Octa-arginine and Octa-lysine Promote Cell Adhesion through Heparan Sulfate Proteoglycans and Integrins

Yuji Yamada,* Toru Onda, Keisuke Hamada, Yamato Kikkawa, and Motoyoshi Nomizu

Department of Clinical Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan.

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Octa-arginine (R8) has been extensively studied as a cell-penetrating peptide. R8 binds to diverse transmembrane heparan sulfate proteoglycans (HSPGs), including syndecans, and is internalized by cells. R8 is also reported to bind to integrin β1. In this study, we evaluated the biological activities of R8 and octa-lysine (K8), a peptide similar to R8, with a focus on cell adhesion. R8 and K8 were immobilized on aldehyde-agarose matrices via covalent conjugation, and the effect of these peptides on cell attachment, spreading, and proliferation was examined using human dermal fibroblasts. The results indicated that R8- and K8-matrices mediate cell adhesion mainly via HSPGs. Moreover, R8- and K8-matrices interacted with integrin β1 and promote cell spreading and proliferation. These results are useful for further understanding of the R8-membrane interactions and the cellular uptake mechanisms. In addition, the R8- and K8-matrices may potentially be used as a multi-functional biomaterial to promote cell adhesion, spreading, and proliferation.

Key words octa-arginine; octa-lysine; heparan sulfate proteoglycan; integrin; cell attachment; cell spreading

INTRODUCTION

Transmembrane heparan sulfate proteoglycans (HSPGs) interact with extracellular proteins, such as extracellular matrix (ECM) proteins and growth factors, and regulate diverse biological functions.1) Electrostatic interactions and hydrogen bonding between sulfate groups in heparan sulfate (HS) and basic amino acids in extracellular proteins contribute largely to HS-protein binding.2,3) Therefore, HS binding sites in proteins are generally rich in arginine and lysine residues.2) Arginine shows a stronger interaction with sulfate groups than lysine because of the strong hydrogen bonding of the guanidino group.2) Thus, HS binding sites are assumed to consist of only arginine residues. Actually, a cell-penetrating peptide octa-arginine (R8) binds to diverse transmembrane HSPGs, including syndecans, and is internalized by cells via endocytosis.5)

Syndecans play a critical role in cell adhesion by interacting with ECM proteins,5) such as laminins.6) We have identified many cell attachment sequences by screening a laminin-derived synthetic peptide library.5,9) These peptides mediate cell attachment via specific receptors, such as syndecans and integrins, when immobilized on cell culture substrates. AG73 (mouse laminin α1 chain, RKRLQVQLSIRT) is a representative of the laminin-derived cell adhesive peptides and binds to syndecans.10,11) AG73 promotes attachment and differentiation of various types of cells.12,13) These studies indicate that syndecan-binding peptides, such as AG73, are useful as cell adhesion molecules for cell culture. Considering its syndecan-binding ability, R8 may also act as a promising cell adhesion molecule. R8 has also been reported to bind to integrin β1, the transmembrane receptor that facilitates cell-ECM adhesion.14) However, to date, no study has reported the cell attachment activity of R8.

In this study, the biological functions of R8 and octa-lysine (K8), a peptide with the same net charge as R8, were analyzed with a focus on cell adhesion. Synthetic peptides were conjugated to aldehyde-functionalized agarose matrices via thiozolidine formation.15) Then cell attachment, spreading, and proliferation on the peptide-agarose matrices were evaluated using human dermal fibroblasts (HDFs). Further, we examined the effect of heparin and ethylenediaminetetraacetic acid (EDTA) on attachment of cells to the R8- and K8-matrices to analyze the mode of cell adhesion to these peptides.

MATERIALS AND METHODS

Peptide Synthesis All peptides were manually synthesized using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy with a C-terminal amide form. The resulting protected peptides were deprotected and cleaved from the resin using trifluoroacetic acid (TFA)/1,3-dimethoxybenzene/thioanisole/m-cresol/ethanedithiol/H2O (85 : 3 : 3 : 3 : 3, v/v). Crude peptides were purified using reverse-phase HPLC on a COSMOSIL 5C18-AR-II column (Nacalai Tesque, Kyoto, Japan) using a gradient elution with water/acetonitrile containing 0.1% TFA. Purity and mass of the peptides were confirmed using analytical HPLC and electrospray ionization mass spectrometry at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences.

Peptide Conjugation to Aldehyde-Functionalized Agarose Matrices Aldehyde-functionalized agarose was synthesized from agarose (Agarose S, NIPPON GENE, Tokyo, Japan) via (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) oxidation as previously reported.15) The aldehyde content of the aldehyde-agarose was 19.2% agarobiose unit. The aldehyde-agarose dissolved in water at 100 µg/mL was added to tissue culture treated 96-well plates (AGC Techno Glass, Shizukuoka, Japan, 100 µL/well) and allowed to dry for preparation of aldehyde-agarose matrices. Then, cysteine-containing peptides in 100 mM acetic buffer at pH 5 (100 µL/well) were added and the plates were incubated for 2 h. The wells were
washed with phosphate-buffered saline (PBS) and used for cell attachment and cell proliferation assays.

