Effects of an E-cadherin–Derived Peptide on the Gene Expression of Caco-2 Cells

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Purpose. The goal of this study was to determine the effects of exposure to an HAV peptide (Ac-SHAVSS-NH_2) on the protein and gene expression in Caco-2 cells, a model for the intestinal mucosa.
Methods. Caco-2 cells were incubated with either 100 or 500 μM of the hexapeptide then evaluated over a 48-h time period.
Results. Cell detachment from the monolayer was seen only after 48 h of exposure to the peptide, with the greatest effects occurring with a peptide concentration of 500 μM. Total protein expression of E-cadherin showed a decrease of nearly 20% at the 24-h time point for each concentration examined, whereas no significant changes were detected at the other time points studied. Short term exposure to a 500 μM solution of Ac-SHAVSS-NH_2 caused few changes in gene expression as determined by Affymetrix GeneChip® microarrays; however, longer exposure periods produced numerous changes in the treated cells. The variations in mRNA expression indicate that this HAV peptide has an effect in the E-cadherin signaling pathways. The greatest increases in mRNA expression were found in genes regulating excretion or degradation of the peptide.
Conclusions. This work suggests that this HAV peptide produces effects that reach beyond modulation of adhesion.

KEY WORDS: Caco-2 cells; E-cadherin; gene expression; HAV peptide; microarrays.

INTRODUCTION

The passage of drugs via the paracellular route is regulated by the adhesion molecule E-cadherin, which is located within the *zonula adherens*. E-cadherin forms cis-dimers on the cell surface, and cis-dimers from one cell interact in a homotypic manner with E-cadherin dimers from adjacent cells in the presence of calcium (1). The structure of this molecule is composed of three distinct regions. The amino terminus extends out of the cell, and this first region is further divided into five repeats consisting of 110 amino acids (2). These repeats are referred to as EC1 through EC5. The EC1 repeat contains the conserved His-Ala-Val (HAV) sequence, which is involved in the homotypic binding of the dimers. E-cadherin also contains a single transmembrane domain and one cytoplasmic domain. The cytoplasmic region binds to β-catenin, an adherens junction protein and transcriptional coactivator in the Wnt signaling pathway. The protein α-catenin attaches to the E-cadherin/β-catenin complex and tethers it to the actin cytoskeleton. This interaction has been shown to be important in the adhesion process as well as in intracellular signaling (3,4).

It has been shown that peptides containing the sequence His-Ala-Val can inhibit the homotypic binding of E-cadherin molecules (5–9). We have evaluated the effects of several HAV peptides on decreasing transepithelial electrical resistance (TER) and on increasing the flux of the paracellular marker mannitol (10,11). We hypothesize that the addition of these HAV peptides to cell monolayers perturbs the homophilic binding of E-cadherin. Consequently, this disturbance increases paracellular permeability as confirmed by our TER and molecular marker flux studies (10,11).

In our previous studies using MDCK cells, we reported that after exposure to various HAV hexapeptides, there was an increase in the amount of total E-cadherin protein concentration as seen in Western blotting assays (11). In the MDCK and Caco-2 cell culture systems, we were able to show that our original HAV hexapeptide, Ac-SHAVSS-NH_2, can penetrate the tight junction and elicit a response from the apical side (10). Thus, in these studies, we used the hexapeptide Ac-SHAVSS-NH_2 to investigate its effects on Caco-2 cells at the protein and mRNA expression levels. Because exposure of cells to this peptide has not been extensively examined at the gene expression level, we treated Caco-2 cell monolayers for various times at 37°C with 500 μM of this E-cadherin-derived peptide. This concentration has been shown to modulate the intercellular junctions in Caco-2 cells and in MDCK cells (11). Gene expression analysis was then used to determine the effects of this exposure. Peptides derived from the second extracellular loop of occludin were shown to reduce TER in EpH4 mammary epithelial cells and to increase the mRNA expression of c-*myc,* a downstream target of the β-catenin/TCF/LEF signaling pathway (12). These results prompted us to examine a variety of genes using oligonucleotide microarrays, which have emerged as a powerful technique to investigate gene expression. More than 12,000 probe sets are present on this chip, which permits gene expression profiling of thousands of genes in a single experiment. This afforded us the ability to investigate changes in the gene expression level of thousands of genes in Caco-2 cells as a function of the presence of an E-cadherin-derived peptide.

MATERIALS AND METHODS

Cell Culture

Caco-2 cells were purchased from the ATCC (Rockville, MD, USA). The culture medium contained the following: Minimum Essential Eagle's Medium with 2 mM L-glutamine and Earle's BSS with 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate, and 20% fetal bovine serum. Media components were purchased

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from Gibco (Carlsbad, CA, USA). Cells were grown in 150 cm^2 Corning cell culture flasks (Corning, NY, USA) at 37°C and 5% CO_2. Cells were fed every other day. At confluency, the cells were dissociated with trypsin-EDTA from Gibco as per ATCC subculturing protocol and seeded in 6-well Corning Costar polystyrene plates at a density of 460,000 cells per well. Cells were grown in 6-well plates instead of Transwells to ensure that the necessary amounts of RNA for the GeneChip expression probe arrays would be isolated and to facilitate the isolation of RNA.

