B, lane 4). However, we have not been able to amplify any other VEGF cDNA including the cDNA encoding VEGF₂₀₆ (Fig. 1B, lane 8) with this pair of primers under the conditions that we used to amplify the VEGF₁₆₂ cDNA derived from the A431 cells. This experiment therefore indicates that the VEGF₁₆₂ cDNA was indeed generated from a natural mRNA species encoding VEGF₁₆₂.
Recombinant VEGF<sub>162</sub> Induces Proliferation of Endothelial Cells in Vitro—To study the biological properties of VEGF<sub>162</sub> and to compare them with those of other VEGF splice forms, we produced recombinant VEGF<sub>162</sub>. The VEGF<sub>162</sub> cDNA was cloned into the PECE expression vector (22) and co-transfected into CHO DHFR<sup>-</sup> cells (23). Clones expressing relatively large amounts of VEGF<sub>162</sub> were initially isolated using a selective medium lacking nucleosides, and the integrated VEGF<sub>162</sub> cDNA was further amplified using methotrexate (23). Conditioned medium from such cells, but not conditioned medium from empty vector-transfected cells, contained immunoreactive VEGF<sub>162</sub> (Fig. 2A). The VEGF<sub>162</sub> was partially purified using a heparin-Sepharose affinity column. It was eluted from the column with 0.8 M NaCl and used for additional experiments. At this stage, it was about 80% pure (Fig. 2B, lane 3). VEGF<sub>162</sub> migrated as two bands in reduced SDS-PAGE gels, as do all the other VEGF forms (see, for example, VEGF<sub>165</sub>, Fig. 2B, lane 1) (4, 29). The upper band probably contains glycosylated VEGF<sub>162</sub>, as is the case with VEGF<sub>165</sub> (29). VEGF<sub>162</sub> was biologically active and induced proliferation of human umbilical vein-derived endothelial cells with an ED<sub>50</sub> of about 10 ng/ml (Fig. 3). This value is somewhat lower than the ED<sub>50</sub> of VEGF<sub>145</sub> (30 ng/ml) (4), indicating that these two VEGF forms, which differ only by the presence of exon 6B in VEGF<sub>162</sub>, have similar biological potencies (4).

Exon 6B Inhibits the Binding of VEGF<sub>162</sub> to Extracellular Matrix as Compared with VEGF<sub>145</sub>—Although VEGF<sub>145</sub> and VEGF<sub>165</sub> display an almost identical affinity toward heparin, VEGF<sub>165</sub> binds less efficiently than VEGF<sub>145</sub> to a native basement membrane produced by corneal endothelial cells (Fig. 4B) (4). The presence of exon 6B is the only difference that distinguishes VEGF<sub>162</sub> from VEGF<sub>145</sub>. To find out whether exon 6B influences the ability to bind to this basement membrane, we incubated basement membrane-coated 24-well dishes with increasing concentrations of VEGF<sub>145</sub> or VEGF<sub>162</sub> as described previously using antibodies directed against VEGF to detect bound VEGF (4). VEGF<sub>145</sub> bound to the basement membrane more efficiently at low concentrations, and at the maximal concentration tested (1 μg/ml), the amount of VEGF<sub>145</sub> that bound to the basement membrane was 2–2.5-fold higher than
the amount of bound VEGF162 (Fig. 4, A and B). In contrast, at this concentration, VEGF138 did not bind at all to the basement membrane despite the presence of exon 6B in this artificial VEGF form, indicating that exon 6B does not interact with extracellular matrices on its own. In that respect, VEGF138 behaved like VEGF121 (4). VEGF165 was able to bind to the basement membrane as reported previously (4), although it did so less efficiently than either VEGF145 or VEGF162 (Fig. 4B) (4).

This result indicates that the peptide encoded by exon 6B may modulate exon 6A-mediated binding to basement membranes.

**VEGF162 Induces Angiogenesis in Vivo**—To find out whether VEGF162 induces angiogenesis, we used the alginate bead assay (4), with modifications (28). Cells were cultured in alginate beads for 1 week to remove impurities from the alginate and to verify growth factor production and release. VEGF162 secretion into the medium was verified by Western blot analysis (data not shown). Clusters of alginate beads were injected subcutaneously into the flanks of nude mice. After 6 days, the animals were sacrificed. The clusters of alginate beads were removed and photographed. It can be seen that blood vessels have penetrated the clusters of beads containing VEGF162-producing cells (Fig. 5C) but not clusters of beads containing equal concentrations of empty vector-transfected cells (Fig. 5A). The inner surface of the skin adjacent to the clusters of beads containing VEGF162-expressing cells contained a higher density of blood vessels. At places in which new blood vessels that grew into the cluster of alginate beads were ripped, bloody leaks can be seen (Fig. 5D). The skin covering the clusters of control beads contained a lower density of blood vessels. Furthermore, the vessels were intact, and no ripped blood vessels were observed in any of the control animals (Fig. 5B). These results indicate that VEGF162 is able to induce angiogenesis in vivo.

