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A fibronectin mimetic motif improves integrin mediated cell biding to recombinant spider silk matrices

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ABSTRACT

The cell binding motif RGD is the most widely used peptide to improve cell binding properties of various biomaterials, including recombinant spider silk. In this paper we use genetic engineering to further enhance the cell supportive capacity of spider silk by presenting the RGD motif as a turn loop, similar to the one found in fibronectin (FN), but in the silk stabilized by cysteines, and therefore denoted FN_{CC}. Human primary cells cultured on FN_{CC}-silk showed increased attachment, spreading, stress fiber formation and focal adhesions, not only compared to RGD-silk, but also to silk fused with linear controls of the RGD containing motif from fibronectin. Cell binding to FN_{CC}-silk was shown to involve the $\alpha5\beta1$ integrin, and to support proliferation and migration of keratinocytes. The FN_{CC}-silk protein allowed efficient assembly, and could even be transformed into free standing films, on which keratinocytes could readily form a monolayer culture. The results hold promise for future applications within tissue engineering.

1. Introduction

Spider silk, natural as well as recombinantly produced, has been shown possible to use as matrix for culture of mammalian cells [1,2]. However, as shown also for numerous other substrates, the inclusion of cell adhesion motifs from the extracellular matrix (ECM) into silk materials highly increases cell attachment and proliferation [3,4]. Replication of the natural integrin-ECM interaction is highly important in order to obtain a functional cell culture. The integrins do not just confer the physical connection between cells and the surrounding, but also mediate signals controlling for example cell growth, polarity, proliferation and survival. Moreover, the integrins are essential for cell migration by acting as the cells "feet".

The most widely characterized cell adhesion motif is the RGD peptide, first discovered in fibronectin [5], and since then widely utilized for improvement of various biomaterials [6]. The RGD motif is found also in many other molecules of the natural ECM, for example in vitronectin, fibrinogen and in cryptic sites of both collagen I and several of the laminin α chains [7]. Almost half of the known integrins, including $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_{6a}$ and $\alpha_v\beta_8$, have been shown to bind ECM in a RGD-dependent manner [6–8]. However, after initial proofs of RGD as general cell adhesion motif, it soon became clear that integrins in general bind with magnitudes higher affinity to larger RGD containing proteins than to short RGD peptides [9]. The preferred conditions for binding also seem to vary between different integrins. It is believed that the specificity and affinity for each integrin-ligand pair is affected by 1) adjacent amino acid residues, especially the residue following RGD [10], and 2) the conformation of the peptide chain [11]. Extensive work have been put on design and selection of synthetic RGD containing peptides with varying flanking residues and cyclizations to provide conformational restraints in order to achieve best possible affinity and selectivity for a specific integrin [11–13].

Most integrins display affinity for a diverse set of ligands. For example, the integrin $\alpha_v\beta_3$, although originally denoted the vitronectin receptor, has a broad natural ligand binding specificity [14] and has also been shown to bind a large variety of RGD peptides [7]. However, by screening of synthetic peptides it seems as $\alpha_v\beta_3$ prefers the amino acids S/A/T as residue following the RGD sequence [11,12], and that several peptidomimetics where the RGD residues are tightly restrained by cyclisation increase the $\alpha_v\beta_3$-ligand affinity [11,13].

Fibronectin is recognized by at least 10 of the cell surface

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receptors of the integrin family, among which 5 ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$) include the β_1 subunit [9]. The α_5 subunit is found only in combination with β_1 [9] and the $\alpha_5\beta_1$ integrin is unique since it is specialized for binding of fibronectin only [15], and therefore originally denoted the fibronectin receptor [16]. The specific interaction between $\alpha_5\beta_1$ and fibronectin seem to be fundamental for vertebrate development since lack of either $\alpha_5\beta_1$ or fibronectin results in early embryonic lethality [17].

The RGD sequence in the fibronectin molecule forms a slightly deformed type II′ β hairpin turn loop bound by β strands near the C-terminus of the tenth type III domain [18–20]. The decrease in binding affinity of α5β1 for the fibronectin-derived GRGDSP peptide as compared to full length fibronectin is 100–1000 fold [10,15]. It is not completely clear how the $\alpha_5\beta_1$ integrin so specifically recognizes fibronectin, but the surface around the PHSRN motif situated in the 9th domain has been suggested as synergistic for high affinity binding [21–23]. It has also been speculated that the overall structure of fibronectin stabilize the particular conformation of the RGD loop required for $\alpha_5\beta_1$ affinity [19].

Herein we investigated the possibility to imitate the $\alpha_5\beta_1$-specific RGD loop motif of fibronectin by positioning cysteines adjacent to the RGD sequence to allow formation of a disulphide-bridge to constrain the chain into a similar type of turn loop. In this way we could increase the cell adhesion efficacy to a matrix made of a recombinantly produced spider silk protein.

