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Syndecan- and integrin-binding peptides synergistically accelerate cell adhesion

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ABSTRACT

Integrins and syndecans mediate cell adhesion to extracellular matrix and their synergistic cooperation is implicated in cell adhesion processes. We previously identified two active peptides, AG73 and EF1, from the laminin α1 chain LG4 module, that promote cell attachment through syndecan- and α2β1 integrin-binding, respectively. Here, we examined time-dependent cell attachment on the mixed peptides AG73/EF1. The AG73/EF1 promoted stronger and more rapid cell attachment, spreading, FAK phosphorylation that reached a maximum at 20 min than that on AG73 (40 min) or EF1 (90 min) supplied singly. Thus, the syndecan- and α2β1 integrin-binding peptides synergistically affect cells and accelerate cell adhesion.

1. Introduction

Cell attachment and spreading on extracellular matrix (ECM) are essential cellular processes for the promotion of cell growth, migration, and invasion [1]. These interactions play key roles in a variety of cellular processes, including embryonic development, tumor metastasis, and wound healing. Cell attachment and spreading processes on ECM involve rapid colocalization of cell surface receptors and cytoplasmic proteins as focal contacts (FCs), which contain integrin, a heterodimeric cell adhesive receptor [2]. Integrin activation is the initial step for promotion and stabilization of FC formation, and "inside-out" signals via cytoplasmic domains of integrin which mediate its activation [3,4]. Activated integrin physically binds to the ECM via ectodomains, and fibrillar organization of the actin cytoskeleton occurs through binding to various components via cytoplasmic domains, such as focal adhesion kinase (FAK), talin, and vinculin during adhesion maturation [5].

Recently, several reports have suggested that integrins can cooperate with other receptors in cell adhesion to the ECM. For example, integrins and syndecans, with both components promoting cell adhesion in concert, to form an adhesion complex through attachment with the ECM proteins [6,7]. However, a time course for syndecan- and integrin-mediated cell attachment and spreading has not yet been established.

Previously, we identified two active synthetic peptides, AG73 (RKRLQVQLSIRT, mouse laminin α1 chain 2719-2730) and EF1 (DYATLQLQEGRLHFMFDLG, mouse laminin α1 chain 2747-2765), in the α1 chain LG4 module [8–11]. AG73 binds to syndecan and promotes cell adhesion with membrane ruffling. EF1 binds to α2β1 integrin and promotes cell spreading with well-organized actin stress fibers and FCs [11]. Site-directed mutagenesis analysis in recombinant LG4 has confirmed that the AG73 site is critical for cell attachment through syndecans and that the EF1 site is mainly involved in cell spreading through α2β1 integrin [12]. When AG73 and EF1 were mixed and conjugated on a chitosan membrane, synergistic effects of cell attachment were observed [13]. We hypothesize that active cell attachment involves multiple binding to ECM via different binding sites and ligands. We view laminin as a useful bioactive protein for initiating the analysis of these types of multiple cooperative interactions, since many of its active sites have been defined and their cellular receptors have been identified.

In the present paper, we study the synergy effect of syndecan- and integrin-mediated cell attachment using AG73 and EF1. We evaluated cell attachment and spreading by time course analysis using varying ratios of the peptides.

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2. Materials and methods

2.1. Cell attachment and spreading assays

Cell attachment and spreading assays were quantified as previously described with minor modifications [12–14]. Human dermal fibroblasts (HDFs) (Iwaki) were maintained in DMEM containing 10% FBS (Invitrogen). The peptide-coated plates were washed and blocked with 1% BSA (Sigma–Aldrich) in DMEM. Then 100 μl HDFs (5×10^3 cells) in DMEM containing 0.1% BSA were added to each well and incubated at 37 °C for 10, 20, 40, 60, 90, and 120 min. The cells were fixed by addition of prewarmed 10% formaldehyde and the attached cells were stained with 0.2% crystal violet aqueous solution in 20% methanol. The attached cells were photographed using a BZ-8000 microscope (Keyence) and six randomly selected fields (0.67 mm^2) were counted. The cell area was calculated using BZ-analyzer software, with the cells over 600 μm^2 were classified as 'spread cells'. Immunostaining of actin cytoskeleton was followed in previous report [13].

2.2. Immunoblotting of FAK signaling

After HDFs were incubated on the various peptide-coated plates (2×10^4 cells/well) for 90 min, the cells were lysed with 50 μl of SDS sample buffer, resolved by 7.5% SDS–PAGE. Then, western blotting analysis was performed using anti phospho-FAK (Tyr397) or anti FAK polyclonal antibody (cell signaling). The relative phosphorylation of FAK Tyr397 was assessed using NIH image 1.63 software.

