Inhibition of Cell Adhesion by Peptides Derived from the EC-4 Domain of E-Cadherin

By

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Derived from the EC-4 Domain of E-Cadherin

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Abstract

The objective of this project was to evaluate the biological activity of peptides derived from the EC-4 domain of E-cadherin in inhibiting E-cadherin-mediated cell-cell adhesion. The activity of these peptides was determined by inhibition of Caco-2 single cells that bind to a Caco-2 monolayer and inhibition of junction resealing of MDCK II cell monolayers. The results showed that peptide 6 (LVVQAADLQG) derived from the EC-2 domain and peptide 8 derived from the EC-4 domain (YTALIIATDN) of E-cadherin were more effective in inhibiting single cell adhesion than peptides from other domains (EC-1, EC-3) of E-cadherin. Peptide 8 had better activity than peptide 6. From overlapping hexapeptides derived from peptide 8, it was found that peptide 12 with ALIIAT sequence had the best activity, suggesting that it was the activity sequence of peptide 8. Alanine-scanning and mutation experiments found that peptide 22 (TEIIAT) had the best activity compared to other hexapeptides derived from peptide 8 in inhibiting cell adhesion of Caco-2 cells and junction resealing of MDCK-II cell monolayers.

Introduction

Cell-cell adhesion processes in the intercellular junctions of biological barriers (i.e., intestinal mucosa barrier and blood-brain barrier (BBB)) are indispensable in the integrity of the intercellular junctions of these barriers [1]. The intercellular junctions are segregated into tight junctions, adherens junctions, and desmosomes and by protein-protein interactions at the extracellular and cytoplasmic domains [1, 2]. The tight junctions are mediated by membrane proteins such as claudin and occluding and junctional adhesion molecules (JAM) proteins [1-3]. The tight junction proteins interact with cytoplasmic domain proteins such as zonula occludin-1 and -2 (ZO-1, ZO-2) [3]. The adherens junctions are constructed with calcium-binding E-cadherin and non-calcium-binding nectin proteins. At the cytoplasmic domain, E-cadherins bind to alpha-, beta-, and gamma-catenins, which connect them to cytoskeletal proteins[4]. The desmosomes are mediated by desmosomal cadherins called desmogleins and desmocollins, and they bind to cytoplasmic plaque proteins [2].

The highly conserved His-Ala-Val (HAV) sequence in the extracellular-1 (EC-1) domain was reported to be important for homophilic binding between cadherins [5]. Our group designed HAV and Ala-Asp-Pro (ADT) peptides derived from the respective groove and bulge regions of the EC-1 domain of E-cadherin [6]. These peptides modulated the intercellular junctions of MDCK II cell monolayers as measured by the lowering of the trans-epithelial electrical resistance (TEER) values of the monolayers [7, 8]. Cadherin peptides improved the penetration of ^{14}C-mannitol through the MDCK II cell monolayers as well as that of ^{14}C-mannitol and ^{3}H-daunomycin across the BBB in *in-situ* rat brain perfusion [7-9]. Recently, HAV and ADT peptides have been shown to improve

in vivo brain delivery of a gadopentetic acid, a contrast agent for magnetic resonance (MRI), into the brains of Balb/c mice [6, 10].

Although HAV and ADT peptides were active in modulating cell-cell adhesion in the intercellular junctions, there is no information as to whether peptides from the groove and bulge regions of other domains (i.e., EC-2, -3, and -4) are active in inhibiting E-cadherin-mediated cell-cell adhesion. Therefore, the study was focused on evaluating the activities of peptides 1–8 derived from the bulge and groove regions of the EC-1 (Table 1), EC-2, EC-3, and EC-4 domains of human E-cadherin to inhibit E-cadherin-mediated Caco-2 single cells and Caco-2 cell monolayers. This study was also done to evaluate the inhibition activity of hexapeptides (peptides 10–24) derived from peptide 8 in inhibiting E-cadherin-mediated cell-cell adhesion.

Materials and Methods

Cells and Reagents

Fmoc amino acids, activating agents, and solvents for peptide synthesis were purchased from Bachem, Inc. (Torrance, CA). Other chemicals and solvents were purchased from Sigma Chemicals (St. Louis, MO). Caco-2 and Madin-Darby Canine Kidney (MDCK II) cells were purchased from ATCC (Manassas, VA). 10% fetal bovine serum (FBS) containing Dulbecco's modified Eagle's medium (DMEM), anti-E-cadherin rat monoclonal antibody (U3254), anti-rat IgG-FITC conjugate antibody (F1763), and anti-mouse IgG-FITC conjugate antibody (F4143) were purchased from Sigma. An anti-E-cadherin monoclonal antibody (SHE78-7) was purchased from PanVera (Madison,

WI). The fluorescence marker (2',7'-bis(2-carboxyethyl)-5(and-6)-carboxy-fluorescein acetoxymethyl ester or BCECF-AM) was purchased from Molecular Probes (Eugene, OR). Dimethyl sulfoxide (DMSO), Triton X-100 and other reagents were purchased from Sigma.

Synthesis and Purification of Peptides

All the peptides were synthesized using a solid-phase peptide synthesis method utilizing Fmoc chemistry in a Pioneer Peptide Synthesizer. After cleavage from the resin, the peptide was precipitated in cold ether to produce a crude product. Then, the crude product was purified using a semi-preparative C18 HPLC column and the separated compounds were collected using fraction collectors. The fractions containing the desired peptide were determined using analytical HPLC and a C18 column; these pure fractions were removed, and the solution of pure peptide was lyophilized. The identity of the pure peptide was determined by mass spectrometry.

Inhibition of Caco-2 Single Cell Adhesion to Caco-2 Cell Monolayers by Cadherin Peptides

Caco-2 cells were maintained in 10% FBS containing DMEM (FBS/DMEM) in T-75 plastic flasks (Falcon) at 37ºC in a humidified 5% CO_2 atmosphere. Culture medium was changed every other day. Cells were subcultured at least once a week so that the cells did not reach confluency. Caco-2 cells were seeded on 48-well culture dishes and,

after the cells monolayers were confluent, the medium was replaced with BSA/DMEM. Another Caco-2 cell layer in a T-75 flask was treated with Ca^{2+}- and Mg^{2+}-free Hank's balanced saline solution (HBSS-) to obtain the Caco-2 single cells. The isolated Caco-2 single cells were incubated with 5.0 μg/mL of BCECF-AM containing FBS/DMEM for 90 min at 37ºC under the humidified 5% CO_2 atmosphere to label the single cells with the fluorescence marker BCECF. After the incubation, BCECF-labeled cells were washed extensively with PBS- (80 mM Na_2HPO_4, 20 mM KH_2PO_4, 140 mM NaCl, 10 mM KCl, pH 7.4) to remove excess fluorescence marker. BCECF-Caco-2 cells were re-suspended in BSA/DMEM at a density of 4×10^5 cells/mL.

The BCECF-Caco-2 single cell suspension was mixed with peptide solution at 1:1 dilution; the BCECF-Caco-2 cells were then added to Caco-2 cell monolayers on a 48-well culture plate (5×10^4 cells/200 μL/well). Single cells and the cell monolayers were co-incubated for 2 h in the dark at 37ºC under the humidified 5% CO_2 atmosphere. After the incubation, the wells were washed three times with PBS+ (0.63 mM $CaCl_2$, 0.74 mM $MgCl_2$, and 1.0 mg/mL glucose in PBS-) to wash away non-bound cells. The cell layers with bound BCECF-Caco-2 cells were lysed with 350 μL/well of 3.0% Triton X-100 solution. Lysates were collected in micro-centrifuge tubes, and centrifuged at 10,000 rpm for 10 min at room temperature. The supernatants (150 μL/well) were transferred to black 96-well plates in duplicate for measuring the fluorescence intensities (excitation at 485 nm; emission at 530 nm) using a Fluorescence Microplate Reader (FL600, Bio-Tek).

