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Vascular endothelial growth factor (VEGF) fails to improve blood flow and to promote collateralization in a diabetic mouse ischemic hindlimb model

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Abstract

Background: Angiogenic therapy with vascular endothelial growth factor (VEGF) has been proposed as a treatment paradigm for patients suffering from an insufficiency of collateral vessels. Diabetes is associated with increase in the production of VEGF and therefore additional VEGF may not be beneficial. Accordingly, we sought to determine the efficacy of VEGF therapy to augment collateral formation and tissue perfusion in a diabetic mouse ischemic hindlimb model.

Methods: Diabetic and non-diabetic mice were studied in parallel for the efficacy of VEGF administration. Diabetes was induced with streptozotocin. Hindlimb ischemia was produced by severing the left iliac artery. An outlet tube from an osmotic infusion pump with placebo/ 500 micrograms of plasmid-DNA encoding VEGF was fenestrated and tunneled into the left quadriceps muscle.

Results: VEGF induced more rapid and complete restoration of blood flow in normal mice. However, in the setting of diabetes there was no difference between VEGF Vs. placebo in the rate or adequacy of flow restoration. There was a significant increase in smooth muscle actin and Factor-VIII antigen densities in diabetic animals and in animals which received VEGF.

Conclusions: Angiogenic therapy with VEGF in the setting of diabetes does not appear to have the beneficial effects seen in the absence of diabetes.

Introduction

Considerable variability exists between individuals in the extent and functional utility of collateral vascular channels serving to effectively bypass obstructive atherosclerotic lesions [1,2]. This variability has been demonstrated in numerous prospective studies to be of primary importance in determining the amount and severity of ischemia and necrosis which occurs subsequent to vessel occlusion. The importance of angiogenesis and arteriogenesis in the development of these collateral channels is now well established [3]. Accordingly, angiogenic therapy with angiogenic cytokines has been proposed as a paradigm to treat all patients with an apparent inadequacy of collateral vessels [4].

However, diabetes mellitus (DM) is associated with a marked impairment in collateral formation and yet angiogenesis is markedly increased in several vascular beds in this disorder most notably in the retina [5,6]. Moreover, there does not appear to be a defect in angiogenic growth factor production in the setting of diabetes, but rather an excess of growth factors production, such as vascular endothelial growth factor (VEGF) which has been directly linked to pathological angiogenesis in the diabetic patient [7]. It has been suggested that impairment in collateral formation in the diabetic patient is due to a defect in the chemotaxis and in the signal transduction apparatus mediating the response to VEGF in the monocyte [7,8]. Therefore, it is not clear why or if, additional VEGF would be beneficial in restoring blood flow in the setting of diabetes. This question is of fundamental importance as the diabetic patients represent a sizable fraction of those presently characterized as having inadequate collaterals [9]. In this study we set out to determine if mice with diabetes respond to angiogenic therapy with VEGF in the same manner as non-diabetic mice.

Methods

Induction of Experimental Diabetes

All procedures and animal studies were approved by the animal board and safety committee of the Faculty of Medicine, Technion – Israel Institute of Technology and comply with all safety and regulatory guidelines. Streptozotocin selectively destroys insulin-producing beta islet cells of the pancreas providing a model of type I diabetes. Streptozotocin (Sigma, Rehovot, Israel) dissolved at 10 mg/ml in 0.1 M sodium citrate buffer (pH 5.5) was injected intraperitonealy to 10 week-old male c57 mice (35–40 gram) at a dose of 80 mg/kg. The mouse was considered diabetic and included in the study if the plasma glucose levels at 72 hours was above 250 mg/dl in 2 repeated measurements, and remained elevated. An elevated glucose level was found in 90% of mice injected with streptozotocin.