**Cell Culture** HDFs (Kurabo, Tokyo, Japan) were maintained in low-glucose Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific, Waltham, MA, U.S.A.) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific).

**Cell Attachment Assay** HDFs grown on culture plates were detached using 0.05% trypsin–EDTA solution and suspended in 0.1% bovine serum albumin (BSA)/DMEM. Then, the cells were seeded onto peptide-agarose matrices (5 × 10³ cells/100 µL/well). For the inhibition assay, the cells were seeded in the presence or absence of either 10 µg/mL heparin, 5 mM EDTA, or 10 µg/mL mouse monoclonal antibodies against human integrin β1 (AIIB2), α3 (P1B5), α5 (P1D6), and α6 (P5G10). The cells were incubated for 1 h to evaluate the number of attached cells and 2 h to evaluate cell spreading. After incubation, the attached cells were fixed, stained with 0.2% crystal violet aqueous solution containing 20% methanol, and imaged using a BZ-X810 microscope (Keyence, Osaka, Japan). The numbers of attached cells in nine central fields (0.77 mm² each) were counted and their averages were calculated using BZ-X800 Analyzer software. The area of the attached cells was measured using BZ-X800 Analyzer software.

**Immunostaining** HDFs in 0.1% BSA/DMEM were added to peptide-agarose matrices (5 × 10³ cells/100 µL/well) and incubated for 2 h. The attached cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized using 0.1% Triton X-100, and blocked with 1% BSA in PBS for 1 h. Then, the cells were incubated with anti-vinculin antibody (hVIN-1, MilliporeSigma, St. Louis, MO, U.S.A., 1:1000) in PBS containing 1% BSA and 0.1% Tween 20 for 1 h. Bound antibody and actin filaments were labeled with secondary antibody (Alexa Fluor® 594-labeled goat anti-mouse IgG1 antibody, Thermo Fisher Scientific, 1:1000) and Alexa Fluor® 488-labeled phalloidin (Thermo Fisher Scientific, 1:100) for 60 min at room temperature in the dark. The nuclei were stained with Hoechst 33258 (Thermo Fisher Scientific, 1:10000) in PBS for 15 min at room temperature. Cells were washed with PBS and imaged using a BZ-X810 microscope.

**Cell Proliferation Assay** HDFs in 10% FBS/DMEM were seeded into peptide-agarose matrices (5 × 10³ cells/100 µL/well). After 2 d, cell viability was measured using cell counting kit-8 (CCK8, Dojindo, Kumamoto, Japan). CCK8 was mixed with DMEM at a 1:10 volume ratio and added to the cells (100 µL/well) and incubated for 1 h at 37°C. Then, the absorbance at 450 nm was measured.

**Statistics** Data were analyzed using one-way ANOVA with Tukey’s multiple-comparison test. A confidence level of >95% (p < 0.05) was considered significant.

**RESULTS AND DISCUSSION**

**Cell Attachment on R8- and K8-Matrices** First, we prepared peptide-conjugated-agarose matrices (Fig. 1). Agarose, a neutral polysaccharide, was employed as a scaffold to minimize charge-induced interactions with highly charged peptides. Oligo-peptides with eight, four, and two arginine or lysine residues were synthesized with a Cys-Gly-Gly (CGG) sequence at the N-terminus (Table 1). The cysteine residue was used for conjugation to the aldehyde group, and two glycine residues were used as a spacer. The syndecan-binding peptide AG7311,12) was used as a positive control. In addition, A99 (mouse laminin α1 chain, AGTFALRGDNPQG),16,17) which has the RGD motif and binds to integrin αvβ3, was used as a control. The peptides were covalently conjugated to agarose-agarose matrices via thiazolidine formation as previously reported.15) Cell attachment activity of the peptide-matrices was evaluated using HDFs under serum-free conditions (Fig. 2). All oligo-arginine- and oligo-lysine-matrices showed dose-dependent cell attachment activity whereas the agarose matrix without peptides showed no cell attachment. Notably, more cells attached to the R8- and K8-matrices than

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![Fig. 1. Schematic Illustration of the Peptide Immobilization Process Using CGG-Peptides and Aldehyde-Functionalized Agarose Matrices](image_url)

**Table 1. Sequences of Synthetic Peptides**

| Peptide | Sequence<sup>a</sup> |
|---------|---------------------|
| R8      | CGG-RRRRRRRR        |
| R4      | CGG-RRRR            |
| R2      | CGG-RR              |
| K8      | CGG-KKKKKKKK        |
| K4      | CGG-KKK             |
| K2      | CGG-KK              |
| AG73    | CGG-RKRLQVLSIRT     |
| A99     | CGG-AGTFAFALGDPQG   |