For activity to inhibit cell-cell adhesion, peptides and antibodies were prepared at a 2-fold concentration for their final concentrations. Antibodies as well as peptides 0, 1, 2,-

3, 4, and 9 were first dissolved in 98.0% volume of 0.1% BSA contained DMEM (BSA/DMEM). Then 2.0% volume of DMSO was added. Due to their hydrophobic nature, peptides 5, 6, 7, and 8 were first dissolved in 2.0% volume of DMSO, followed by addition of 98.0% volume of BSA/DMEM. A 2.0% DMSO containing DMEM was used as a control vehicle. Finally, all samples contained 1.0% DMSO.

Immunofluorescence Staining of E-cadherin Molecules

Caco-2 cell monolayers were cultured on 48-well plates, washed three times with PBS+, and fixed overnight at 4ºC in 4.0% formaldehyde solution. After three-time washing for 5 min in PBS+, several of the cell monolayers were permeabilized by treatment with 0.5% Triton X-100 solution. The cell monolayers, washed three times with PBS+, were treated with blocking buffer (5% skim milk and 1% BSA in PBS+) for 60 min at room temperature to prevent the non-specific binding of antibodies. Cell monolayers were washed again, as above, and incubated with 250 µL/well of 2 µg/mL of anti-E-cadherin monoclonal antibody (SHE78-7) containing blocking buffer at 4ºC overnight. The cell monolayers were then washed three times with washing buffer (0.05% Tween 20 in PBS+) followed by 60 min-treatment at 37ºC with FITC-conjugated antimouse IgG antibody in 1:50 dilution in blocking buffer. The cell monolayers were washed five times with washing buffer, and the localization of E-cadherin molecules was observed under a fluorescence microscope (Nikon).

Inhibition of Junction Resealing of MDCK Cell Monolayers by Cadherin Peptides

MDCK strain-2 cells were cultured using the Eagle's medium (Cellgro, Manassas, VA) containing 1.42 g/L HEPES sodium, 100 units/mL penicillin, 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, VA, USA), and 100 µg/mL streptomycin (Atlanta Biologicals). The cells (100,000 cells/well) were seeded on polyethylene membrane Transwells (0.4 µm; diameter 1.12 cm^2) from Costar (Cambridge, MA, USA) until the cell monolayers were confluent with TEER values above 300 $Ohm.cm^2$ around day 7. Hank's balanced salt solution (HBSS) containing 1% glucose, 10 mM HEPES, and 2.0 mM $CaCl_2$ at pH 7.4 was used to wash the monolayers, and the TEER values of intact MDCK monolayers were measured in HBSS after incubation for 1.5 h for equilibration using EVOM2 (World Precision Instruments, Sarasota, FL, USA). To open the intercellular junctions, the cell monolayers were incubated in calcium-deficient HBSS for 1 h and then the media was switched to calcium-sufficient media in the absence or presence of different concentrations of peptides. The effects of peptide concentrations in inhibiting the resealing of the intercellular junctions were monitored by the TEER values of the cell monolayers. Each experiment was conducted at least in triplicate.

Statistical Analysis

The results were presented as the mean ± SD of 4–6 determinations. Statistical analyses were determined by Dunnett's multiple comparison test when the data were regarded as the parametric data in Bartlet's test, or Dunnett's multiple rank test when

the data were considered as the non-parametric data, with *p* values below 0.05 regarded as significant using a statistical analysis software, SAS.

Results

The Presence of E-cadherin on the Junctions of Permeabilized and Normal Caco-2 Cell Monolayers

The presence of E-cadherin on modified and normal Caco-2 cell monolayers was detected using mouse anti-E-cadherin antibody (mAb) and FITC-labeled secondary IgG antibody. To detect E-cadherin at the adherens junctions of Caco-2 cell monolayers, the monolayers were disrupted using Triton X-100 to allow the penetration of the mAbs through the tight junctions. The results indicated that anti-E-cadherin mAb decorates the intercellular junctions of Caco-2 cell monolayer as shown by fluorescence microscopy (Figures 1A); the cells were still anchored on the polycarbonate membrane on the well after Triton X-100 treatment as revealed by light microscopy (Figure 1B). The normal cell monolayers were treated with the anti-E-cadherin mAb; some E-cadherins were located on the surface of the monolayer (Figure 1C), and the cell monolayer was visualized with light microscopy (Figure 1D). As a negative control, the cell monolayer was incubated with FITC-labeled anti-mouse IgG, and no decoration of fluorescence on the surface due to non-specific binding of the IgG mAb was seen (Figures 1 E&F).

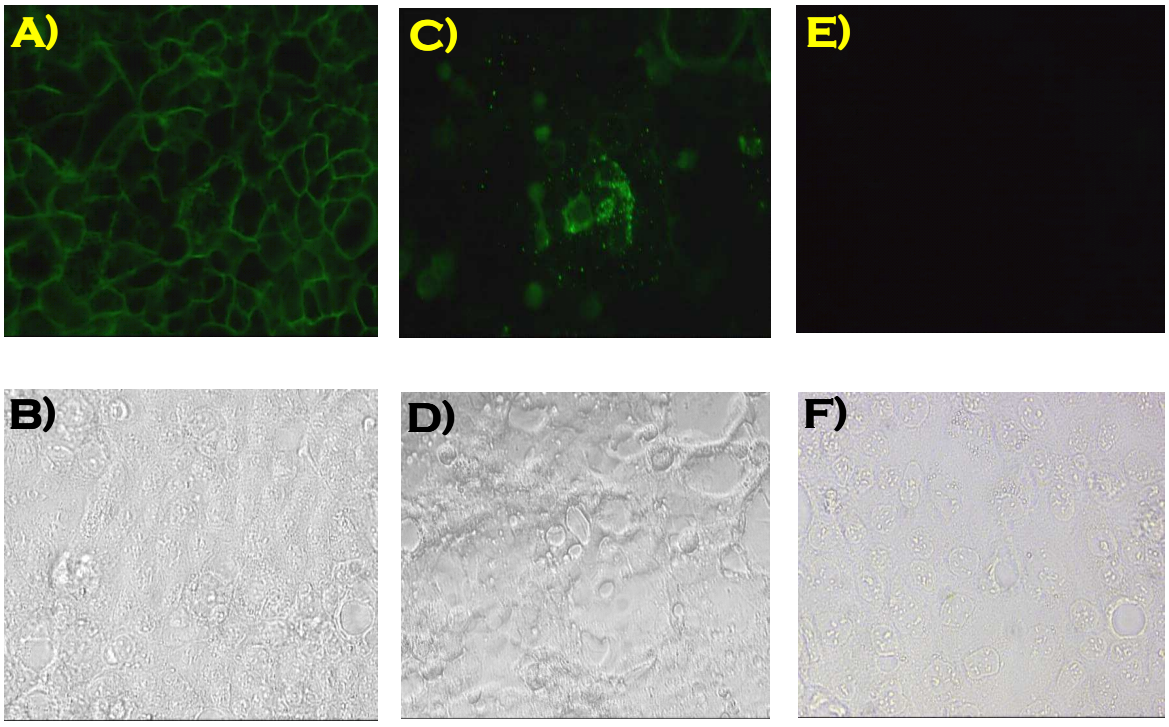

Figure 1: Localization of E-cadherin protein in the adherens junctions of modified and normal Caco-2 cell monolayers. (A) Fluorescence and (B) light contrast microscopy of normal Caco-2 cell monolayers treated with Triton X-100 C followed by treatment with anti-E-cadherin and FITC-anti-mouse IgG Ab. (C) Fluorescence and (D) light contrast microscopy of normal Caco-2 cell monolayers treated with anti-E-cadherin and FITC-anti-mouse IgG Ab. (E) Fluorescence and (F) light contrast microscopy of normal Caco-2 cell monolayers treated with control mouse IgG and FITC-anti-mouse IgG Ab.

