Improving the Selectivity of HAV-Peptides in Modulating E-Cadherin-E-Cadherin Interactions in the Intercellular Junction of MDCK Cell Monolayers

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Purpose. The objective of this work is to understand the sequence specificity of HAV peptides and to improve their selectivity in regulating E-cadherin-E-cadherin interactions in the intercellular junctions.

Methods. Peptide **1** was modified using an alanine scanning method to give peptides **2**–**6**. The ability of these peptides to modulate intercellular junctions was evaluated using Madin-Darby Canine Kidney (MDCK) cell monolayers on Transwell™ membranes from either the apical (AP) or the basolateral (BL) side. Modulation of the intercellular junctions was measured by the ability to lower the transepithelial electrical resistance (TEER) of MDCK monolayers and by the increase in mannitol flux. Molecular docking experiments were performed to model the binding properties of these peptides to the EC1 domain of E-cadherin.

Results. Peptides **5** (Ac-SHAVAS-NH_2) and **6** (Ac-SHAVSA-NH_2) were found to be more effective than the parent peptide **1** in decreasing the resistance of the cell monolayer. Furthermore, comparative studies with the control and the weak inhibitor peptide **2** indicate that peptide **5** displayed a significant increase in mannitol flux. Molecular docking of peptides **1**, **2** and **5** to the EC1 domain suggests that peptide **5** has the lowest binding energy.

Conclusions. HAV peptides have the ability to modulate E-cadherin-E-cadherin interactions in the intercellular junctions of the MDCK cell monolayer, thus indirectly increasing the permeability of the tight junctions. This observation indicates that residues flanking the HAV sequence are important in the binding selectivity of HAV peptides to E-cadherin. Molecular docking can further aid in the design of peptides with better selectivity to the EC1 domain of E-cadherin.

KEY WORDS: E-cadherin; intercellular junctions; HAV-peptides; modulation of intercellular junctions; MDCK monolayers.

INTRODUCTION

The passage of solutes across biological barriers such as the intestinal mucosa and the blood brain barrier (BBB) can be mediated through the paracellular pathway, which allows the passive diffusion of solutes smaller than 11Å across the intercellular junctions (1). Recent studies indicate that the homophilic cell adhesion molecule E-cadherin, a major component of the *zonula adherens* (adheren junctions), has been shown to initiate, regulate and maintain the formation of the intercellular junctional complex (2–5).

E-cadherin contains an extracellular (EC) domain that consists of five tandem repeat domains (EC-1 to EC-5) (6–8). Active conformation of these domains depends on Ca^{2+} ions and their removal leads to a rapid opening of tight junctions in both epithelial and endothelial cells (8–10). Site-directed mutagenesis studies of the highly conserved His-Ala-Val (HAV) sequence revealed the importance of this tripeptide sequence for homophilic cadherin-cadherin interaction (11,12). Furthermore, a decapeptide derived from N-cadherin bearing an HAV sequence has been shown to inhibit neurite outgrowth and mouse embryo compaction due to its binding to N-cadherin and E-cadherin, respectively (13). Another study revealed that E-cadherin 17-mer HAV peptide inhibited E-cadherin-mediated fusion of monocytes during the formation of multinucleated bone-resorbing osteoclasts (14).

Based on this knowledge, our goal is to design an hexapeptide small enough to permeate the tight junction and able to recognize E-cadherin by perturbing cadherin homophilic interactions. It is hoped that this effect would indirectly result in the loosening of occludin interaction at the tight junction. The increase in pore size would, in turn, allow the rapid passage of the drug of interest. Subsequent removal of the modulating peptide would reseal the tight junction, returning it to its initial condition (15).

Previously, we found that peptides derived from the HAV region inhibited E-cadherin-mediated cell-cell aggregation of bovine brain microvessel endothelial cells (BBMEC) in a concentration-dependent manner (16). The fluorescence-labeled HAV peptides have also been shown to bind E-cadherin BBMEC monolayers and single cells (17). Furthermore, the inhibition of BBMEC aggregation using HAV peptides depends on the flanking residues of the HAV sequence for selectivity to bind to E-cadherin (16,18,19). These data support the hypothesis that residues flanking HAV can also directly influence the binding specificity of cadherin-cadherin interactions (11,12).

As part of our effort to improve the selectivity of HAV peptides in modulating E-cadherin interactions, we carried out a full alanine scanning of the parent HAV peptide **1** (Ac-SHAVSS-NH_2) to assess and determine the contribution of each individual amino side chain in perturbing E-cadherin interaction and the effect on the permeability of the tight junction. The Madin Darby Canine Kidney (MDCK) cell monolayer was used as a model to investigate the effects of HAV peptides in modulating intercellular junctions; this included measuring both the decrease in transepithelial electrical resistance (TEER) of cell monolayers and the increase in the paracellular transport of a marker molecule (^{14}C-mannitol) across the monolayers. To further assess the possible interaction of alanine scanned HAV peptides with E-cadherin, a molecular docking simulation was performed. These experiments will improve our understanding of the mechanism of action of HAV peptides in modulating E-cadherin-mediated intercellular junctions.