2.3. Peptide coated plate

The peptides AG73 and EF1 synthesis [11,14] and the coating efficiency of synthetic peptides on the plate [15] were described in previous paper.

3. Results

3.1. Cell attachment activity of peptides and time course analysis of cell attachment

AG73, EF1, and mixtures of both peptides at various ratios were coated on plates and the attachment activity of HDFs was examined at 60 min (Fig. 1). The AG73 peptide alone promoted cell attachment (Fig. 1A) and the cells were round with ruffling membranes as shown previously (Fig. 1B(a)) [10]. The EF1 peptide alone promoted cell attachment and spreading, and the cells appeared thin and elongated (Fig. 1B(c)). EF1-mediated attachment activity was lower than that of AG73 (Fig. 1A). The mixture of AG73 and EF1 at various molar ratios (9:1, 4:1, 1:1, 1:4, and 1:9) showed stronger cell attachment activity compared with that of AG73 (Fig. 1A). The strongest cell attachment and cell spreading activities were observed at the equimolar ratio (AG73/EF1), indicating that the AG73 and EF1 peptides were synergistically enhancing cell attachment and spreading by 60 min (Fig. 1A and B).

Next, we analyzed the time course of cell attachment using AG73, EF1, and AG73/EF1 (AG73:EF1 = 1:1) (Fig. 1C). HDFs attached faster to AG73 with a half-maximal attachment at 20 min. The EF1 peptide promoted slow attachment with a half-maximal attachment at 45 min. The peptide mixture AG73/EF1 promoted faster cell attachment over the two individual peptides with a half maximum at 10 min. These results suggest that the peptide mixture accelerates cell attachment through both syndecan and integrin-binding processes.

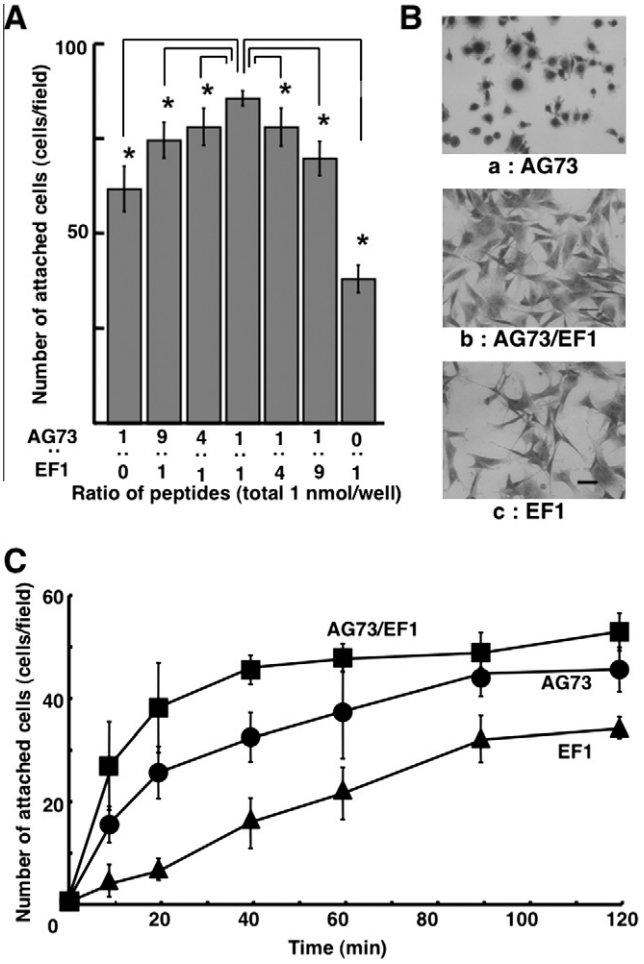

Fig. 1. Cell attachment activities of AG73 and EF1. AG73, EF1, and the peptide mixture in a various ratios were used to coat 96-well plates as described in Section 2. (A) The peptides were coated on the plate in various molar ratios for totally 1 nmol/well mixture. HDFs were allowed to attach to the peptides for 1 h, then were stained. The attached cells in six randomly selected fields were counted. (B) Appearance of cells on peptide substrates. Bar; 50 μm. (C) AG73 (circle), EF1 (triangle), and a mixed AG73/EF1 (square) molar ratio of 1 nmol peptide were coated onto 96-well plates and HDFs were allowed to attach. The attached cells were fixed with 10% formaldehyde after 10, 20, 40, 60, 90, and 120 min incubations. Each value represents the mean ± S.D. of triplicate experiments. * Denotes a P value of <0.05 against AG73/EF1 (1:1).