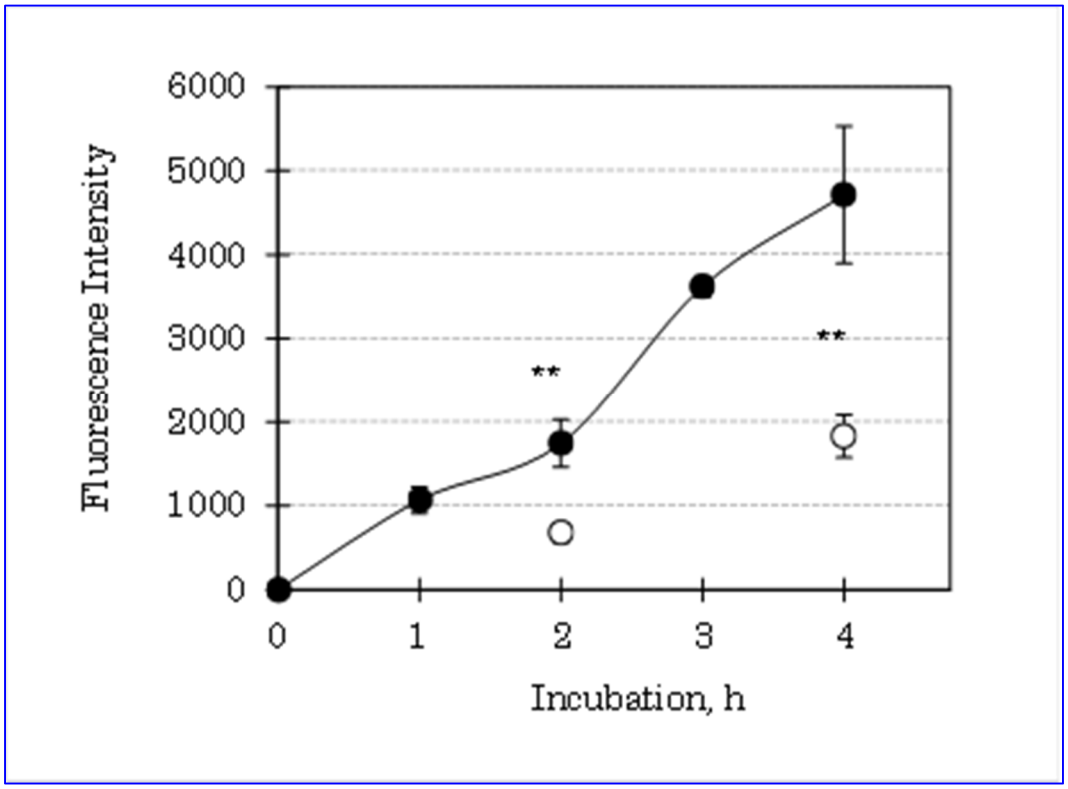

Figure 2: Time course for the adhesion of BCECF-labeled Caco-2 single cells to modified Caco-2 cell monolayers. BCECF-labeled Caco-2 single cells were incubated onto modified Caco-2 cell monolayers in the absence (closed circles) or presence (open circles) of anti-E-cadherin mAb (50x). The culture medium contained 1.0% DMSO. Data are shown as the mean ± SD of 6 determinations. The anti-E-cadherin mAb blocked the adhesion of single cells to cell monolayers, suggesting that the cell adhesion was partially mediated by E-cadherins. **: $p<0.01$ significantly different from the control group, which did not contain anti-E-Cadherin mAb, in the same incubation period.

Table 1. Amino acid sequence of E-cadherin-related peptides.

Peptide No.	Origin	Region	Amino Acid Sequences
0	EC-1 domain	Scramble	HSASVA
1	EC-1 domain	Bulge	Q^{42}GADTPPVGV^{51}
2	EC-2 domain	Bulge	Q^{154}DPELPDKNM^{163}
3	EC-3 domain	Bulge	N^{264}DDGGQFVVT^{273}
4	EC-4 domain	Bulge	T^{368}YRIWRDT*A*^{*1}N^{377}
5	EC-1 domain	Groove	L^{77}FSHAVSSNG^{86}
6	EC-2 domain	Groove	L^{190}VVQAADLQG^{199}
7	EC-3 domain	Groove	Y^{297}ILHVAVTNV^{306}
8	EC-4 domain	Groove	Y^{407}TALIIATDN^{416}
9	EC-1 domain	Groove	D^{68}RERIATYTLFSHAVSSNGNAVED^{91}

^{*1}: The original sequence is R^{376}

Table 2. Inhibitory activities of E-Cadherin-derived peptides in Caco-2 single cell adhesion to Caco-2 cell monolayer. NS: No significant change from control.

Peptide No.	Origin	Conc. mM	n	Fluorescence Intensity Mean ± SD	Inh. %	Stat. vs cont. p. value
Control	-	-	6	1,915.0 ± 93.37	-	-
Peptide-0	N/R	1.0	6	1,798.8 ± 139.42	6.1	NS
Peptide-1	EC-1	1.0	6	1,940.3 ± 130.60	-1.3	NS
Peptide-2	EC-2	1.0	6	2,033.3 ± 63.12	-6.2	NS
Peptide-3	EC-3	1.0	6	1,978.5 ± 75.54	-3.3	NS
Peptide-4	EC-4	1.0	6	1,970.0 ± 100.11	-2.9	NS
Peptide-5	EC-1	1.0	6	1,468.8 ± 35.51	23.3	NS
Peptide-9	EC-1	0.5	6	800.2 ± 108.34	58.2	$p < 0.01$
Peptide-6	EC-2	1.0	6	1,014.5 ± 124.08	47.0	$p < 0.01$
Peptide-7	EC-3	1.0	6	1,919.8 ± 58.40	-0.3	NS
Peptide-8	EC-4	1.0	6	1,047.7 ± 77.43	45.3	$p < 0.05$
E-Cad. mAb.	-	50 x	6	1,005.8 ± 88.02	47.5	$p < 0.01$

E-cadherin-mediated Caco-2 Single Cell Adhesion to Modified Caco-2 Cell Monolayers

To evaluate the activity of cadherin peptides, a cell adhesion assay was performed using E-cadherin-mediated BCECF-Caco-2 single cells adhesion onto modified Caco-2 cell monolayers. The amount of single cell adhesion was assumed to be proportionate to the fluorescence intensity in the well from the adhering single cells. As shown in Figure 2, the amount of adhesion of BCECF-Caco-2 single cells onto the cell monolayer was dependent on the incubation time of single cells as determined by the increase in fluorescence intensity. At 2- and 4-h time points, the single cells were incubated onto cell monolayers in the presence of anti-E-cadherin mAb. The fluorescence intensities from the single cells were significantly lower in the presence of anti-E-cadherin mAb than without mAb (Figure 2) at both the 2- and 4-h time points. The result suggested that E-cadherin was involved in the adhesion of single cells to the cell monolayers.

Using this assay, the ability of various cadherin peptides (Table 1) to inhibit single cells adhesion to cell monolayers was evaluated by measuring the inhibition of fluorescence intensities from labeled Caco-2 single cells (Table 2). Peptides 1–4 were derived from the respective bulge regions of the EC-1, EC-2, EC-3, and EC-4 domains of E-cadherin, and peptides 5–8 were derived from the groove regions of the EC-1, EC-2, EC-3, and EC-4 domains. Peptide 0 is a scrambled peptide derived from the HAV groove region (SHAVSS) of the EC-1 domain of E-cadherin. Peptide 9, which served as a positive control, was derived from the HAV groove region of EC1. As a positive control, the results showed that peptide 9 produced the strongest inhibition of cell-cell adhesion (58.2%) at 0.5 mM concentration (Table 2). Another positive control was anti-

E-cadherin mAb, which inhibited cell adhesion by 47.5%. As a negative control, peptide 0 did not have any effect on inhibiting cell-cell adhesion at 1.0 mM concentration. At 1.0 mM concentration, peptides 6 and 8, which were obtained from the respective groove regions of EC-2 and -4 domains, significantly blocked the cell-to-cell adhesion at 47% and 45% inhibition, respectively (Table 2). Peptide 5, which contained the HAV sequence, had weak inhibitory activity (23%) in this cell adhesion assay, and peptide 7, derived from the groove region of the EC-3 domain, did not inhibit cell-to-cell adhesion. In this assay, peptides 1–4 from the respective bulge regions of EC-1, -2, -3, and -4 did not show any inhibitory activity.

