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An epidermal growth factor derivative with binding affinity for hydroxyapatite and titanium surfaces

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ABSTRACT

An epidermal growth factor (EGF) derivative with affinity for apatite and titanium surfaces was designed using a peptide moiety derived from salivary statherin, a protein that adheres to hydroxyapatite. Since the active sequence has two phosphoserine residues, the EGF derivative was prepared by organic synthesis, and a 54 residue peptide was successfully prepared using this method. Circular dichroism spectra indicated that the conformation of EGF was not significantly altered by the addition of the affinity peptide sequence and the mitogenic activity was only slightly reduced when compared with the wild-type protein. However, the binding affinity of the modified EGF to hydroxyapatite and titanium was significantly higher than the unmodified EGF. The phosphate groups in the affinity sequence contributed to the affinity of modified EGF to both apatite and titanium. The modified EGF significantly enhanced the growth of cells on hydroxyapatite and titanium. It was also demonstrated that the bound EGF enhanced the signal transduction for longer periods than unbound EGF. In conclusion, the modified EGF had significantly higher binding affinity for apatite and titanium than soluble EGF, and the bound EGF significantly enhanced cell growth by long-lasting activation of intracellular signal transduction.

1. Introduction

Growth factors play important roles in wound healing and tissue regeneration. They are naturally occurring proteins capable of stimulating cellular growth, proliferation and cellular differentiation. Growth factors are important for the regulation of a variety of cellular processes and typically act as signaling molecules between cells. They often promote cell differentiation and maturation, and this varies among individual growth factors. In the field of tissue engineering, growth factors are a fundamental requirement, in addition to cells and materials (either synthetic substrates or naturally occurring matrices) [1–3]. The prolonged retention and activity of growth factors on cells or their surrounding environment (e.g., extracellular matrices (ECM) or artificial implant scaffolds) during the initial repair or even complete healing process are considered to be advantageous in regenerative medicine applications.

Therefore, many researchers have studied growth factor immobilization. Following the demonstration that immobilized insulin enhanced cell growth significantly [4,5], epidermal growth factor (EGF) was also immobilized onto surfaces [6,7]. Recently, many kinds of growth factors have been immobilized and the effects of immobilized growth factors have been discussed [8–10]. The effectiveness of immobilized growth factors for enhancing cell growth has been confirmed using several methods, including micropattern immobilization, antibody blocking, radioisotope labeling and microarray-based comparison with other proteins [8–10]. The effect of immobilized growth factors has been shown to be higher than their soluble counterparts [8–12]. Compared with soluble EGF, a lower amount of immobilized EGF was sufficient for cell growth, and that the maximal cell growth-enhancing effect of immobilized EGF

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was higher than that of soluble EGF. This observation can be explained by the high local concentration of growth factors and the multivalency of immobilized growth factors. In comparison to the effects of soluble EGF, the long-lasting cell activation by immobilized EGF may arise through inhibition of down-regulating mechanisms [8–16]. Activation has been shown to be enhanced by co-immobilization of growth factors with adhesion factors [17,18]. Besides the quantitative effects, such as enhanced growth, immobilized EGF induced effects qualitatively distinct from those of soluble EGF for some cells [19].

In addition to chemical immobilization of growth factors, binding growth factors have also been developed using genetic engineering [20–23]. A prime strategy for the design of these growth factors is their fusion with polypeptide sequences that have an affinity for target proteins or materials. The resulting proteins exhibit collagen-binding, fibrin-binding, or cell-binding affinities. Although this methodology requires steps for elaborate production of recombinant proteins, once the binding growth factors are generated, one can simply add them to the target materials for binding without harmful treatment. Moreover, it is possible to achieve uniform orientation of these molecules on their target surfaces.

The binding domains discussed above were extracted from natural proteins consisting of the 20 canonical (natural) amino acids. In contrast, some binding proteins have active sites consisting of non-canonical amino acids that are modified after translational synthesis of the polypeptides *in vivo*. For example, underwater adhesive proteins containing non-canonical amino acids such as phosphorylated serine from salivary statherin, which is adhesive to hydroxyapatite of teeth, and 3,4-dihydroxyphenylalanine (DOPA) from a mussel protein, play important roles in adhesion to various materials, including polymers, metals and ceramics [24]. Herein we employed another bio-inspired approach, using a peptide derived from salivary statherin, which has noncanonical amino acid phosphoserine residues, and binds readily to hydroxyapatite (HA) [25–27].

In this study, the sequence of EGF was conjugated to the binding peptide sequence from statherin to immobilize active EGF on HA or titanium (Ti). Because the sequence contains two phosphate serines, organic synthesis using a solid phase method was necessary to add the sequence to EGF. The EGF derivative was designed to cover only the active regions of EGF and statherin to ensure precise and productive synthesis.

