Effects of VEGF\textsubscript{165} and VEGF\textsubscript{121} on vasculogenesis and angiogenesis in cultured embryonic quail hearts

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Yue, Xiping, and Robert J. Tomanek. Effects of VEGF\textsubscript{165} and VEGF\textsubscript{121} on vasculogenesis and angiogenesis in cultured embryonic quail hearts. Am J Physiol Heart Circ Physiol 280: H2240–H2247, 2001.—It has been documented that hypoxia enhances coronary vasculogenesis and angiogenesis in cultured embryonic quail hearts via the upregulation of vascular endothelial growth factor (VEGF). In this study, we compared the functions of two VEGF splice variants. Ventricles from 6-day-old embryonic quail hearts were cultured on three-dimensional collagen gels. Recombinant human VEGF\textsubscript{121} or VEGF\textsubscript{165} were added to the culture medium for 48 h, and vascular growth was visualized by immunostaining with a quail-specific endothelial cell (EC) marker, QH1. VEGF\textsubscript{165} enhanced vascular growth in a dose-dependent manner: 5 ng/ml of VEGF\textsubscript{165} slightly increased the number of ECs, 10 ng/ml of VEGF\textsubscript{165} increased the incorporation of ECs into tubular structures, and at 20 ng/ml of VEGF\textsubscript{165} wider tubes were formed. This pattern plateaued at the 50 ng/ml dose. In contrast, VEGF\textsubscript{121} did not enhance either the number of ECs or tube formation at these or higher dosages. Combined effects of hypoxia and exogenous VEGF\textsubscript{165} were then compared. Tube formation from the heart explants treated with both hypoxia and 50 ng/ml of VEGF\textsubscript{165} had a morphology intermediate to those treated with hypoxia or VEGF\textsubscript{165} alone. Immunocytochemistry study revealed EC lumenization under all culture conditions. However, the addition of VEGF\textsubscript{165} stimulated the coalescence of ECs to form larger vessels. We conclude the following: 1) VEGF\textsubscript{121} and VEGF\textsubscript{165} induced by hypoxia have different functions on coronary vascular growth, 2) unknown factors induced by hypoxia can modify the effect of VEGF\textsubscript{165}, and 3) EC lumenization observed in the heart explant culture closely mimics in vivo coronary vasculogenesis.

Vascular endothelial growth factor (VEGF) was initially known as vascular permeability factor because of its capacity to increase the permeability of microvessels to plasma and plasma proteins (6, 22). Ten years later, the same protein was independently identified as an endothelial cell mitogen in conditioned medium of pituitary follicular cells (3, 7, 11) and was therefore named VEGF.

VEGF is a 34- to 46-kDa homodimeric glycoprotein. Five human VEGF mRNA species encoding VEGF isoforms of 121, 145, 165, 189, and 206 amino acids are produced by alternative splicing of the VEGF mRNA from a single gene (13, 17, 19). Transcripts encoding VEGF\textsubscript{121} and VEGF\textsubscript{165} are detected in the majority of cells and tissues expressing the VEGF gene. VEGF\textsubscript{121} lacks the amino acids encoded by exon 7 of the VEGF gene, which is present in VEGF\textsubscript{165} and enables the ability of VEGF\textsubscript{165} to bind to heparin and heparan sulfate. A recent discovery showed that this exon 7-encoded domain also mediates isoform-specific receptor binding (24, 26). This VEGF\textsubscript{165} isoform-specific receptor is neuropilin-1. It was originally identified as a receptor that mediates the chemorepulsive activity of the collapsin-1/semaphorin III complex, which functions in repulsive growth cone and axon guidance in the developing embryo (12, 16). It has been reported (26) that neuropilin-1 can enhance both the binding of VEGF\textsubscript{165} to the Flk-1/KDR receptor and VEGF\textsubscript{165}-mediated chemotaxis. However, the function of neuropilin-1 in the development of the coronary vasculature is not clear.

Heparin-binding VEGF\textsubscript{165} is the best-characterized VEGF species. The binding of VEGF\textsubscript{165} to the VEGF receptors of vascular endothelial cells is modulated by the addition of exogenous heparin or heparan sulfate and inhibited after digestion of endothelial cells with heparinase (10, 25). The potency of VEGF\textsubscript{165} as an endothelial cell mitogen has been shown to be much higher than VEGF\textsubscript{121} by some researchers (14), whereas others report smaller differences in activity (8, 21, 23).