MATERIALS AND METHODS

Peptide Synthesis and Purification

Peptides (Table I) were synthesized using a Rainin automated peptide synthesizer (PS 390) employing standard

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Table I. Sequence Obtained from Alanine Scanning of Peptide **1**[a]

Peptide	Sequence	MW
1	Ac-SHAVSS-NH_2	627
2	Ac-**A**HAVSS-NH_2[c]	611
3	Ac-S**A**AVSS-NH_2	561
4	Ac-SHAA**S**S-NH_2	599
5	Ac-SHAV**A**S-NH_2	611
6	Ac-SHAVS**A**-NH_2	611
7[b]	ITGEATDSG	883

[a] Peptide **1** origin is from human E-cadherin.
[b] Control peptide unrelated to cadherin sequence
[c] Bold A represents the alanine substitution.

Fmoc-amino acid chemistry with amide carboxyl termini using Dod-resin (Bachem, Switzerland). The crude lyophilized peptides were purified by reversed-phase HPLC using a Rainin C_{18} column with a gradient of solvent system A (95:5 = 0.1% TFA/H_2O:acetonitrile) and solvent B (100% acetonitrile). Nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometry (FAB-MS) were used to determine the identities of the synthetic peptides.

Cell Culture

The MDCK cell line (ATCC, Rockville, MD) with passage numbers 53–65 was used in these experiments. The culture medium consisted of Earle's balanced salts (1.87 g/l), 0.5% lactalbumin hydrolysate, benzylpenicillin (G) (160 U/ml), streptomycin sulfate (100 μg/ml), sodium bicarbonate (0.85 g/l), and 5% newborn calf serum. Cells were cultured in a 150-cm^2 treated polystyrene flask at a density of 5.0×10^4 cells/cm^2 and incubated at 37°C under 5% CO_2 and air atmosphere. Cells were harvested with trypsin-EDTA and seeded on polycarbonate filters (0.4 μm pores, 1 cm^2 growth area) inside Transwell™ cell culture chambers (Costar, Cambridge, MA) at a density of 5×10^4 cells/filter. The culture medium was replaced after 48 h and thereafter every 24 h. Monolayers reached confluency 6–7 days after seeding, and were subsequently used for experiments.

Measurement of Transepithelial Electrical Resistance (TEER)

TEER values were measured using an EVOM A ERS meter device (World Precision Instruments, Sarasota, FL) before and during treatment with the respective peptide; a nonrelated peptide was used as a negative control. One hour before the experiment, transport medium consisting of Hank's balanced salt solution (HBSS) containing 25 mM glucose, 2.0 mM $CaCl_2$, 0.75 mM $MgSO_4$, and 10 mM HEPES buffer (pH 7.4) was appropriately added to both sides of the Transwells™. The experiments were initiated by addition of transport medium containing 500 μM of the respective peptide to either the apical (AP) or basolateral (BL) side of the Transwells™. All experiments were done in triplicate at 37°C. After addition of peptide, the TEER values were measured every hour for 7 h.

For the reversibility study, a similar experiment was performed. After 3 h exposure the peptide was removed and replaced by fresh transport medium. The TEER values were then measured every hour for another 4 h. Average TEER values for untreated cell monolayers were in the range of 170–210 ohms.cm^2 after substracting the resistance of the blank filters. TEER values were measured at desired time points in triplicate for each peptide. Percent change of the TEER value was calculated relative to the initial value at time zero.

Quantitative Western Blotting

MDCK cells were plated onto wells in triplicate and maintained until confluency as indicated above; the wells were then washed once with cold PBS. Cells were lysed by adding 300 μl of M-PER™ mammalian protein extraction reagent (Pierce, Rockford, IL) containing 150 mM NaCl, 25 mM KCl, 2.0 mM EDTA and Complete™ Mini protease inhibitors (Boehringer Mannheim GmbH, Mannheim, Germany) for 5 min at 4°C. The whole lysates were centrifuged at $10{,}000 \times g$ for 10 min. The supernatant was collected as the detergent-soluble fraction (S). The pellet, designated as the detergent-insoluble fractions (I), was resuspended and sonicated briefly in aqueous solution containing 10 mM phosphate, pH 7.1, 1.0 mM DTT, and 1% SDS. Protein concentration was determined using bovine serum albumin (BSA) as standard with the BCA kit (Pierce, Rockford, IL).

At each time point, addition of equal buffer volumes for both extracts S and I were used. The amount loaded per lane of either fraction S or I onto the 4–12% gradient SDS-polyacrylamide gel was normalized by comparing the total protein content of the respective soluble or insoluble fraction found in all time points. Thus, variability in protein was controlled. Western blotting was performed with the mouse anti-human-E-cadherin antibody (Transduction Laboratories, Lexington, KY). Band densities from duplicate experiments were quantified using a densitometer (FX Molecular Imager, Biorad, Hercules, CA) and expressed in arbitrary densitometric units. Differences among density readings were analyzed for significance using the student *t*-test.