Mouse hindlimb ischemia

Animal surgery: For surgical procedures and laser Doppler analysis of hindlimb blood flow C57 mice were anesthetized by intraperitoneal pentobarbital injection (160 mg/kg). The surgical procedure was performed 4 weeks after the induction of DM and was done under a microscope. Skin incisions were performed at the groin of the left hindlimb overlying the iliac artery, and carried out downward. The iliac artery was then ligated proximally and distally with 4-0 silk ligatures, and excised. All accessory arteries were ligated and cut. Prior to closure, a 200 μl osmotic infusion pump (Alzet osmotic pumps, Durect corporation, Cupertino, CA, USA) containing a 5% glucose solution with or without 500 micrograms of plasmid DNA encoding for VEGF was implanted intra-abdominally with an outlet tube from the pump fenestrated and tunneled into the left quadriceps muscle. We have previously performed a dose response curve (0–500 micrograms of VEGF DNA) using the hindlimb ischemia model described above and the VEGF DNA vector described below and have determined that the maximal effect is seen at a dose of 500 microgram of the plasmid DNA. The pump rate has been documented to be 1 μl per hour, which given a 200 μl reservoir would allow continuous perimuscular pumping for approximately seven days. The overlying skin was then closed with continuous surgical sutures using 3-0 silk ligatures.

Study groups

In this study there were 4 groups: Normal-VEGF, Normal-placebo, DM-VEGF and DM-placebo. In each group 18–21 mice were included. The operation as well as all blood flow measurements and blood vessel density calculations were done without knowledge of the treatment given.

Mole rat VEGF

The coding sequence for VEGF used in this study was cloned from the Israeli mole rat (*Spalax ehrenbergi*), which is indigenous only to Egypt, Israel, and Syria [10]. The coding sequence of mole rat VEGF was previously described [11]. The entire approximately 1 kb of 5' UTR of mole rat VEGF mRNA was cloned by RT-PCR and juxtaposed to the mole rat VEGF165 amino acid isoform (VEGF-165) coding sequence. The sequence homology of mole rat, rat, mouse and human VEGF is over 90% in the coding region [11].

Vector/regulatory sequences used

The isolated Spalax cDNA for VEGF-165 with its 1 kb of 5' UTR were cloned into pTRE (Clontech, Palo Alto, CA, USA) and is hereafter referred to as the VEGF vector. This vector utilized the 763 bp cytomegalovirus promoter / enhancer element to drive VEGF transcription and downstream of the VEGF cDNA sequences there was an intron and a polyadenylation signal sequence derived from SV

40. Vector was purified using Qiagen columns (Hilden, Germany) and resuspended in a 5% glucose solution at a concentration of 2.5 μg/microliter. Placebo animals received only 5% glucose in the osmotic pump. We also examined in the past, a second "placebo" group which included only the backbone pTRE vector without the VEGF gene, which resulted in similar results as the placebo (data not reported here).

Monitoring hindlimb blood flow

Ischemic (left) / normal (right) limb blood flow ratio was measured using a laser Doppler blood flow meter (Perimed-4000, Jarfalla, Sweden). Measurements were performed on a multichannel laser-Doppler flowmeter (PeriFlux 4001 Master; Perimed AB, Jarfalla, Sweden) at a wavelength of 780 nm, using two probes (PF 415:42) with fiber separation of 0.5 mm. The laser Doppler uses a 12-mW helium neon laser beam that sequentially scans a defined surface area. A photodiode in the scanner collects the backscattered light emitted by the laser and the shift in the frequency of the incident light is transformed into voltage variations in the range of 0–10 V which is directly related to blood flow distribution. Previous studies have validated that Doppler flow velocity correlates with capillary density in the ischemic limb [12]. For measurements of capillary perfusion, the probes were placed perpendicular to the foot. Recordings were obtained continuously for 2 minutes each, at 5 different points over the mouse foot. The capillary blood flow was calculated, using Perisoft software (version-5.10C2), by multiplying the concentration of moving blood cells (concentration units) by mean velocity of the cells (velocity units) and expressed as perfusion units [13]. Hindlimb blood flow was monitored every week until reaching a plateau or a normal value.

Immunohistochemistry

Animals were sacrificed at predetermined time points after surgery by an overdose of sodium pentobarbital. For each limb 2 sections were selected for analysis, a proximal area (mid quadriceps muscle) and a distal area (mid gastrocnemius muscle), presented hereafter as proximal and distal.