3.2. Time course analysis of cell spreading

Time course cell spreading activity in response to AG73, EF1, and AG73/EF1 were examined (Fig. 2). The ratio of spread cells on AG73 was approx. 30% after a 120 min-incubation (Fig. 2A). On EF1, the ratio of spread cells was higher than that on AG73 (Fig. 2A). Cells on AG73/EF1 spread rapidly and the highest number of spread cells 90% at 60 min was observed (Fig. 2A). Cell size following growth on the peptides was also determined (Fig. 2B). The cell area reached a plateau at 90 min after cell seeding. The cell areas were AG73 < EF1 < AG73/EF1, indicating that the peptide mixture AG73/EF1 promoted not only accelerated spreading but also increased cell area.

3.3. Immunostaining of actin cytoskeleton

The integrin cytoplasmic domain associates with the actin cytoskeleton and modulates cell morphology. We also evaluated the

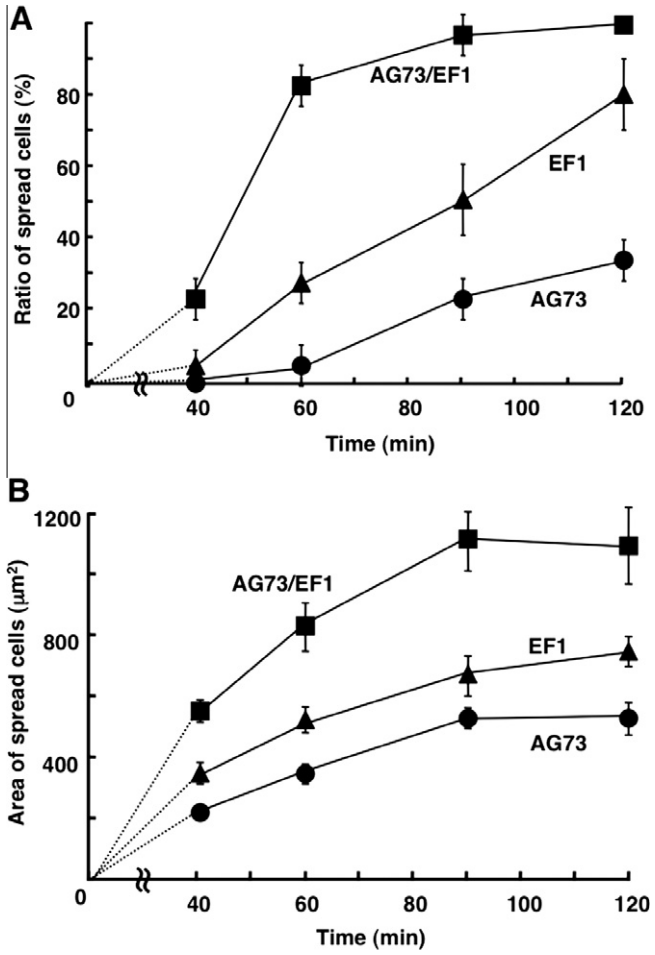

Fig. 2. Time course analysis of cell spreading activity in response to mixed peptides. AG73 (circle), EF1 (triangle), and a mixed AG73/EF1 (square) were coated onto 96-well plates and HDFs were allowed to attach. The attached cells were fixed with 10% formaldehyde addition after 10, 20, 40, 60, 90, and 120 min incubations and then stained with crystal violet. (A) The spread cells (exceeded 600 μm^2) were counted. (B) The area of cell spreading was analyzed. Each value represents the mean ± S.D. of triplicate experiments. $n = 30$.

organization of the actin cytoskeleton of HDFs attached to AG73, EF1, and AG73/EF1 (Fig. 3). HDFs on AG73 showed actin accumulation at the edges of the cells with ruffling on the membranes. In contrast, HDFs on EF1 formed actin stress fibers and an elongated cell morphology (Fig. 3). When HDFs on AG73/EF1 were immunostained, well-organized actin stress fibers and the more extensive cell spreading were observed (Fig. 3). These results demonstrate that the cellular responses to the two peptides involve rapid cell attachment on AG73 and accelerated cell spreading via EF1.