^{14}C-Mannitol Transport

The integrity of the intercellular junction was checked by measuring TEER values prior to the experiment. All experiments were performed in triplicate at 37°C. The mannitol transport experiments were initiated by replacing the apical medium with 0.5 ml of transport buffer in the absence and presence of 0.5 mM final peptide concentration. Solutions containing the ^{14}C-mannitol were added on the AP side of the monolayers and 30 μl samples were taken from the BL side at 20 min intervals over a period of 3 h. Samples taken from the BL side were replaced with an equal volume of clean buffer. The apparent permeability coefficient (P_{app}, expressed as cm/sec) was determined according to the following equation: $P = dQ/dt \cdot 1/AC_o$, where dQ/dt is the permeability rate (steady-state flux in mole/sec), C_o is the initial concentration in the AP side of cell monolayers (mole/mL), and A is the surface area of the porous membrane (cm^2).

Effect of Peptides on Cell Morphology

MDCK cells (5×10^4 cells/cm^2) were seeded into 12-well plates; after they reached confluency, the culture medium was removed and replaced with 2.5 ml transport medium containing 2.0 mM of peptide **5**. A medium solution without calcium

was used as a positive control and a solution of peptide **7** was utilized as a negative control. The change in cell morphology was recorded by taking photographs at 0, 3, 6, and 9 h time points using a Nikon camera attached to the microscope.

Molecular Docking

The conformation and intermolecular interactions of HAV peptides bound to EC1 domain of E-cadherin (Protein Data Bank code 1EDH) were determined using molecular docking AUTODOCK (version 2.4) (20). During the docking experiments, torsion angles in the peptides were allowed to rotate freely. One hundred lowest energy structures based on the force field scoring were stored for further analysis. The acetyl group at the N-terminal and the amide group at the C-terminal of the HAV peptide were added using Insight-II software (MSI). Swiss PDB software package was used to generate the structures of peptides **2** and **5** from peptide **1**. No structural minimization was performed before running the docking experiments.

RESULTS

Effect of Peptides on Tight Junction Permeability

The alanine scanning of the parent peptide **1** was performed replacing each residue with L-alanine to yield peptides **2–6** (Table I). AP side addition of peptides **1**, **2**, **3**, and **4** decreased the TEER values to 65–75% in the first 3 h (Figure 1a). Following this incubation period, the monolayers treated with peptides **2** and **4** displayed a reversal, increasing TEER values to about 90% (Figure 1a) after 7 h of incubation. Similarly, monolayers treated with peptides **1** and **3** returned to TEER values of 80%. In contrast, peptides **5** and **6** were found to be most effective in disrupting the intercellular junctions. These peptides reduced the TEER values to 50–60%, and these values remained constant after 7 h.

The effect of the alanine scanning peptides added from the BL side displayed a similar trend (Figure 1b). Although peptides **1–4** reduced the TEER values to 60–70%, the TEER values were not reversed over the course of 7-h incubation as was seen in the AP side treatment. Both peptides **5** and **6** brought the TEER values to 50%, indicating that these peptides were most effective in disrupting the intercellular junctions. This observation was consistent with the results obtained from the AP side.

To ascertain the specificity of these peptides, a nonrelated peptide (peptide **7**) derived from a different cell adhesion molecule (ICAM-1) was used as a negative control. It showed no perturbation effect from either the AP or BL side. EDTA was used as a positive control to ascertain the integrity of the tight junction of the MDCK cell monolayer. When EDTA was added from the AP side, the TEER values gradually decreased to 30% in 3 h and 0% after 7 h. Addition of EDTA from the BL side was more dramatic, with the TEER values reduced to 0% after only 1-h treatment. A similar result was previously observed by other investigators (10).

The reversibility effect on the tight junctions of MDCK cell monolayers by one of the most potent peptides was evaluated by comparing the effect before and after the removal of peptide **5** and EDTA (Figure 2). The MDCK cell monolayer was incubated with peptide **5** from either the AP or BL side

