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Vascular endothelial growth factor immobilized in collagen scaffold promotes penetration and proliferation of endothelial cells

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Abstract

A key challenge in engineering functional tissues in vitro is the limited transport capacity of oxygen and nutrients into the tissue. Inducing vascularization within engineered tissues is a key strategy to improving their survival in vitro and in vivo. The presence of vascular endothelial growth factor (VEGF) in a three-dimensional porous collagen scaffold may provide a useful strategy to promote vascularization of the engineered tissue in a controlled manner. To this end, we investigated whether immobilized VEGF could promote the invasion and assembly of endothelial cells (ECs) into the collagen scaffolds. We conjugated VEGF onto collagen scaffolds using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride chemistry, and measured the concentrations of immobilized VEGF in collagen scaffolds by direct VEGF enzyme-linked immunosorbent assay. We demonstrated that immobilized VEGF (relative to soluble VEGF) promoted the penetration and proliferation of ECs in the collagen scaffold, based on results of cell density analysis in histological sections, immunohistochemistry, XTT proliferation assay, glucose consumption and lactate production. Furthermore, we observed increased viability of ECs cultured in scaffolds with immobilized VEGF relative to soluble VEGF. This research demonstrates that immobilization of VEGF is a useful strategy to promote the invasion and proliferation of ECs into a scaffold, which may in turn lead to a vascularized scaffold.

Keywords: Collagen scaffold; Endothelial cell; VEGF; Covalent immobilization; Tissue engineering

1. Introduction

One of the main goals of tissue engineering is to design tissue scaffolds that can support the survival, growth and assembly of cells into functional tissues. A key challenge in engineering functional tissues is the limited transport capacity of oxygen and nutrients into the tissue. For example, in hypoxia-sensitive cardiac tissue engineered in vitro, conventional medium diffusion can provide oxygen at a distance of up to 100 μm, yet oxygen transport beyond this distance is limited [1,2]. As a result, the interior of a cardiac construct has low cell density and viability [3,4]. Oxygen gradients were shown to correlate with cell density and cell viability in engineered cardiac constructs [5].

Inducing vascularization is a key strategy to improving the survival of engineered tissues both in vitro and in vivo [6,7]. The most studied approach is the delivery of angiogenic growth factors to promote neovascularization [8]. Vascular endothelial growth factor (VEGF) is a potent inducer of endothelial cell (EC) proliferation, migration and tube formation, and is a key mediator in the process of angiogenesis [8,9]. There exist a family of VEGFs,

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VEGF-A through VEGF-E, of which an isoform of VEGF-A, $VEGF_{165}$, is the most prevalent [8]. Two distinct VEGF receptor tyrosine kinases, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), have been identified on ECs [10–12]. It is thought that flk-1 functions in inducing EC proliferation, while flt-1 functions in differentiation and vascular organization [11]. Binding of VEGF to VEGF receptor leads to receptor dimerization and autophosphorylation, which allows for the recruitment of signaling proteins containing src-homology-2 (SH2) domains to specific phosphorylated tyrosines [11].

There has been tremendous success in using biomaterials for the controlled delivery of VEGF to induce neovascularization [13–16], as well as to support cell survival and differentiation [17–19]. Alternately, growth factors such as VEGF can be immobilized onto the biomaterial scaffolds for tissue engineering applications. Immobilized growth factors can promote the desired cell-material interactions, and render synthetic materials bioactive [20,21]. Several studies have shown enhanced proliferation of ECs on two-dimensional substrates with immobilized VEGF. Taguchi and colleagues immobilized VEGF onto poly(acrylic acid) grafted poly(ethylene) films using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) chemistry, and found that VEGF and fibronectin co-immobilized surface greatly promoted the growth of human umbilical vein endothelial cells (HUVECs) [22]. Ito and colleagues photoimmobilized VEGF onto gelatin surfaces, and showed that immobilized VEGF greatly enhanced the proliferation and surface coverage of HUVECs [23]. Backer and colleagues engineered a cysteine tag into VEGF and then conjugated VEGF onto fibronectin-coated surfaces via the cysteine tag, and observed growth stimulation of VEGFR-2 overexpressing cells [24]. Zisch and colleagues engineered a Factor XIIIa substrate sequence into VEGF and crosslinked VEGF into the fibrin gel through a coagulation process, and subsequently demonstrated growth enhancement of HUVECs cultured on VEGF–fibrin surfaces [25].