For immunohistochemical analysis, five-micrometer thick sections were prepared from paraffin embedded tissue samples of the limbs. Sections were cut with the muscle fibers oriented transversely. Identification of endothelial cells was performed by immunostaining for Factor VIII related antigen using a polyclonal anti-factor VIII antiserum at a 1/20 dilution (Zymed, San Francisco, CA). Immunostaining for smooth muscle actin using a mouse monoclonal antibody at 1/100 dilution (Zymed) was used to identify smooth muscle cells. Immunohistochemistry was performed on an automated system (Ventanu Medical Systems, Tucson, Az). Capillary density in both ischemic and non-ischemic limbs was determined by counting endothelial cells positively stained with Factor VIII under light microscopy. Analysis of smooth muscle cells was performed in order to differentiate arterioles from capillaries or venules. The microvessel density (obtained by either Factor VIII or smooth muscle actin staining) was computed using a computerized image analysis system composed of a trichip RGB video-camera (Sony, Japan), installed on a light microscope (Zeiss, Germany) and attached to personal computer, equipped with a frame grabber. Histological images were captured, digitized and displayed on a high-resolution monitor. Five microscopic fields were analyzed, using a medium sized magnifying lens (× 200) and were loaded on a 760 × 570 pixels buffer area. The microvessel density parameter was measured using the Image Pro-Plus 4 image analysis software (Media Cybernetics, Silver Spring, MD, USA). Thirty randomly chosen high-power fields in two different light microscopic sections from the 4 sites in each limb of each animal were counted and blood vessel density expressed as number of blood vessels/mm^2. A ratio in each level, between the legs was then calculated.

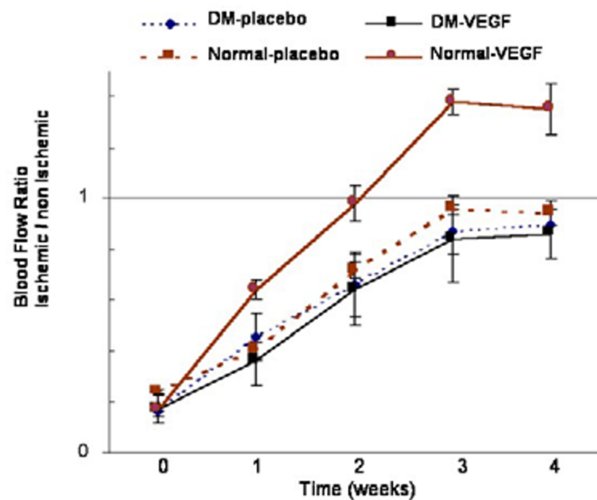

Figure 1
Blood flow ratio with time. Time dependent increase in blood flow in normal and diabetic mice with and without VEGF. Each group at each time point contains 18–21 animals. The Normal VEGF is statistically significant by 1-week as compared to all other treatment arms. There is no statistically significant difference between all other treatment groups.

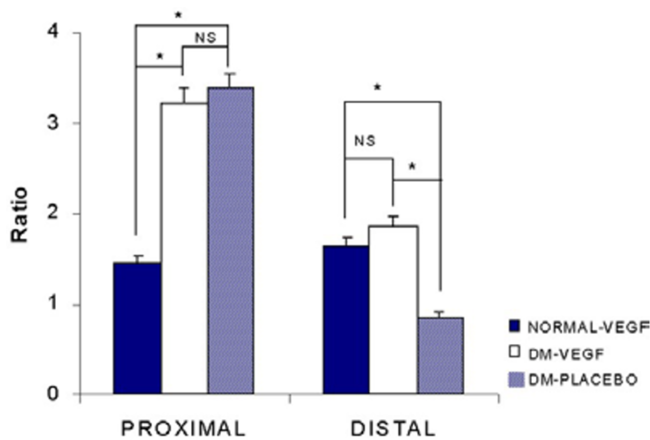

Figure 2
Ratio of factor VIII positive cells in the ischemic to nonischemic limb in the proximal and distal limb segments. Ratios (Proximal; Distal) Normal VEGF (1.4; 1.6), DM VEGF (3.2; 1.9), DM placebo (3.4; 0.9). Each group contains 4 or 5 animals. [* – p < 0.01].

