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Human corneal epithelial cell response to epidermal growth factor tethered via coiled-coil interactions

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ABSTRACT

The development of new strategies for protein immobilization to control cell adhesion, growth and differentiation is of prime interest in the field of tissue engineering. Here we propose a versatile approach based on the interaction between two *de novo* designed peptides, Ecoil and Kcoil, for oriented immobilization of epidermal growth factor (EGF) on polyethylene terephthalate (PET) films. After amination of PET surfaces by ammonia plasma treatment, Kcoil peptides were covalently grafted in an oriented fashion using succinimidyl 6-[30-(2-pyridyldithio)-propionamido] hexanoate (LC-SPDP) linker, and the Kcoil-functionalized films were characterized by X-ray photoelectron spectroscopy (XPS). Bioactivity of Ecoil-EGF captured on Kcoil-functionalized PET via coiled-coil interactions was confirmed by EGF receptor phosphorylation analysis following A-431 cell attachment. We also demonstrated cell biological effects where tethered EGF enhanced adhesion, spreading and proliferation of human corneal epithelial cells compared to EGF that was either physically adsorbed or present in solution. Tethered EGF effects were most likely linked to the prolonged activation of both mitogen-activated protein kinase and phosphoinositidine 3-kinase pathways. Taken together, our results indicate that coiled-coil-based oriented immobilization is a powerful method to specifically tailor biomaterial surfaces for tissue engineering applications.

1. Introduction

Eye diseases affecting the cornea are one of the major causes of blindness worldwide. Whether the underlying etiology is trachoma or corneal ulceration, an eye that is blind from corneal scarring or vascularization typically remains blind throughout life [1]. A favored treatment is corneal transplantation. However, due to the limited availability of potential donors, the development of artificial corneas has become a subject of intense investigation. Within the last three decades, noticeable progress has been made in both design and material used for corneal implant in order to promote tight integration of the host tissue within the peripheral region of the implant, and thus ensure appropriate anchorage [2]. A highly desirable but yet unmet property for corneal devices resides in the enhancement of their ability to promote epithelialization over their entire anterior surface. Indeed, complete epithelialization will maintain a tear film and provide a natural barrier against bacterial infection while protecting the stroma from collagenase and proteinase enzymatic activity for longer implant stability [3]. The development of new strategies allowing for both migration and proliferation of epithelial cells on implanted eye devices is thus highly desired.

In this context, epidermal growth factor (EGF) is a promising candidate to stimulate the formation of an epithelial layer. In mammals, EGF is synthesized as a membrane-spanning precursor molecule that is proteolytically processed to become fully active. In its soluble active form, EGF is a 6-kDa protein that directly interacts with several cell surface receptors from the ErbB receptor tyrosine kinase family. EGF exerts its activity by promoting the formation of signaling-competent receptor homo- or hetero-dimers at the cell surface. EGF-mediated receptor dimerization triggers receptor cytoplasmic domain autophosphorylation, in turn leading to the activation of two major signaling pathways, i.e., the mitogen-activated protein kinase (MAPK) and the phosphoinositidine 3-kinase

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(PI3K) pathways. EGF has been demonstrated to be directly implicated in almost all fundamental cellular processes, including survival, proliferation, migration, differentiation and metabolism regulation, and in different cell lines. Interestingly, in the case of a damaged corneal epithelium, EGF has been shown to be absolutely required to induce cell motility, a key component of the epithelial response for repair [4].

Material surface properties (e.g., hydrophobicity, porosity, topography) are key factors to control for optimal implant integration; in the context of a corneal device, material properties may be further enhanced by grafting appropriate growth factors such as EGF at its surface, as outlined by Klenkler and Sheardown [5]. Towards this end, covalent grafting of EGF on various supports such as glass [6] or polydimethylsiloxane (PDMS) [7] substrates through the use of PEG linkers has been reported to have clear effects on cell growth, while adsorbed EGF showed no biological activity [6]. Nonetheless, covalent grafting combined with the use of a PEG linker presents several caveats. Indeed, in a recent study, Klenkler and colleagues [8] reported that the formation of a confluent layer of corneal epithelial cells could not be achieved using an EGF-tethering strategy based on PEG, most likely because PEG limits the adsorption of adhesion molecules. Furthermore, covalent coupling strategies that rely on the use of reactive amine groups present at the EGF N-terminus or on its lysine side-chain (at position 28 and 48 for human EGF) have been shown to negatively impact EGF bioactivity [9–11]. Alternative strategies exploring other types of linkers while at the same time promoting the oriented immobilization of EGF, should therefore be examined.

In the current study, we have immobilized a chimeric protein (Ecoil-EGF) corresponding to human EGF fused at its N-terminus to a *de novo* designed peptide, namely Ecoil, in order to promote EGF tethering in an oriented fashion through the interaction of the Ecoil peptide moiety with its interacting peptide partner, i.e., Kcoil peptide. The E/K peptides have been shown to form a stable heterodimeric complex in a highly specific fashion [12]. This coiled-coil heterodimerizing pair has been previously used in many applications such as virus retargeting [13], protein purification and detection [14,15], or protein capture on glass [16] and biosensor [17] surfaces. In this work, X-ray photoelectron spectroscopy (XPS) was used to characterize each step leading to the covalent grafting of Kcoil peptides on ammonia plasma-treated polyethylene terephthalate (PET) through the use of a small linker (LC-SPDP, Fig. 1). Ecoil-EGF capture on Kcoil-derivatized PET surfaces was then achieved by simple incubation. The effect of tethered EGF upon adhesion, spreading, proliferation and activation of both mitogen-activated protein kinase and phosphoinositidine 3-kinase pathways of human corneal epithelial cells were investigated and compared to those related to adsorbed or in-solution EGF.