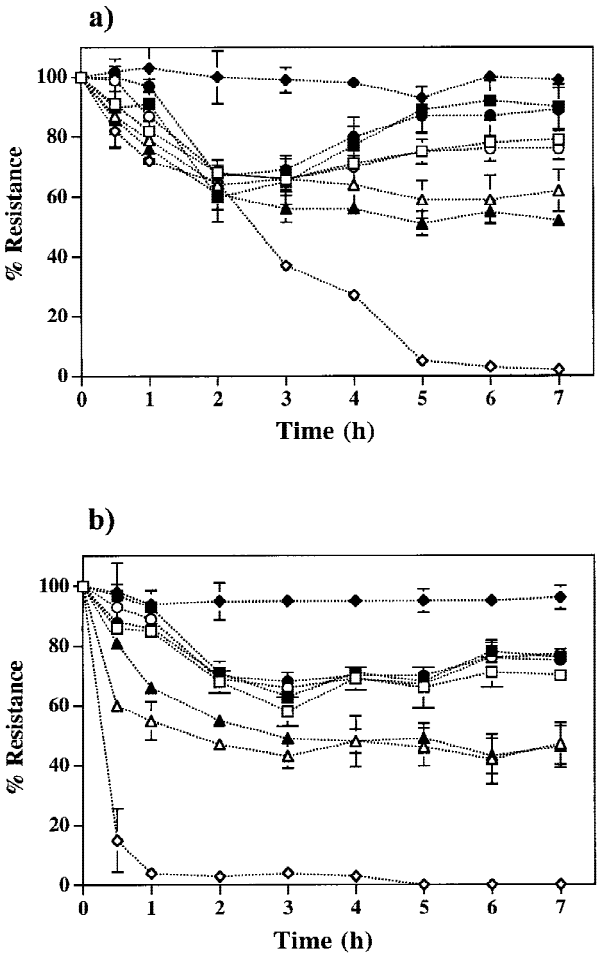

Fig. 1. Effects of the respective peptides on TEER values when administered from the (a) apical (AP) or (b) basolateral (BL) side of the MDCK cell monolayers. TEER values were measured at different time points after incubating 500 μM of each peptide added either on the AP or BL side. The percentage was calculated relative to initial TEER values. Values are the mean of triplicate measurements ± s.d. Peptide **1** = □; Peptide **2** = ■; Peptide **3** = ○; Peptide **4** = ●; Peptide **5** = △; Peptide **6** = ▲; Peptide **7** = ◆; EDTA = ◇.

for a period of 4-h to bring the TEER values down to 50–60%. Following this treatment, peptide **5** was removed by repeated washing and substituted in either the AP or BL side with fresh medium. The results showed that removal of peptide **5** from either the AP or BL side increased the TEER values to near the original resistance in the next four hours (at the 8-h time point). Similarly, treatment of the MDCK cell monolayer with EDTA from either the AP or BL side for the first 4-h reduced significantly the resistance of the tight junctions and displayed a near complete resealing of the tight junctions subsequent to removal of EDTA. Staining with trypan blue after completion of the experiments did not yield in any visible color reaction, suggesting that cell monolayers were viable and peptide **5** was non-toxic.

Effect of Peptide on ^{14}C-Mannitol Flux

To further demonstrate the effectiveness of peptide **5** in modulating intercellular junctions, the change in mannitol flux across the cell monolayers was also evaluated. The weak inhibitor peptide **2** was used for comparative purposes. Table

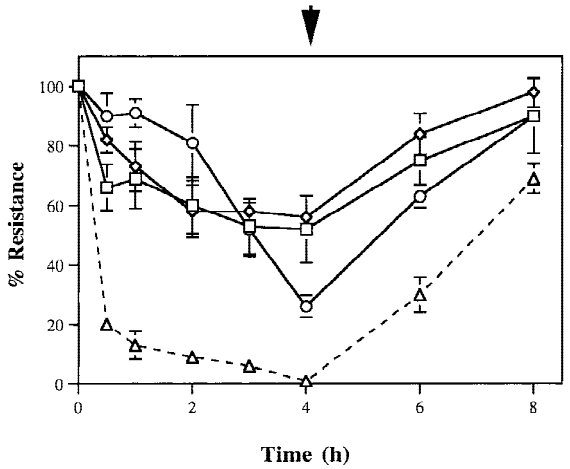

Fig. 2. Reversibility of the decrease in TEER values of MDCK cell monolayers when treated with peptide **5** and washed from the monolayers. After 3 h exposure at either AP (□) or BL (◇), peptide **5** was removed by replacing the medium with fresh medium. Subsequent to this change of medium, the TEER of intercellular junctions was restored to close to its initial value. TEER was determined at the indicated time. EDTA added at either the AP (△) or BL (○) side served as a positive control. The values shown (means ± s.d. of triplicate) have been expressed relative to the initial TEER values. The medium change is indicated by the arrow.

II shows the effect of peptides **2** and **5** on the permeation of mannitol via the paracellular route. In the absence of any peptide, mannitol flux was minimal, as shown in Table II. In contrast, peptide **2**- and peptide **5**-treated cell monolayers showed an increase in apparent permeability of eight and eighteen times greater than the non-treated monolayers, respectively (Table II). These results correlate well with the effect of peptides **2** and **5** from our previous TEER studies. It can be said that peptide **5** is more selective than peptide **2** in modulating E-cadherin interactions, which in turn affect the tight junctions.

Effect of Peptides on E-Cadherin Distribution

The collected whole-cell lysates were partitioned into detergent-soluble (S) and detergent-insoluble (I) fractions. The S and I fractions represent the cytosol and the cell-surface E-cadherin, respectively. Western blotting of E-cadherin in either soluble or insoluble fractions displayed different degrees of band intensities at their respective time points (Figure 3). The intensities for I, S and total (I + S) fractions of E-cadherin are plotted in Figure 4. Total E-cadherin expression increased with time when incubated with either peptide **2** or **5**. A significant increase in expression was found at 2–4 h and 0–2 h by peptides **2** and **5**, respectively. The increase in E-cadherin reached a stationary level following those indicated times. However, the change in the I fraction from peptide **2**-treated cell monolayer was higher than the effect produced by peptide **5**. This suggests that the monolayers respond to peptide modulation by increasing E-cadherin expression.