Cells were allowed to grow for 14 days, then washed twice with PBS prior to incubation with the E-cadherin peptide. Control cells were incubated with Hank's Balanced Salt Solution (HBSS), and the treated cells were incubated with an HAV peptide solution in HBSS of either 100 or 500 μM for up to 48 h. The peptide solution was changed at 24 h for the cells treated for 48 h. Due to the instability of the peptide in serum, serum was not used. Light microscope images of the cells were taken at various time points using a Leica (Bannockburn, IL) DMRBE microscope with a 2.5 × Leica lense. Images were captured using a Nikon (Melville, NY, USA) DXM1200 digital camera, utilizing the Nikon imaging program Act-1.

Peptide Synthesis and Purification

A Pioneer peptide synthesizer (Applied Bio Systems, Foster City, CA, USA) was used to synthesize the E-cadherin-derived peptide by solid-phase peptide synthesis with Fmoc chemistry as previously described in greater detail (10,11). Fmoc-amino acids and resin were obtained from Bachem Biosciences (King of Prussia, PA, USA). The peptide was acetylated and amidated at the N- and C-terminus, respectively. Reversed-phase HPLC with a Varian Dynamax Microsorb C_{18} column (Palo Alto, CA, USA) was used to purify the crude peptide. The gradient system was solvent A (5% acetonitrile in water containing 0.1% TFA) and solvent B (100% acetonitrile). The pure peptide fractions were verified by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometry (FAB-MS). Peptide purity was estimated at > 96%.

Re-aggregation Studies

The activity of the synthetic cadherin peptide to inhibit the re-aggregation of Caco-2 single cells was evaluated using the following procedure. Cell monolayers grown in 24-well plates were washed 3 times with Ca^{2+} and Mg^{2+} free HBSS (pH 7.4) and then incubated in the same solution for 3 h to disrupt the tight intercellular junctions and to form single cells. Control monolayers were incubated in HBSS containing 2.0 mM $CaCl_2$. Following junction modulation, the cells were induced to re-aggregate by replacing the medium with HBSS containing 2.0 mM $CaCl_2$ in the absence or presence of 1.0 mM peptide. Monolayers were observed under phase-contrast microscope for up to 5 h of incubation with this calcium-containing medium. Pictures were taken at several time points.

RNA and Protein Isolation

RNA and protein were isolated from the 6-well plates at 8, 24, and 48 h of exposure to either 500 μM peptide or control vehicle. The medium and any detached cells were removed from the wells. RNA isolation was performed on the cells that remained attached using the Qiagen Rneasy kit (Valencia, CA, USA) as per the manufacturer's protocol. RNA samples were isolated in duplicate. The pure RNA was quantified using a spectrophotometer and Softmax Pro software. The integrity of the RNA was verified using the Agilent 2100 Bioanalyzer (Palo Alto, CA, USA) with the Eukaryote Total RNA assay. The RNA extracts were stored at −80°C until needed.

Protein was isolated from treated and control wells as follows. The medium and any detached cells were aspirated, and the cells were washed with PBS from Gibco. Lysis buffer containing 50 mM Tris, pH 7.0, 1% Triton X 100, and one tablet of Complete Mini Protease Inhibitor (Roche, Indianapolis, IN, USA) was added for 15 min while plates were kept on ice. The wells were scraped using a Nalgene Nunc no. 23 cell scraper (Rochester, NY, USA) and then transferred to clean vials. The lysate was then sonicated before DNase (Qiagen) was added. The total protein content was determined using the Pierce BCA Protein Assay kit (Rockford, IL, USA). The protein was kept at −80°C until needed for the Western blotting studies.

GeneChip® Eukaryotic Expression Arrays

The isolated RNA was used with the GeneChip® Sample Cleanup Module to prepare biotinylated eukaryotic cRNA to hybridize to the GeneChip® expression probe arrays. The detailed instructions provided by the manufacturer (Qiagen) were followed. In brief, double-stranded cDNA is prepared from the isolated RNA using the Invitrogen Life Technologies (Carlsbad, CA, USA) SuperScript system with a T7-$(dT)_{24}$ primer. This double-stranded cDNA is used to make the biotin-labeled cRNA by *in vitro* transcription using a labeling kit from Enzo (Farmingdale, NY, USA). The biotinylated cRNA is fragmented for 35 min at 94°C in the provided fragmentation buffer and then hybridized to the Affymetrix GeneChip® U95Av2 (Santa Clara, CA, USA) arrays for 16 h at 45°C. The hybridization mixture also contained the following internal controls to ensure efficient hybridization: control oligonucleotide B2 50 pM, bioB 1.5 pM, bioC 5 pM, bioD 25 pM, cre 100 pM, and herring sperm DNA 0.1 mg/ml. Each probe array was then washed and stained prior to being scanned in replicate using the GeneArray Scanner according to the manufacturer's procedures (Affymetrix, Santa Clara, CA, USA).