2. Materials and methods

2.1. Materials

HA plates (15 mm wide and 1 mm thick) were purchased from Pentax (Tokyo, Japan). Ti plates were prepared by vacuum-deposition (Osaka Vacuum Industries; Osaka, Japan) of titanium onto glass plates (15 mm wide and 1 mm thick) using a 400 nm (±25%) electron beam after the glass plate was cleaned nine times by ultrasonication in ultrapure water and dried under a stream of heated air. The coated plate is referred to as Ti. Anti-EGF and fluorescein isocyanate (FITC)-conjugated secondary antibodies were purchased from Cappel Research Reagents (Costa Mesa, CA, USA).

The synthesized peptide is shown in Fig. 1. The active sequence of statherin for binding was added to the C-terminus of truncated EGF by a solid-phase peptide synthesis method using 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids. Low density of amino acid-loaded resin Fmoc-Lys(Boc)-NovaSynR TGA (0.2 mmol/g) was purchased from Novabiochem® (San Diego, CA, USA). In addition to conventional Fmoc-protected canonical amino acids, Ser[PO(Obzl)OH] purchased from Bachem (Bubendorf, Switzerland) was used. The peptide synthesizer ABI433A (Life Technologies, Japan) was employed. After purification of the synthesized peptide using high performance liquid chromatography (HPLC) equipped with a YMC-Triart C18 column (250 × 4.6 mm I.D.) (Supplementary data Fig. S1), the formulations were confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass analysis (MS: m/z 6341.6 $[M + H]^+$, average mass) (Supplementary data Fig. S2). The phosphorylated binding region peptide is referred to as N8P, and the nonphosphorylated peptide is referred to as N8. The EGF derivative is referred to as EGFN8P.

2.2. Circular dichroism (CD) measurements

EGF and synthesized peptides were dissolved in phosphate-buffered saline (PBS, pH 7.4, 200 μg/mL). CD spectra were recorded using a JASCO J-720 spectropolarimeter (Tokyo, Japan) and a 0.1 mm path-length cell with a response time of 0.5 s, a bandwidth of 1 nm, a scan speed of 100 nm/min, and at 20 °C, with an accumulation of eight scans.

2.3. Binding assay

To assay binding of N8 and N8P, the HA or Ti plates were incubated in PBS containing FITC-conjugated N8 or N8P2 at 37 °C. After 3 h, unbound peptide was removed by washing with PBS. The amount of FITC was measured using an Axio Observer Z1 fluorescent microscope (Carl Zeiss, Göttingen, Germany) fitted with a charge-coupled device camera (Cool SNAP HQ, Trenton, NJ, USA) and AxioVision digital image processing software V 4.8.2 (Carl Zeiss, Göttingen, Germany) was used to process the images. To construct a calibration curve, various concentrations of FITC-conjugated peptide were spotted onto the surface.

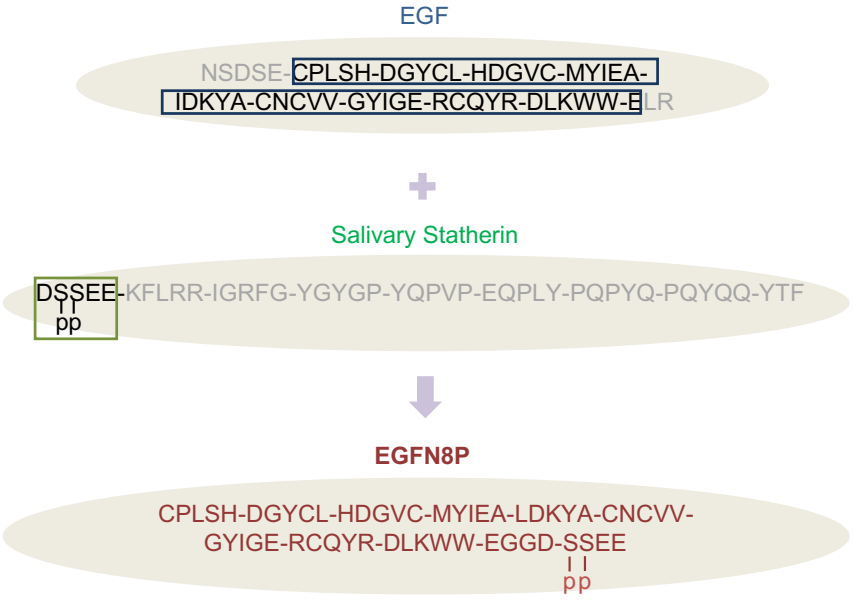

Fig. 1. Peptide sequence and illustration.