The concentration-dependent activities of peptides 6, 8, and 9 were evaluated and compared to control buffer, peptide 0, and anti-E-cadherin mAb (Figures 3–5). The activity of peptide 9 was concentration-dependent and similar to that of anti-E-cadherin mAb (50x) at 500 μM (Figure 3). Peptide 6 showed a trend of concentration-dependent inhibition of cell adhesion, and significant inhibition of cell adhesion was observed at the peptide concentration of 750 μM with maximum activity at 1000 μM (Figure 4). The activity of peptide 8 was also concentration-dependent, and inhibition was observed as low as 250 μM concentration with the maximum activity around 750μM (Figure 5).

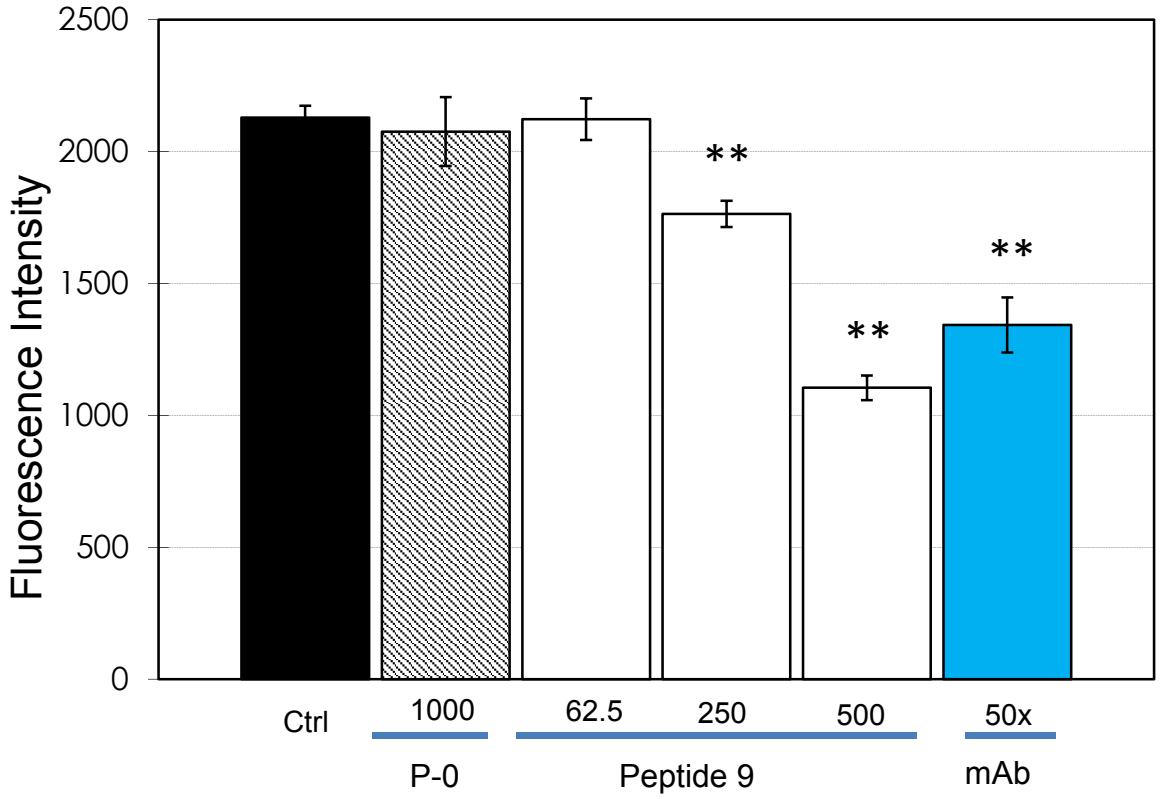

Figure 3: Concentration-dependent inhibitory activity of peptide 9 on Caco-2 single cell adhesion to Caco-2 cell monolayer. Data are shown as the mean ± SD of 6 determinations. *: $p<0.05$ and **: $p<0.01$ significantly different from the control group in Dunnett's multiple rank test. Ctrl: Control, P-0: peptide 0, mAb: Anti-E-cadherin mAb.

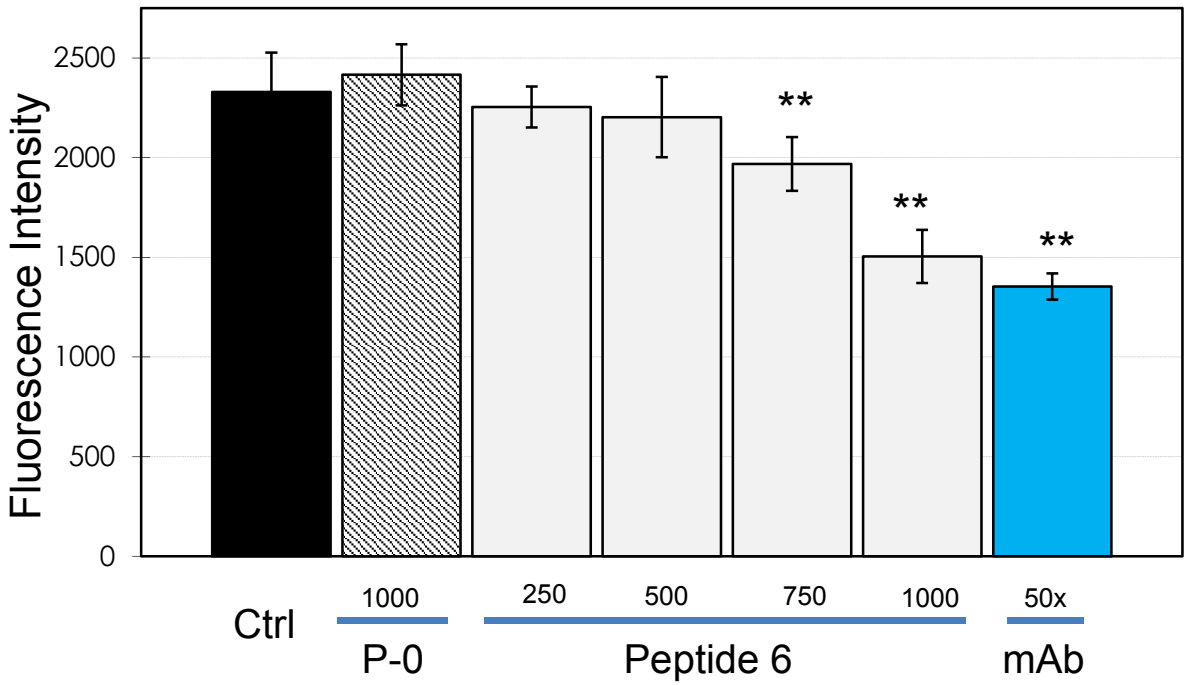

Figure 4: Concentration-dependent inhibitory activity of peptide 6 on Caco-2 single cell adhesion to Caco-2 cell monolayer. Data are shown as the mean ± SD of 6 determinations. **: $p<0.01$ significantly different from the control group in Dunnett's multiple rank test. Ctrl: Control Group; P-0: peptide 0; mAb: Anti-E-cadherin mAb