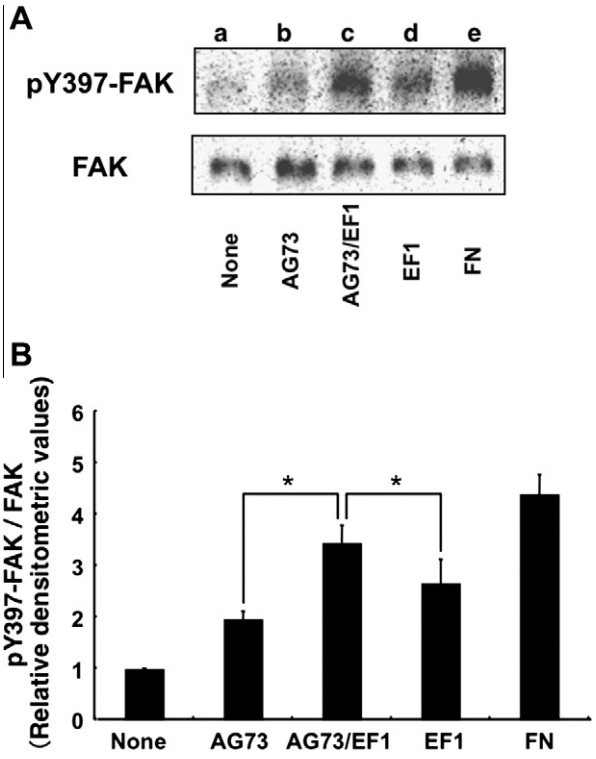

Fig. 4. FAK Tyr397 phosphorylation on mixed peptides. (A) FAK Tyr397 phosphorylation and FAK of HDFs on (a) control; (b) 1 nmol of AG73, (c) 1 nmol each of AG73/EF1, (d) 1 nmol of EF1, and (e) 1 mg/ml of fibronectin. The HDFs were incubated on the various peptide-coated plates for 90 min, then were lysed by addition of 50 μl of SDS sample buffer and assessed by Western blotting. (B) Quantification of (A).

3.4. FAK signaling

FAK is an important component of FCs and FAK in FCs undergo autophosphorylation of its major phosphorylation site tyrosine 397. Tyr397 phosphorylation of FAK is critical for FCs and actin cytoskeleton formation [4]. When we evaluated the Tyr397 phosphorylation of FAK, both the syndecan-binding AG73 and α2β1 integrin-binding EF1 peptides promoted Tyr397 phosphorylation. AG73 induced weaker phosphorylation than that on EF1 (Fig. 4). The peptide mixture AG73/EF1 promoted the strongest phosphorylation, suggesting that the dual-ligation of both syndecan and α2β1 integrin enhances FAK signaling through the cross-interaction of the receptors.

4. Discussion

Cell adhesion to ECM occurs through a wide variety of cell surface receptors, including integrins, syndecans, and growth factor

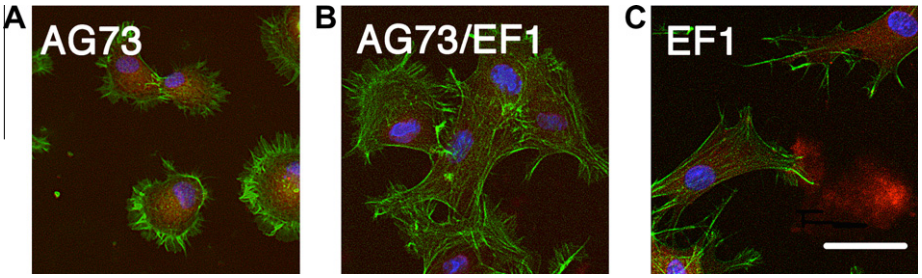

Fig. 3. Organization of actin stress fibers. HDFs were plated on 8-well glass chambers coated with 2 nmol/well peptide for 2 h. Cells were fixed, and then stained with phalloidin, anti-vinculin antibody, and DAPI for actin filaments (green), vinculin (red), and nucleus (blue). (A) AG73; (B) AG73/EF1; and (C) EF1. Bar: 20 μm.

receptors. Integrins, a major class of cell surface receptors, and several integrins bind to laminins. Syndecans, a family of transmembrane heparan sulfate proteoglycans, have multiple functions and also bind to laminins. Syndecans are involved in promotion of cell motility, FC assembly, and regulation of growth factors [16–18]. Recent studies have also revealed that cooperation with integrins is one of major function of the syndecans [6,7,19,20], however, the progression mechanism of the two different receptors has not been well defined. Here, we focus on the order of syndecan- and integrin-mediated cell attachment process using synthetic peptides derived from laminin.

Cell attachment on AG73 was faster than that on EF1. However, EF1 more effectively promoted cell spreading than AG73. AG73/EF1 strongly accelerated and promoted both cell attachment and cell spreading. The number of attached cells on AG73/EF1 (total 1 nmol/well) was two fold greater than the sum of the attached cells on AG73 and EF1 (each 0.5 nmol/well) (data not shown), implying that AG73 and EF1 cooperatively interact with cells and synergistically accelerated cell attachment. AG73/EF1 promoted cell attachment, cell spreading, actin cytoskeleton organization, and FAK Tyr397 phosphorylation of HDFs. Taken together, rapid AG73 binding improves late EF1 binding, and AG73/EF1 may effectively accelerate the activation of integrins by syndecan binding.