In a previous study, we found that VEGF signaling is involved in hypoxia-induced coronary vasculogenesis and angiogenesis in cultured embryonic quail heart explants. Three VEGF isoforms including VEGF\textsubscript{122}, VEGF\textsubscript{166}, and VEGF\textsubscript{190} (the homologues of human VEGF\textsubscript{121}, VEGF\textsubscript{165}, and VEGF\textsubscript{190}) were upregulated proportionally. But the possible different functions of these isoforms could not be differentiated by the previous study. Quail VEGFs share high homology with human VEGFs. At the protein level, quail VEGF\textsubscript{122} is

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70% homologous to human VEGF₁₂₁, and quail VEGF₁₆₆ is 75% homologous to human VEGF₁₆₅. Comparable effects on vascular growth have been demonstrated in the quail with either the administration of recombinant human VEGF or retroviral application of quail VEGF (1, 5, 9, 23, 28). In the present study, we tested the hypothesis that VEGF₁₆₅ and VEGF₁₂₁ have different functions in the development of coronary vasculature. The same heart explant model was used in this study. Recombinant human VEGF₁₆₅ and VEGF₁₂₁ were added to the culture medium singly or in combination under either normoxic or hypoxic conditions, and vascular growth was examined and compared.

**MATERIALS AND METHODS**

Preparation of three-dimensional collagen gels. Collagen gels were prepared according to the method described by the manufacturer with some modifications. Described briefly, a neutralized collagen mixture was prepared by mixing stock collagen (type I rat tail collagen, Collaborative Research; Bedford, MA) with 2× medium 199 (M199, Life Technologies; Grand Island, NY) and 1 N NaOH. The final collagen concentration was 1.5 mg/ml. The mixture was poured into four-well tissue culture plates (400 μl/well) and allowed to gel in an incubator containing 5% CO₂-95% ambient air at 37°C for 1 h. The gels were then incubated with M199 supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C for 30 min before use.

Embryonic heart explant culture. Fertilized quail eggs (Coturnix coturnix japonica) were incubated in a humidified egg incubator at 38°C for 6 days. At the end of the sixth day, ventricles were dissected from the embryonic hearts and placed onto the surface of collagen gels with the cut ends facing the gel. The experimental design is summarized in Fig. 1. The explants were first incubated at 37°C without the addition of medium in the CO₂ incubator for 4–6 h to allow the explants to attach to the gel surface. After this interval, M199 supplemented with 10% heat-inactivated FBS was added to each well, and care was taken to avoid dislodging the adhering explants. The cultures were allowed to grow under these conditions for 2 days and were then changed to low-serum M199 (0.5% heat-inactivated FBS) supplemented with insulin-transferin-selenium-A (Life Technologies) for another day. Cells (mostly mesenchymal cells at this time) began slowly proliferating and migrating from the explants during the first 2 days. The growth accelerated on the third day even though the explants were changed to low-serum medium. Treatments of the heart explants with VEGF₁₆₅ and/or VEGF₁₂₁ were started at the beginning of the fourth day (time 0). Immunostaining with the endothelial cell marker QH1 has verified that endothelial cells start to proliferate and migrate from the heart explants at this time (29).

Treatment of heart explants with VEGF. Recombinant human VEGF₁₆₅ (R&D Systems; Minneapolis, MN) and/or VEGF₁₂₁ (R&D Systems and Scios; Sunnyvale, CA) at various dosages were added to the culture medium at time 0, and the treatments were carried out for 48 h. The effects of VEGF₁₆₅ at 20 and 50 ng/ml doses were also tested in the presence or absence of 10 ng/ml to 1 mg/ml of heparin (Sigma; St. Louis, MO) and in the presence or absence of 0.1 or 0.5 U/ml of heparinase I, II, or III (Sigma) singly or in combination (0.05, 0.2, or 0.5 U/ml of each heparinase). In one group of experiments, the effects of 50 ng/ml of VEGF₁₆₅ were also studied at different time points (24, 48, and 72 h) with either a single addition of 50 ng/ml of VEGF₁₆₅ at time 0 without medium change thereafter or with the replacement of fresh medium supplemented with 50 ng/ml of VEGF₁₆₅ each day.