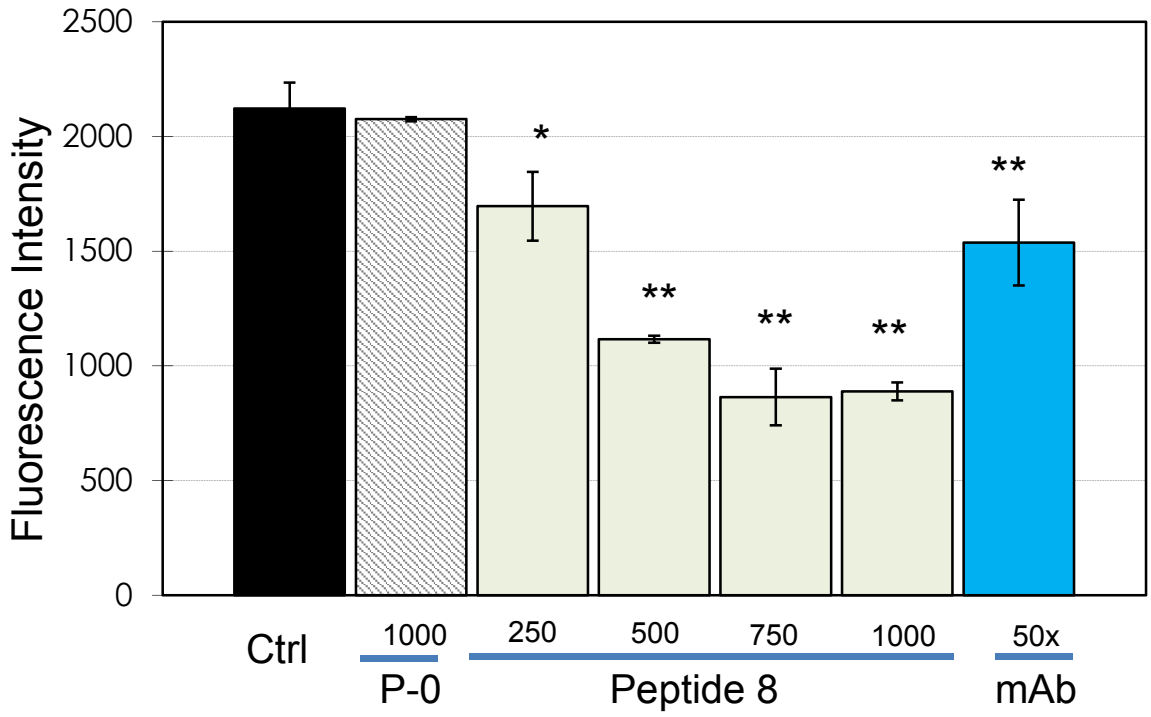

Figure 5: Concentration-dependent inhibitory activity of peptide 8 on Caco-2 single cell adhesion to Caco-2 cell monolayer. Data are shown as the mean ± SD of 6 determinations. *: $p < 0.05$ and **: $p<0.01$ significantly different from the control group in Dunnett's multiple rank test. Ctrl: Control, P-0: peptide 0, mAb: Anti-E-cadherin mAb.

Table 3. Sequence of hexapeptides from EC-4 domain.

Peptide No.	Origin	Nature	Amino Acid Sequences
8	EC-4 domain	Parent	YTALIIATDN
10	EC-4 domain	Overlapping	YTALII
11	EC-4 domain	Overlapping	TALIIA
12	EC-4 domain	Overlapping	ALIIAT
13	EC-4 domain	Overlapping	LIIATD
14	EC-4 domain	Overlapping	IIATDN
15	EC-4 domain	Ala-Scan	A**A**IIAT
16	EC-4 domain	Ala-Scan	AL**A**IAT
17	EC-4 domain	Ala-Scan	ALI**A**AT
18	EC-4 domain	Ala-Scan	ALIIA**A**
19	EC-4 domain	Mutant	**TL**IIAT
20	EC-4 domain	Mutant	**TA**IIAT
21	EC-4 domain	Mutant	**TF**IIAT
22	EC-4 domain	Mutant	**TE**IIAT
23	EC-4 domain	Mutant	**TN**IIAT
24	EC-4 domain	Mutant	**TR**IIAT

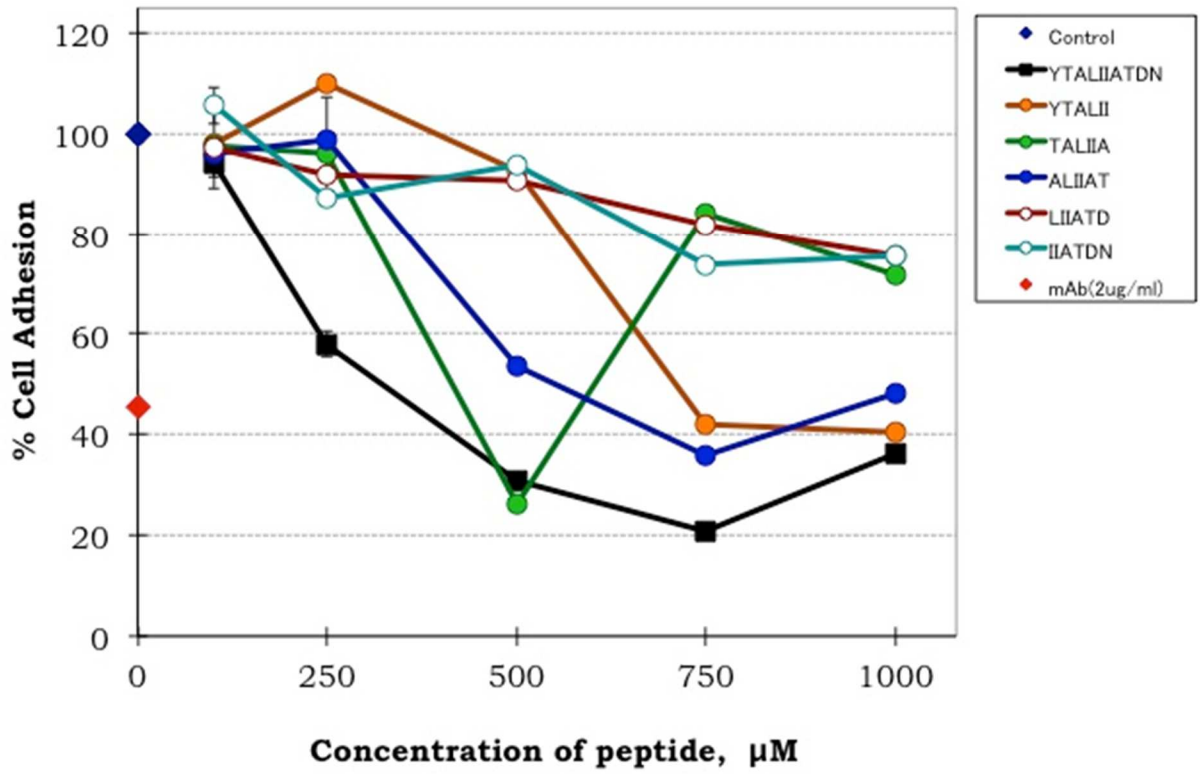

Figure 6: Concentration-dependent inhibitory activity of peptide 8 and its derivatives on Caco-2 single cell adhesion to Caco-2 cell monolayer. Data are shown as mean of 4 determinations for each concentration.

Reduction of Peptide 8 Decapeptide to Hexapeptides

To evaluate the important sequence and to reduce the size of peptide 8, five overlapping hexapeptides were synthesized (Table 3) and their ability to inhibit cell adhesion were determined in a concentration-dependent manner (Figure 7). Peptide 8 (YTALIIATDN) was the most potent in inhibiting cell adhesion, and its inhibition effect was concentration-dependent. The ALIIAT hexapeptide (peptide 12) had consistent inhibitory activity at 500, 750, and 1000 μM concentrations, and it had better activity than all other hexapeptides at 750 and 1000 μM.

Next, alanine scanning was carried out on ALIIAT to determine amino acids that were important for the biological activity (Figure 8). Replacement of the Leu-2, Ile-4, and Thr-6 residues in the alanine residue enhanced the activities, suggesting that these residues are important for activity. One of the best peptides was ALIAAT (peptide 17), in which the Ile-4 residue was replaced with Ala-4. To evaluate the effect of Ala-1 on ALIIAT, the first residue was replaced with Thr-1 to make TLIIAT (peptide 19), and the presence of a hydroxyl group in Thr-1 was to enhance the solubility of the peptide. Mutation of Ala-1 to Thr-1 to make TLIIAT did not improve the inhibitory activity at the given concentration. Because the Leu-2 was important for activity, as shown from the alanine scanning, the Leu-2 residue in TLIIAT was mutated to Ala-2, Phe-2, Glu-2 Asn-2, and Arg-2 to give TAIIAT, TFIIAT, TEIIAT, TNIIAT, and TEIIAT (Table 3). The results showed that replacing Leu-2 with hydrophobic Ala-2 (TAIIAT; peptide 20) and Phe-2 (TFIIAT; peptide 21) improved peptide activities compared to those of the parent TLIIAT. Replacing Leu-2 with acid Glu-2, neutral Asn-2, and basic Arg-2 also improved the peptide activity, and the best activity was seen in TEIIAT with Glu-2 residue. Thus,

TEIIAT was used for a further study to inhibit junction resealing of MDCK cell monolayers.

