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Modulating Notch Signaling to Enhance Neovascularization and Reperfusion in Diabetic Mice

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Abstract

Diabetes can diminish the responsiveness to angiogenic factors (e.g., VEGF) important for wound healing and the treatment of ischemic diseases, and this study investigated the hypothesis that this effect can be reversed by altering Notch signaling. Aortic endothelial cells (ECs) isolated from diabetic mice demonstrated reduced sprouting capability *in vitro*, but adding a Notch inhibitor (DAPT) led to cell-density and VEGF-dose dependent enhancement of proliferation, migration and sprouting, in both 2-D and 3-D cultures, as compared to VEGF alone. The *in vivo* effects of VEGF and DAPT were tested in the ischemic hindlimbs of diabetic mice. Combining VEGF and DAPT delivery resulted in increased blood vessel density (~150%) and improved tissue perfusion (~160%), as compared to VEGF alone. To examine if DAPT would interfere with vessel maturation, DAPT was also delivered with a combination of VEGF and platelet derived growth factor (PDGF). DAPT and PDGF did not interfere with the effects of the other, and highly functional and mature networks of vessels could be formed with appropriate delivery. In summary, modulating Notch signaling enhances neovascularization and perfusion recovery in diabetic mice suffering from ischemia, suggesting this approach could have utility for human diabetics.

INTRODUCTION

Disease-specific strategies will likely be needed to appropriately promote neovascularization for the treatment of ischemic diseases, and likely will be multifactorial. For example, the significantly increased risk of vascular diseases with diabetes [1] likely results from dysfunctions of endothelial cells, endothelial progenitor cells, monocytes, and vascular smooth muscle cells[2–7], abnormal extracellular matrix[8], and impaired growth factor signaling, including decreased expression of VEGF and VEGF receptor 2 (VEGFR2) and defects in VEGF receptor mediated signal transduction in the cardiac and peripheral vasculature[9–11]. Approaches to promote angiogenesis that do not address the diminished host VEGF responsiveness will likely not be effective in the context of diabetes[12]. Moreover, broad up-

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regulation of neovascularization (e.g., systemic delivery of exogenous growth factors) may introduce excessive angiogenesis in non-targeted organs (e.g., eyes and kidneys) where endogenous levels of angiogenic factors are already high, and lead to retinopathy or nephropathy. Therefore local induction of neovascularization only at the site of ischemia will likely be required.

It may be possible to recover the impaired responsiveness of diabetic endothelial cells to angiogenic stimuli such as vascular endothelial growth factor (VEGF) by interfering with Notch signaling. Notch signaling is required for arterial-venous differentiation, embryonic/postnatal angiogenesis and arteriogenesis, and tumor angiogenesis [13–17]. A key role of Notch signaling in postnatal angiogenesis has recently been recognized, as this signaling maintains the quiescent state of the endothelium by suppressing endothelial cell proliferation, inducing endothelial cell contact inhibition, and regulating endothelial tip cell formation and vessel branching [18–23]. VEGF signaling lies upstream of the Notch pathway, and activation of VEGF signaling (e.g., binding of VEGF to its VEGFR2 receptor) activates Notch signaling by increasing the expression of Notch ligands such as Dll4 [18,24–25]. Upregulation of Notch ligands and their binding to neighboring Notch receptors in turn then downregulate VEGFR2 expression[26–27]. Thus, Notch signaling is able to assist in pruning and patterning vascular networks by locally regulating endothelial cell responsiveness to global pro-angiogenic stimuli, particularly VEGF[28–30]. Previous studies from this lab have shown that a sustained and localized delivery of a Notch inhibitor could enhance the responsiveness of ECs in normal mice to VEGF, and promote angiogenesis without causing systemic side effects[31].

This study is based on the hypothesis that the impaired angiogenic response in diabetics to VEGF could be rescued by appropriate exposure to drugs modulating Notch signaling. This hypothesis was first tested *in vitro* with aortic ECs isolated from insulin deficient mice (induced by streptozotocin), and subsequently *in vivo* with the same diabetic mice model subject to surgically induced hindlimb ischemia by femoral artery ligation. Streptozotocin (STZ) induces diabetes mellitus by causing pancreatic insulinitis and destruction of insulin-secreting beta cells, and STZ-induced diabetes is a commonly used diabetic animal model[32–33]. This murine model of hindlimb ischemia mimicking peripheral arterial disease[34], is a widely used model in studies of limb revascularization strategies.

