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Controlled co-immobilization of EGF and VEGF to optimize vascular cell survival

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ABSTRACT

Growth factors (GFs) are potent signaling molecules that act in a coordinated manner in physiological processes such as tissue healing or angiogenesis. Co-immobilizing GFs on materials while preserving their bioactivity still represents a major challenge in the field of tissue regeneration and bioactive implants. In this study, we explore the potential of an oriented immobilization technique based on two high affinity peptides, namely the Ecoil and Kcoil, to allow for the simultaneous capture of the epidermal growth factor (EGF) and the vascular endothelial growth factor (VEGF) on a chondroitin sulfate coating. This glycosaminoglycan layer was selected as it promotes cell adhesion but reduces non-specific adsorption of plasma proteins. We demonstrate here that both Ecoil-tagged GFs can be successfully immobilized on chondroitin sulfate surfaces that had been pre-decorated with the Kcoil peptide. As shown by direct ELISA, changing the incubation concentration of the various GFs enabled to control their grafted amount. Moreover, cell survival studies with endothelial and smooth muscle cells confirmed that our oriented tethering strategy preserved GF bioactivity. Of salient interest, co-immobilizing EGF and VEGF led to better cell survival compared to each GF captured alone, suggesting a synergistic effect of these GFs. Altogether, these results demonstrate the potential of coiled-coil oriented GF tethering for the co-immobilization of macromolecules; it thus open the way to the generation of biomaterials surfaces with fine-tuned biological properties.

Statement of significance

Growth factors are potent signaling molecules that act in a coordinated manner in physiological processes such as tissue healing or angiogenesis. Controlled coimmobilization of growth factors on biomaterials while preserving their bioactivity represents a major challenge in the field of tissue regeneration and bioactive implants. This study demonstrates the potential of an oriented immobilization technique based on two high affinity peptides to allow for the simultaneous capture of epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). Our system allowed an efficient control on growth factor immobilization by adjusting the incubation concentrations of EGF and VEGF. Of salient interest, co-immobilizing of specific ratios of EGF and VEGF demonstrated a synergistic effect on cell survival compared to each GF captured alone.

1. Introduction

Conferring bioactive properties to synthetic materials via specific signaling molecules such as growth factors is a promising way to predict and control biological response to implants. Growth factors (GFs) are naturally present in the body as soluble or extracellular matrix (ECM)-bound cues for the cells. Accordingly, many efforts have been dedicated to incorporate GFs into materials in order to take advantage of their natural regenerative action either via their progressive release [1–3] or their immobilization on

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various substrates [4–6]. Such GF-modified biomaterials have been shown to promote *in vitro* cell proliferation [7,8], migration [8,9] and differentiation [10,11].

However, most of the studies on the biological modification of materials rely on the addition of a single GF, while in their physiological environment, GFs are often combined, and complex processes such as wound healing or angiogenesis typically involve several signaling proteins possessing synergistic or complementary actions [12–15]. This generated a growing interest in biomimetic materials delivering or displaying multiple GFs [15–18]. Studies have shown that GF combination in biomaterials can improve angiogenesis [17,19–22], osteogenesis [23], directed migration [24] or cell differentiation [23] when compared to a single GF. However, major hurdles still limit the use of systems involving multiple GF, such as the huge quantities of GF required for large size implants/scaffolds, or the lack of characterization of potential synergies existing between GF that could be exploited for enhanced implant biological activity. The search towards an efficient, well-controlled immobilization platform for GF combination is thus still in progress.

To reach that aim, stable immobilization appears advantageous since it provides the substrate with a high local concentration of GF that is not released over time, thereby inducing an enhanced and sustainable cell response compared to free growth factor [25–27]. Moreover, lower amounts of immobilized GFs are required to trigger cell stimulation when compared to their supply in a diffusible form, given that large quantities need to be provided by a sustainable source (either medium renewal *in vitro* or controlled release) [28]. Finally, for blood-contacting devices, stable immobilization enables a localized and durable effect [25] as it prevents GF release into the blood stream. Another challenge is the choice of GF immobilization method to preserve its biological activity. Electrostatic binding can alter protein structure and negatively impact GF bioactivity as a result of denaturation and/or a random presentation on the surface, with active sites not being fully accessible for recognition by cell surface receptors [29]. Furthermore, in physiological conditions, the long term availability of the GF may also be limited due to the weak attachment of molecules on the surface [29]. Covalent immobilization involving irreversible binding of reactive groups present on side chain residues to chemical moieties available at the surface is durable but can also lead to loss of bioactivity due to conformational changes and random orientation of the GF upon grafting [5,29].