**DISCUSSION**

VEGF206 was identified as a nonsecreted VEGF form in which alternative splicing in the region of exon 6 of the VEGF mRNA resulted in a 41-amino acid insertion relative to the
more widely distributed VEGF<sub>165</sub>. The insertion included the 24 amino acids found in VEGF<sub>165</sub> (2) and 17 additional amino acids. These two different portions of exon 6 have become known as exons 6A and 6B. VEGF<sub>206</sub> is a relatively rare form of VEGF that is not released into the conditioned medium of producing cells and is therefore difficult to study. The presence of exon 6B in VEGF<sub>206</sub> indicated that exon 6B may be present in additional forms and that the properties of such forms may perhaps be studied more readily. We therefore set out to look for such forms.

We have found that A431 squamous carcinoma cells contain mRNA encoding an additional exon 6B-containing VEGF form. This form is identical to VEGF<sub>145</sub> (4), except for the added amino acids encoded by exon 6B that lead to the production of a 162-amino acid-long VEGF form. This form may have been wrongly identified as VEGF<sub>165</sub> because the mass of VEGF<sub>162</sub> and the size of the mRNA encoding VEGF<sub>162</sub> closely resemble VEGF<sub>165</sub>. Recombinant VEGF<sub>162</sub> was secreted from CHO cells and was biologically active as determined by endothelial cell proliferation assays and by its ability to induce angiogenesis in vivo. In these properties, it does not appear to be significantly different from VEGF<sub>145</sub>. VEGF<sub>162</sub> bound to a native basement membrane produced by corneal endothelial cells. It bound to the basement membrane less efficiently than VEGF<sub>145</sub> but better than VEGF<sub>165</sub>, which binds to such matrices relatively inefficiently, despite the substantial affinity that it displays toward heparin (4, 7). The synthetic VEGF form VEGF<sub>138</sub>, which contains exon 6B but lacks exon 6A, was not able to bind to this basement membrane, behaving very similarly in this respect to VEGF<sub>121</sub> (4). It can therefore be concluded that exon 6B does not contribute to VEGF binding to the extracellular matrix. Rather, exon 6B seems to interfere with the interaction of exon 6A with basement membrane components, leading to a decreased extracellular matrix binding ability as compared with VEGF<sub>145</sub>. However, the interaction of VEGF<sub>162</sub> with the basement membrane is still somewhat stronger than that of VEGF<sub>165</sub>.

The evidence gathered in the past decade indicates that apparently insignificant differences in the properties of the VEGF splice forms have turned out to be biologically meaningful. Mice expressing only VEGF<sub>120</sub> or VEGF<sub>188</sub> develop abnormally, even if these VEGF forms are expressed at levels comparable with the expression levels of all VEGF forms put together (17–19, 30). These changes are the result of differential affinities to heparin sulfate proteoglycans and extracellular matrix components, differential recognition of VEGF receptors, and differential susceptibility to reactive oxygen species (8, 14–16, 31). These differences imply that certain forms of VEGF may act more efficiently than other forms in specific microenvironments and suggest that the differential splicing of VEGF may be more tightly regulated than is currently appreciated. Some evidence supporting such tight regulation is already available. For example, it was found that progesterone selectively up-regulates the expression of VEGF<sub>165</sub> in decidual cells (32).

To conclude, we have characterized a new, secreted, biologically active VEGF splice form. Whether this VEGF splice form has a biological role distinct from that of other VEGF forms is unclear at the moment, and it is not known whether there exist specific mechanisms that regulate the synthesis of the VEGF<sub>162</sub> mRNA. Tools that allow easy discrimination between the expression patterns of VEGF<sub>165</sub> and the other splice forms will have to be developed to study the in vivo expression patterns of VEGF<sub>162</sub>. The elucidation of these questions, as well as the design of experiments aimed at the identification of the specific biological roles of VEGF<sub>162</sub> will most likely be the focus for the continuation of the research in the near future.

Acknowledgments—We thank Dr. OfrA Kessler for critical comments and excellent technical tips. We thank Dr. Israel Vlodavsky for invaluable help with basement membrane binding experiments.

REFERENCES

VEGF A New Heparin-binding VEGF Splice Form
VEGF_{162}, A New Heparin-binding Vascular Endothelial Growth Factor Splice Form That Is Expressed in Transformed Human Cells

Tali Lange, Noga Guttmann-Raviv, Limor Baruch, Marcelle Machluf and Gera Neufeld

doi: 10.1074/jbc.M212224200 originally published online February 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212224200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 16 of which can be accessed free at http://www.jbc.org/content/278/19/17164.full.html#ref-list-1