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Brief communication

Presentation of fibronectin fragments using affinity protein interactions for enhanced retention and function

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ABSTRACT

We present a protein immobilization system, based on the Src Homology 3 (SH3) affinity domain, allowing for a transient interaction between a fibronectin ligand and a biomaterial surface. This strategy leads to enhanced retention of the fibronectin fragment over adsorbed fibronectin, and increased cellular proliferation and motility over either covalently immobilized or adsorbed fibronectin. The results indicate that intermediate affinity protein immobilization could provide benefits for tissue engineering beyond the traditional immobilization techniques, adsorption or covalent attachment.

1. Introduction

The presentation of proteins and oligopeptides to cells and tissues has emerged as a promising approach in the modulation of biological activities for tissue engineering. In particular, the presentation of fibronectin fragments or the RGD cell binding peptide from fibronectin has been extensively studied for promoting cellular wound healing behaviors, including adhesion, proliferation and motility [1,2]. Commonly, these studies have bound fibronectin either covalently by amine or thiol coupling, or recombinantly through genetic fusions, or non-covalently via adsorption [1,3–5]. These techniques have been shown to be successful for enhancing cellular behaviors for wound healing, but they have limitations. Studies utilizing adsorption have shown that cellular activities and the degree of fibronectin adsorption are highly dependent on the host material surface properties [6,7]. Covalent immobilization and genetic fusion generally show higher levels of bioactivity than adsorption, due in part to higher retention of the protein on the surface [8], but also due to the ability to control the conformation of the protein by site-specific immobilization [9].

However, both adsorption and covalent approaches deprive cells of their native ability to remodel their immediate surroundings. Clustering of RGD cell binding domains promotes cell adhesion and motility via integrins, which is natively achieved by cellular remodeling of extracellular matrix (ECM) ligands [10]. This remodeling is associated with higher rates of cell motility [11]. Covalently immobilized proteins disallow this sort of remodeling due to their irreversible nature. In vivo, remodeling is achieved by transient associations between full-length fibronectin and structural components such as collagen, heparin and other fibronectin molecules [12]. However, in regions of large wounds these components are destroyed or altered in function [13]. In this paper, we show that the use of affinity protein–protein interactions to promote transient interactions with a surface, thus allowing ligand remodeling, leads to high retention of fibronectin and enhanced cellular activities compared to adsorbed and covalent fibronectin, which can be useful in wound healing or tissue engineering.

In this study, we demonstrate the use of a transient protein immobilization system built on the high affinity interaction of the Src Homology 3 (SH3) domain and its associated SH3 binding peptides (SBPs). SH3 domains are widely found in adapter proteins and enzymes in intracellular signaling pathways, and thus SH3 domains are likely to be biocompatible in mammalian systems while having no native function in the extracellular context. In this work, we use the SH3 domain from the hematopoietic "Mona/Gads" adaptor and the SLP-76 T-cell receptor signal transducer peptide. Harkiolaki et al. also identified several variants of the SLP-76 peptide that exhibited different affinities to the Mona/Gads SH3 domain, thus allowing us to tune our system to a desired affinity [14]. This interaction has previously been used as a tunable method of extended release for growth factors [15], and as a proof of concept for monobody engineering [16].

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2. Materials and methods

2.1. Protein fabrication

Recombinant proteins were cloned via PCR assembly and site directed mutagenesis followed by ligation into a PQE60 vector (Qiagen, Valencia, CA) and verified by sequencing. Expression was performed in BL21 *Escherichia coli* with isopropyl β-D-1-thio-galactopyranoside (IPTG) induction for 3–4 h. Proteins were purified non-natively with Ni-NTA chromatography followed by dialysis and lyophilization. For more details, including protein and primer sequences, see Supplemental information.

2.2. Affinity measurement

A Biacore T200 was used for all surface plasmon resonance experiments. EDC/NHS/PDEA chemistry was used to immobilize an elastin-like polypeptide (ELP-SH3) on a CM5 chip (GE Biacore) according to the manufacturer's protocol, utilizing the N-terminal cysteine of ELP-SH3. Six concentrations of each FN10-SBP (tenth type III repeat of fibronectin) between 10 nM and 5 μM in HBS-EP (0.1 M HEPES, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid, 0.005% v/v Tween 20) were prepared. 60 s association and dissociation times were used for each measurement. Surface regeneration was performed twice with 0.5% v/v sodium dodecyl sulfate followed by a 3 min recovery. Biacore kinetics evaluation software was used to fit k_a and K_D for each FN10-SBP. The dissociation kinetic parameter, k_d, was calculated from the fit parameters.