MATERIALS AND METHODS

Induction of diabetic mice

Insulin deficient diabetes was induced in 4 to 6 week-old male C57 mice by intraperitoneal injection with streptozotocin (100 mg/kg) (Sigma, St Louis, MO) on 2 consecutive days after overnight fasting. Diabetes syndrome was confirmed by measuring the blood glucose level using a glucometer (Glucometer Elite XL; Bayer, Elkhart, IN) following collection of around 2 microliters of blood from the tail vein. A blood glucose level larger than 250 mg/dL was considered to represent diabetes, as shown previously[35]. Body weight of the diabetic mice was measured once a week and insulin injection was given subcutaneously at a dose of 0.1–0.2 units per mouse 2 to 3 times a week only when there was a $\geq 10\%$ weight loss. After induction of diabetes, diabetes syndrome had been maintained for 8 weeks before the diabetic mice were subject to any experiments, and the blood glucose levels of diabetic mice were maintained above 250 mg/dL during all experimentation.

Isolation of endothelial cells

The method of isolating endothelial cell has been previously published [36] which results in 99% CD31-positive endothelial cells. Briefly, Mice under deep anaesthesia were perfused with PBS through the left ventricle. The descending aorta was dissected free from adventitial tissue,

and opened longitudinally with scissors. The aorta was then removed by cutting from remaining adventitial tissue, transferred to a collagenase (Worthington Biochemical Corp, NJ) solution (0.2% w/v in DMEM/0.1% BSA), and incubated at 37 °C with rocking for one hour. The digest was passed through a 40 µm cell strainer and then incubated with Dynabeads (Invitrogen, CA) conjugated with sheep anti-rat IgG and subsequently coated with CD31 anti-mouse monoclonal antibody (BD Pharmingen, CA). A ratio of ~5 beads per endothelial cell was used, and the solution was rotated at 4 °C for 30–60 minutes. Suspensions were then placed in a magnet for approximately 30 seconds to separate bound cells. The solution was decanted while maintained in magnet. DMEM/0.1% BSA was added to resuspend cells coupled to the magnets, by inverting the tube several times. The magnetic isolation was repeated twice. Cells were then resuspended in growth medium containing DMEM (low glucose), 10% v/v horse serum, 50 µg/mL endothelial cell growth substance (ECGS, Biomedical Technologies, MA), and 100 µg/mL heparin. A diabetic phenotype is retained in the cultured primary mouse endothelial cells[37]. We therefore used growth medium with non-diabetic concentrations of glucose so that differences between groups would reflect abnormalities of diabetes rather than differences in culture conditions. Cells were then counted and plated at a density of 15000/cm² in 0.1% collagen-coated well plates. When cells were 70–80% confluent, they were trypsinized and sorted again with Dynabeads coated with ICAM2/CD102 rat anti-mouse IgG2α monoclonal antibody, to further increase the purity of endothelial cells. Sorted cells were confirmed to exhibit endothelial cell phenotype by CD31 staining (99% CD31-positive endothelial cells), as we have previously published[36]. In addition to CD31, cells isolated by this method express eNOS, KDR/VEGFR2, and VE-cadherin at the protein level. Cells used for the subsequent experiments were between passage 5 and 7. Cells were also isolated from age-matched non-diabetic mice as controls.

Cell proliferation, migration and sprouting assays

For cell proliferation assays, endothelial cells isolated from diabetic mice were seeded in 24-well plates at varied seeding densities. Media was supplemented with recombinant human VEGF-A₁₆₅ (10ng/mL) (R&D systems, MN) and/or 0.1% (v/v) of solutions with different concentrations of gamma secretase inhibitor IX (also named DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester) (EMD Chemicals, NJ). DAPT did not affect cell adhesion[31]. For cell migration analysis in transwells, 5×10⁵ cells in 100µl EGM-2MV (Lonza, MD) were seeded in the top well of transwell dishes (Costar, MA) with a pore size of 5 µm. Cell migration towards the bottom well (containing 600µl endothelial basal medium supplemented with 50ng/mL VEGF and 50ng/mL recombinant hepatocyte growth factor (HGF) (R&D systems, MN)) was quantified by a coulter counter after 24 hours. For O-ring proliferation and migration assays, PDMS O-rings (PDMS films with 5 mm diameter holes punched in the center) were placed in 12-well plates, and ECs were seeded (at a seeding density of 26,000/cm²) only inside the O-rings. The O-rings were subsequently removed 1–2 days after cells reached confluence, and the cells were simultaneously exposed to DAPT and VEGF, and were able to proliferate and migrate in concert. For sprouting assays, endothelial cells were seeded onto Cytodex 3 microcarriers (Amersham Biosciences, NJ) at a ratio of 3× 10⁶ cells per 40mg beads and cultured on a shaker until cells reached confluence. The beads were then mixed with a solution of human fibrinogen solution (4mg/mL) and aprotinin (500µg/mL) at a volume ratio of 1: 3:0.4. The beads-fibrinogen mixture was then placed into 24-well plates to which human thrombin (Sigma, MO) (25 units/mL) was added at a volume ratio of 4:5 and mixed thoroughly by pipetting. The mixtures of beads, fibrinogen, thrombin and aprotinin were kept in the wells at room temperature for 5 min before being transferred to a 37°C incubator for 10 min to form hydrogels. Fresh EGM-2 media was placed on top of the gel for 30min, removed and replaced with EGM basal medium containing different concentrations of VEGF and Notch inhibitor. After 6 days, media was removed and gels were washed twice with PBS, and fixed with 4% formaldehyde overnight at 4°C. The formaldehyde