<sup>a</sup>) All peptides were synthesized with a C-terminal amide form.
Heparin and EDTA, respectively. Cell attachment to the R8- and K8-matrices was completely inhibited by heparin or EDTA to determine the mode of attachment mediated by HSPGs, such as syndecans. EDTA reduced cell attachment using heparin or EDTA to the R8- and K8-matrices was also completely inhibited by heparin, although few HDFs had well-organized fibers at 2 h. These results indicated that cell attachment to the R8- and K8-matrices was mainly mediated via HSPGs, but partially via divalent cation-dependent adhesion, such as integrin-mediated adhesion. The binding of R8 and K8 to integrins has been previously reported. In 1993, using immunoprecipitation, Vogel et al. found that R8 and K8 bind to β1 integrins. Therefore, the divalent cation-dependent cell attachment to the R8- and K8-matrices is most likely mediated by integrin β1. In the same experiment using HeLa cells which express integrin β1, heparin completely inhibited cell attachment to R8- and K8-matrices, but EDTA did not show significant reduction (Supplementary Fig. S1). The contribution of integrin β1 was assumed to vary depending on the balance with the expression level of HSPGs because HeLa cells express high levels of HSPGs.

**Cell Spreading on R8- and K8-Matrices** Morphology of the cells cultured on each peptide-matrix was observed (Fig. 4). As previously reported, HDFs cultured on the AG73-matrix were round, while HDFs cultured on the A99-matrix spread and showed typical fibroblast-like morphology, which is a characteristic of integrin-mediated adhesion. Further, HDFs cultured on the R8- and K8-matrices spread and showed fibroblast-like morphology; area of the cells cultured on the R8- and K8-matrices was larger than that of the cells cultured on the AG73-matrix and comparable with that on the A99-matrix. There was no significant difference in cell spreading between the R8- and K8-matrices. Next, we examined the effect of EDTA and the anti-integrin antibodies on the cell morphology. EDTA and the antibodies did not affect the cell morphology on the AG73-matrix. In the case of the A99-matrix, EDTA completely blocked cell attachment, while the anti-integrin β1 antibody only slightly reduced cell spreading. This occurred because A99 binds mainly to integrin αvβ3 and less to integrin β1. EDTA and the anti-integrin β1 antibody dramatically reduced cell spreading on the R8- and K8-matrices. The cell area was comparable to that on the AG73-matrix. These results indicate that HDFs spread in an integrin β1-dependent manner on the R8- and K8-matrices. The number of attached cells under these conditions was measured, and no statistically significant decrease was observed for the integrin β1 antibody treatment (Supplementary Fig. S2). These results indicate that for the R8- and K8-matrices, quantification of cell area, rather than number of attached cells, is more suitable to analyze the contribution of integrins. When HDFs were seeded to plates coated with polylysine, a polymer of lysine that is commonly used for cell adhesion, the cells did not spread and EDTA did not affect the cell area (Supplementary Fig. S3). These results indicate that the contribution of integrins was minimal in the adhesion of cells to polylysine-coated plates.

**Formation of Actin Stress Fibers and Focal Adhesions on R8- and K8-Matrices** Next, we observed the formation of actin stress fibers and focal adhesions, which are characteristic of integrin-mediated cell adhesion (Fig. 5). As previously reported, HDFs cultured on the A99-matrix showed well-organized actin stress fibers with vinculin accumulation, indicating formation of focal adhesions. HDFs cultured on the AG73-matrix were round with membrane ruffling at 2 h and then weakly spread at 18 h, but actin stress fibers were hardly organized. HDFs cultured on the R8- and K8-matrices formed well-organized actin stress fibers with focal adhesion at 18 h, although few HDFs had well-organized fibers at 2 h. These results indicated that cell attachment to the R8- and K8-matrices was mainly mediated via HSPGs, but partially via divalent cation-dependent adhesion, such as integrin-mediated adhesion. The binding of R8 and K8 to integrins has been previously reported. In 1993, using immunoprecipitation, Vogel et al. found that R8 and K8 bind to β1 integrins. Therefore, the divalent cation-dependent cell attachment to the R8- and K8-matrices is most likely mediated by integrin β1. In the same experiment using HeLa cells which express integrin β1, heparin completely inhibited cell attachment to R8- and K8-matrices, but EDTA did not show significant reduction (Supplementary Fig. S1). The contribution of integrin β1 was assumed to vary depending on the balance with the expression level of HSPGs because HeLa cells express high levels of HSPGs.