Western Blotting

Equivalent amounts of protein (5 μg) were prepared under reducing conditions (with NuPage LDS sample and NuPage reducing buffer) and boiled for 2.5 min. A Bio-Rad (Hercules, CA, USA) Criterion precast gel 7.5% Tris-HCl 1.0 mm was used to separate the proteins using SDS-PAGE. One gel was stained with Coomassie blue (Bio-Rad) for 20 min and then destained overnight with Bio-Rad destain solution. This Coomassie blue-stained gel verified equal protein loading (data not shown). A Hoefer Easy Breeze drying frame (San Francisco, CA, USA) was used to dry this gel. The electrophoresed proteins from the second gel were transferred to an Immun-Blot PVDF membrane (Bio-Rad); then nonspe-

cific binding was then blocked with 5% nonfat dried milk solution (Bio-Rad). The blot was cut, and the appropriate sections probed with either mouse anti-human E-cadherin antibody (clone 36, Transduction Laboratories, Lexington, KY, USA) at 1:2500 dilution or mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA, USA) at 1:8000 dilution overnight at 4°C with gentle agitation. A secondary antibody, goat anti-mouse IgG:horseradish peroxidase conjugate (Transduction Laboratories) at 1:5000 dilution, was used for 1 h at room temperature. Amersham Biosciences ECL Western blotting detection reagents (Piscataway, NJ, USA) were used to detect the signal as indicated. The blot was exposed to Hyperfilm within a Hypercassette by Amersham Pharmacia Biotech (Piscataway, NJ) for various times. A Konica SRX-101A film processor (Scarborough, ME, USA) was used to develop the film.

Analysis of GeneChip® Data

Affymetrix Microarray Suite 5.0 software (MAS5) was used to examine the expression data. Absent/Present calls were used to identify genes deemed present in particular cultures. Genes absent in all cultures were excluded from further analysis. Unnormalized signal data was exported from MAS5 and normalized by dividing by the magnitude of each array vector (Fostel and Vidmar, unpublished data), then imported into Spotfire Decision Site software (Spotfire, Somerville, MA, USA) for clustering and comparison of groups of arrays. The distinction calculation for each treated and untreated pair was computed in Spotfire; the genes with greatest distinction value for each comparison were then assessed in the context of their expression variability over the entire 48-h time course. Probe sets with significant change in expression for a particular time point are presented in Tables I–III. A 1.5-fold change was chosen as the criteria for significance differences so that changes found consistently at more than one time point could be reported. A Hierarchical Clustering using cosine correlation as the distance metric and the complete linkage joining method was performed using Spotfire Functional Genomics software.

RESULTS

Inhibition of Re-aggregation in Caco-2 Cells

Re-aggregation studies were used to evaluate the ability of the synthetic peptide to perturb cadherin-cadherin interactions. In these studies, Caco-2 monolayers were treated with calcium-free medium for 3 h to dissociate the monolayers. The monolayers were then incubated for 5 h in HBSS containing 2.0 mM $CaCl_2$ in the presence or absence of 1 mM HAV6. In the absence of peptide, nearly all cells re-aggregated during the 1 h recovery period in the presence of $CaCl_2$ (Fig. 1A), and the results at the later time points were identical to the re-aggregated shown in Fig. 1A (results not shown). On the other hand, the re-aggregation of Caco-2 cells was inhibited when HAV6 was also present in the calcium containing medium (Fig. 1B). Figures 1C and 1D show the re-aggregation of Caco-2 cells in the presence of HAV6 at 4 and 5 h time points, respectively. Fewer single cells were found at 5-h compared to the 4-h time point. These results suggest that this peptide can block the E-cadherin-mediated re-aggregation process.

Detachment of Cells Following 48 h of Exposure to Ac-SHAVSS-NH_2

Light microscopy images were captured at various time points to investigate any changes that occurred to the monolayers upon exposure to the E-cadherin-derived peptide, Ac-SHAVSS-NH_2. Three time points were chosen to evaluate the cells in all studies. In previous work, the cell incubation period with our peptides was 8 h; therefore, this was our first time point. Two later time points, 24 and 48 h, were also evaluated because others have shown that this length of incubation without serum did not cause detrimental effects to Caco-2 cells (13). Thus, any effects seen should be due to the peptide and not the lack of serum. Two concentrations, 100 and 500 μM, were used because Ac-SHAVSS-NH_2 previously displayed activity in Caco-2 cells at 500 μM. A HBSS control that contained 1.3 mM calcium was also used. No visual changes can be detected between the treatments with either concentration of the peptide and the control cells at 8 and 24

Table I. Gene Expression Summary for 8 and 24 h

Gene name	Function	Fold increase
Increased for treated after 8 h		
Prostate differentiation factor	Signal transduction	1.6
Increased for treated after 24 h		
Chromodomain helicase DNA binding protein 1-like	Transcription regulation	1.6
Myxovirus (influenza virus) resistance 2 (mouse)[a]	Defense response	2.3
Decreased for treated after 24 h		
Tight junction protein 2 (zonula occludens 2)	Adhesion	2.4
Hairy homolog (Drosophila)	Cell-cell signaling	2.1
Hypothetical protein KIAA1164	Function unknown	3.7
Guanine nucleotide exchange factor for Rap1	Signal transduction	2.3
Hypothetical protein FLJ20259[b]	Function unknown	2.4
Bullous pemphigoid antigen 1	Cytoskeleton organization & biogenesis	2.7
Ligase IV, DNA, ATP-dependent[b]	Single-strand break repair	2.0

[a] Expressed in treated and not expressed in control.
[b] Not expressed in treated and expressed in control.