solution was then aspirated, the gels were washed twice with PBS, and the number of sprouts were counted and normalized to the number of beads. (150–250 beads analyzed for each experimental condition). Sprouts were defined as a linear extension containing more than one cell. To quantify the number of ECs in the 3-D gels, fibrin gels were dissolved with a plasmin solution (1 µg/µL), and ECs adhered on microbeads were detached with a 0.2% collagenase solution and counted with a hemocytometer or coulter counter.

Murine ischemic hindlimb model

All protocols were approved by Harvard's Institutional Animal Care and Use Committee. The animals used were diabetic C57BL/6J mice (Jackson Laboratory, ME) of which diabetes syndrome had been maintained for 8 weeks. Unilateral hindlimb ischemia was created surgically. In brief, the animals were anesthetized by intraperitoneal injections of ketamine (80 mg kg⁻¹) and xylazine (5 mg kg⁻¹). The external iliac and femoral artery and vein were ligated, and 50 µL alginate hydrogel (prepared by the protocol described previously[31]) incorporating 3 µg VEGF, and/or 3 µg PDGF, and/or 860 ng DAPT was injected near the distal end of the ligation site. Incisions were closed by 5-0 Ethilon sutures (Johnson & Johnson, NJ). Blood flow in the hindlimb was monitored for 3–4 weeks by a laser Doppler perfusion imaging (LDPI) system (Perimed AB, Sweden) and the results were normalized to the control unligated limb of the same animal, as shown previously[31]. 5–7 mice were used as replicates for each condition group. Tissue necrosis of hindlimbs subjected to surgery was visually examined and grouped as normal (displaying limb integrity from non-ischemic hindlimbs of the same animal), presenting one necrotic toe, or multiple necrotic toes.

Histology and immunohistochemistry

Hindlimb muscle tissues between the two suture knots defining the ligation site were dissected and fixed by Z-fix (Anatech, MI) overnight and changed into 70% EtOH for storage prior to histology processing. Samples were embedded in paraffin and sectioned (5 µm thick) onto slides by Paragon (Paragon Bioservices, MD). For CD31 staining, sections were incubated with primary anti-mouse CD31 antibody (1:250) (Pharmingen, CA), followed by incubation with an anti-rat mouse biotinylated secondary (1:200) (Vector Laboratories, CA), and amplified by a Tyramide Signal Amplification (TSA) Biotin System (Perkin Elmer Life Sciences, MA). Staining was developed using DAB+ substrate chromogen (DAKO, CA) and counterstained with Mayer's Hematoxylin. Capillary densities were quantified by counting the CD31 positive capillary numbers, normalized to the tissue area, in 30 randomly chosen high-power (200x, 400x) fields. For α -Smooth muscle actin (SMA) staining, sections were incubated with an alkaline phosphatase-conjugated antibody to SMA (Sigma) (1:50) for 2 h at room temperature. Staining was detected through incubation with Vector Red substrate solution (Vector Laboratories) for 20 min. Sections were counterstained with hematoxylin. Images were captured with an Olympus-IX81 light microscope connected to an Olympus DP70 digital image capture system.

STATISTICAL ANALYSIS

All statistical comparisons were performed using Student's t-test (two-tail comparisons). Differences between conditions were considered significant only for $p < 0.05$.

RESULTS

Diminished VEGF responsiveness of diabetic aortic ECs

The diminished angiogenic response to VEGF in diabetics was first confirmed *in vitro* using aortic endothelial cells (ECs) isolated from insulin deficient diabetic mice. As compared to ECs from non-diabetic controls, the diabetic ECs exhibited a considerable reduction in sprout

formation, a key early step in angiogenesis, in response to VEGF stimulation, (Fig 1a and Fig S1), and also exhibited diminished proliferation and migration (Fig 1b–1c).