Table II. Effect of Peptide-treated MDCK Cell Monolayer on [^{14}C]-Mannitol Permeation

Peptide	$P_{app} \times 10^{-6}$ (cm/s)
Control	2.03 ± 1.5
Peptide **2**	17.0 ± 0.40
Peptide **5**	36.2 ± 0.20

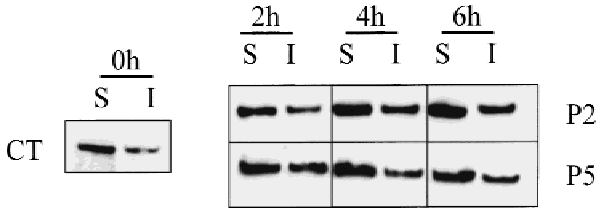

Fig. 3. Western blot analysis of E-cadherin fractionated from the detergent-soluble (S) and -insoluble (I) fractions of MDCK cells. Cell monolayers were treated with peptide **2** or peptide **5**. Cells were fractionated into detergent-soluble (S) and -insoluble (I) fractions and were normalized by comparing the total protein content of the respective soluble or insoluble fraction found in all time points. The molecular mass for E-cadherin corresponds to 135 kD.

Effect of Peptide 5 on Morphology of MDCK Cell Monolayer

The effect on the morphological cell appearance by peptide **5** was followed at several time points. The non-treated MDCK monolayer displayed a normal compact polygonal structure with little light being transmitted through the monolayers (Figure 5a). As a positive control, medium lacking Ca^{2+} resulted in the dissociation of cell-cell interaction with some regions of the cell monolayer yielding round single cell clusters (Figure 5b). On the other hand, treatment with peptide **5** did not result in the formation of single cell clustering but rather induced the cell monolayer to become more elongated; this presumably caused a slight increase in pore size of the tight junction, as indicated by the increase in light transmission across the cell monolayers at 3- and 6-h incubation times (Figures 5c and 5d).

Docking of HAV Peptides on EC1 of E-Cadherin

The low-energy docked models of the alanine scanned peptides (**1**, **2**, and **5**) to the EC-1 region of E-cadherin seems to indicate that these peptides may bind to a single site on the EC1 domain of E-cadherin (Figure 6). The docking energies of peptides **1**, **2** and **5** were –39.8, –35.5 and –41.3 kcal/mol, respectively. Although it was not possible to correlate the computational docking energy with the experimentally determined association constant, it is inferred that the ranking of preferable binding of HAV peptides onto EC1 of E-cadherin is peptide **5** > peptide **1** > peptide **2**.

All three peptides were found to bind to the upper part of the loop connecting the C-strand and the D-strand (residues 45–52) of EC1. A hydrophobic pocket was found around the Pro47, Val50, Leu66, Ala70, Ile71, and Tyr74 residues, which may be important for the high affinity of ligand binding. Peptides **1** and **5** bind to the EC1 domain in the same orientation. In contrast, peptide **2** binds to EC-1 cadherin in an orientation opposite to that of peptide **1**.

In peptide **1**, the side chain of the His2 residue formed hydrogen bonds with Gly49 and Thr63 of EC-1 while the His2

