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Covalently immobilized VEGF-mimicking peptide with gelatin methacrylate enhances microvascularization of endothelial cells

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ABSTRACT

Clinically usable tissue-engineered constructs are currently limited due to their inability of forming microvascular networks necessary for adequate cellular oxygen and nutrient supply upon implantation. The aim of this study is to investigate the conditions necessary for microvascularization in a tissue-engineered construct using vascular endothelial growth factor (VEGF). The construct was made of gelatin methacrylate (GelMA) based cell-laden hydrogel system, which was then covalently linked with VEGF-mimicking peptide (AcQK), using human umbilical vein endothelial cells (HUVECs) as the model cell. The results of the mechanics and gene expression analysis indicated significant changes in mechanical properties and upregulation of vascular-specific genes. The major finding of this study is that the increased expression of vascular-specific genes could be achieved by employing AcQK in the GelMA based hydrogel system, leading to accelerated microvascularization. We conclude that GelMA with covalently-linked angiogenic peptide is a useful tissue engineered construct suitable for microvascularization.

Statement of Significance

(1) This study reports the conditions necessary for microvascularization in a tissue-engineered construct using vascular endothelial growth factor (VEGF). (2) The construct was made of gelatin methacrylate based cell-laden hydrogel system. (3) There is a significant change observed in mechanical properties and upregulation of vascular-specific genes, in particular CD34, when AcQK is used. (4) The major finding of this study is that the increased expression of vascular-specific genes, i.e., CD34 could be achieved by employing AcQK in the GelMA based hydrogel system, leading to accelerated microvascularization.

1. Introduction

Tissue engineering is emerging as a viable approach to address the shortage of donor organs in regenerative medicine. However, clinically usable tissue-engineered constructs are currently limited due to their inability of forming microvascular networks necessary for adequate cellular oxygen and nutrient supply upon implantation. Microvasculature is one of the key components, which determines the functional aspects of engineered tissues and organs. Currently, the main limitation associated with engineering functional thick hydrogel construct (>110 μm in thickness) is their inability to provide microvasculature system necessary for the survival and integration of the constructs within the host tissues [1–3]. Prevascularized hydrogel constructs are ideal for implantation, as they can quickly develop interconnections (inosculation) with the host vasculature to establish blood flow [4]. Prevascularization involves the formation of well-connected vascular networks which involves the formation of new vessels from pre-existing blood vessels – a phenomenon termed as angiogenesis [5]. To facilitate angiogenesis, the hydrogel construct should be engineered with angiogenesis promoting factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Therefore, to integrate such angiogenesis promoting factors, the choice of the material used as an extracellular matrix (ECM) mimicking scaffold

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is of paramount importance as it controls most of the cellular functions.

Hydrogels are crosslinked polymer networks with high water content and are widely employed as matrices for studying and recapitulating cell's *in vivo* behavior in a 3D environment [6–8]. The physical and chemical constituent of a hydrogel for mimicking ECM should match the natural physiological environment as closely as possible [9]. Native ECM is primarily composed of water, proteins and polysaccharides. Collagen is one of the main ECM proteins which provides structural support to cells and thus tissues and organs. Gelatin, a natural polymer derived from collagen is ideal for biomedical applications owing to its biodegradability and biocompatibility in physiological environment [10]. To make gelatin as a photopolymerizable hydrogel, methacrylate groups are added to the amine-containing side-groups of gelatin to yield gelatin methacrylate (GelMA) [11]. The GelMA hydrogel is tunable and provides cell-responsive characteristics like adhesion sites and proteolytic degradability [12–14]. Nevertheless, GelMA hydrogels have been extensively used to demonstrate directed 3D alignment of fibroblast cells [15], directional control of endothelial cell morphogenesis [16], generation of an extensive functional capillary network using human blood-endothelial colony-forming cells (ECFCs) and bone marrow-derived mesenchymal stem cells (MSCs) [17], and as a promising hydrogel for patterning cells using dielectrophoresis method [18].