Effects of Notch inhibition on EC in 2-D

Previous studies have demonstrated that the VEGF responsiveness of normal EC can be enhanced by interfering with Notch signaling via small molecules such as gamma secretase inhibitor IX (also named DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester)[31]. To investigate the effects of DAPT, the proliferation and migration of diabetic ECs in standard 2-D culture were then investigated. DAPT did not demonstrate any significant effects on cell phenotype in the absence of VEGF, regardless of EC seeding density, but unexpectedly, exhibited a dose-dependent inhibitory effect on EC proliferation in the presence of VEGF, when cells were plated at a typical seeding density (5000/cm²) in culture (Fig 2a). However, the DAPT dose that did not affect proliferation of ECs at a low seeding density (5000/cm²) did lead to an increase of cell proliferation at a higher seeding density (15000/cm², Fig 2b). To better mimic the confluent nature of ECs *in vivo*, and their ability to simultaneously migrate and proliferate during angiogenesis, cells were then seeded at confluence on surfaces initially confined by a PDMS Oring. The O-ring was subsequently removed to simultaneously expose cells to DAPT and VEGF, to allow cells to proliferate and migrate in concert (illustration in Fig 2c). Exposure to media or DAPT alone led cells in the center of the culture region to become sub-confluent (Fig 2c), probably due to cell apoptosis. Adding VEGF allowed cells in the center region to proliferate, except at the highest VEGF concentration (100ng/mL), where cells started to detach. VEGF enhanced outward cell migration from the boundary of the O-ring, in a dose-dependent manner. Low concentrations of DAPT ($\leq 2\mu\text{M}$) did not exhibit a significant effect on cell proliferation regardless of the VEGF concentration, but enhanced cell migration at a low concentration of VEGF (50ng/mL). Increasing the DAPT concentration (10 μM) greatly enhanced cell proliferation at the intermediate VEGF concentration (50ng/mL), and cell migration at high VEGF concentrations (50–100ng/mL). The increase in cell number reflected the contributions of proliferating cells both within and outside of the original culture area (defined by the O-ring). However, the major increase in cell number resulted from cell proliferation within the original culture areas and not migrating cells, as only a small fraction of the total cells were outside of the boundary (<30% of the initial seeding number). Interestingly, ECs in the center region assembled into spiral structures when treated with a combination of VEGF and DAPT (Fig 2c), and this was not seen with VEGF treatment alone.

Effects of Notch inhibition on EC in 3-D

The effects of DAPT on diabetic ECs were next examined in more physiologically relevant 3-D *in vitro* systems, and the initial cell seeding density as well as the doses of VEGF and DAPT were again varied to examine their roles. First, cells were simply encapsulated as individual cells in 3-D fibrin gels at two cell seeding densities. At both densities, cells did not exhibit significant cell-cell contact (Fig S2A). VEGF alone enhanced cell proliferation at both cell densities, but the effect was diminished for the lower density condition. Interestingly, combining DAPT with an optimal level of VEGF enhanced cell proliferation in the sub-confluent 3-D condition (Fig 3a–3b). This finding suggests an effect of DAPT in 3-D culture, and also supports the VEGF-dose dependency of Notch inhibition effects on EC proliferation seen in 2-D studies. In a follow-up experiment, to mimic the confluent nature of ECs *in vivo*, cells were first seeded onto microbeads and allowed to reach confluence, and then microbeads were embedded in 3-D fibrin gels. Sprout formation, which involves the coordination of proliferation and migration, occurred with VEGF stimulation as expected. (Fig S2B). VEGF alone enhanced both cell proliferation and sprout formation, in a dose-dependent manner (Fig 3c–3d). Combining VEGF and DAPT further increased cell proliferation, and the effect peaked at the highest concentration of DAPT (10 μM) with VEGF concentrations of 50 and 100ng/mL. A significant enhancement of sprout formation, however, occurred only at the intermediate

VEGF concentration (50ng/mL) when combined with the highest DAPT concentration (10 μ M).

Effect of VEGF&DAPT *in vivo* in diabetic mice

The influence of combined VEGF and Notch signaling was then examined *in vivo* in diabetic mice with surgically induced hindlimb ischemia. Either VEGF alone or DAPT alone, and a combination of both were released from alginate gels injected at the ischemic site to provide a local and sustained presentation. The number of blood vessels in the ischemic tissue, and the overall blood perfusion level were quantified. Delivery of VEGF alone increased the vessel density 2-fold above blank controls, while DAPT alone did not lead to a significant increase. However, combining VEGF and DAPT led to a higher vessel density than VEGF alone (Fig 4a–4b). This combination of VEGF and DAPT also exhibited the fastest recovery of blood flow, as compared to delivery of either factor alone (Fig 4c). Delivery of VEGF led to a higher perfusion level than blank controls, while DAPT delivery alone led to a similar level of perfusion as VEGF delivery at Day 7, but levels subsequently subsided to similar levels as blank controls at later time-points. Histological analysis of the tissues in the ischemic region indicated that no observable edema resulted with the delivery of either VEGF or DAPT, or their combination with this approach (Fig S3). Furthermore, we have previously shown that DAPT *in vivo* delivery using our system did not result in systemic effects, as indicated by analyzing the crypt cells in the small intestine[31], since the crypt cells have been previously identified as a target of circulating DAPT[17, 38].