Expression of heart explants to hypoxia. Tissue-culture plates with heart explants were placed in custom-made oxygen chambers (University of Iowa Medical Instrument Shop) and flushed with gas mixtures containing 5% O₂-5% CO₂-90% N₂ for 5 min at 10 l/min. The chambers were then closed and placed in the CO₂ incubator at 37°C and regassed daily. The medium was preequilibrated with the 5% O₂ gas mixture overnight before the hypoxic experiments were performed. Hearts cultured under normoxia were maintained in the same CO₂ incubator (5% CO₂-95% ambient air).

Immunostaining and confocal microscopy. The heart explants with the collagen gels were rinsed twice in ice-cold PBS and then fixed overnight in 4% paraformaldehyde in PBS at 4°C. After three washes in PBS, the samples were incubated with 1% BSA + 0.5% Triton X-100 in PBS for 30 min to block nonspecific binding. Samples were then incubated with the quail-specific endothelial cell marker QH1 (18) (Hybridoma Bank, University of Iowa, Iowa City, IA) for 2 h at room temperature or overnight at 4°C. After a second block with 5% normal goat serum in PBS, the binding of QH1 was detected by FITC-labeled goat anti-mouse secondary antibody (Sigma). The samples were examined and scanned with a confocal microscope using Bio-Rad Laser Sharp software. Vascular growth was then quantified by counting the number of endothelial cells and the number of tubes and measuring the total and average lengths of the tubes using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The average width at half-length was also calculated from five randomly chosen tubes from each heart explant.

Electron microscopy and immunocytochemistry. Areas of collagen gels with the heart explants were excised, rinsed with 0.1 M sodium cacodylate buffer (pH 7.4), and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The samples were then rinsed again with sodium cacodylate buffer, postfixed in 1% OsO₄ for 1 h at room temperature, dehydrated through a series of acetone, and embedded in spurs. Thin sections were cut perpendicular to the culture plane with an ultramicrotome (Reichert-Jung), stained with uranyl acetate and lead citrate, and then examined with a Hitachi 7000 transmission electron microscope.

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**Fig. 1. Heart explant culture design.** Ventricles from 6-day-old quail embryos were cultured on three-dimensional collagen gels. For the first 2 days, heart explants were cultured in medium 199 (M199) supplemented with 10% heat-inactivated fetal bovine serum (FBS) under normoxia; media were then changed to M199 supplemented with 0.5% heat-inactivated FBS and insulin-transferin-selenium-A under normoxia for another day. Treatment of heart explants with recombinant human vascular endothelial growth factor (VEGF) isoforms (VEGF₁₆₅ and/or VEGF₁₂₁) under normoxic or hypoxic (5% O₂) conditions was started at the beginning of the fourth day (time 0) and was carried out for 48 h.
For immunocytochemistry study, heart explants with the collagen gels or hearts from 4-, 5-, and 6-day-old normal quail embryos were rinsed twice in ice-cold PBS and fixed in 2% paraformaldehyde + 1% glutaraldehyde in PBS for 2 h at 4°C. The samples were then dehydrated through a series of ethanol and embedded in LR white. Thin sections were cut and incubated with 0.05 M glycine in PBS for 15 min before being blocked with 5% normal goat serum + 0.5% BSA in PBS for 30 min. The sections were then incubated on drops of QH1 in 0.5% BSA in PBS at 4°C overnight. On the next day, the sections were washed and incubated with gold-conjugated (gold particles 10 nm in diameter) secondary antibody (a 1:20 ratio; Sigma) at room temperature for 1–2 h and were then washed and postfixed in 2% glutaraldehyde in PBS for 5 min. Finally, the sections were counterstained with 5% aqueous uranyl acetate and lead citrate and examined with the electron microscope.

Statistics. Each experimental group consisted of at least 10 samples. Data are presented as means ± SE. For comparisons of two means, statistical significance was evaluated by unpaired Student’s t-tests. For multiple comparisons, one-way ANOVA and Bonferroni’s multiple comparisons versus the control group were used. Differences were considered significant at \( P < 0.05 \).

