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Diabetes impairs arterio-venous specification in engineered vascular tissues in a perivascular cell recruitment-dependent manner

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ABSTRACT

Cell-based tissue engineering is a potential treatment alternative for organ replacement. However, the lack of a robust vasculature, especially in the context of diseases such as diabetes, is a major hindrance to its success. Despite extensive research on the effects of diabetes in angiogenic sprouting, its effects on vessel arterio-venous (AV) specification have not been addressed. Using an engineered tissue that yields functional vessels with characteristic AV identities, we demonstrate that type 1 diabetes negatively affects vessel AV specification and perivascular cell (PVC) coverage. Blockage of PVC recruitment in normoglycemia does not affect blood flow parameters, but recapitulates the vascular immaturity found in diabetes, suggesting a role for PVCs in AV specification. The downregulation of Jagged1 and Notch3, key modulators of endothelial-perivascular interaction, observed in diabetes support this assertion. Co-culture assays indicate that PVCs induce arterial identity specification by inducing EphrinB2 and downregulating EphB4. This is antagonized by high glucose or blockage of endothelial Jagged1. Engineered tissues composed of microvessels from diabetic mice display normal PVC coverage and Jagged1/Notch3 gene expression when implanted into non-diabetic hosts. These indicate a lack of legacy effect and support the use of a more aggressive treatment of diabetes in patients undergoing revascularization therapies.

1. Introduction

Cell-based tissue engineering strategies are a potential treatment alternative for tissue and organ replacement. Multiple strategies, such as the co-culture of different vascular cell types or addition of angiogenic growth factors (reviewed in Refs. [1,2]), have been utilized to develop engineered vascular tissues. These strategies can accelerate vascularization upon implantation and promote blood perfusion. However, obtaining a robust, functional vasculature remains a major challenge for the creation of complex engineered tissues, especially in the context of diseases such as diabetes; which can have a direct impact in endothelial function and neovascularization [1]. Despite extensive investigation on the effects of diabetes in angiogenic sprouting, one of the major obstacles related to vascularization strategies for regenerative medicine that has been systematically underappreciated is the achievement of a mature vascular network with acquisition of specific arterial and venous identities and characteristic microvessel hierarchy.

The formation of a new vascular bed is characterized by complex vascular adaptation including: (i) angiogenic events such as endothelial proliferation and migration that lead to the formation of immature vessels devoid of perivascular cell (PVC) coverage [3], (ii) the initiation of blood flow, and (iii) vascular wall stabilization mediated by the recruitment of PVCs such as pericytes and smooth muscle cells (SMCs) [4,5]. In addition, the systemic vasculature is subdivided in 2 structurally and functionally distinct, but interconnected, compartments: the arterial compartment characterized by the presence of high blood flow, pressures and velocities, robust

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PVC coverage, and selective expression of arterial markers (i.e. EphrinB2 [6–8]) and the venous compartment characterized by lower blood flow, pressures and velocities, modest PVC coverage and the selective expression of venous markers (i.e. EphB4 [7,8]). The specification of arterial and venous identities and the formation of a hierarchical tree with specific inflow/outflow compartments are essential to the formation of a functional vascular network [9]. Although it is becoming clear that molecular (i.e. genetic pre-specification [7,10]) and environmental factors (i.e. hemodynamics [11]) help regulate the acquisition of arterio-venous (AV) identity in the embryo, the mechanisms that modulate acquisition and maintenance of AV phenotype in adult neovascularization are completely unknown. The elucidation of the mechanisms controlling vessel plasticity in the adult may be of critical importance to therapeutic vessel formation in ischemic tissues and graft transplants. In the case of coronary artery bypass grafts using saphenous veins, for example, inefficient adaptation to arterial blood flow (evidenced by intimal hyperplasia and accelerated atherosclerosis) leads to graft failure [12].

We have demonstrated that an engineered vascular tissue created with adipose or brain tissue-derived microvessels embedded in collagen type I hydrogel can form a hierarchical, blood-perfused vascular network when implanted subcutaneously in vivo [13]. Despite the initial loss of AV identity in early neovascularization (week 1) [14], characterized by the absence of stereotypical tree structure, lack of PVC coverage and ambiguous AV identity (ubiquitous expression of EphrinB2 and EphB4 AV genes; also present in newly formed vessels in embryo and retina [14–16]), neovessels gradually mature and acquire specific arterial or venous identity at later time points (weeks 4–6, compartmentalized expression of arterial or venous markers). These characteristics make this pre-vascularized engineered tissue ideal to test the effect of diabetes on vessel maturation and AV specification and to interrogate which micro-environmental factor(s) are involved in AV specialization in tissue engineered implants.