Vessel maturation

Angiogenesis is a multi-stage process that includes not only the formation of new blood vessels, but also the recruitment and association of perivascular cells as the vessels mature. The effect of DAPT on the maturation of blood vessels was next analyzed. Previous studies have shown a sequential delivery of VEGF followed by platelet derived growth factor (PDGF) increases the recruitment of mural cells and generates a more matured vasculature than delivery of VEGF alone[39]. Therefore the influence of DAPT on vessel formation and maturation was examined in this system. Dual delivery of VEGF/DAPT led to a similar capillary density as triple delivery of VEGF/PDGF/DAPT, and both of these conditions were significantly higher than VEGF/PDGF, VEGF alone, or PDGF alone (Fig 5a–5b), suggesting that the presence of PDGF did not interfere with the vessel forming ability of VEGF and DAPT. It should be noted that VEGF and DAPT are both released quickly with the system, while PDGF release is delayed[40], leading to sequential, not simultaneous exposure of tissues to VEGF, DAPT and PDGF. The very low percentage (based on weight) of the gel comprised by any of these three agents (VEGF – 6%, PDGF – 6%, DAPT – 0.1%; mass percentages) makes it unlikely any of these would influence the release of the other, or that release would vary with changes in the dose, as found experimentally[31]. The association of perivascular cells with vessels, as an indication of vessel maturation, was next examined via staining for α -smooth muscle actin (SMA). The presence of PDGF, either in combination with VEGF or with VEGF/DAPT, significantly increased the number of SMA positive cells, as compared to VEGF alone, DAPT alone, or a combination of VEGF and DAPT (Fig 5c–5d). This finding supported past reports of PDGF's effects on maturation of vessels induced by exogenous VEGF[39]. There was no significant difference in the number of SMA positive cells between VEGF/PDGF and VEGF/PDGF/DAPT, however, suggesting that DAPT and PDGF did not interfere with the effects of each other. The tissue perfusion was then measured as a test of the function of the regenerated vasculature. Dual delivery of VEGF/PDGF led to higher perfusion levels than VEGF or PDGF alone, indicating that the maturation of blood vessels induced by PDGF contributed to functional recovery. Interestingly, the combination of DAPT and VEGF, although exhibiting a lower number of SMA positive cells than the combination of VEGF and PDGF, led to a similar perfusion level as the VEGF/PDGF/DAPT condition, and was statistically higher than the

VEGF/PDGF condition (Fig 5e). As a result, VEGF/DAPT led to a lower degree of tissue and toe necrosis than VEGF or VEGF/PDGF (Fig S4).

DISCUSSION

Enhancing the responsiveness of endothelial cells in diabetics to VEGF may provide a new approach to treat macrovascular complications of diabetes. The reduction of VEGFR2 level and impaired downstream signaling in diabetes are associated with the insufficient angiogenesis implicated in diabetic ulcers and ischemic diseases[8–9]. Localized modulation of Notch signaling provides the possibility to alter regional angiogenesis processes (e.g., enhance vessel densities in ischemic heart and legs) while not disturbing distant organs (e.g., eyes). Interestingly, it has been suggested that the impaired angiogenic responses in ischemic diseases in both type 1 and type 2 diabetic models are also associated with the diminished VEGF responsiveness of endothelial progenitor cells, monocytes and cardiomyocytes[10,12, 41–45]. Although the role of the Notch pathway on VEGF signaling was tested on ECs in the current study, it is likely that Notch inhibition may also affect other cell types. If so, the general idea of recovering the responsiveness to angiogenic stimuli may have an even broader impact.

The findings in this study that DAPT could lead to functional blood flow recovery are in sharp contrast to the previous tumor angiogenesis studies in which Notch inhibition, via bolus systemic injection of Notch inhibitors, led to excessive and dysfunctional vasculature[16–17]. The differences between the current and past studies likely relate to the local and optimal level of Notch inhibition accomplished with localized gel delivery in the current study. A previous study indicates that an excessive amount of Notch inhibitor, even with local delivery, can increase capillary densities, but fails to enhance tissue perfusion[31], is consistent with past tumor angiogenesis studies[16–17]. In addition, the findings that the effects of Notch signaling on ECs are cell-density and VEGF-dose dependent may explain seemingly contradictory previous observations. For example, some reports suggest that Notch inhibition promotes endothelial cell proliferation and sprout formation [17,46], while other studies suggest that inhibiting Notch signaling decreases endothelial cell proliferation and migration [47–48]. Our results suggest that both may be correct, but depend on the exact experiment conditions (e.g., 2-D or 3-D, cell density). Altogether, these findings suggest that the role of Notch signaling on angiogenesis is highly context dependent; however, the mechanisms of these effects need further investigation.