The coating efficiencies of AG73 and EF1 were 5.2 ± 1.7% and 36.0 ± 7.0%, respectively, suggesting that AG73/EF1 would contain AG73 and EF1 at a 1:7 molar ratio. We previously reported that chitosan, a polysaccharide, is a useful scaffold and that peptide-conjugated chitosan membrane can be used to analyze the biological functions of cell-adhesive peptides and mimic the protein functions [13,14,21]. When we covalently conjugated both the AG73 and EF1 peptides to a chitosan membrane, cell attachment and cell spreading activity were accelerated at the AG73:EF1 molar ratio of 1:4–1:9 [13]. The effective molar ratios of syndecan- and integrin-binding peptides on cell adhesion are comparable in the previous and current studies. The effective molar ratio for cell adhesion may be due to the receptor-ligand affinity but that has not yet been demonstrated. However, the finding of effective molar ratios is useful for evaluating the mechanism of syndecan- and integrin-mediated cell adhesion caused by ECM molecules and for designing biomaterials for cell engineering.

The cooperation between syndecan- and integrin-mediated cell attachment has been described. We previously reported that mutations in the syndecan-binding sites of the laminin α1 chain LG4 module result in a significant reduction in cell attachment, whereas mutations in the α2β1 integrin-binding site retain cell attachment activity of the module but reduce cell spreading activity [12]. In this paper we used AG73 and EF1 derived from laminin α1 chain LG4 module that specifically bound to syndecan and α2β1 integrin, respectively, and found an acceleration of cell attachment by the synergistic effect of syndecan- and integrin-mediated cell attachment (Fig. 5). Syndecan-mediated cell attachment is faster than integrin-mediated attachment (Fig. 5). These suggest that cell attachment to the laminin α1 chain LG4 module is initiated by syndecan-binding and followed by integrin binding, and then promotes cell spreading efficiently. The receptor-specific peptides have various advantages and are a powerful tool for evaluating the mechanism of the multi-receptor interactions, including cell-ECM interactions.

In this paper, we used a syndecan-binding peptide, AG73, and an α2β1 integrin-binding peptide, EF1, to study the temporal ligation of these receptors in the cell attachment processes by time course analysis. Both cell attachment and cell spreading were accelerated when cells were plated on AG73/EF1, implying that rapid cell binding via syndecan may occur primarily by an ionic charge interaction that initiates subsequent ECM-integrin-binding through intracellular activation. We conclude that the two peptides elicited a synergistic response that involved cooperation between syndecan and integrin, and simultaneous cellular interactions with two receptors could be critical for cell adhesion and spreading. The mixed peptide approach is useful for evaluating multi-receptor crosstalk and for mimicking multifunctional proteins, including ECM molecules.

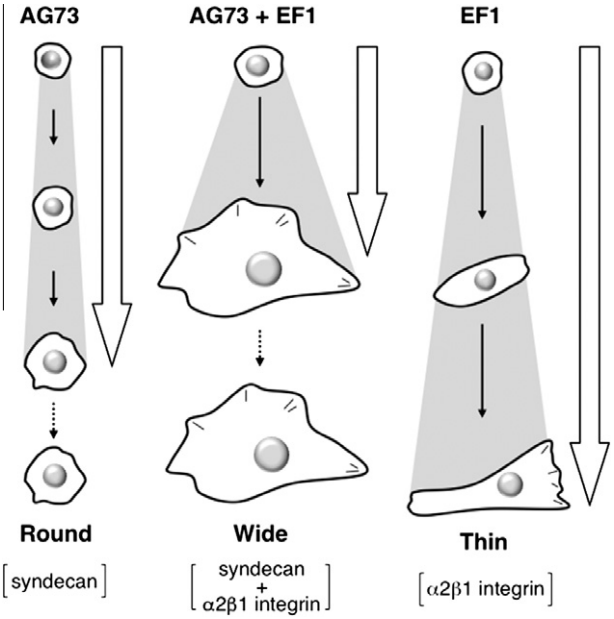

Fig. 5. Schematic models of cell adhesion via syndecan, integrin, and a combination of both receptors. HDFs on AG73 showed a round cell morphology and on EF1 the cells showed an extended morphology. AG73/EF1 promoted the most extensive cell spreading, with organization of the actin cytoskeleton and FC formation. Cell areas are indicated by shadows.

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