We show for the first time that type 1 diabetes, a risk factor for ischemic diseases such as myocardial infarction and lower limb ischemia, impinges on microvessel AV specification and maturation in a transplantation model, by decreasing PVC coverage. Blockage of PVC recruitment in normoglycemic conditions recapitulates the events shown in transplants performed into diabetic animals; and demonstrate the influence of PVC recruitment, a known inducer of vessel maturation, in the modulation of arterial identity acquisition. Analysis of the hemodynamic forces in the implants demonstrate that they do not appear to be responsible for the defects observed in implants lacking PVC coverage. We also demonstrate that Jagged1 and Notch3, molecules involved in AV specification and endothelial-perivascular cell-cell interactions are downregulated in diabetic conditions. In vitro assays show that perivascular-endothelial cell-cell contact promotes arterial phenotype on endothelial cells, and that blockage of endothelial Jagged1 or high glucose prevents it. Furthermore, we determined that the effects of diabetes in donor cells are not maintained when engineered tissues are transplanted into non-diabetic hosts. This is the first study that systematically evaluates vessel AV identity in engineered, pre-vascularized constructs in both health and diabetes; dissecting the contributions of micro-environmental cues.

2. Materials and methods

2.1. Mice

All animal experiments were performed in compliance with institutional guidelines and were approved by University Health Network Institutional Animal Care and Use Committee procedures and policies (#2420 and #2427 protocols). ***EphrinB2* reporter mice (arterial reporter)**: *B6; 129S4-Efnb2^{tm2Sor}/J* mouse contains green fluorescent protein under the control of the EphrinB2 gene [6]. Heterozygous mice develop normally and were used as reporter for the specific gene activity. Offspring were genotyped using standard PCR techniques and primers: 5′–3′ AAG TTC ATC TGG ACC ACC G and TCC TTG AAG AAG ATG GTG CG. **Rag1 mice**: *B6.129S7-Rag1^{tm1Mom}/J*-lack mature T and B cells and were used as host for transplantation studies. ***DsRed*-expressing mice**: *B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J*-this strain ubiquitously express DsRed under the beta actin promoter.

2.2. Diabetic mouse model

Type 1 diabetes model was based on previously published methods [45]. After fasting for 4 h, eight to twelve week-old transgenic, wild type black 6 or Rag1 mice were injected intraperitoneally once with 150 mg/kg STZ (Sigma-Aldrich) in 10 mM sodium citrate buffer at pH 4.5. Blood glucose measurements were performed 1 week after initial injection and every week thereafter after fasting. Mice that did not reach hyperglycemia (blood glucose >14.5 mmol; ~35%) within 1 week following initial injection and remained non-diabetic were used as controls.

2.3. Microvessel isolation

Microvessel fragments (MFs) were isolated from mouse brain tissue as previously described [13,14,47]. Briefly, tissue was minced and submitted to a limited digestion with 2 mg/ml collagenase type I (Worthington) and 1 mg/ml DNAse-1 (Sigma-Aldrich) in DPBS containing 0.1% BSA at 37 °C under agitation. Digested tissues underwent selective size screening for MFs using 500 μm and 30 μm filter screens. Isolated MFs were suspended in type I collagen gels (3 mg/ml) (**BD Biosciences**) at 20,000 MF/ml of gel, cast into 48-well plates and allowed to polymerize for 30 min at 37 °C.

For in vivo neovascularization assays, collagen gels containing MFs were implanted in subcutaneous skin pockets into Rag1 mouse (one per flank) and harvested at the indicated time points as before [2,13,14]. To prevent PVC recruitment to newly formed microvessels, 5 μg of anti-PDGFRβ (clone APB5; eBioscience) [18] or 200 μM of a PDGFRβ inhibitor (DMPQ dihydrochloride, Tocris Biosciences) [48] were injected daily from days 13–22 post-implantation. Injections were performed with a 30Ga needle around the implantation site, being careful to not disrupt the implant. An IgG matched irrelevant antibody (5 μg, eBioscience) was injected as control. At endpoints implants were harvested for qPCR or mice were perfused intravenously with Rhodamin-Dextran (Sigma-Aldrich)and implants were removed for imaging using an Olympus fluoView IX81 inverted confocal microscope (Olympus-Life science). For the assessment of PVC coverage, endothelial cells were stained with GSI-TRITC (Vectorlabs) and the PVC were stained with anti-smooth muscle actin (Sigma Aldrich) followed by a FITC conjugated antibody. Confocal images were analyzed on Image J software (each channel separately) and, after adjusting the threshold manually, the AND function was used to measure the area of overlap as previously [49]. For the assessment of shear stress, the implants were imaged in situ using live-imaging two-photon microscope (LSM710 NLO, Zeiss) with the help of a blood tracer (Rhodamin-Dextran; Sigma-Aldrich). Vessel diameter was measured using Zen imaging software (Zeiss). Blood shear stress was calculated with Haagen Poisseuille equation [50] and viscosity was assumed to be 1.3 cP [51].