The findings that DAPT and PDGF did not interfere with the effects of each other, and the delivery of VEGF/DAPT led to a faster perfusion recovery than the delivery of VEGF/PDGF opens up new possibilities in designing strategies to enhance angiogenesis, and suggests future studies investigating the relative contribution of vessel density and maturation to ultimate tissue perfusion. Altogether, this report provides a new approach of modulating Notch signaling to promote neovascularization and enhance perfusion in the ischemic tissues in diabetics, where simple administration of single angiogenic factors may not be effective due to the impaired responsiveness resulting from diabetes.

CONCLUSIONS

The results of these studies demonstrate that modulating Notch signaling via a local and sustained delivery of a Notch inhibitor, DAPT, allows one to rescue the impaired VEGF responsiveness of ECs in diabetic mice, promote angiogenesis, and achieve effective tissue perfusion. Further, DAPT did not seem to interfere with vessel maturation or cause edema. This report also demonstrates, for the first time, that the effects of Notch signaling on ECs are cell-density and VEGF-dose dependent. In the presence of VEGF, DAPT could exhibit either inhibitory or enhancing effects on EC proliferation and migration, depending on the cell

density. The relative strength of VEGF to Notch inhibition is also important in determining EC phenotype, both in 2-D and 3-D culture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, et al. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. *Circulation* 1999;100:1134–46. [PubMed: 10477542]
2. Rask-Madsen C, King GL. Mechanisms of Disease: endothelial dysfunction in insulin resistance and diabetes. *Nat Clin Pract Endocrinol Metab* 2007;3:46–56. [PubMed: 17179929]
3. Mäkimattila S, Yki-Järvinen H. Endothelial dysfunction in human diabetes. *Curr Diab Rep* 2002;2:26–36. [PubMed: 12643120]
4. Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes* 2004;53:195–9. [PubMed: 14693715]
5. Tamarat R, Silvestre JS, Le Ricousse-Roussanne S, Barateau V, Lecomte-Raclet L, Clergue M, et al. Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. *Am J Pathol* 2004;164:457–66. [PubMed: 14742252]
6. Ding H, Triggle CR. Endothelial cell dysfunction and the vascular complications associated with type 2 diabetes: assessing the health of the endothelium. *Vasc Health Risk Manag* 2005;1:55–71. [PubMed: 17319098]
7. Loomans CJ, De Koning EJ, Staal FJ, Rabelink TJ, Zonneveld AJ. Endothelial progenitor cell dysfunction in type 1 diabetes: another consequence of oxidative stress? *Antioxid Redox Signal* 2005;7:1468–75. [PubMed: 16356109]
8. Martin A, Komada MR, Sane DC. Abnormal angiogenesis in diabetes mellitus. *Med Res Rev* 2003;23:117–45. [PubMed: 12500286]
9. Simons M. Angiogenesis, arteriogenesis, and diabetes: paradigm reassessed? *J Am Coll Cardiol* 2005;46:835–7. [PubMed: 16139133]
10. Hochberg IHA, Levy AP. Regulation of VEGF in diabetic patients with critical limb ischemia. *Ann Vasc Surg* 2001;15:388–92. [PubMed: 11414092]
11. He Z, Opland DM, Way KJ, Ueki K, Bodyak N, Kang PM, et al. Regulation of vascular endothelial growth factor expression and vascularization in the myocardium by insulin receptor and PI3K/Akt pathways in insulin resistance and ischemia. *Arterioscler Thromb Vasc Biol* 2006;26:787–93. [PubMed: 16469952]
12. Roguin A, Nitecki S, Rubinstein I, Nevo E, Avivi A, Levy NS, et al. Vascular endothelial growth factor (VEGF) fails to improve blood flow and to promote collateralization in a diabetic mouse ischemic hindlimb model. *Cardiovasc Diabetol* 2003;2:18. [PubMed: 14683529]
13. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999;284:770–6. [PubMed: 10221902]
14. Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, et al. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* 2001;128:3675–83. [PubMed: 11585794]