**Effects of Heparin and EDTA on Cell Attachment to R8- and K8-Matrices** We examined inhibition of cell attachment using heparin or EDTA to determine the mode of adhesion of the peptides to cells (Fig. 3). Heparin blocks cell attachment mediated by HSPGs, such as syndecans. EDTA inhibits divalent cation-dependent adhesion, such as integrin-mediated attachment. As previously reported, cell attachment to the AG73- and A99-matrices was completely inhibited by heparin and EDTA, respectively. Cell attachment to the R8- and K8-matrices was also completely inhibited by heparin, similar to that to the AG73-matrix, indicating that the cells attached to the R8- and K8-matrices via HSPGs. However, EDTA also reduced cell attachment to the R8- (77%) and K8- (71%) matrices. These results indicated that cell attachment to the R8- and K8-matrices was mainly mediated via HSPGs, but partially via divalent cation-dependent adhesion, such as integrin-mediated adhesion. The binding of R8 and K8 to integrins has been previously reported. In 1993, using immunoprecipitation, Vogel et al. found that R8 and K8 bind to β1 integrins. Therefore, the divalent cation-dependent cell attachment to the R8- and K8-matrices is most likely mediated by integrin β1. In the same experiment using HeLa cells which express integrin β1, heparin completely inhibited cell attachment to R8- and K8-matrices, but EDTA did not show significant reduction (Supplementary Fig. S1). The contribution of integrin β1 was assumed to vary depending on the balance with the expression level of HSPGs because HeLa cells express high levels of HSPGs.
Fig. 4. Cell Spreading on R8- and K8-Matrices

The peptides (100µM) were conjugated to the aldehyde-agarose matrices. HDFs in 0.1% BSA/DMEM (5 x 10^3 cells/well) were seeded into the wells in the presence or absence of 5 mM EDTA or 10 µg/mL anti-integrin antibodies (β1, α3, α5, and α6) and incubated for 2 h. (A) Morphology of HDFs attached to peptides. Scale bar = 100 µm. (B) Area of HDFs attached to the peptides. The values are shown as means of more than 30 measurements ± standard deviation (S.D.). *p < 0.05 vs. None.

Fig. 5. Cytoskeletal Organization of Cells Cultured on R8- and K8-Matrices

The peptides (100µM) were conjugated to the aldehyde-agarose matrices. HDFs in 0.1% BSA/DMEM (5 x 10^3 cells/well) were seeded into the wells and incubated for 2 and 18 h. Actin stress fibers and focal adhesions in the cells were visualized by staining for actin (green), vinculin (red), and nuclei (blue). Scale bar = 100 µm. Insets are enlarged images of dotted squares showing focal adhesions (vinculin accumulation).
results also support the involvement of integrin signaling in cell attachment to the R8- and K8-matrices.

**Cell Proliferation on R8- and K8-Matrices** Finally, we analyzed the difference in proliferation of the cells cultured on different peptide-matrices under serum-containing conditions (Fig. 6). As previously reported, the cells did not proliferate on the AG73-matrix but proliferated on the A99-matrix.15) Cell proliferation was significantly higher on the R8- and K8-matrices than on the AG73-matrix. There was no significant difference in cell proliferation between the R8-, K8-, and A99-matrices. Integrins promote cell proliferation via the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein extracellular kinase/extracellular signal-regulated kinase (MEK/Erk) pathways.20) Therefore, the high cell proliferation on the R8- and K8-matrices was most likely because of integrin signaling. These results demonstrated that the R8- and K8-matrices are useful as cell culture scaffolds.

In summary, immobilized R8 and K8 promoted cell attachment, spreading, and proliferation. The adhesion to the R8- and K8-matrices was mainly mediated by HSPGs but also by integrin β1, and the integrin signaling promoted cell spreading and proliferation. However, it should be considered that HSPGs, particularly syndecans, also play an important role in cell adhesion.21,22) Syndecans also act as co-receptors for integrins and activate integrins.23) Thus, it is possible that there is a synergistic effect of syndecans and integrins on cell adhesion, spreading, and proliferation on the R8- and K8-matrices. Although the R8-matrix showed slightly higher cell attachment activity than the K8-matrix, there was no major difference in biological functions between the R8- and K8-matrices. These results are consistent with previous studies showing no difference in cellular uptake of R8- and K8-modified nanoparticles.24) In general, positively charged material surfaces have excellent cell adhesion properties.25,26) For example, culture substrates are commonly coated with polylysine for cell culturing.27) Polylysine is normally coated by physical adsorption, whereas R8 and K8 can be immobilized on material surfaces by covalent bonding, as shown in the present study, resulting in controlled peptide modification. Therefore, R8 and K8 are promising as multi-functional cell adhesion molecules for cell culture and may help in development of biomaterials. Moreover, the findings of this study may be useful for further understanding of the R8-membrane interactions and the cellular uptake mechanisms.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** This article contains supplementary materials.

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