Table II. Gene Expression Summary for 48 h

Gene name	Function	Fold increase
Increased for treated after 48 h		
Cytochrome P450, subfamily I polypeptide 1	Monooxygenase; oncogenesis	5.3
Heat shock 70-kDa protein 6	Protein folding; stress response	3.8
Adenosine kinase	Ribonucleoside monophosphate biosynthesis	2.6
Protein kinase, AMP-activated, alpha 2 catalytic subunit[a]	protein phosphorylation	6.1
Thra1/BTR fusion gene[a]	Transcription, DNA-dependent	6.4
Microseminoprotein, beta-endothelin 3[a]	Inhibits the secretin of FSH by pituitary cells development, signal transduction	2.8 2.6
ATP-binding cassette, sub-family ABC A (ABC1), member 5[a]	Small molecule transport; unknown substrate	14.8
Nephronophthisis 1 (juvenile)[a]	Excretion	2.8
Decreased for treated after 48 h		
Estrogen-related receptor gamma[b]	Transcription	2.0
Human glucose transporter pseudogene[b]	Function unknown	2.1
Coagulation factor II (thrombin) receptor-like 1	G-protein coupled protein signaling; cytostolic calcium ion concentration elevation	1.8
S-adenosylmethionine decarboxylase 1	Polyamine biosynthesis	2.1
Aspartate beta-hydroxylase[b]	Calcium homeostasis; muscle contraction	2.0
Frequently rearranged in advanced T-cell lymphomas 2[b]	Positive regulator of WNT signaling	2.5
KIAA0750 gene product	Flavoprotein oxidoreductase	2.0

[a] Expressed in treated and not expressed in control.
[b] Not expressed in treated and expressed in control.

h (Fig. 2, top and middle panels). After 48 h, the controls cells show some loss of cells, however not to the extent of the two treated groups (Fig. 2, bottom panel). The cells exposed to the hexapeptide Ac-SHAVSS-NH_2 at 100 μM also showed some cell detachment from the monolayer; but, the greatest detachment occurred with the 500 μM treatment. The 500-μM treatment produced cells that had dispersed from the monolayer and could be seen floating as small clusters. This suggests that the HAV peptide is responsible for cell detachment from the monolayer in a concentration-dependent manner.

Table III. Genes That Changed in More Than One Time Point

Gene name	Function
Increased for treated after 8 and 48 h	
F-box and leucine-rich repeat protein 4[a]	Ubiquitin-dependent protein degradation
Decreased for treated after 8 and 24 h	
KIAA0982 protein	Function unknown
Beta-amyloid binding protein precursor	May be involved in G protein–regulated signaling
Decreased for treated after 24 and 48 h	
Transforming growth factor, beta receptor II	Protein modification; cell-cell signaling

[a] Gene expressed in treated cells and not expressed in the control cells.

The HAV Hexapeptide Slightly Alters E-cadherin Protein Expression

In MDCK cells, we observed an increase in E-cadherin expression following treatment with several HAV hexapeptides at 500 μM (11). To examine if any alterations in E-cadherin protein expression would occur in Caco-2 cells following treatment with the HAV peptide, Western Blotting studies were performed. Protein was isolated from control cells and Caco-2 cells treated with two concentrations of the hexapeptide Ac-SHAVSS-NH_2 at 8, 24, and 48 h. The E-cadherin signal corresponds to a 120 kDa band; the control for the Western blot was actin, the band at 42 kDa (Fig. 3A). The signal for E-cadherin was normalized to the signal for actin, and the treated samples were compared to the control cells at each time point. No difference in protein expression of E-cadherin was found after 8 h of treatment with the two concentrations of the E-cadherin-derived peptide. At 24 h of exposure, there was a decrease in protein expression of E-cadherin for both peptide concentrations with the greatest decrease, 18%, occurring with 24 h exposure to 100 μM Ac-SHAVSS-NH_2 (Fig. 3B). A decrease in E-cadherin expression of 14% for the cells treated with 500 μM Ac-SHAVSS-NH_2 was found after 24 h of treatment. At the 48-h time point, the protein expression levels of E-cadherin for cells treated with 100 μM Ac-SHAVSS-NH_2 were approximately 97% of the control expression levels, whereas treatment with the higher concentration produced expression levels of about 90% of that of the control cells (Fig. 3B).