15. Shawber CJ, Kitajewski J. Notch function in the vasculature: insights from zebrafish, mouse and man. *BioEssays* 2004;26:225–34. [PubMed: 14988924]
16. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale N, et al. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 2006;444:1032–7. [PubMed: 17183313]
17. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 2006;444:1083–7. [PubMed: 17183323]
18. Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 2006;7:678–89. [PubMed: 16921404]
19. Sainson RC, Harris AL. Regulation of angiogenesis by homotypic and heterotypic notch signalling in endothelial cells and pericytes: from basic research to potential therapies. *Angiogenesis* 2008;11:41–51. [PubMed: 18256896]
20. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003;161:1163–77. [PubMed: 12810700]
21. Nosedá M, Chang L, McLean G, Grim JE, Clurman BE, Smith LL, et al. Notch activation induces endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. *Mol Cell Biol* 2004;24:8813–22. [PubMed: 15456857]
22. Hellström M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* 2007;445:776–80. [PubMed: 17259973]
23. Suchting S, Freitas C, le Noble F, Benedito R, Breant C, Duarte A, et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc Natl Acad Sci U S A* 2007;104:3225–30. [PubMed: 17296941]
24. Gridley T. Notch signaling in vascular development and physiology. *Development* 2007;134:2709–18. [PubMed: 17611219]
25. Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, et al. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A* 2007;104:3219–24. [PubMed: 17296940]
26. Williams CK, Li JL, Murga M, Harris AL, Tosato G. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood* 2006;107:931–9. [PubMed: 16219802]
27. Harrington LS, Sainson RC, Williams CK, Taylor JM, Shi W, Li JL, et al. Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells. *Microvasc Res* 2008;75:144–54. [PubMed: 17692341]
28. Uyttendaele H, Ho J, Rossant J, Kitajewski J. Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *Proc Natl Acad Sci U S A* 2001;98:5643–8. [PubMed: 11344305]
29. Roca C, Adams RH. Regulation of vascular morphogenesis by Notch signaling. *Genes Dev* 2007;21:2511–24. [PubMed: 17938237]
30. Shawber CJ, Funahashi Y, Francisco E, Vorontchikhina M, Kitamura Y, Stowell S, et al. Notch alters VEGF responsiveness in human and murine endothelial cells by direct regulation of VEGFR-3 expression. *J Clin Invest* 2007;117:3369–82. [PubMed: 17948123]
31. Cao L, Arany PR, Wang YS, Mooney DJ. Promoting angiogenesis via manipulation of VEGF responsiveness with notch signaling. *Biomaterials* 2009;30:4085–93. [PubMed: 19481797]
32. Like AA, Rossini AA. Streptozotocin-induced pancreatic insulinitis: new model of diabetes mellitus. *Science* 1976;193:415–7. [PubMed: 180605]
33. Rees DA, Alcolado JC. Animal models of diabetes mellitus. *Diabet Med* 2005;22:359–70. [PubMed: 15787657]
34. Couffignal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM. Mouse model of angiogenesis. *Am J Pathol* 1998;152:1667–79. [PubMed: 9626071]
35. Way KJ, Isshiki K, Suzuma K, Yokota T, Zvagelsky D, Schoen FJ, et al. Expression of connective tissue growth factor is increased in injured myocardium associated with protein kinase C beta2 activation and diabetes. *Diabetes* 2002;51:2709–18. [PubMed: 12196463]

36. Rask-Madsen C, Li Q, Freund B, Feather D, Abramov R, Wu IH, et al. Loss of insulin signaling in vascular endothelial cells accelerates atherosclerosis in apolipoprotein E null mice. *Cell Metab* 2010;11:379–89. [PubMed: 20444418]
37. Hatley ME, Srinivasan S, Reilly KB, Bolick DT, Hedrick CC. Increased production of 12/15 lipoxygenase eicosanoids accelerates monocyte/endothelial interactions in diabetic db/db mice. *J Biol Chem* 2003;278:25369–75. [PubMed: 12734208]
38. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* 2005;435:959–63. [PubMed: 15959515]
39. Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. *Nat Biotechnol* 2001;19:1029–34. [PubMed: 11689847]
40. Hao X, Silva EA, Mansson-Broberg A, Grinnemo KH, Siddiqui AJ, Dellgren G, et al. Angiogenic effects of sequential release of VEGF-A(165) and PDGF-BB with alginate hydrogels after myocardial infarction. *Cardiovasc Res* 2007;75:178–85. [PubMed: 17481597]
41. Gallagher KA, Goldstein LJ, Thom SR, Velazquez OC. Hyperbaric oxygen and bone marrow-derived endothelial progenitor cells in diabetic wound healing. *Vascular* 2006;14:328–37. [PubMed: 17150153]
42. Tchaikovski V, Olieslagers S, Bohmer FD, Waltenberger J. Diabetes mellitus activates signal transduction pathways resulting in vascular endothelial growth factor resistance of human monocytes. *Circulation* 2009;120:150–9. [PubMed: 19564559]
43. Waltenberger J, Lange J, Kranz A. Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus: A potential predictor for the individual capacity to develop collaterals. *Circulation* 2000;102:185–90. [PubMed: 10889129]
44. Waltenberger J. Impaired collateral vessel development in diabetes: potential cellular mechanisms and therapeutic implications. *Cardiovasc Res* 2001;49:554–60. [PubMed: 11166268]
45. Sasso FC, Torella D, Carbonara O, Ellison GM, Torella M, Scardone M, et al. Increased vascular endothelial growth factor expression but impaired vascular endothelial growth factor receptor signaling in the myocardium of type 2 diabetic patients with chronic coronary heart disease. *J Am Coll Cardiol* 2005;46:827–34. [PubMed: 16139132]
46. Sainson RC, Aoto J, Nakatsu MN, Holderfield M, Conn E, Koller E, et al. Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis. *FASEB J* 2005;19:1027–9. [PubMed: 15774577]
47. Takeshita K, Satoh M, Ii M, Silver M, Limbourg FP, Mukai Y, et al. Critical role of endothelial Notch1 signaling in postnatal angiogenesis. *Circ Res* 2007;100:70–8. [PubMed: 17158336]
48. Chigurupati S, Arumugam TV, Son TG, Lathia JD, Jameel S, Mughal MR, et al. Involvement of notch signaling in wound healing. *PLoS ONE* 2007;2:e1167. [PubMed: 18000539]