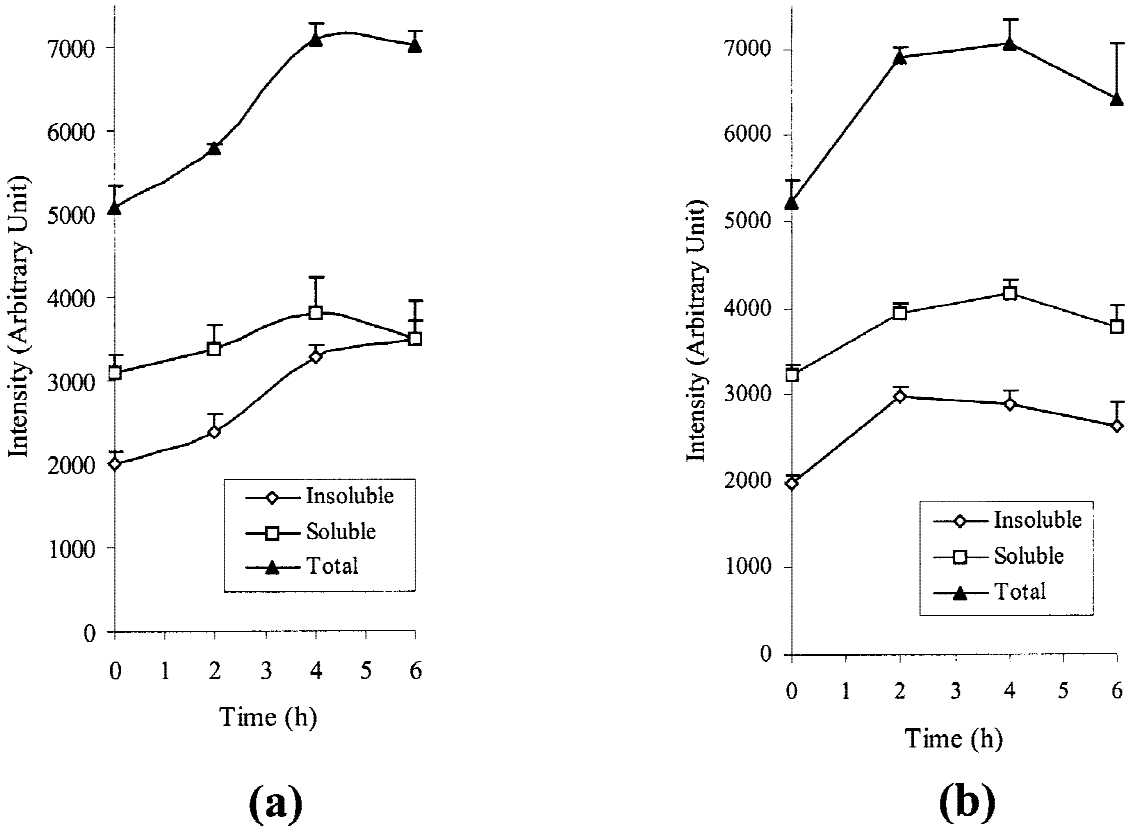

Fig. 4. The effect of peptides **2** (a) and **5** (b) on the distribution of soluble and insoluble E-cadherin on MDCK cells. Soluble and insoluble readings in arbitrary units were derived from the densitometric readings of the gel obtained in Figure 3. The total E-cadherin was calculated by adding the soluble and insoluble fractions of E-cadherin.

side chain of peptide **5** formed a hydrophobic interaction with Pro47 of EC-1 in the hydrophobic pocket. The His2 side chain of peptide **2** is at the top of the hydrophobic pocket of EC-1 and interacts with the Ala70 residue. The Val4 residue in peptide **1** points away from the hydrophobic pocket but interacts with another hydrophobic residue, Pro65 in EC-1. In contrast, the Val4 in peptide **5** is buried in the hydrophobic pocket and interacts with the side chains of Pro47, Val 50, Ala70, and Ile71 of EC-1. Extensive hydrophobic contacts on Val4 in peptide **2** were not observed. Thus, the additional hydrophobic interaction in peptide **5** may lower the binding energy of this peptide to the EC-1 domain.

DISCUSSION

Our previous work indicated that different lengths of HAV peptides derived from the contact region of cadherin-cadherin interactions were able to inhibit E-cadherin-mediated cell-cell adhesion and increase the porosity of intercellular junctions (16,17). In the present work, the effect of alanine scanned 6-mer peptides (**1**–**6**; Table I) on disrupting E-cadherin-E-cadherin interactions was judged by the changes in TEER, which correlate directly with a change in the tight junction permeability (21). Thus, a decrease in TEER values is due to an increase in the paracellular porosity across the tight junctions. The alanine scanning identified the importance of the residues flanking the HAV sequence. Replacement of a hydrophilic residue (Ser1) with a hydrophobic residue (Ala1) in peptide **2** decreased the ability of peptide **2** to modulate cadherin-cadherin interactions; after 7 h of incubation, the TEER value displayed a near complete resealing. The activity of peptide **3** (Ac-SAAVSS-NH_2) resembles that of the parent peptide **1** and supports our earlier assumption that mutation of the His residue to Ala in the decapeptide (ILYSAAVSSN) had less impact on the anti-E-cadherin antibody binding to the peptide (7). Peptide **4** had an inhibitory effect smaller than the parent peptide **1** when Val4 was replaced by Ala4. NMR structure of the EC1 domain (7) and the X-ray structure of the EC1-EC2 domains of E-cadherin (8) show that Val4 of the HAV sequence protrudes to the exterior surface, which indicates that Val4 is important for binding selectivity.

In addition to the low binding selectivity by peptides **1**–**4**, the recovery in resistance in cell monolayers treated with peptides **1**–**4** at a later stage may be due to the internalization of these peptides by E-cadherin in response to peptide binding by E-cadherin. FITC-peptide internalization by E-cadherin was previously observed at 37°C using the BBMEC monolayer as a cell model for the blood brain barrier (17). Through an unknown mechanism, this internalization may cause an increase in E-cadherin expression to counter the presence of HAV peptides as observed in the effect of peptide **2** on the E-cadherin equilibrium between the cell surface and the cytosol (Figure 4).