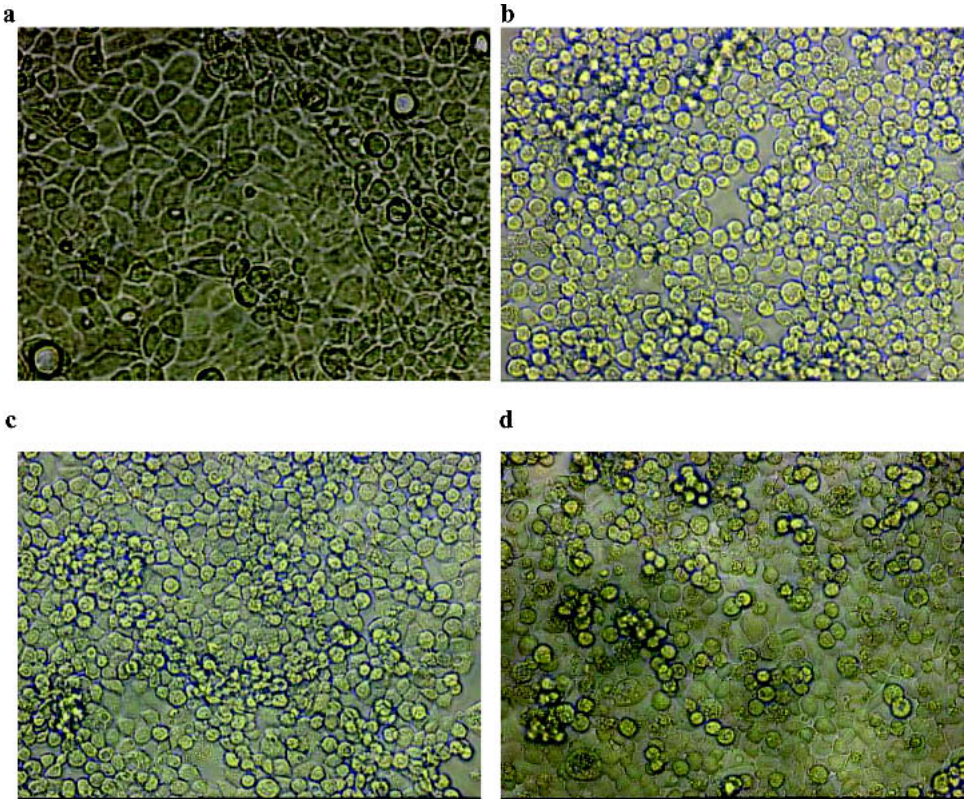

Fig. 1. Time dependent re-aggregation studies with single Caco-2 cells evaluating HAV6. (a) Single Caco-2 cells were allowed to re-aggregate for 1 h in the absence of HAV6. (b) HAV6 blocked cell to cell adhesion in a medium containing 2 mM calcium. (c) More than 50% re-aggregation had occurred after a 4 h incubation with HAV6 in the presence of 2 mM calcium. (d) At the 5 h mark, nearly a full monolayer was formed with only a few single cells floating in the medium containing HAV6 and 2 mM calcium.

HAV Hexapeptide Exposure Results in Gene Expression Changes

Previously, we (7,8,10,11) and others (5,6,9) have shown that HAV peptides inhibit adhesion in various cell lines; however, no one has examined the effects on gene expression following treatment with any HAV peptide. Affymetrix GeneChips® were used to see if any changes occurred after exposure to our HAV peptide. RNA was isolated from Caco-2 cells treated with HBSS as the control and 500 μM Ac-SHAVSS-NH_2, at 8, 24, and 48 h. The patterns in gene expression profiles across the different experimental conditions are shown in the dendogram in Fig. 4. The treatments were normalized against the expression levels of the control cells prior to performing the hierarchical clustering. The expression profiles for the 8- and 24-h treated cells form a cluster while the 48-h replicates form their own cluster.

Table I lists the probe sets that show a significant change at the early time points, 8 and 24 h. No probe sets were decreased in the treated cells compared to the control cells at 8 h. Prostate differentiation factor was the only probe set at 8 h that showed a slight increase in gene expression. The 24-h exposure data display a greater number of probe sets that show a decrease in genomic expression compared to the control cells. Two of these probe sets, hypothetical protein FLJ20259 and ATP-dependent DNA ligase IV, are not expressed in the treated cells; however, they are expressed in the control cells. There are only two probe sets that exhibit an increase in expression at this time point. The probe set for myxovirus (influenza virus) resistance 2 (mouse), which functions as a defense response, is turned on in the treated cells.

Table II displays the probe sets at 48 h that demonstrate a change of 1.5-fold or greater. The greatest number of probe sets was changed after 48 h of exposure to the hexapeptide Ac-SHAVSS-NH_2. In each grouping of probe sets, exposure to the hexapeptide caused several genes to be turned either on or off. The largest increases at 48 h are displayed by genes that were turned on in treated cells. These probe sets include AMP-activated, alpha 2 catalytic subunit protein kinase, Thra1/BTR fusion gene, endothelin 3, ATP-binding cassette, sub-family ABC-A (ABC1), member 5, and nephronophthisis 1 (juvenile).

Probe sets that are increased or decreased at more than one time point are given in Table III. No probe sets were either increased or decreased at all three time points. Only F-box and leucine-rich repeat protein 4 was increased at two time points, 8 and 48 h. In addition, this gene was expressed only in the treated cells. Decreased gene expression was also exhibited in several probe sets at more than one time point after exposure to the hexapeptide Ac-SHAVSS-NH_2. Beta receptor II transforming growth factor was the only probe set to decrease at both 24 and 48 h for the treated cells.

DISCUSSION

HAV peptides have been implicated in modulating the intercellular junction by interfering with the homotypic interactions of the cell adhesion molecule E-cadherin. This highly