Figure 1. In vitro angiogenic responses of diabetic endothelial cells

a), quantification of sprouts in 3-D fibrin gels formed by endothelial cells isolated from type 1 diabetic mice (DM) and non-diabetic controls (NDM) 6 days after cell seeding. b), proliferation c), migration of endothelial cells in a transwell towards chemoattractants (a mixture of 50ng/mL VEGF and 50ng/mL HGF). The number of sprouts were counted and normalized to the number of microcarriers. Values represent mean $\pm$ SD. N=5. *p<0.05.

Figure 2. Effects of Notch inhibition on the proliferation and migration of endothelial cells in 2-D cultures

a), Cell proliferation (cell number normalized by initial seeding number) after 6 days following an initial seeding density of 5000/cm², and with exposure to different treatments: media alone (B), 10ng/mL VEGF (V), 2μM DAPT (D), 10ng/mL VEGF combined with 0.2μM DAPT (V+D), 2μM DAPT (V+10D), and 20μM DAPT (V+100D). b) Effect of DAPT on cell proliferation at an initial low (5000/cm², Low SD) and high cell seeding density (15000/cm², High SD) in culture with media alone (Media), 10ng/mL VEGF (VEGF), and 10ng/mL VEGF combined with 2μM DAPT (VEGF+DAPT). c) Schematic illustration of the cell assay using o-rings and representative images of the condition with media alone (Media), 10μM DAPT (DAPT), 50ng/mL VEGF (VEGF) and a combination of both (VEGF+DAPT). Red bands are drawn to indicate the boundaries of the o-rings. Arrows point to the direction of outward migration from within the region defined initially by the o-rings. 10x microscope objectives. d), Quantification of total cell number normalized by the initial seeding number. e)

Quantification of the number of migrated cells outside of the boundary defined by the O-ring, normalized by the initial cell seeding number. Cells were treated with VEGF, without DAPT (−DAPT), or with 2μM (+DAPT) or 10μM DAPT (++DAPT). Values are mean ± SD. N=4. *p<0.05. NS, not statistically different.

Figure 3. In vitro effects of Notch inhibition on the proliferation and sprout formation of endothelial cells in 3-D cultures

EC proliferation after starting with an initial low seeding density (10000 per well in 24-well plates) (a), a high seeding density (27,000 per well in 24-well plates) (b), and confluent density (c) with cells pre-seeded on microbeads, with 2 μ M DAPT (+DAPT) or without DAPT (–DAPT). (d) Quantification of EC sprout formation, starting from confluent ECs on microbeads. Cells were treated with VEGF, without DAPT (–DAPT), or with 2 μ M (+DAPT) or 10 μ M DAPT (++DAPT). Values represent mean $\pm$ SD. N=4. *p<0.05.

Figure 4. Effect of a combination of DAPT and VEGF *in vivo* on type 1 diabetic mice with induced hindlimb ischemia

a), representative images of CD31 staining and b) quantification of capillary number in the ischemic muscle tissue regions with different treatments: blank gels (B), gels incorporating 3 μ g VEGF (V), gels incorporating 860ng DAPT (D), and a combination of both (V+D). c), quantification of blood perfusion in the ischemic hindlimb, as a ratio of the non-ischemic counter-lateral limb. Scale bar, 100 μ m. Values represent mean $\pm$ SD. N=5–7. * p <0.05.

Figure 5. Maturation of blood vessels was assessed by adding a third factor, PDGF that is able to recruit perivascular cells to mature newly formed vessels

Treatment conditions include delivery of gels containing 3 μg VEGF (V), 3 μg PDGF (P), 860 ng DAPT (D), and a combination of two (V+D, V+P) or three of them (V+P+D). Capillary density was examined by CD31 immunostaining of muscle tissues (a) and quantified (b). α-smooth muscle actin staining, as an indicator of perivascular cell association was then examined (c) and quantified (d). e) blood perfusion in the ischemic hindlimb as a ratio of the non-ischemic counter-lateral limb was also quantified. Scale bar, 100 μm. Values represent mean ± SD. N=5–7. *p<0.05. NS, not statistically different.