Modulating Notch Signaling to Enhance Neovascularization and Reperfusion in Diabetic Mice

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Introduction: Disease-specific strategies will likely be needed to appropriately promote neovascularization for the treatment of ischemic diseases, or engineering vascularized tissues, and likely will be multifactorial. Diabetes can diminish the responsiveness to angiogenic factors (e.g., VEGF) important for wound healing and the treatment of ischemic diseases, but it may be possible to reverse this effect by altering Notch signaling. 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 (For example, Notch inhibitors such as DAPT). This hypothesis was first tested *in vitro* with aortic ECs isolated from type 1 diabetic mice (induced by streptozotocin), and subsequently *in vivo* with the same diabetic mice model subject to surgically induced hindlimb ischemia by femoral artery ligation.

Methods:

Induction of diabetic mice

Type 1 diabetes was induced in C57 mice by intraperitoneal injection with streptozotocin. A blood glucose level larger than 250 mg/dL was considered to represent diabetes.

Isolation of aortic endothelial cells

The aorta was digested by collagenase solution and incubated with magnetic Dynabeads conjugated with IgG and subsequently coated with CD31 anti-mouse monoclonal antibody. Separated cells were plated, trypsinized and sorted again with Dynabeads coated with ICAM2/CD102 IgG2 antibody, to further increase the purity of endothelial cells.

Cell proliferation, migration and sprouting assays

ECs seeded at varied densities were exposed to different concentrations of VEGF, DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester), or a combination of both. To mimic the confluence nature of ECs *in vivo*, PDMS O-rings (5 mm diameter) were placed in 12-well plates, and ECs were seeded only inside the O-rings. The O-rings were subsequently removed after cells reached confluence, and the cells were simultaneously exposed to DAPT and VEGF, and were able to proliferate and migrate in concert. For 3-D sprouting assays, endothelial cells were seeded onto Cytodex 3 microcarriers and subsequently embedded in fibrin gels to allow cells to form sprouts.

Western Blots

Endothelial cells exposed to VEGF, DAPT, or a combination of both were lysised. Blots were incubated with primary antibodies for total VEGFR2, Phospho c-Raf, and Actin followed by appropriate species-specific secondary antibodies.

Murine ischemic hindlimb model

Unilateral hindlimb ischemia was created surgically. The external iliac and femoral artery and vein were ligated, and alginate hydrogels incorporating VEGF, PDGF, and/or DAPT were injected. Blood flow in the hindlimb was monitored by a laser Doppler perfusion imaging (LDPI) system.

Histology and immunohistochemistry

Hindlimb muscle tissues were paraformaldehyde fixed, and paraffin embedded. Sections were incubated with primary anti-mouse CD31 antibody or alkaline phosphatase-conjugated antibody to smooth muscle actin. Staining was amplified and developed.

Results: Aortic endothelial cells (ECs) isolated from type 1 diabetic mice demonstrated reduced sprouting capability *in vitro* in response to VEGF, but adding a Notch inhibitor, DAPT, led to cell-density and VEGF-dose dependent enhancement of sprouting. In particular, high cell densities, but not low cell densities, significantly enhance the proliferation, migration and sprout formation of ECs in both 2-D and 3-D culture in response to VEGF and DAPT exposure, as compared to VEGF alone. Combining VEGF and DAPT delivery resulted in increased blood vessel density and improved tissue perfusion than delivering VEGF alone in the ischemic limbs of diabetic mice (Fig1). 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.

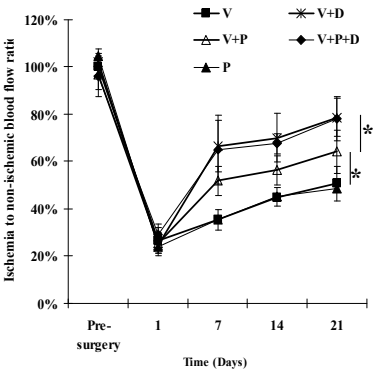

Fig 1. Blood perfusion in the ischemic hindlimb as a ratio of the non-ischemic counter-lateral limb. V, VEGF, D, DAPT, P, PDGF.

Conclusions:

The results of these studies demonstrate that modulating Notch signaling *via* a local and sustained delivery of Notch inhibitors, DAPT, allows one to rescue the impaired VEGF responsiveness of aortic ECs in diabetic mice, promote angiogenesis, and achieve effective tissue perfusion; and DAPT did not seem to interfere with the vessel maturation or cause vessel leakiness. 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.

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