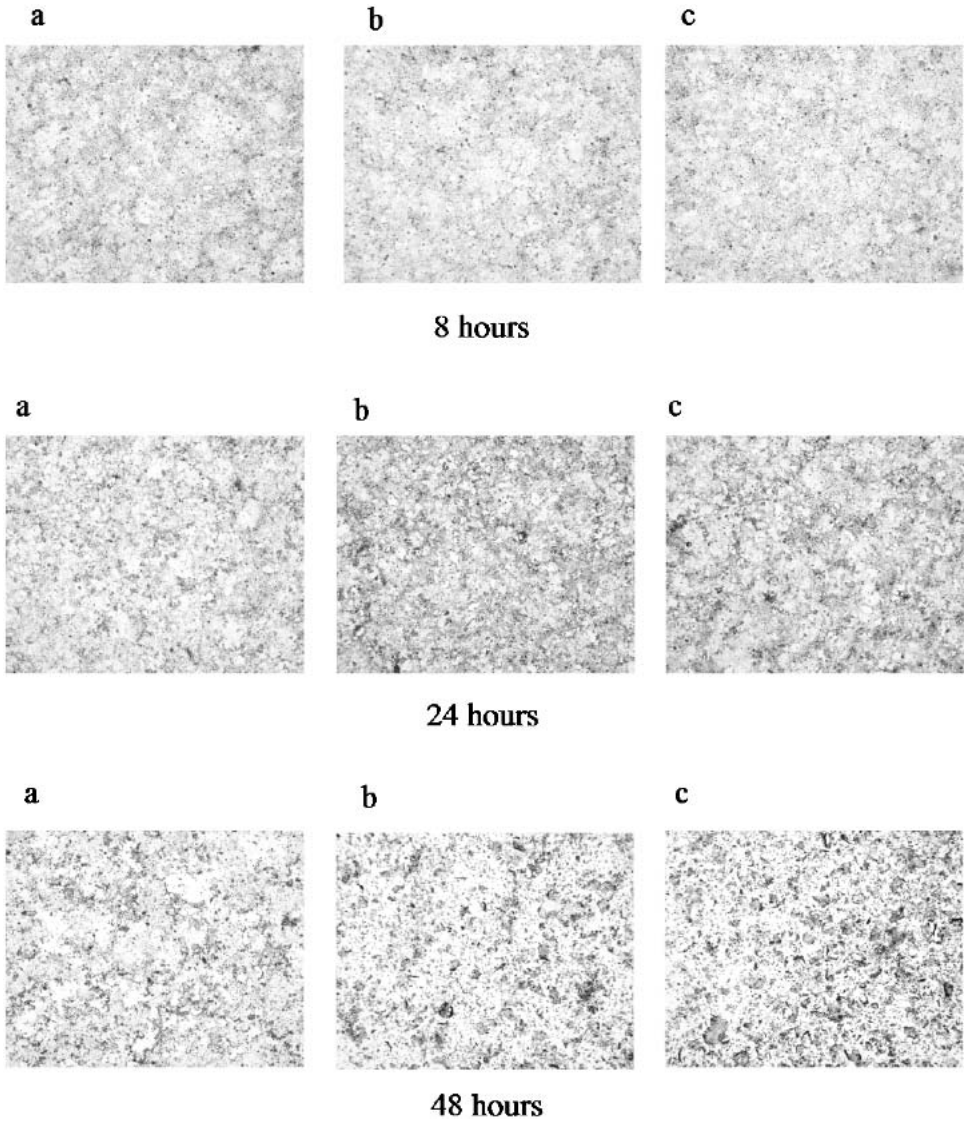

Fig. 2. Light microscopy images of Caco-2 cells after treatment. Top panel: These images were captured after 8 h of treatment with one of the three treatment conditions. In all panels, treatment conditions are as follows: (a) the control vehicle, which is HBSS, (b) 100 μM HAV6, or (c) 500 μM HAV6. Middle panel: These images were captured after 24 h of treatment. Bottom panel: These images were captured after 48 h of treatment. (magnification ×2.5).

conserved sequence is present in the cadherin superfamily (14). The use of peptides containing this sequence has been proposed to enhance the permeability of the paracellular pathway. Our re-aggregation studies suggest that this hexapeptide inhibits E-cadherin-mediated adhesion in Caco-2 cells (Fig. 1). The aim of this work is to determine if exposure to such a peptide will cause any alterations in the expression at the protein level for E-cadherin or at the mRNA level for of a host of probe sets present on the Affymetrix GeneChip® U95Av2. An occludin-derived peptide, which also increases tight junction permeability, has been shown to activate transcription of β-catenin/TCF/LEF genes (12). This result and the lack of studies examining gene expression effects of HAV peptides led us to evaluate the hexapeptide Ac-SHAVSS-NH_2.

Treatment with the hexapeptide Ac-SHAVSS-NH_2 did not cause any visible changes to the cell monolayers after short exposure times of 8 and 24 h. The cell monolayer that had been grown for 14 days prior to treatment showed no variations when inspected under the light microscope (Fig. 2, top and middle panels). At the final time point of 48 h, cell detachment was seen with both concentrations; however, the greatest effect was seen at 500 μM. These results agree with our previous work, which showed that this peptide was also able to dissociate BBMEC and MDCK monolayers (7). In the BBMEC and MDCK models, this peptide at a concentration of 2 mM could dissociate the monolayer grown for 8 days and 2 days, respectively, similar to the anti-E-cadherin antibody used in those studies. The seeding conditions varied in each of these studies; yet, the cell monolayers were at confluency at the time of the dissociation assay in each study. Subsequent work indicated that a 500 μM concentration of this peptide was effective in lowering the transepithelial electrical resistance and increasing mannitol flux in MDCK cells (11). In this work, the concentration was significantly less than that used in the study by Lutz *et al.* (7). Therefore, the longer exposure