The main objective of this work was to immobilize VEGF into a porous three-dimensional (3D) collagen scaffold to promote the penetration and assembly of ECs into the scaffold. We chose to use porous collagen scaffolds because they are widely used in tissue engineering to support the growth of various cell types, and are also used for surgical wound dressings. We demonstrated that VEGF maintained biological activity upon immobilization and promoted the infiltration and proliferation of ECs in the 3D collagen scaffold. This research is the first step in assembling a high density of ECs in a 3D scaffold, in order to promote vascularization within the engineered tissue constructs.

2. Materials and methods

2.1. Collagen scaffolds

Scaffolds were discs (7 mm diameter × 2 mm thick) punched from sheets of commercially available Ultrafoam™ collagen hemostat (Davol Inc., Cranston, RI). According to the manufacturer's specifications, Ultrafoam™ is a water-insoluble, partial HCl salt of purified bovine dermal (corium) collagen formed as a sponge with interconnected pores. Dry collagen scaffold was cross-sectioned using a sharp razor balder, sputter coated with gold and imaged in face and cross-section using a Hitachi S-3400N variable pressure scanning electron microscope.

To assess the level of collagen scaffold degradation in the culture medium, the scaffolds ($n = 3$ per group per time point) were placed in a 12-well plate and maintained in 1 ml of D4T cell culture medium for 10 min, 7 days and 12 days. Culture medium was changed by 100% every other day. The samples were immersed in distilled water for 10 min before being freeze dried in liquid nitrogen for 10 min. The samples were then placed into the lyophilizer for 24 h to dry under vacuum followed by measurements of dry weight.

2.2. Immobilization of VEGF in collagen scaffold

Mouse recombinant $VEGF_{165}$ was immobilized into the porous collagen scaffold using EDC chemistry. The collagen scaffold (7 mm diameter, 2 mm thickness, cut out from the collagen sponge sheet using a circular metal borer) was immersed in 150 μl of EDC (Sigma) and *N*-hydroxysulfosuccinimide (sulfo-NHS) (Pierce Chemicals) (EDC/sulfo-NHS concentrations of 24 mg/60 mg ml^{-1} or 4.8 mg/12 mg ml^{-1}) dissolved in PBS (filter sterilized for cell culture) in a 96-well plate. The reaction proceeded for 20 min at room temperature. The scaffold was then removed from the EDC/sulfo-NHS soaking solution and immersed completely in 100 μl of VEGF (concentrations of 500 ng ml^{-1} or 1 μg ml^{-1}) dissolved in phosphate-buffered saline (PBS). The reaction proceeded for 1 h at room temperature. The scaffold was then subjected to successive soaks in fresh PBS (eight washes of at least 5 min each) to remove any uncrosslinked VEGF and EDC/sulfo-NHS.

2.3. Quantification of immobilized VEGF

The amount of immobilized VEGF throughout the depth of the scaffold ($n = 3$) was quantified by a direct VEGF enzyme-linked immunosorbent assay (ELISA) (BioVision VEGF ELISA kit) technique, in which the standard VEGF ELISA procedure in the kit was followed using the scaffold as the primary substance. Briefly, the scaffold was blocked in 1% bovine serum albumin (BSA) in PBS for 1 h in a 96-well plate at room temperature, after which it was washed (1% Tween-20 in PBS) for 1 min. The scaffold was then immersed in biotinylated anti-mVEGF detection antibody (0.025 μg ml^{-1}) in diluent (0.05% Tween-20, 0.1% BSA in PBS) for 1 h at 37 °C, after which it was washed repeatedly (six washes of at least 3 min each on a shaker). The scaffold was immersed in Avidin peroxidase conjugate (0.05% in diluent) for 30 min at room temperature, after which it was washed repeatedly. The wet scaffolds were placed in a

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