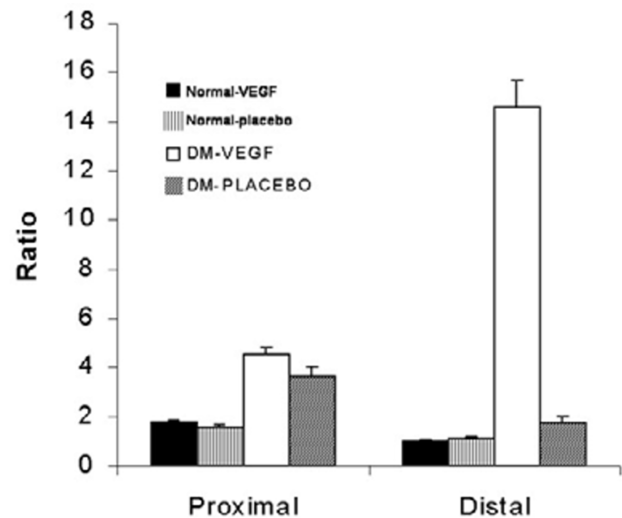

Figure 3
Ratio of smooth muscle actin positive cells in the ischemic to nonischemic limb in the proximal and distal limb segments. Each group contains 4 or 5 animals. Ratio and P Values: Proximal: VEGF arm (1.8) Vs. Placebo (1.5) p < 0.05, Vs both DM arms p < 0.001, Normal placebo Vs. both DM arms p < 0.001, DM VEGF (4.5) Vs DM-Placebo (3.6) p < 0.05. Distal: VEGF arm (1.0) Vs. Placebo (1.1) p = NS, Vs DM-VEGF p < 0.001, Vs. DM-Placebo p < 0.01, DM VEGF (14.6) Vs DM-Placebo (1.7) p < 0.01.

Statistical Analysis
Multiple parametric groups were compared using the one-way ANOVA test followed by the Bonferroni post-hoc test. P values of 0.05 or less were considered to be statistically significant

Results
Restoration of Flow
Pre-operatively the ratio of blood flow between the two hindlimbs for all animals used in this study was 1.0. Immediately after the operation in all animals the operated limb developed a blue-gray color and the ratio of the blood flow between the ischemic and normal limb was less than 0.2 (figure 1).

A more complete and rapid restoration of blood flow was observed in the normal-VEGF treated mice as compared to the other three study groups (normal placebo, Diabetic-VEGF and Diabetic placebo). In normal mice receiving VEGF there was a statistically significant improvement in flow that was evident as early as 1-week after beginning infusion with VEGF as compared to the other three groups ($p < 0.05$). There was no difference in the rate of restoration of flow between the normal placebo, diabetic placebo and diabetic VEGF groups. Restoration of flow in all groups reached a plateau by 3 weeks after which no further differences in the ratio of the ischemic to non ischemic limb was noted (Figure 1). This 21-day time point was therefore indicative of the completeness of restoration of blood flow. Normal mice treated with VEGF not only returned flow to normal at this time point but even surpassed that in the normal limb at the 3-week time point (ratio greater than 1.0). However, in all three other groups the ratio never returned to 1.0 but rather plateaued only to a level of 0.8–0.9 at 3-weeks.

Vessel Density Ratio
Immunohistochemical methods were used to identify the Factor VIII related antigen present on endothelial cells and thus representing capillaries as well as arterioles, and to identify smooth muscle actin which is found only on non-capillary vessels. Angiogenesis, or new blood vessel formation occurs at the level of the capillary. These new vessels as well as other preexisting capillaries may mature into larger vessels by the process of arteriogenesis. An increase in the number of endothelial cells was therefore interpreted as being indicative of angiogenesis while an increase in smooth muscle cells was interpreted as being indicative of arteriogenesis. There was a significant increase in factor VIII and actin staining in DM animals who did receive VEGF compared to DM animals who did not receive VEGF. VEGF in the setting of diabetes resulted in an increase in vessel density distally (Figure 2, Factor VIII staining) and an increase in smooth muscle actin staining (Figure 3) proximally and distally in comparison

to diabetic animals that did not receive VEGF. The ratio for factor VIII for normal-placebo could not be calculated due to excessive necrosis.