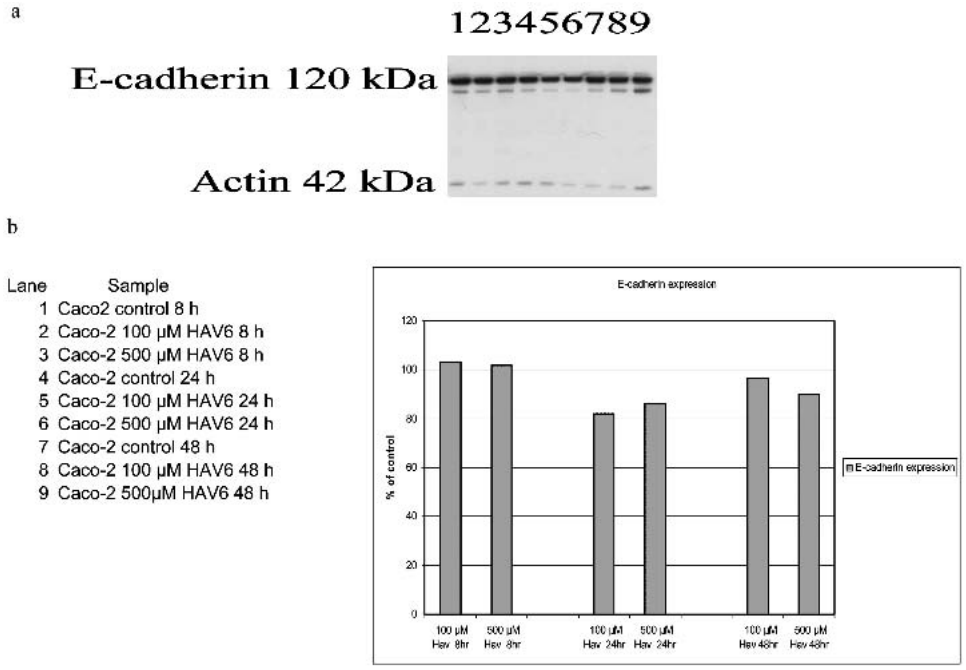

Fig. 3. Total protein expression of E-cadherin. (a) Depicted is Western blot for E-cadherin (120 kDa) and actin (42 kDa); the total protein loaded was equivalent for each lane. The legend below indicates the samples present within each lane. (b) The comparison of E-cadherin signal for control and treated cells is presented in this graph.

periods may be necessary to disrupt the interactions of E-cadherin that have been allowed to mature at confluency for a cell line that has been shown to form tighter monolayers than MDCK cells (15,16). The control vehicle of HBSS does contain calcium at a concentration of 1.3 mM; however, after 48 h of incubation with the control vehicle some cell detachment was observed. The greatest dissociation of cells was seen in the 500-μM Ac-SHAVSS-NH_2 treatment. This suggests that the HAV peptide is responsible for the dissociation of the cells due to the disruption of E-cadherin interactions. It has been shown that no differences in survival or in expression levels of anti- and pro-apoptotic Bcl-2 homologs were found when comparing cells maintained in the absence of serum for 48 h and cells maintained in 10% fetal bovine serum for the same time period (13). The function of Caco-2 cells has also been reported to be unchanged in the absence of serum for 48 h (13). Therefore, the conditions in this study should not adversely affect the cells.

In this study, only adherent cells were included in the protein and gene expression analyses. Total protein expression levels for E-cadherin were evaluated at 8, 24 and 48 h. No significant changes in E-cadherin protein expression were found for the two treated cells compared to the control at 8 h. This again may be due to the maturity of the intercellular junctions as was seen in the lack of changes in the appearance of the monolayers. At the 24-h point, a 20% decrease in the total E-cadherin protein level was noted for the cells treated with 100 μM peptide (Fig. 3B). The 500 μM concentration produced a decrease of about 14%. The cause of this decrease has yet to be determined; however, the gene expression levels for E-cadherin at 8 and 24 h show no change compared to the control cells (data not shown). It is possible that the degradation of E-cadherin may be increased by the HAV peptide as it disrupts the homotypic interactions of E-cadherin, causing E-cadherin to be internalized and then degraded. Further understanding of this disruption by the HAV peptide is necessary to elucidate the mechanism of this decrease in protein expression. The protein levels returned to nearly 100% of the control levels at 48 h after treatment with 100 μM Ac-SHAVSS-NH_2; yet, the 500 μM treatment remained 10% below the levels of the control (Fig. 3B). In our previous work in MDCK cells grown for 6–7 days, exposure to two related HAV peptides, Ac-SHAVAS-NH_2 and Ac-ASHAVSS-NH_2 at 500 μM, caused an increase in total protein expression of E-cadherin after 6 h of treatment (11). This difference may be attributed to the differences between cell types and the maturity of the adherens junction. The cells treated with 500 μM were further assessed for changes in gene expression.

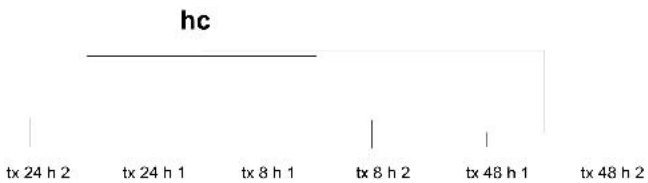

Fig. 4. Hierarchical clustering of all microarrays. This technique uses cosine correlation as the distance metric and the complete linkage joining method. Three groups were determined from the various experimental conditions. Group one, located on the left, contains the all the Caco-2 treatments at the early stages (8 and 24 h). The numbers below the dendrogram indicate the sample.

Interestingly, as exposure time to 500 μM Ac-SHAVSS-NH_2 increased, the number of stress-induced genes that were upregulated was also increased. After 8 h of treatment, prostate differentiation factor, a member of the transforming growth factor-beta superfamily, was the only probe set of 12,625 on the microarray to display a change in expression compared to the mRNA levels of the controls (Table I). The

transforming growth factor-beta superfamily controls growth and proliferation of cells and phosphorylates SMADs, which regulate transcription. Prostate differentiation factor is also upregulated in response to apoptotic signaling resulting from DNA damage (17,18); the increase found in our work also appears to be related to upregulation by apoptotic signaling. Another defense response gene is upregulated at the 24-h point. The homolog of the mouse myxovirus (influenza virus) resistance 2 gene was turned on in the treated cells, and it was not expressed in the control cells at this time point. This gene is a member of the dynamin and large GTPase family and is implicated in antiviral responses (19).