As an important angiogenesis promoting factor, VEGF plays a crucial role in regulating vasculogenesis and angiogenesis [19]. The VEGF family of growth factors occurs as homodimeric and heterodimeric polypeptides such as, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). Among these, VEGF-A is necessary for growth of new blood vessels during normal and pathological conditions. VEGF-A occurs as VEGF-121, VEGF-165, VEGF-189, and VEGF-206 isoforms due to alternative exon splicing [20]. Among these four isoforms, VEGF-121 is freely diffusible, VEGF-189 and VEGF-206 are completely bound to heparin-like moieties on the cell surface or extracellular matrix, and VEGF-165 has intermediate heparin binding properties. To study VEGF-receptor interactions, various synthetic peptides have been established to mimic VEGF isoforms *in vitro* [21]. To this end, D'Andrea et al. [22] designed an agonist peptide, QK, reproducing the helix region 17–25 of VEGF, and showed that QK recapitulated the effect of isoform VEGFA-165 on VEGF receptors. QK adopts a helical conformation in pure water which enables it to bind to VEGF receptors, thereby inducing angiogenesis. Interestingly, it has been shown that endothelial cells respond distinctly to the soluble and matrix-bound VEGF [23]. Hence, it is rational to integrate QK in the ECM, as QK will improve receptor-cell interaction and its associated functions. Previous research concerning immobilization of QK peptide in hydrogels has been performed to promote microvasculature formation [24,25]. For instance, Chan et al. [24] modified QK to include a collagen-mimetic peptide (CMP) sequence and designed a novel bifunctional peptide, QKCMP which was used to control endothelial cell morphogenesis in collagen scaffolds. In another study, Leslie-Barbick et al. [25] covalently linked QK to poly(ethylene glycol) (PEG) based hydrogels and demonstrated improved bioactivity and superior ability of QK for promotion of angiogenesis *in vitro*. These studies have shown that covalent conjugation of QK peptide onto the hydrogel matrix does not affect its biological function.

The central motivation of the current work was to take advantage of the cell-responsive properties of GelMA hydrogel and interlink it with angiogenic properties of QK peptide. This interlinking will engineer the GelMA hydrogel as an angiogenic ECM mimicking scaffold for the presentation of a matrix-bound, non-soluble cell-signaling moiety to promote capillary formation of endothelial cells. Matrix-bound VEGF facilitate stronger interactions between the VEGF molecules and its receptors located on the surface of endothelial cells leading to overall improvement of cellular response [23]. Although GelMA supports vascular network formation, the absence of angiogenic molecules in the matrix hastens the endothelial cells migration and lumen formation. The free VEGF molecules in the growth medium cannot provide the sustained cell-signaling needed to accelerate vascular network formation. Further, VEGF-A isoforms occur physiologically both as matrix-bound and freely diffusible molecules [20]. Hence, the QK peptide is presented as a matrix-bound entity to reap the full benefits of GelMA hydrogel. The QK peptide is linked to the GelMA backbone through a photo-initiated crosslinking mechanism – the same mechanism which governs the crosslinking of GelMA. Therefore, the peptide QK interferes with the crosslinking of GelMA to directly affect mechanical properties of the hydrogel. Hence, we suspect that covalent linking of the peptide QK to GelMA will not only elicit enhanced cellular response but also aid in tailoring the mechanics of the hydrogel. It has been found that the activity of QK peptide will be at its maximum during the initial stage of vascular morphogenesis, where the individual endothelial cells migrate and begin to form a network leading to the formation of endothelial tubes with patent lumen. Therefore, the expression of genes responsible for such fundamental processes like endothelial migration, cord and lumen formation needs to be quantified. The logic behind the above statement can be understood when we consider that the peptide QK interacts with kinase-insert domain receptor, KDR (VEGFR2/FLK-1) of endothelial cells, which is responsible for a multitude of endothelial functions [19]. Therefore, more attention needs to be given to the changes occurring during the initial stages of capillary formation.

The objective of this research was to study the effect of matrix-bound QK on microvascularization of human umbilical vein endothelial cells (HUVECs) and its vascular-specific gene expression under three-dimensional (3D) conditions. We hypothesized that the covalent conjugation of peptide QK to GelMA will up-regulate VEGF interaction with endothelial cell receptors, particularly KDR, thereby promoting vascular morphogenesis. We tested this hypothesis by encapsulating HUVECs in peptide-linked GelMA hydrogel and studied the resultant gene expression in different GelMA systems.

2. Experimental

2.1. Preparation of GelMA

GelMA was synthesized according to the procedure described by Nichol et al. [14]. Type-A porcine skin gelatin (Sigma-Aldrich) was dissolved in Dulbecco's phosphate buffered saline (DPBS) (Gibco, Life Technologies) at 60 °C. To this solution, methacrylic anhydride (MA) (Sigma-Aldrich) was added at a rate of 0.5 mL min^{-1} under stirring to a final MA concentration of 1% (v/v). After reaction for 3 h at 50 °C, the GelMA solution was dialyzed against deionized water using 12–14 kDa cut-off dialysis tubes for a week at 50 °C. Following dialysis, the GelMA solution was frozen at −80 °C, lyophilized, and stored at −20 °C.

2.2. Preparation of peptide-linked GelMA

Acrylated peptide (Ac-K-{Acryl}-LTWQELYQLK(Ac)YK(Ac)GI-NH2) (MW: 2090.35; CH-2802W, ChinaPeptides Co., Ltd., China) was dissolved in DPBS and stored as 50 μL aliquots. The freeze-dried GelMA (5 w/v% final) was dissolved in DPBS along with the photoinitiator (Irgacure 2959, 0.5 w/v%) at 70 °C. To this prepolymer, the peptide was added at a concentration of 200 μg/mL. This precursor hydrogel solution was then exposed to 7 mW/cm^2 UV