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Engineered human vascularized constructs accelerate diabetic wound healing

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ABSTRACT

Stem cell-based therapy is emerging as a promising approach for chronic diabetic wounds, but strategies for optimizing both cellular differentiation and delivery remain as major obstacles. Here, we study bioengineered vascularized constructs as a therapeutic modality for diabetic wound healing. We developed a wound model in immunodeficient rodent and treated it with engineered vascularized constructs from endothelial progenitors or early vascular cells-derived from human induced pluripotent stem cells (hiPSCs) reprogrammed either from healthy donor or type-1 diabetic patient. We found that all vascularized constructs expedited wound closure and reperfusion, with endothelial progenitor constructs having the earliest maximum closure rate followed closely by healthy and diabetic hiPSC-derivative constructs. This was accompanied by rapid granulation layer formation and regression in all vascularized construct groups. Macrophage infiltration into the hydrogel matrix occurred during early stages of healing, seeming to facilitate rapid neovascularization of the wound that could then better persist in the vascularized constructs. Blood perfusion of the human vasculature could be detected after three days, indicating rapid integration with the host vasculature. Overall, we propose a potential therapeutic strategy using allograft or autologous vascularized constructs to treat type-1 diabetic wounds. This approach highlights the unprecedented prospects of designing patient-specific stem cell therapy.

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1. Introduction

Individuals with diabetes exhibit significant impairments in wound healing, which underlies high incidence of diabetic foot ulcers (DFUs), as well as morbidity and mortality associated with this condition. The estimated lifetime risk for developing foot ulcers is 25% in diabetics, and more than 14–24% of these patients will require amputation as a result of progressive disease. The current standard of treatment includes debridement of the wound, infection control, and application of various wound dressings to facilitate healing [1]. However, in many cases, using these treatments is not efficient to facilitate ideal healing, as non-healing diabetic ulcers have become a leading cause of non-traumatic amputation in the U.S [2].

In humans, wound healing is a continuum of processes, which consists of several overlapping stages. These stages include inflammation, cell proliferation, migration, angiogenesis, re-epithelialization, and reconstruction of the extracellular matrix [3]. Specifically, neovascularization and angiogenesis are critical determinants of wound-healing outcomes [4]. Newly formed blood vessels, comprised of endothelial cells (ECs) and supporting cells, such as pericytes, participate in the healing process, providing nutrition and oxygen to growing tissues [5].

In uncontrolled diabetic patients (both type-1 and type-2 diabetes), constant hyperglycemia can cause dysfunction at several stages throughout the wound healing process. For example, inflammation is prolonged and intensified due to the intrinsic high glucose environment, as the ability of macrophages to remove necrotic cells is diminished [6]. Additionally, perpetual high glucose

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levels in the blood prevent proper blood flow to the extremities, which causes a lack of oxygen and nutrient supply, thereby limiting angiogenesis and altering the permeability of the vasculature [7]. Importantly, diabetes-induced abnormalities in endothelial progenitor cell function likely aggravates the impairment in neovascularization in diabetic wounds [8] [9]. Thus, patients often suffer from an impaired wound healing process and exhibit non-healing wounds, which consequently can lead to ulcers, especially in the lower extremities.

Recently, an approach for wound treatment has emerged where biomaterials are used as a scaffold to induce new vascular growth in damaged vasculatures [10]. However, in diabetes patients, who suffer from a slow regenerative capacity, it is imperative to augment the endogenous pathways by delivering the vascular components to upsurge regeneration [11].

Vascular engineering encompasses rebuilding vessels *in vitro* within hydrogel matrix, utilizing cues inherent to the synthesized cells or present in the material to regulate the morphogenesis of ECs to create patent microvascular networks for subsequent transplantation. Previously, our group has engineered hyaluronic-acid (HA) hydrogel material to induce vasculogenesis of endothelial colony forming cells (ECFCs) resulting in functional human microvascular networks in a deliverable matrix [12,13]. In more recent works, we demonstrated the derivation of early vascular cells (EVCs) from human induced pluripotent stem cell (hiPSC). These hiPSC-EVCs can mature into ECs and pericytes and self-assemble to functional multicellular microvascular networks in the HA hydrogels [14,15]. The generation of vascular networks containing pericytes is highly relevant given its likely importance in wound healing in both normal and diabetic environments [16]. Moreover, hiPSCs might be less susceptible to diabetes-induced impairments, which further increases their attractiveness as a source for vascular cells for diabetic wound healing [17]. In that context, we have recently derived EVCs from hiPSCs reprogramed from type 1 diabetic (T1D) patients and demonstrated their functionality and ability to form networks in HA hydrogels as well as in response to hypoxic conditions [17].

Engineering vascularized constructs in which stem cell-derivatives pre-formed vascular networks in a deliverable matrix offer unmet opportunities to treat diabetic wounds. In this study, we hypothesized that engineered vascularized constructs would survive implantation into diabetic wounds and integrate with the diabetic host to reestablish blood flow more efficiently to improve the wound healing rate of diabetic ulcers compared to controls. In this light, we examined the effectiveness of both ECFCs and hiPSC-EVCs as the cellular bases for these constructs, given the potential advantages that each offers, and we compared the effectiveness of diabetic and non-diabetic hiPSCs.