In contrast, peptides **5** and **6** were more effective than the other peptides in lowering the TEER values from either the AP or BL side. Thus, substitution of the Ser residues with Ala at either position 5 or 6, respectively, produced a more potent

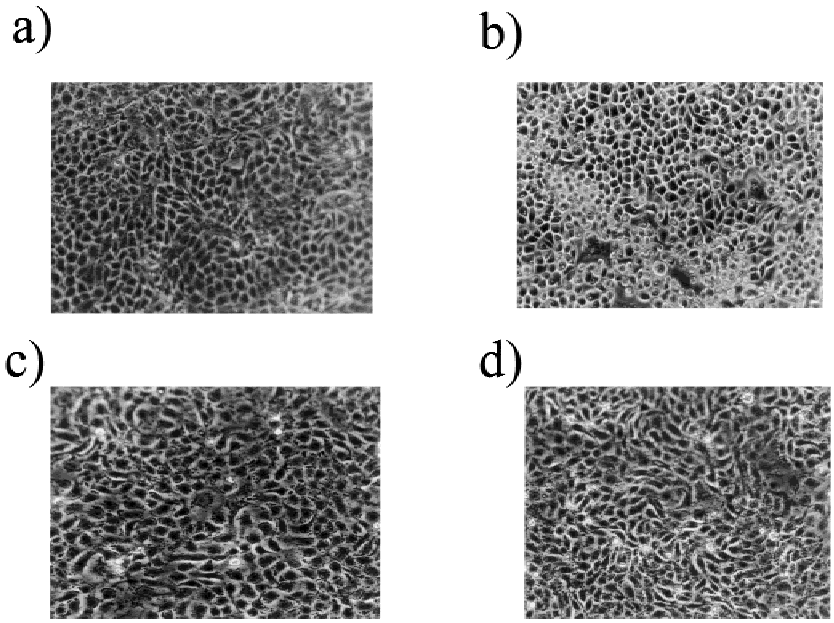

Fig. 5. Effect of peptide **5** on the morphological appearance of MDCK cell monolayers. (a) control peptide; (b) Ca^{2+}-lacking cell monolayer serves as a positive control; (c–d) peptide **5**-treated cell monolayers at 3 h and 6 h, respectively. At 6 h, the inhibitory effect results in the cell shape becoming more elongated and having less intercellular contact, as indicated by the increase in light transmission.

peptide than peptide **1**. Presumably, a small hydrophobic residue is preferred over a neutral hydrophilic residue at position 5 or 6. These results confirm our previous finding that residue 5 is important for the selectivity of HAV peptide to E-cadherin recognition; for example, mutation of residue 5 in peptide **1** to the Asp, Lys or Phe residue decreased the inhibitory activity of HAV peptides on E-cadherin-mediated BBMEC cell aggregation (16). Thus, the notion that mutation of flanking residues to the HAV sequence in E-cadherins on the cell surface can affect the cell adhesion properties suggests that the HAV sequence and its flanking residues are important for the selectivity of HAV peptides in modulating E-cadherin interactions (12,13).

Further assessment of HAV peptides to increase the porosity of the tight junction was performed by measuring ^{14}C-mannitol flux across MDCK monolayers. Peptides **2** and **5** were used to represent low and high selectivity peptides. The results are shown in Table II. In the non-treated MDCK monolayers, the mannitol flux was very low. The data, however, indicate that peptide **5** was more effective than peptide **2** at increasing paracellular porosity and agree with the observed TEER experiments. In addition, peptide **5** has a reversible binding characteristic since its removal and fresh medium replacement from either the AP or BL led to a return to initial TEER values in treated MDCK monolayers. The rapid resealing suggests an equilibrium binding property between peptide **5** and E-cadherin in the intercellular junction with the components of the intercellular junctions remaining functional. Hence, peptide **5** was proven to be a potent modulator of the tight junction.

The morphological appearance of MDCK cell monolayer treated with peptide **5** appears to have a slight effect on the intercellular junctions as evidenced by the increment in light transmission across it at 3 and 6 h incubation times (Figures 5c–d). In contrast, the lack of Ca^{2+} ions in the medium caused E-cadherins to become completely non-functional and affected the integrity of the cell monolayer as demonstrated by the gradual formation of single cell clusters (Figure 5b). It is conceivable that the binding of peptide **5** to E-cadherin triggered a contraction of the cytoskeleton that perturbed the interactions among proteins within the tight junction and, hence, led to a small opening of the tight junction without destroying the overall integrity of the cell monolayer. Slight morphological changes were also observed when MDCK cell monolayers were treated with compounds such as phenylarsine oxide (22) or cadmium (26) that perturbed the tight junctions but still retained cell-cell contacts.