RESULTS

Treatment of heart explants with VEGF165 or VEGF121. Recombinant human VEGF121 and VEGF165 were first added to the culture medium singly at various doses, and vascular growth was examined after 48 h. VEGF165 increased vascular growth in a dose-dependant manner (Fig. 2). At the 5 ng/ml dose, VEGF165 slightly increased the number of individual endothelial cells (Fig. 2B), whereas at the 10 ng/ml dose, VEGF165 increased the incorporation of endothelial cells into tubular structures (Fig. 2C). The vascular characteristics of these tubular structures have been documented in our previous work (29). The total length of the tubes normalized to the 1-mm perimeter of the heart explant was 1,407.7 ± 272.4 μm with 10 ng/ml of VEGF165 compared with 576.6 ± 111.2 μm (\( P = 0.002 \)) in control explants. When the concentration was increased to 20 ng/ml of VEGF165, tubes with greater diameters were formed (Fig. 2D), and this effect plateaued at the 50 ng/ml dose (Fig. 2, E and F). Width at half-length increased with increased concentration of VEGF165 from 9.67 ± 0.44 μm under control conditions to 22.01 ± 1.66 μm at 100 ng/ml of VEGF165 (Table 1). At higher concentrations of VEGF165 (10–100 ng/ml), the number of individual or free endothelial cells tended to decrease with statistical significance observed at the highest concentration (100 ng/ml) compared with the controls (Fig. 3). However, because the endothelial cells on or in the collagen gels exist in two populations, i.e., as individual endothelial cells or as components of the tubular structures, the total number of endothelial cells was likely increased after VEGF165 treatment. A time course study with 50 ng/ml of
Table 1. Comparison between control heart explants and those treated with VEGF165, cultured with O2, and treated with both VEGF165 and O2.

<table>
<thead>
<tr>
<th></th>
<th>No. of Tubes</th>
<th>Average Length</th>
<th>Width at Half-Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.3 ± 3.4</td>
<td>120.7 ± 5.3</td>
<td>9.67 ± 0.44</td>
</tr>
<tr>
<td>VEGF165</td>
<td>10 ng/ml</td>
<td>11.5 ± 4.1</td>
<td>116.4 ± 11.7</td>
</tr>
<tr>
<td></td>
<td>20 ng/ml</td>
<td>41.6 ± 9.4</td>
<td>137.0 ± 12.1</td>
</tr>
<tr>
<td></td>
<td>50 ng/ml</td>
<td>48.5 ± 8.7*</td>
<td>143.3 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>100 ng/ml</td>
<td>48.0 ± 6.2†</td>
<td>139.3 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>5% O2</td>
<td>38.3 ± 7.2</td>
<td>132.9 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>5% O2 + VEGF165</td>
<td>36.8 ± 13.2</td>
<td>137.0 ± 16.0</td>
</tr>
<tr>
<td></td>
<td>50 ng/ml</td>
<td>30.9 ± 8.4</td>
<td>143.6 ± 14.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. VEGF, vascular endothelial growth factor. Comparisons were made using one-way ANOVA and Bonferroni’s multiple comparisons vs. control. Differences considered significant at \( P < 0.05 \) are marked as *\( P = 0.02 \) and †\( P = 0.001 \) vs. control.

VEGF165 showed that VEGF165 induced the formation of wide tubes as early as 24 h into the study and that these tubes remained as wide tubular structures at 72 h if fresh media supplemented with 50 ng/ml of VEGF165 were changed every day. In contrast, with only a single addition of 50 ng/ml of VEGF165 at time 0, thinner and longer tubes were observed at 72 h (data not shown).

In contrast to VEGF165, VEGF121 did not increase either the number of free endothelial cells or tube formation at the same dosages. When the concentra-
tion of VEGF121 was increased to 500 and 1,000 ng/ml, there was a trend toward enhanced tube formation but no statistical significance was attained. The effects of VEGF121 from two sources (R&D Systems and Scios) used in this study were similar.