Discussion

We have previously demonstrated that angiogenic therapy with VEGF results in a significant improvement in the rate and completeness of restoring blood flow to an ischemic limb in normal animals [10]. The chief objective of the present study was to determine whether VEGF could improve blood flow to an ischemic limb in the setting of diabetes. We found that the answer to this question is no. We therefore sought to determine whether this failure of VEGF to stimulate blood flow in a diabetic animal was due to (1) an impairment in the ability to deliver VEGF to the diabetic ischemic limb or (2) an impairment in the ability of VEGF to stimulate endothelial growth (angiogenesis) and/or new smooth muscles (arteriogenesis). Our data clearly demonstrates that neither of these potential explanations can explain why VEGF fails to increase blood flow in the setting of diabetes. Despite the apparent benefit from VEGF as evidenced from an increase in factor VIII and smooth muscle actin in the DM animals who received VEGF as compared to those DM animals who did not receive VEGF there was no benefit in terms of blood flow.

Our findings stand in conflict with those previously described by Rivard et al [14] who reported that angiogenic therapy with VEGF results in an amelioration of blood flow to diabetic animals with an ischemic limb. Rivard et al attributed the success of providing additional VEGF in the setting of diabetes to an impairment in VEGF production in the diabetic ischemic limb. However the findings reported by Rivard et al are not consistent with a growing body of literature demonstrating that there is an increased production of VEGF in the setting of diabetes [15]. Cooper et al [16] have shown in diabetic rats an increase in VEGF production as well as an upregulation of VEGF receptors. Hyperglycemia results in a dramatic upregulation of VEGF expression in a variety of cell types [17,18], which is due to a transcriptional activation of the VEGF gene (Levy et al, unpublished observations). This increase in VEGF production in the setting of diabetes is consistent with an increase in angiogenesis in diabetic animals and humans at sites in which ischemia is present such as in the retina.

Since there does not appear to be a defect in angiogenesis or arteriogenesis in the setting of increased VEGF in diabetic animals but there is an impairment in the restoration of blood flow we need to consider other pathogenic mechanisms which do not involve a deficiency in VEGF production [19]. One of the key features involved in the maturation of blood vessels into larger conduits is a remodeling of the vessel by monocyte/macrophages. The ability of monocytes to migrate towards a gradient of VEGF is severely impaired in diabetic individuals [7]. A second defect present in diabetic individuals that could result in a decreased blood flow despite an adequate number of blood vessels is an impairment in endothelial dependent relaxation [5,9]. Recently, Tepper et al [20] reported that circulating endothelial progenitor cells from type II diabetes patients had decreased adherence to human umbilical vein endothelial cells activated by tumor necrosis factor-α (TNF-α) and were less likely to participate in tubule formation compared with controls. Further research on the interaction between DM and angiogenesis is warranted.

Conclusions

In diabetes, the VEGF dependent angiogenic pathway may be activated to maximum, therefore giving more of this factor may not further augment blood flow.

List of abbreviations

Diabetes mellitus – DM

Smooth muscle actin – SMA

Tumor necrosis factor-α (TNF-α)

Vascular endothelial growth factor – VEGF

Authors' contributions

Study concept and design: AR, SN, AH, APL.

Acquisition of data: AR, SN, IR, ZAA, AA, ES, OL, MF.

Analysis and interpretation of data: AR, IR, NSL, MF, AH, APL.

Drafting of the manuscript: AR, SN, IR, EN, AH, APL

Statistical expertise: ES.

Study supervision: AH, APL.

Critical revision of the manuscript for important intellectual content: All authors

All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflicts of interest.

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