Consequently, gene expression was highly upregulated for genes that play a protective role for the cell following 48 h of treatment with 500 μM HAV peptide. Many of these protective genes were turned on following exposure to the peptide; these include AMP-activated protein kinase alpha 2 catalytic subunit, Thra1/BTR fusion gene, endothelin, ATP-binding cassette sub-family ABCA member 5, and nephronophthisis 1. The probe set presenting the greatest increase was the gene for ABCA5, a small molecule transporter (20). It is possible that these genes have been activated in response to the peptide in order to eliminate it from the cell as several of these genes encode proteins that regulate excretion (20,21). Other genes encoding proteins in response to stress such as heat shock protein 6 (22) and genes for drug-metabolizing enzymes such as cytochrome P1-450 1A1 (23) also display a significant increase. This further supports the hypothesis that the cell is upregulating genes that will protect the cell in response to modulation of the intercellular junctions by this HAV peptide. AMP-activated protein kinase has been reported to protect cells from stresses that cause ATP depletion by switching off ATP-consuming biosynthetic pathways (24); in this work, it shows an increase of over 6-fold. Similarly, a 17mer HAV peptide has been shown to inhibit phosphatidylinositol 3-kinase activity and induce apoptosis in primary mouse proximal tubular cells in the absence of cell-matrix attachment (25). Although our studies did not prevent cell-matrix attachment, data from Bergin *et al.* support our work and suggest that the action of the HAV peptide plays a negative role in cell survival (25). It is necessary to further evaluate these changes at the protein and functional level to confirm that exposure to the HAV peptide causes upregulation of these protective genes.

Changes in gene expression were also seen for various E-cadherin-related signal transduction pathways. First, the expression of several adhesion regulators was altered following exposure to this HAV peptide. Tight junction protein 2, also known as ZO-2, and bullous pemphigoid antigen 1, a member of the plakin adhesion family, are two genes that encode proteins involved in cell adhesion that exhibited a decrease in the levels of mRNA expression following 24-h treatment times (26,27). Second, treatment with this HAV peptide for 48 h turned off several of the probe sets (Table II), which include a nuclear hormone receptor, a regulator of calcium homeostasis, and a binding protein for GSK-3 that also acts as a positive regulator of the Wnt pathway (28–31). The thrombin receptor like 1 gene has been reported to be involved in ion transport in response to increases in intercellular calcium (32,33). Thus, there may be a relationship between this HAV peptide and the down regulation of genes implicated in the regulation of calcium levels and the β-catenin/Wnt pathway. Additional studies are necessary to confirm this. In response to this exposure, the positive regulator of the Wnt pathway, frequently rearranged in advanced T-cell lymphomas 2, may be turned off. Treatment with the peptide caused a decrease in gene expression of β-catenin at 8 and 24 h but an increase at 48 h (data not shown). In addition, decreases in expression were found at 8 and 24 h for the beta-amyloid binding protein precursor, which appears to modulate the apoptotic response to beta-amyloid through a mechanism dependent on caspases and G-proteins (Table III); however, the exact mechanism is unknown (34). Finally, at the two later time points, only the gene for the transforming growth factor beta receptor II is decreased. This gene functions in signal transduction through its kinase activity and regulation of SMAD transcriptional regulators. A relationship between TGF signaling and β-catenin/Wnt signaling has also been observed (35). It was recently determined that the transforming growth factor beta receptor II interacts with E-cadherin within the adherens junction; yet, it dissociates from E-cadherin when the adherens junction disassembles (36). Although several pathways have been shown to be affected by this peptide, further studies are necessary to decipher the exact mechanisms of this regulation.

Here again, a relationship between the degradation of E-cadherin and exposure to the HAV peptide may exist. The mRNA for the F-box and leucine-rich repeat protein 4 was detected only in the treated cells at 8 and 48 h; its expression was absent from the control cells at those time points. This gene encodes a protein that is a component of the modular E3 ubiquitin protein ligase (37). Recent reports suggest that an E3 ubiquitin ligase is responsible for the endocytosis of E-cadherin in a phosphorylation-dependent manner (38). It is possible that the F-box and leucine-rich repeat protein 4 may play a role in E-cadherin removal from the cell surface; however, additional studies are necessary to confirm this.

Although relationships between E-cadherin and many of the probe sets on the microarray are apparent, correlations with several probe sets and the E-cadherin-derived peptide are less obvious. For instance, at 8 and 24 h, KIAA0982 protein demonstrated a decrease in expression following peptide treatment; however, the function of KIAA0982 is currently unknown (39). Furthermore, several other hypothetical proteins with unknown functions also demonstrated changes in gene expression; to better understand these changes, the identification of these proteins and their respective functions is necessary.

In this work, long exposure times to Ac-SHAVSS-NH_2 caused several changes in Caco-2 cells. Detachment of cells from the monolayer and changes in protein expression levels of E-cadherin were seen following treatment with this HAV peptide. Most interestingly, exposure to this E-cadherin-derived hexapeptide over extended periods of time resulted in significant increases in the mRNA expression of various genes that serve to protect the cell from harm. This implies that long exposure (48 h) to this peptide may be viewed as harmful to the cell. In addition, various genes involved in cell signaling pathways such as the Wnt pathway responded to the presence of this peptide. In conclusion, HAV peptides not only can modulate intercellular junctions but can also perturb various intercellular pathways that are involved in a variety of E-cadherin related phenomena.

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