2.3. Fluorescent dye conjugation

Alexa-Fluor 488 SDP ester was purchased from Invitrogen and used to label FN10-containing fusion proteins. In a typical labeling reaction, 5 mg of protein was dissolved in 75 μl 4.5 M GuHCl. 350 μl of 0.2 M NaH_2PO_4 at pH 7.8 was slowly added to the protein solution for a final pH of 7.0. 0.5 mg of dye was dissolved in 50 μl dimethyl sulfoxide and added to the protein solution. The solution was vortexed in the dark for 4 h. Ultrafiltration (Pall, Microsep, 3 kDa MWCO) followed by concentration into phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) was used to remove unconjugated dye and guanidinium.

2.4. Self-assembled monolayer (SAM) synthesis

Circular glass coverslips were purchased from Fisher and cleaned by incubation in 1 M potassium hydroxide for 1 h. They were rinsed and sonicated twice in deionized water. Residual water was removed with an ethanol rinse, and the cover slips were allowed to air-dry. A 3% v/v (3-mercaptopropyl)trimethoxysilane (3-MPTS, Sigma-Aldrich) solution in toluene was prepared, and added to the glass slides for thiol functionalization. After 2 min, the 3-MPTS solution was removed and the slides were washed twice with toluene and air-dried. The slides were incubated with ELP-SH3 or ELP-FN10 (0.33 mg ml^{-1} in 2.5 M GuHCl in PBS) overnight at 4 °C to saturation. The protein solution was removed and surfaces were washed twice with 2.5 M GuHCl in PBS. The bound proteins were refolded through a gradient of decreasing GuHCl concentrations in PBS. After refolding, FN10-SBPs were added (0.25 mg ml^{-1} in PBS) overnight at 4 °C to saturation. Surface saturation was confirmed by incubation with more dilute concentrations of either ELP containing proteins or FN10-SBPs, which did not affect initial coverage.

2.5. Protein retention assay

Protein monolayers were prepared as described above, except that fluorescently labeled ELP-FN10, FN10-SBP2, FN10-SBP13, FN10-SBP14 and FN10 were used instead of their unlabeled counterparts. After overnight incubation of the fluorescent proteins, the surfaces were washed twice with PBS, and imaged with a Typhoon Imager (GE) to obtain an initial fluorescence measurement. Then, the surfaces were incubated in 5 mg ml^{-1} bovine serum albumin (BSA) in PBS. Daily for 5 days, surfaces were washed, imaged and re-incubated in fresh BSA solution. Fluorescence images were quantified with ImageQuantTL (GE).

2.6. Cell culture

NIH 3T3 murine embryonic fibroblasts (ATCC CRL1658) were used for all cell experiments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Corning) supplemented with 10% calf serum (ATCC) and 1% penicillin-streptomycin at 37 °C, 5% CO_2 and high humidity. Cells were used for experiments between passages 5 and 25.

2.7. Cell motility assay

Protein surfaces were synthesized as described above. If ELP-FN10 surfaces were to be used, they were first incubated in 5 mg ml^{-1} BSA in PBS for 3 days to remove any nonspecifically bound protein. Surfaces were then placed in a live-cell microscopy incubation chamber. Cells were seeded at 75 cells mm^{-2} on the surfaces in DMEM supplemented with 10% calf serum and 1% penicillin-streptomycin. The incubation chamber was maintained at 37 °C and 5% CO_2 with high humidity. The cells were allowed to adhere for 4 h, after which all cells were attached to the substrate, and a Zeiss Axiovert inverted phase contrast microscope was used to obtain time lapse images every 10 min for 8 h. At least 25 cells per surface condition per experiment were manually tracked using Zeiss Axiovision tracking software or ImageJ (NIH). Data are reported as the mean average speed of the cells. Each surface condition was repeated at least once.

2.8. Proliferation assay

BrdU (Invitrogen) and Anti-BrdU (Abcam ab136650) and rhodamine-conjugated anti-mouse (Rockland) antibodies were purchased. Surfaces were prepared as described above. Cells were seeded at 100 cells mm^{-2} on surfaces in DMEM supplemented with 10% calf serum and 1% penicillin-streptomycin. Cells were allowed to adhere for 6 h, and fresh medium with BrdU (3.1 μg ml^{-1}) was added. After 160 min, the cells were fixed in 70% ice-cold ethanol for 10 min. DNA was denatured in 4 M HCl for 20 min and then neutralized with 50 mM NaCl in 100 mM Tris-HCl (pH 7.4). The cells were blocked with 5% fetal bovine serum and 1% BSA for 1 h and incubated with anti-BrdU (10 μg ml^{-1}) overnight. Cells were washed and incubated with rhodamine-conjugated anti-mouse antibody (1:20) for 1 h. Cells were manually scored for BrdU incorporation. Six representative, non-overlapping fields were used for each surface. Data are reported as the average percentage of BrdU incorporation ($N = 4$ surfaces).

2.9. Statistical analysis

Data are reported as mean ± standard deviation, unless otherwise noted. Groups were compared using one-way analysis of variance to determine significance between groups. P values <0.05 were characterized as significant.