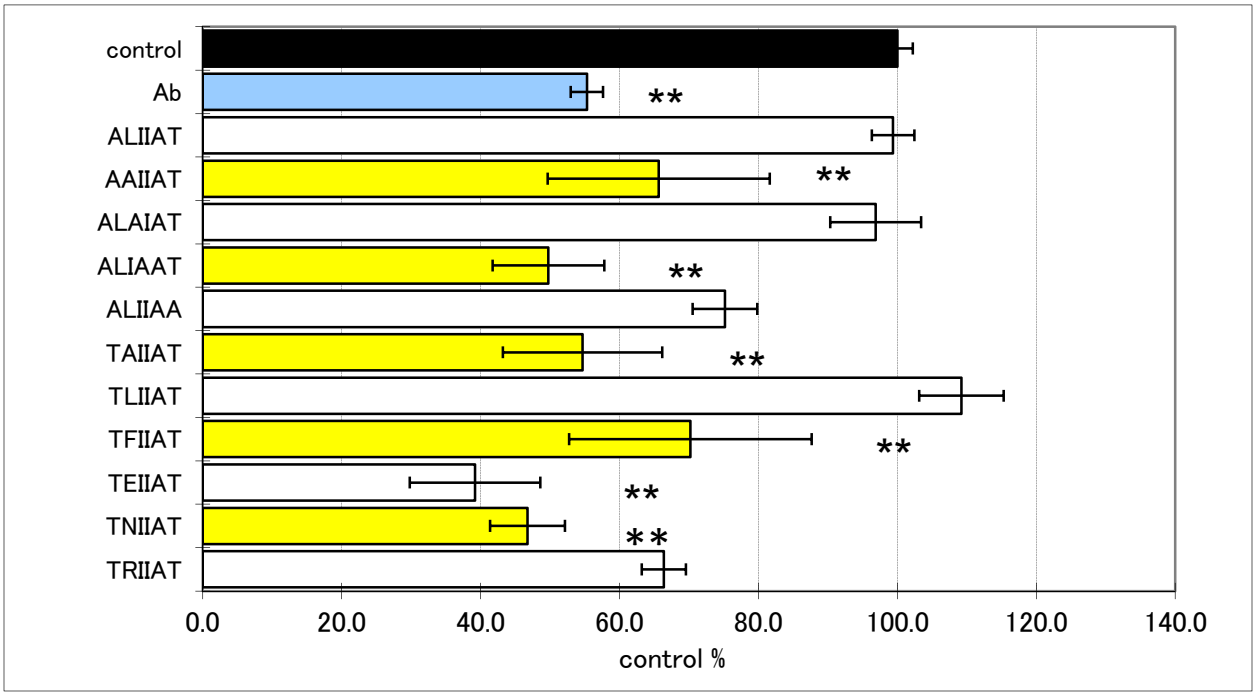

Figure 7: Evaluation of the effect of alanine scanning and residue mutation of ALIIAT peptide using Caco-2 single cell adhesion to Caco-2 cell monolayers at 250 μM peptide concentration. The inhibition of cell adhesion was measured by fluorescence intensities from the single cells, and the results were shown as percent of cell adhesion in the presence of peptide compared to the negative control. The positive control is anti-E-cadherin mAb. Data are shown as the mean ± SD of 6 determinations. **: $p<0.01$ significantly different from the control group in Dunnett's multiple rank test. Ctrl: Control, mAb: Anti-E-cadherin mAb.

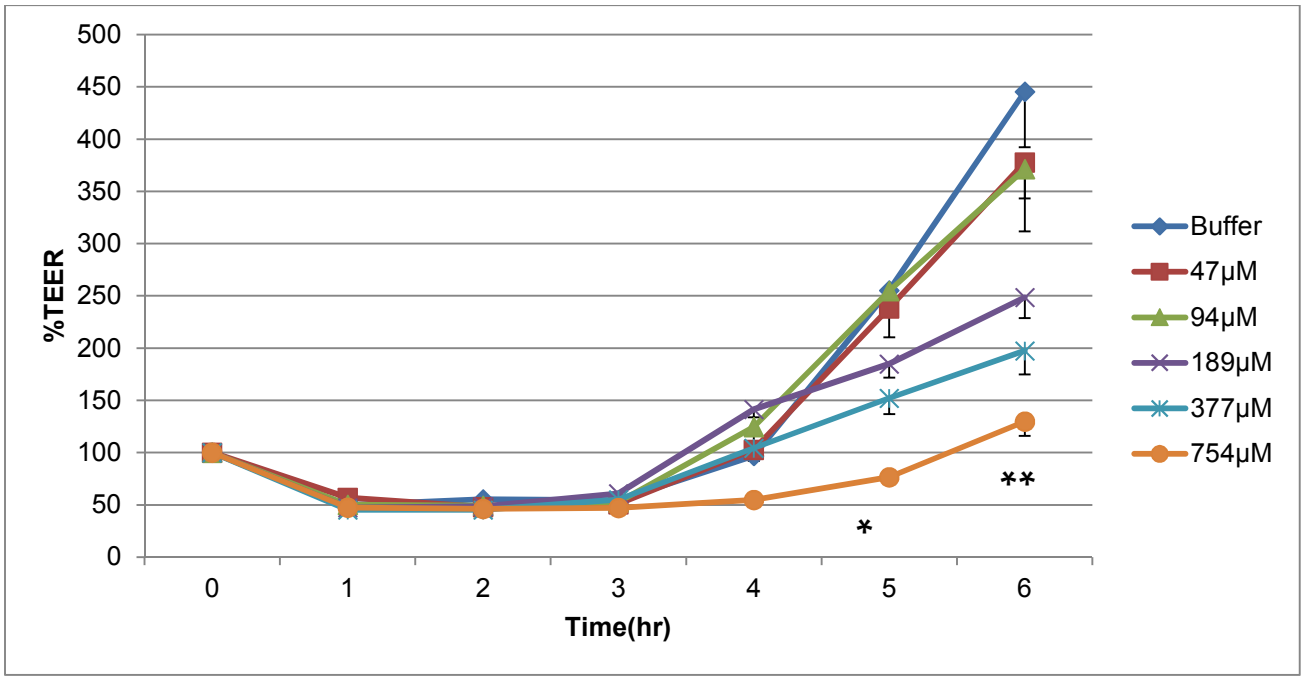

Figure 8: Concentration-dependent inhibition of junction resealing of MDCK monolayers by TEIIAT. Data are shown as mean ± SD of 3 determinations. **; p <0.01 and *: p<0.05 statistically significant from the control group.