The effect of HAV peptides **2** and **5** on the E-cadherin level on the cell surface and in the cytosol was evaluated by measuring the insoluble (I) and soluble (S) E-cadherin concentrations. E-cadherin from the S fraction represents that localized in the cytosol while the I fraction represents E-cadherin found on the cell surface (24,25). During the steady-state growth of epithelial cell monolayers, it was observed that the expression level of E-cadherin remained in equilibrium between E-cadherins in cytosol and on the cell surface. Treatment of the cell monolayer with either of these peptides produced an increase in insoluble E-cadherin (I fraction), especially in cell monolayers treated with peptide **2** (Figure 4). Thus, these peptides may trigger the expression and secretion of cytosol E-cadherin to the cell surface membrane of the MDCK cell monolayer in order to counteract the perturbed intercellular junctions. Although we did not examine the distribution of other intercellular junction-associated proteins, it is probable that α-catenin, β-catenin and p120, which mediate E-cadherin linkage to actin, are involved in the signaling pathways that stabilize the resealing in MDCK cell monolayers (26).

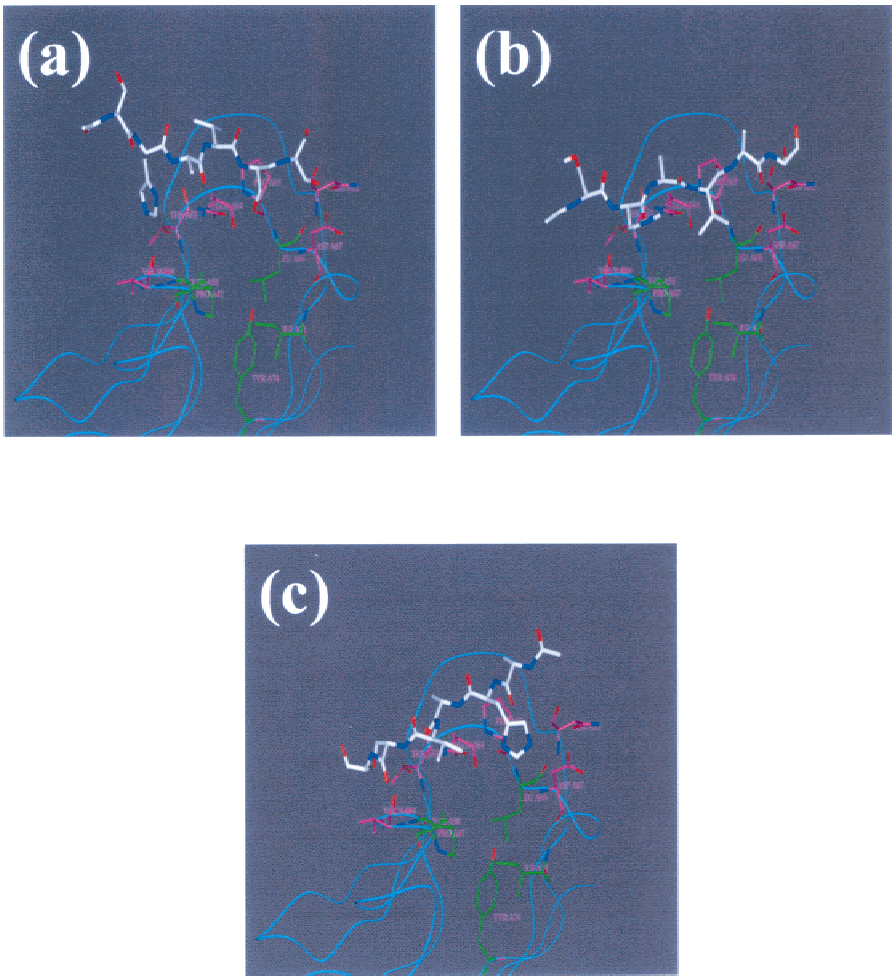

Fig. 6. Molecular interactions between HAV peptides **1** (a), **5** (b), and **2** (c) and EC1 of E-cadherin. The residues interacting with the peptide are shown in magenta or green lines. The residues in magenta form hydrophilic interactions with E-cadherin, while the residues in green stick form a hydrophobic binding pocket, which may play important role for high affinity. H-bonds are in green dotted lines.

Docking experiments suggest that the highest binding energies of these peptides were ranked as **2** > **1** > **5**. This result agrees with the TEER effects whose perturbation effect was in the order of peptides **5** > **1** > **2**. These peptides bind to the C and D loop of the EC1 domain. Peptides **5** and **1** were found to bind in a similar arrangement, while peptide **2** bound in the opposite arrangement (Figure 6). The side chains of the His2 and Val4 residues in peptide **5** formed hydrogen bonds and hydrophobic interactions with the EC1 domain more efficiently than did peptides **1** and **2**. Thus, the efficient interaction between peptide **5** and EC1 may be used to design a better derivative to modulate E-cadherin-E-cadherin interactions in the intercellular junctions.

In conclusion, our results suggest the possibility of using HAV peptides to modulate tight junctions to increase paracellular porosity and improve drug delivery across biological barriers. The alanine scanning studies revealed the importance of flanking residues surrounding the HAV region for the selectivity of peptide binding to E-cadherins. The docking experiments provide the possible binding region of HAV peptides to the EC1 region of E-cadherin with modulation of the intercellular junctions by peptide **5** being the most potent.

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