2. Materials and methods

2.1. Chemicals and reagents

Cysteine (99+% purity) and sodium chloride (99.99% purity) were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON). Succinimidyl 6-[30-(2-pyridyldithio)-propionamido]hexanoate (LC-SPDP, 95+% purity) was obtained from Pierce Biotechnology, Inc. (Rockford, IL). Cysteine-tagged Kcoil peptides were synthesized by the peptide facility at University of Colorado (Denver, CO). Untagged recombinant human EGF (carrier-free) was purchased from R&D Systems (Minneapolis, MN).

2.2. Ammonia plasma treatment

Polyethylene terephthalate (PET) film samples (50 μm-thick DuPont Mylar®, DuPont Teijin Films, Hopewell, VA) were aminated with covalently-bound nitrogen (N) groups, preferably primary amines, C–NH_2, by exposing the films to ammonia (NH_3) plasma treatment, adapting the procedure previously described [18]. Briefly, plasma treatments were carried out for 15 min in a cylindrical aluminium/steel vacuum chamber of approximately 20 cm in diameter and 20 cm in height. A turbo-molecular pump, backed by a two-stage rotary vane pump was used to evacuate the chamber to a base pressure of $<10^{-4}$ Pa (7.5×10^{-5} mTorr), as measured by a Pirani gauge. A flow of anhydrous ammonia (99.99%, Air Liquide, Canada) was then fed into the chamber using an electronic flow meter/controller (Vacuum General Inc.), through a 'shower head' gas distributor (10 cm in diameter) at the top of the chamber. The ammonia flow rate was kept constant at 20 standard cm^3/min (sccm). During plasma treatment, the operating pressure was set and maintained constant at 80 Pa (600 mTorr) by a 'butterfly' throttle valve combined with a capacitive pressure gauge (Baratron, MKS Instruments). The capacitively coupled radio-frequency (r.f., 13.56 MHz) plasma was generated with the help of a power supply (ENI) and an automatic impedance matching network (Advanced Energy), connected to a 10-cm diameter powered electrode/sample holder in the center of the chamber, the walls acting as the grounded electrode. The distance between the bottom of the shower head (see above) and the r.f.-driven electrode was 15 cm. The power fed to the plasma was 10 W, resulting in a negative d.c. bias voltage at the powered electrode, $V_B = -40$ V.

2.3. Grafting procedures

2.3.1. Kcoil peptide grafting on aminated PET

Kcoil peptides were immobilized on aminated PET surfaces by adapting the experimental protocol previously described [19]. Aminated PET samples (1,13 cm^2) were first covered with a 2-mM LC-SPDP solution (100 μL) for 2 h at room temperature, to allow for LC-SPDP amine-reactive N-hydroxysuccinimide ester covalent binding to free -NH_2 groups on the aminated PET surfaces (Fig. 1). The surfaces were then extensively rinsed with MilliQ water and incubated with cysteine-tagged Kcoil peptides (10 μM, 100 μL) for 2 h at room temperature, in order to form a covalent disulfide bond between the pyridyl disulfide group of LC-SPDP and the thiol group of cysteine-tagged Kcoil peptide. After rinsing in MilliQ water, blockage of unreacted LC-SPDP pyridyl disulfide groups was achieved with 50 mM cysteine solution (1 M NaCl in 0.1 M sodium acetate, pH 4.0) for 45 min (100 μL). Each surface was finally

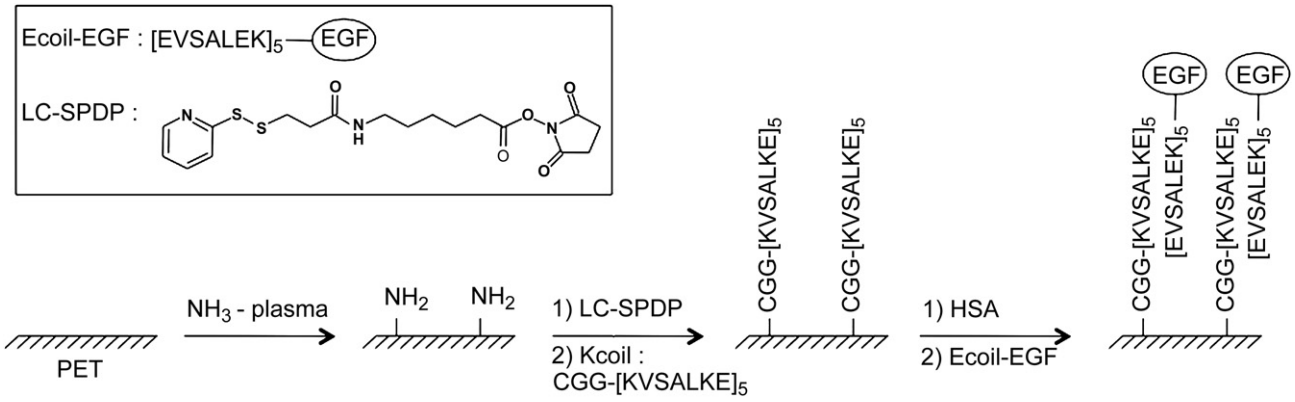

Fig. 1. Schematic diagram of the chemical grafting procedure used for coiled-coil mediated EGF tethering. After amination of PET by NH_3 plasma treatment, cysteine-tagged Kcoil peptide was covalently bound to PET via LC-SPDP linker. Human serum albumin (HSA) treatment was then performed to prevent non-specific adsorption of EGF during subsequent steps. Ecoil-EGF was immobilized onto Kcoil-functionalized PET surfaces via coiled-coil interactions.