2. Materials and methods

2.1. Cell culture and differentiation

EVC differentiation from BC1 and T1D hiPSC lines followed our established protocols [14,15,17]. Briefly, undifferentiated hiPSC (BC1 and T1D) were maintained on inactivated mouse embryonic fibroblast feeder layers in growth medium with 80% ES-EMEM/F12 (Global Stem), 20% serum replacement (Invitrogen), and 10 ng/ml basic fibroblast growth factor (bFGF). For differentiation, hiPSCs were collected through digestion with EDTA (Promega), single cells were seeded and plated onto collagen IV (Trevigen)-coated cell culture plates with a concentration of 5×10^4 cell/cm^2 supplemented with 10 μM ROCK inhibitor Y-27632 (Stemcell Technologies). Cells were cultured for 6 days in a differentiation medium composed of alpha-MEM (Invitrogen), 10% FBS (HyClone), and 0.1 mM β-mercaptoethanol (β-ME), with the medium changed daily. Differentiated cells were collected through digestion with TrypLE (Invitrogen) on day 6 and seeded at a concentration of 2×10^4 cells/cm^2 on collagen type IV-coated plates in endothelial cell growth media (ECGM) (PromoCell) supplemented with 2% FBS, 50 ng/mL VEGF with 10 μM SB431542 (Tocris) for 6 days. The medium was changed every other day. ECFCs (Lonza) were cultured under the manufacturer's protocol. Briefly, cells were seeded and expanded in endothelial growth media EGM-2 (Lonza) and cells with passages 6 through 8 were used for experiments, as we previously described [12,13,18].

2.2. Synthesis of acrylated hyaluronic acid (AHA) hydrogels macromer

AHA macromer synthesis was conducted as previously reported [12,19,20]. In brief, AHA was synthesized in two steps: (1) the tetrabutyl-ammonium salt of HA (HA-TBA) was formed by reacting sodium hyaluronate (90 kDa, LifeCore Biomedical) with the ion exchange resin Dowex-100 (Sigma) and neutralized with 0.2 M TBA-OF (Sigma); (2) acrylic acid (3 eq) and HA-TBA (1 eq) were coupled in the presence of dimethylaminopyridine (DMAP; 0.15 eq) and di-tert-butyl dicarbonate (3 eq) in DMSO, followed by dialysis and lyophilization. ^{1}H NMR spectra were used to confirm the modification of the AHA. The cell-adhesive peptide GCGYGRGDSPG (RGDS, molecular weight [MW]: 1025.1 Da) and MMP-sensitive peptide cross-linker GCRDGPQGWGQDRCG (MMP, MW: 1754 Da) were purchased (Genscript, > 95% purity per manufacturer HPLC analysis.)

2.3. Generation of human vascular constructs from ECFCs and EVCs

We engineered human vascular networks within the AHA hydrogels similar to our previous studies [12,13]. AHA macromer was dissolved in triethanolamine buffered saline (TEOA buffer: 0.2 M TEOA) at 3 wt%. Cell adhesive peptides (RGDS, GenScript) were dissolved in TEOA buffer and added to the AHA solution to the final peptide concentration of 4.8 mM Recombinant human VEGF$_{165}$ (Pierce), bFGF (Invitrogen), Ang-1 (R&D Systems), TNF-alpha (R&D Systems), and stromal cell-derived factor-1 (SDF-1; R&D Systems) were also added into the AHA-RGD mixture at 50 ng/ml. The mixture was allowed to react for 1 h with gentle shaking. For crosslinking, an MMP cross linker (MMP, GenScript) dissolved in TEOA buffer was added to a final concentration of 5.15 mM. ECFCs or EVCs were encapsulated in AHA hydrogels at a density of 5×10^6 cells/ml by suspending the cells in the AHA-RGD solution as described above prior to the addition of the MMP crosslinker. A 50 μl volume of the final mixture was pipetted into sterile molds (5 mm diameter, 2 mm height) and allowed to react for 10 min at room temperature in a laminar flow hood. The constructs were then cultured in ECFC culture media in the conditions described throughout the study. Cured AHA constructs without cells cultured in the same media and duration, served as acellular controls.

2.4. Streptozotocin (STZ) induced diabetic immunodeficient mice

The standard protocol from the American Models of Diabetic Complications Consortium was used to induce diabetes in mice [7,21,22]. Female nude mice (nu/nu) at about 2–2.5 months of age (about 20–25 g body weight) were randomly assigned to a diabetic or a non-diabetic group. Animals in the diabetic group received 5 daily intraperitoneal injections of STZ at a dose of 50 mg/kg body weight (STZ is freshly dissolved in citrate buffer, pH 4.5). Prior to injection, mice were fasted for 4–6 h. The weight and blood glucose of each mouse were checked daily for up to 2 weeks after the STZ injection. Successful induction of diabetes was confirmed by two

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