In this context, several groups have developed site-specific, oriented protein tethering systems that maintain GF bioactivity. Those include the use of ECM binding tags that can be genetically fused to GFs, as well as pairs of high affinity peptides with variable stabilities in which one peptide is bound to the substrate of interest while the other is fused to GFs by genetic engineering [17,30–33]. On that note, we have investigated the potential of two peptides, the Ecoil and Kcoil, of respective amino acid sequence $[EVSALEK]_5$ and $[KVSALKE]_5$ that bind to each other to form stable coiled-coil structures [34]. Ecoil/Kcoil mediated capture has been successfully applied to tether various Ecoil-tagged GFs, such as the epidermal growth factor (EGF) [35] and the vascular endothelial growth factor (VEGF) [36], onto substrates that had been predecorated with Kcoil peptides. Careful positioning of the Ecoil tags on the chimeras corresponding to each GF allowed this immobilization strategy to preserve the GF bioactivity after tethering (as deduced from receptor activation [34], increase in adhesion [27], proliferation [27] or survival [36,37] of cells). Of interest, coiled-coil immobilized and free EGF and VEGF led to comparable cell survival when provided at sufficiently high doses [36,37].

Although this system had been limited so far to the immobilization of a single type of GF at a time, we hypothesized that Kcoil-bearing substrates could readily be functionalized with multiple Ecoil-tagged GFs. The objective of this work was therefore to investigate the feasibility of GF co-immobilization, its control and the advantage of the stable coiled-coil capture system as a platform to investigate the collaborative effect between GFs and its potential to create a coating with tailored properties. More specifically, we hypothesized that a controlled combination of EGF and VEGF could help to optimize a coating with pro-survival properties. Indeed, conferring pro-survival properties to biomaterials is an ongoing challenge for several applications such as tissue engineered scaffolds where survival of pre-seeded cells is crucial to counter the cell loss caused by serum deprivation and hypoxia before neoangiogenesis takes place [38,39]. Healing around stent grafts implanted during endovascular repair of abdominal aortic aneurysms may also benefit from pro-survival properties as the vessel wall in aneurysms displays strong vascular cell depletion due to a pro-apoptotic environment [40,41].

To achieve this aim, Ecoil-tagged EGF and Ecoil-tagged VEGF (hereafter designated as E-EGF and E-VEGF, respectively) were co-immobilized using coiled-coil mediated tethering on chondroitin sulfate (CS). When coated on biomaterial's surfaces, this ECM glycosaminoglycan was shown to permit cell adhesion despite low-fouling properties and low-platelet adhesion [42,43], suggesting that it could enhance the bioavailability of the immobilized GF. In an effort to evaluate the potential of a dual GF immobilization for vascular and tissue engineering applications, the effect of surface densities of these GFs (alone and combined), on the survival of two vascular cell types – the human umbilical vein endothelial cells (HUVEC) and human aortic smooth muscle cells (AoSMC) was studied.

2. Materials and methods

2.1. Materials and reagents

Chondroitin-4-sulfate (CS), sodium chloride (99.99% purity), 1-ethyl-3-(3-dimethylaminopropyl)-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid (MES), Dulbecco's Phosphate Buffered Saline (modified PBS, without calcium chloride and magnesium chloride), Tween 20, Cysteine (99+% purity) and resazurin sodium salt were purchased from Sigma–Aldrich (Oakville, ON). EMCH (3,3′-N-[ε-maleimidocaproic acid] hydrazide, trifluoroacetic acid salt) was purchased from Pierce Biotechnology (Rockford, IL). Commercially available DuoSet ELISA kits containing biotinylated goat anti-hEGF antibody and mouse anti-$hVEGF_{165}$ antibody and detection antibodies, streptavidin–horseradish peroxidase (streptavidin–HRP), bovine serum albumin (BSA), substrate solution (hydrogen peroxide/tetramethylbenzidine) was obtained from R&D Systems (Minneapolis, MN). CellBIND® carboxyl-exposing microplates (96-well) were purchased from Corning (Corning, NY).

2.2. Kcoil, Ecoil-tagged EGF and Ecoil-tagged VEGF production

Cysteine-tagged Kcoil peptides were synthesized by the peptide facility at University of Colorado (Denver, CO) [35]. Ecoil-tagged EGF (E-EGF) and Ecoil-tagged $VEGF_{165}$ (E-VEGF) were produced in HEK 293-6E cells and purified by immobilized metal-ion affinity chromatography (IMAC) as previously described [35]. Protein concentration was determined by ELISA. Purified proteins were then stored at −80 °C until use.

2.3. Chondroitin sulfate and growth factor immobilization

2.3.1. Chondroitin sulfate coating preparation

CellBIND® 96 well microplates were used as model surfaces to prepare the coatings and carry out the cell culture assays. To create

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