The effects of VEGF165 at 20 and 50 ng/ml doses were also examined in the presence of different concentrations of heparin or heparinases (heparinase I, II, and III singly or in combination), but no differences were observed compared with the heart explants treated with VEGF165 alone (data not shown).

Similarities and differences between VEGF165 and hypoxia. In a previous study (29) we showed that relative hypoxia (10% and 5% O2 but not 2% O2) can stimulate coronary vascular growth from cultured heart explants, and this stimulation is mediated by VEGF signaling (including VEGF121, VEGF165, and VEGF190). Are hypoxia and VEGF the same? What are the functions of different VEGF isoforms under hypoxic conditions? To answer these questions, we compared the heart explants treated with hypoxia to those treated with VEGF121 or VEGF165. Because VEGF121 did not enhance vascular growth from the heart explants, we first compared the heart explants treated with hypoxia and those treated with VEGF165 under normoxic conditions. We then further treated the heart explants with VEGF121 or VEGF165 under hypoxic conditions and compared these hearts with those treated with hypoxia alone.

We used 5% O2 in these experiments. In the hypoxic environment, although more tubes were formed (29), the width of these tubes as represented by the width at half-length was similar to those formed under normoxic conditions, whereas with the treatment of VEGF165, the tube width increased with increasing concentrations of VEGF165 (Table 1 and Fig. 4). Interestingly, tubes formed from the heart explants treated with both hypoxia and VEGF165 had a morphology intermediate to those treated with hypoxia or VEGF165 alone (Table 1 and Fig. 4D). This indicates that other molecules induced by hypoxia can interact with or oppose the effects of VEGF165. The combined effects of hypoxia and VEGF121 were also tested but we did not find any differences between the heart explants treated with hypoxia and those treated with both hypoxia and exogenous VEGF121. A combination of VEGF165 and VEGF121, both at 20 or 50 ng/ml concentrations, were also given to the heart explants but we could not discern any apparent differences compared with the heart explants treated with VEGF165 at 20 or 50 ng/ml doses alone. However, possible interactions between VEGF165 and VEGF121 cannot be excluded.

Endothelial cell lumenization (via vacuole formation) in vitro mimics in vivo coronary vasculogenesis. To examine some details of this mode of tube growth, the heart explants were also processed for evaluation at the electron microscopic level. Under all culture conditions, a lumenization process (via vacuole formation) was noted in some individual cells. Immunostaining with QH1 primary antibody and gold-labeled secondary antibody confirmed that these vacuolizing cells are indeed endothelial cells (Fig. 5). Immunogold labeling of sections from normal embryonic quail hearts (4–6 days old) showed similar vacuolizing endothelial cells (data not shown). In contrast, addition of VEGF165 stimulated the coalescence of endothelial cells to form larger vessels. Some of the vessels were seen immediately beneath the gel surface (Fig. 6A) and some were found deeper inside the gel (Fig. 6B).
DISCUSSION

This study provides two novel findings regarding coronary vascular tube formation. First, VEGF165 but not VEGF121 induces a marked vascular tube formation, and the tube morphology induced by VEGF165 is dose dependent. Second, although hypoxia enhances vascular tube formation via the up-regulation of VEGF, the morphological characteristics of the tubes formed in the presence of 50 ng/ml of exogenous VEGF165 under hypoxia differ from those induced by 50 ng/ml of VEGF165 under normoxic conditions. This indicates that the effects of hypoxia and VEGF165 are not identical. The differences observed could be due to the presence of other VEGF...
isoforms under hypoxic conditions or other molecules induced by hypoxia.

The dose of VEGF165 is an important determinant of the specific vasculogenic response. In the range from 5 to 100 ng/ml, VEGF165 first slightly increased the number of individual or free endothelial cells and then stimulated the incorporation of endothelial cells into tubular structures; at a concentration \(\geq 20\) ng/ml, VEGF165 induced widening of the vascular tubes, which plateaued at the 50 ng/ml concentration. Electron microscopic observations revealed that this widening of vascular tubes was due to a coalescence of endothelial cells in the tubular structures. This phenomenon has been observed in some earlier studies (4, 27); however, the dose-dependent vascular growth under the influence of VEGF165 had not been previously described in detail.