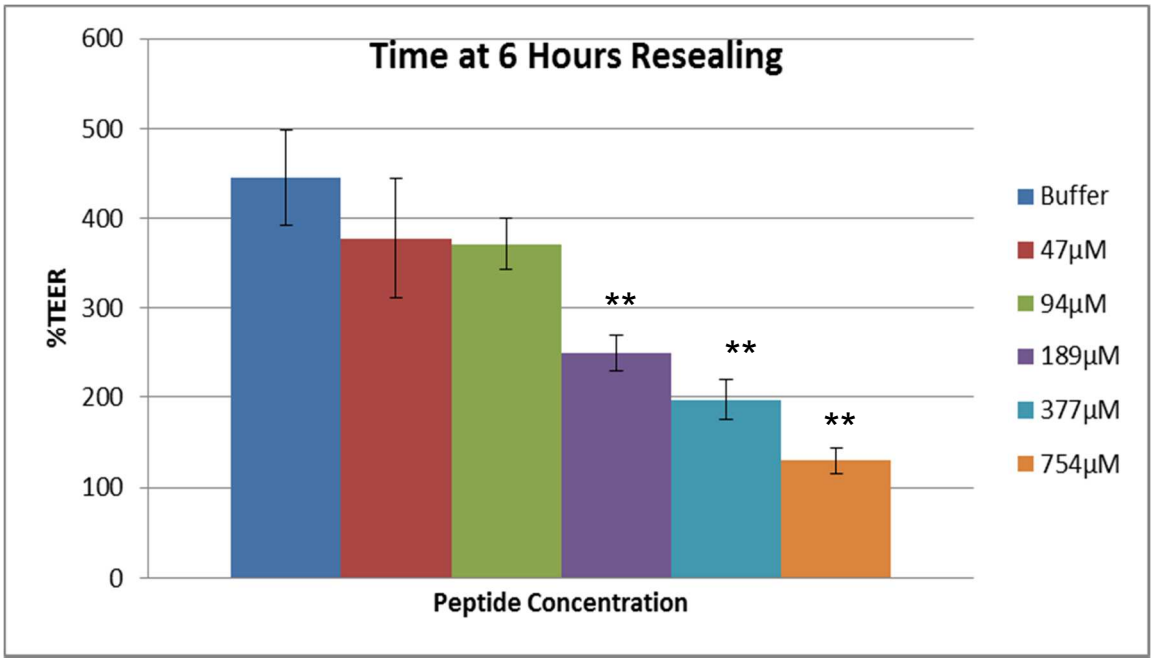

Figure 9: The effect of concentration on junction resealing inhibitory activity of TEIIAT peptide on MDCK-2 cell monolayers at time 6 h. Data are shown as mean ± SD of 3 determinations. **: $p<0.01$ statistically significant from the control group.

Concentration-dependent inhibition of MDCK-2 monolayer resealing by TEIIAT Peptide

The concentration-dependent activities of TEIIAT peptide were also evaluated in inhibiting the resealing of cell-to-cell adhesion at the intercellular junctions of MDCK-2 cell monolayer. In this case, the intercellular junction was disrupted by incubating the monolayer with calcium-deficient medium for 1 h when the TEER value of the cell monolayer dropped. After 1 h, the medium was switch to calcium-sufficient medium (HBSS) to reseal the intercellular junction in the absence (control) and presence of TEIIAT peptide at different concentrations. The TEER values were measured every hour up to 6-h time point. The results showed that the TEER values of monolayers treated with HBSS were increased due to the resealing of the intercellular junctions (Figure 9). In contrast, the monolayers treated with peptides showed inhibition of TEER value increases from 2-h to 6-h time points due to inhibition of resealing of the intercellular junctions. The inhibition TEER value increase was dependent on the peptide concentration. At the 6-h time point, the effects of peptide concentrations were compared, and the peptide was found to significantly inhibit the resealing of the intercellular junction at concentrations of 189 μM or higher compared to HBSS control ($p < 0.01$).

Discussion

The paracellular pathways of the intestinal mucosa and blood-brain barriers (BBB) can be exploited to increase the oral and brain delivery of drugs and diagnostic agents. In this case, the porosity of biological barriers in paracellular pathways can be increased via modulation of protein-protein interactions at the intercellular junctions. Several peptides derived from occludin and claudin at the tight junctions have been shown to modulate the intercellular junctions of the epithelial cell monolayers and improve the paracellular transport of marker (i.e., ^{14}C-mannitol, ^{3}H-inulin) and drug molecules [11, 12]. Our group also designed HAV and ADT peptides from the EC-1 domain of E-cadherin that can modulate *in vitro* and *in vivo* intercellular junctions to enhance delivery of marker and drug molecules in to the brain [9]. Thus, this work was to study whether peptides from other EC domains of E-cadherin could also modulate the intercellular junctions of biological barriers. In this case, the structural similarities of EC domains were evaluated by alignment analysis of EC domains. The alignments were computed using the Tree-base Best-first Iterative Algorithm with Tree-dependent partitioning (TBIAT) protein sequence multiple alignment analysis method [13]. As a result, decapeptides from EC-2, EC-3, and EC-4 that were aligned with the HAV and ADT peptides from the respective groove and bulge regions of EC-1 were synthesized (Table 1). The activities of these peptides in modulating E-cadherin-mediated cell-cell adhesion were evaluated using peptide inhibition of (a) Caco-2 single cell adhesion to Caco-2 cell monolayers and (b) resealing of the intercellular junctions of MDCK cell monolayers.

The hypothesis is that the peptide inhibits cell adhesion by binding to E-cadherin and

blocks cadherin-cadherin interaction in the intercellular junctions to create greater paracellular porosity. E-cadherin is a Ca^{2+}-dependent glycoprotein localized at adherens junctions and is responsible for cell-cell adhesion in the intercellular junctions of biological barriers. E-cadherin homophilic interactions form cell-to-cell adhesion via stable *trans*-homophilic interactions of the domains [14]. The extracellular region of human E-cadherin is composed of 554 amino acid residues with five extracellular repeat domains (EC-1, -2, -3, -4, -5); EC-5 is also called the extracellular anchor (EA) domain [15]. The primary sequence homology values among the EC domains were low when calculated using the TBIAT program. The similarities among EC domains are between 12.4 and 25.5%, with the highest similarity between EC-1 and EC-2 domains (25.5%). The similarities between the EC-5 and other EC domains were low (between 5.8 and 8.4%).

It has been shown that the EC-1 domain was important for E-cadherin-mediated cell adhesion [16]. HAV and ADT peptides derived from the respective groove and bulge regions of EC-1 domain inhibited E-cadherin-mediated cell-cell adhesion to modulate the intercellular junctions of *in vitro* and *in vivo* biological barriers [8]. Thus, the hypothesis is that the peptides derived from the groove and bulge regions of other EC domains (i.e., EC-2, EC-3, EC-4) can inhibit E-cadherin-mediated cell adhesion. Here, the activities of peptides derived from other EC domains were compared in inhibiting adhesion of Caco-2 single cells to Caco-2 cell monolayers. Adhesion of Caco-2 single cells to Caco-2 cell monolayers was mediated between E-cadherins on the single cells and E-cadherins found on the surface of the cell monolayers (Figure 1C). In the permeabilized Caco-2 cell monolayers using Triton X-100, E-cadherin molecules were

recognized by anti-E-cadherin mAb at the individual cell fringes, suggesting that most E-cadherins were located in the intercellular junctions (Figure 1B). On the other hand, some distributed E-cadherin molecules were detected on the surface of the non-permeabilized Caco-2 cell monolayers in an immunofluorescence staining study (Figure 1A). Anti-E-cadherin mAb inhibited Caco-2 single cell adhesion to Caco-2 cell monolayers, suggesting that the cell adhesion was mediated by E-cadherin. As another positive control, peptide 9 with HAV sequence suppressed single cell adhesion in a concentration-dependent manner (Figure 5); this peptide was previously found to effectively block E-cadherin-mediated aggregation of bovine brain endothelial microvessel cells (BBMECs) [17].

In this assay, peptide 6 and peptide 8 from the respective EC-2 and EC-4 domains were significantly active in inhibiting cell adhesion compared to control (HBSS). As a negative control, peptide 0 with scrambled sequences did not show any activity. As a positive control, peptide 9 significantly inhibited cell adhesion at 500 μM (Figure 3). Peptide 6 inhibited cell adhesion in concentration-dependent inhibition manner with significant inhibition at 1000 μM (Figure 5). Interestingly, peptide 8 inhibited cell adhesion as low as 250 μM, which suggested that this peptide had better activity than peptide 6. Because peptide 8 had high activity, further studies were done to reduce the size of the peptide from decapeptides to hexapeptides to find the small active region of this peptide.

The size of cadherin peptides can influence their penetration into the intercellular junctions of the BBB [8, 18]. It has been shown that HAV hexapeptides can penetrate the intercellular junctions of BBB *in vivo* to modulate biological barriers for enhancing

brain delivery of marker molecules [10]. Thus, peptide 8 was reduced to overlapping hexapeptides 10–14 (Table 2), and the results showed that peptide 12 (ALIIAT) had the most consistent activity at 500, 750, and 1000 μM (Figure 6). As expected, peptide 12 had lower activity than the parent peptide 8. A similar trend was previously observed when peptide 9 (HAV peptide) was reduced to deca- and hexapeptides [9]. Lower activities of small peptides were due to the loss of secondary structure and flanking residues from the basic active sequence such as the His-Ala-Val sequence in HAV peptide [19]. Because peptide 12 was the best hexapeptide derivative, it was subjected to alanine scanning and mutation studies to find a better peptide.