2. Materials and methods

2.1. Genetic incorporation of fibronectin derived cell binding motifs into recombinant spider silk

The recombinant spider silk protein 4RepCT [24] (herein denoted WT) was genetically functionalized with the RGD containing cell binding motif from the fibronectin type III module 10, in three slightly different versions (Fig. 1): In the first (FN_{CC}), two amino acids flanking the RGD sequence was substituted for cysteines to enable loop formation of the motif (**C**TGRGDSPA**C**). In the second (FN_{SS}), the introduced cysteines were substituted for serine to create a linear control (**S**TGRGDSPA**S**). Here the amino acid serine was selected due to its resemblance to cysteine, while lacking the ability to form disulfide-bonds. In the third (FN_{VS}), the original sequence of the motif (**V**TGRGDSPA**S**) was used as a linear, native control. The genes encoding the functionalized variants were made by cloning of oligos encoding the different motifs into the vector encoding 4RepCT using restriction enzymes. The new sequences were introduced N-terminally to 4RepCT and confirmed by sequencing. Protein production in *Escherichia coli* and following purification were done essentially as previously described [24,25].

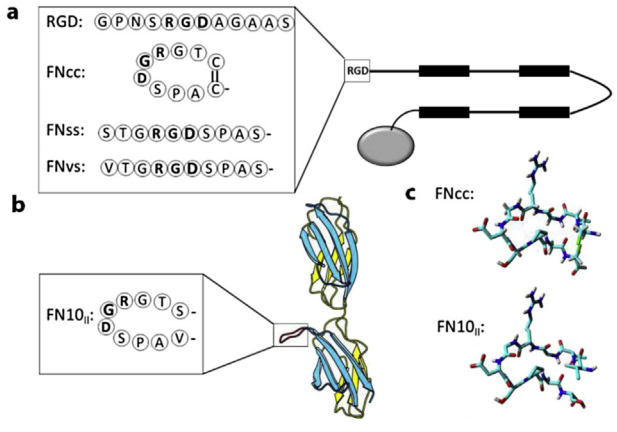

Fig. 1. Silk constructs with cell binding motifs derived from fibronectin. a) Schematics of the silk protein 4RepCT with different RGD motifs genetically introduced to the N-terminus. RGD: the RGD containing peptide used in Ref. [3]. FN_{VS}: the RGD containing decapeptide from fibronectin. FN_{CC}: the same peptide with V and S exchanged to C. FN_{SS}: the same peptide with V and S exchanged to S. b) Structure of the 9th and 10th domain of fibronectin, displaying the turn loop containing the RGD motif. c) Structure model of the RGD loop taken from fibronectin, with the residues V and S mutated to C (adapted from 1FNF.pdb).

2.2. Fabrication of cell culture matrices

After purification, the protein solution was filter sterilized (0.22 μm) and concentrated by centrifugal filtration (Amicon Ultra, Millipore) before preparation of film, as previously described [2,3].

For studies of early attachment and repopulation, solutions of a protein concentration of 0.3 mg/ml were casted into films in 96- and 24 well cell culture plates respectively (Sarstedt, suspension cells) precoated with 1% pluronic to limit cell adhesion to the plastic surface. In control experiments, a reducing agent (either 5 mM dithiothreitol, 20 mM β-mercaptoethanol or 10 mM tris(2-carboxyethyl)phosphine HCl) were added to the protein solutions directly before films were prepared.

For microscopic studies, the proteins were cast as films in chamber glass slides (LabTekII). For proliferation experiments (Alamar blue assay), where whole well coverage is desired, the cell culture wells were coated with a covering protein solution of 0.3 mg/ml for 2 h before the liquid was removed. Films and coated surfaces were allowed to dry over night at 25 °C and 30% relative humidity (rh) under sterile conditions, then washed twice with sterile 20 mM phosphate buffer, pH 7.4, and pre-incubated with complete cell culture medium for 1 h at 37 °C with 5% CO_2 before cell seeding.

The free-standing films were prepared by applying of a droplet of protein solution (3 mg/ml) onto a ~3 mm wide frame of metal wire hanging hooked up in a well of a 96-well plate and allowed to dry over night at 25 °C and 30% rh under sterile conditions.

The control bovine fibronectin (Sigma–Aldrich F1137) was coated at recommended concentration (5 μg/cm^2) over night at 37 °C.

2.3. Structural analysis of matrices

Fourier transform infrared (FTIR) spectra of fibers, casted films and free-standing films were recorded on a FTIR spectrometer (Bruker). The films were placed on a crystal for measuring IR spectra by attenuated total reflection. For each spectrum 100 scans were averaged. The amide I region was further analyzed to compare the peak height of α-helical (1654 cm^{-1}) and β-sheet (1629 cm^{-1}) structures, respectively.

2.4. Cell culture

Human dermal microvascular endothelial cells (EC), (HDMEC, PromoCell GmbH, Germany) isolated from dermis from adult donor were grown in culture flasks coated with gelatin (Sigma Aldrich) in complete endothelial cell media MV, containing 5% fetal bovine serum (PromoCell GmbH, Germany). All experiments with HDMECs were performed in this serum containing medium. Human mesenchymal stem cells (hMSC, Gibco) from bone marrow were grown in culture flasks coated with CELLstart (Gibco) in complete StemPro MSC serum free medium CTS (Gibco) containing 25 ng/μl fibroblast growth factor β (Gibco) and 2 mM Glutamax (Gibco). Normal human epidermal keratinocytes from adult skin (NHEK-ad) were purchased from Lonza. Subculture, proliferation and migration experiments were done in KGM-Gold (Lonza), containing