In contrast, VEGF121 did not increase either the number of individual endothelial cells or the formation of tubular structures at similar or even higher dosages. That two sources of VEGF121 were used in this study with similar results confirms the validity of this finding. In previous studies, however, it has been shown that VEGF121 can induce proliferation of vascular endothelial cells and expression of Flk-1 on the chorioallantoic membrane (28), and retroviral overexpression of quail VEGF122 induces hypervascularization and increased vascular permeability in the limb buds (9, 21). The effects of VEGF121 and VEGF122 were also shown to be comparable to those of VEGF165 and VEGF166 in these studies. That we did not observe any effect of VEGF121 in our heart explant culture could suggest that VEGF isoforms function in an organ-specific fashion.

RT-PCR results from our previous study (29) showed that three VEGF isoforms (VEGF122, VEGF166, and VEGF190) were upregulated proportionally by hypoxia with VEGF166 being the most abundant form (\(-40\%\)) followed by VEGF190 (\(-35\%\)) and VEGF122 (\(-25\%\)). Although this does not reflect the protein level of these different VEGF isoforms, a disproportionately high level of VEGF122 would not be expected under normal conditions. Additional studies on the effects of the relatively low level of VEGF122 and the possible interaction between VEGF122 and other VEGF isoforms are needed.

Several possibilities could explain the differences between VEGF165 and VEGF121 observed in this study. One major difference between these two splice variants is that VEGF165 can bind to heparin or heparan sulfate through the exon 7-encoded domain, whereas VEGF121 cannot. But the fact that heparin or heparinases added to the culture medium neither diminished nor enhanced the effect of VEGF165 indicates that the differences between VEGF121 and VEGF165 observed in this study were not likely due to differing heparin-binding capacities.

A second explanation could be the presence of the isoform-specific receptor neuropilin-1. Relatively high levels of neuropilin-1 are expressed in both embryonic and adult hearts (15, 26). It has been shown that overexpression of neuropilin-1 causes hypervascularization, dilation of blood vessels, and malformed hearts in chimeric mice embryos, which suggests that the VEGF-neuropilin-1 complex may play a role in normal embryonic vascular development (15). From these facts, we speculate that the interaction between VEGF165 and neuropilin-1 could contribute to the differences between VEGF121 and VEGF165 observed in this study. This possibility is currently under investigation.

Finally, differential receptor activation by VEGF165 and VEGF121 could also contribute to the differences observed. Preliminary study in our laboratory using recombinant human VEGF121 and VEGF165 in human umbilical vein endothelial cell and human coronary artery endothelial cell cultures suggests that these two VEGF isoforms differentially activate VEGF receptor 2, Flk-1.

In this study, we also observed a lumenization process (via vacuole formation) in endothelial cells under all culture conditions that is similar to that seen in normal embryonic quail hearts as well as chicken (unpublished data) and rat hearts (20). This supports the notion that endothelial cell lumenization is a normal process in both in vivo and in vitro coronary vasculogenesis. It also indicates that our in vitro system...
closely mimics in vivo coronary vascular development. QH1 has long been used as a marker for quail vascular endothelial cells and endothelial precursor cells (18), but to our knowledge this is the first time that QH1 labeling was utilized at the electron microscopy level. Accordingly, we submit that QH1 can also be used as an excellent tool to study the details of early vasculo-genic process.

In conclusion, VEGF165 induces a dose-dependent coronary vascular growth in cultured embryonic heart explants, whereas VEGF121 has no apparent effects. Whether this reflects what happens in vivo during coronary vascular development is presently unknown. A recent study in mice (2) showed that selective inactivation of the heparin-binding isoforms of VEGF that left one functional isoform (VEGF120) was insufficient for the proper development of the cardiovascular system and resulted in myocardial ischemia and perinatal or early postnatal lethality. It would be enlightening to see the effects of selective VEGF164 or VEGF188 expression, which could reveal whether each VEGF isoform has truly distinct angiogenic roles. Knowledge of the functions of different VEGF isoforms is also important in the clinical application of VEGF in treating ischemic vascular diseases. Is a combination of different VEGF isoforms superior to the application of one single VEGF isoform? What are the optimal dosages of each isoform? These questions need to be answered for us to achieve the best therapeutic effects.

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