To find residues that are important for peptide activity, an alanine-scanning study was performed on peptide 12 (ALIIAT) to make peptides 15–18. The ability to inhibit single cell adhesion was evaluated at 250 μM (Figure 7). This study showed that replacing residues 2, 4, and 6 with alanine enhanced the activity of the peptides, suggesting that reducing the bulkiness of the side chain of these residues improved peptide activities. To test the importance of Ala-1 residue in ALIIAT (peptide 12), it was replaced with Thr-1 to make peptide 19 (TLIIAT); this replacement did not improve the activity compared to that of parent peptide 12 (ALIIAT). Then, residue 2 in peptide 19 was also replaced with aromatic residue Ala-2 (small hydrophobic), Phe-2 (hydrophobic aromatic), Glu-2 (acidic residue), Asn-2 (neutral residue), and Arg-2 (basic residue). As in peptide 15 (AAIIAT), mutation on the Ala-2 residue on peptide 19 to peptide 20 (TAIIAT) enhanced peptide activity. Peptide 22 (TEIIAT) and peptide 23 (TNIIAT) had high inhibitory activity of single cell adhesion, suggesting that the acidic (Glu-2) and neutral hydrophilic (Asn-2) residues were better amino acids for position 2 compared to

hydrophobic and basic residues. In the future, similar mutations (i.e., Ala, Phe, Glu, Asn, Arg) at positions 4 and 6 will be carried out on peptide 22 to improve its activity.

The activity of peptide 23 (TEIIAT) was confirmed with another biological assay called a resealing assay using MDCK II cell monolayers. The idea was that the junctions of monolayers were disrupted by removing calcium ions from the medium followed by resealing the junctions by adding calcium to the medium. During the resealing process, TEIIAT peptide was added at different concentrations. The peptide was very effective in inhibiting the resealing of the intercellular junctions of MDCK II cell monolayers, with 50% inhibition of resealing seen at a concentration of 189 μM. Compared to previous deca- and hexapeptides derived from HAV- and ADT-peptides, peptide 23 (TEIIAT) may have better inhibitory activity in inhibiting E-cadherin-mediated cell adhesion. Further studies need to be done to provide side-by-side comparisons of the activities of peptide 23 and other HAV- and ADT-peptides

Conclusions

This study found that peptides 6 and 8 from the groove region of the respective EC-2 and EC-4 domains of E-cadherin inhibit Caco-2 single cell adhesion to Caco-2 cell monolayers. Peptide 8 had better inhibitory ability than peptide 6. Reduction of peptide 8 to hexapeptides produced peptide 12 (ALIIAT) with better activity than other hexapeptides derived from peptide 8. Alanine-scanning and mutation studies of peptide 12 produced a very active peptide 22 (TEIIAT), which effectively inhibited Caco-2 single cell adhesion to cell monolayers and intercellular junctions of MDCK II cell monolayers. In the future, peptide 22 will be evaluated for enhancing the delivery of MRI contrast

agents (i.e., Gd-DTPA), Gd-DTPA-labeled peptides, and NIR-dye-labeled peptides.

Bibliography

[1] M. Laksitorini, V.D. Prasasty, P.K. Kiptoo, T.J. Siahaan, Pathways and progress in improving drug delivery through the intestinal mucosa and blood-brain barriers, Therapeutic Del., 5 (2014) 1143–1163.

[2] K. Zheng, M. Trivedi, T.J. Siahaan, Structure and function of the intercellular junctions: barrier of paracellular drug delivery, Curr. Pharm. Design, 12 (2006) 2813–2824.

[3] A. Hartsock, W.J. Nelson, Adherens and tight junctions: structure, function and connections to the actin cytoskeleton, Biochim. Biophys. Acta, 1778 (2008) 660–669.

[4] F. van Roy, G. Berx, The cell-cell adhesion molecule E-cadherin, Cell. Mol. Life Sci., 65 (2008) 3756–3788.

[5] V. Noe, J. Willems, J. Vandekerckhove, F.V. Roy, E. Bruyneel, M. Mareel, Inhibition of adhesion and induction of epithelial cell invasion by HAV-containing E-cadherin-specific peptides, J. Cell Sci., 112 (Pt 1) (1999) 127–135.

[6] M.D. Laksitorini, P.K. Kiptoo, N.H. On, J.A. Thliveris, D.W. Miller, T.J. Siahaan, Modulation of Intercellular Junctions by Cyclic-ADT Peptides as a Method to Reversibly Increase Blood-Brain Barrier Permeability, J. Pharm. Sci., 104 (2015) 1065–1075.

[7] I. Makagiansar, M. Avery, Y. Hu, K.L. Audus, T.J. Siahaan, Improving the selectivity of HAV-peptides in modulating E-cadherin-E-cadherin interactions in the intercellular junction of MDCK cell monolayers, Pharm. Res., 18 (2001) 446–553.

[8] E. Sinaga, S.D. Jois, M. Avery, I.T. Makagiansar, U.S. Tambunan, K.L. Audus, T.J. Siahaan, Increasing paracellular porosity by E-cadherin peptides: Discovery of bulge and groove regions in the EC1-domain of E-cadherin, Pharm. Res., 19 (2002) 1170–1179.

[9] P. Kiptoo, E. Sinaga, A.M. Calcagno, H. Zhao, N. Kobayashi, U.S. Tambunan, T.J. Siahaan, Enhancement of drug absorption through the blood-brain barrier and inhibition of intercellular tight junction resealing by E-cadherin peptides, Mol. Pharm., 8 (2011) 239–249.

[10] N.H. On, P. Kiptoo, T.J. Siahaan, D.W. Miller, Modulation of blood-brain barrier permeability in mice using synthetic E-cadherin peptide, Mol. Pharm., 11 (2014) 974–981.

[11] S. Tavelin, K. Hashimoto, J. Malkinson, L. Lazorova, I. Toth, P. Artursson, A new principle for tight junction modulation based on occludin peptides, Mol. Pharmacol., 64 (2003) 1530–1540.

[12] S. Tsukita, M. Furuse, Occludin and claudins in tight-junction strands: Leading or supporting players?, Trends. Cell Biol., 9 (1999) 268–273.

[13] M. Hirosawa, Y. Totoki, M. Hoshida, M. Ishikawa, Comprehensive study on iterative algorithms of multiple sequence alignment, Comp. Appl. Biosci., 11 (1995) 13–18.

[14] A.W. Koch, D. Bozic, O. Pertz, J. Engle, Homophilic adhesion by cadherins, Curr. Opin. Struct. Biol., 9 (1999) 275–281.

[15] M. Overduin, T. Harvey, S. Bagby, K. Tong, P. Yau, M. Takeichi, M. Ikura, Solution structure of the epithelial cadherin domain responsible for selective cell-adhesion, Science, 267 (1995) 386–389.

[16] A. Nose, K. Tsuji, M. Takeichi, Localization of specificity determining sites in cadherin cell adhesion molecules, Cell, 61 (1990) 147–155.

[17] K.L. Lutz, T.J. Siahaan, Molecular structure of the apical junction complex and its contribution to the paracellular barrier, J. Pharm. Sci., 86 (1997) 977–984.

[18] A. Adson, T.J. Raub, P.S. Burton, C.L. Barsuhn, A.R. Hilgers, K.L. Audus, N.F.H. Ho, Quantitative approaches to delineate paracellular diffusion in cultured epithelial cell monolayers, J. Pharm. Sci., 83 (1994) 1529–1536.

[19] K.L. Lutz, S.D. Jois, T.J. Siahaan, Secondary structure of the HAV peptide which regulates cadherin-cadherin interaction, J. Biomol. Struct. Dyn., 13 (1995) 447–455.