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Induction of apoptosis in cultured endothelial cells by a cadherin antagonist peptide: involvement of fibroblast growth factor receptor-mediated signaling

Noam Erez,[a] Eli Zamir,[a] Barbara J. Gour,[b] Orest W. Blaschuk,[c] and Benjamin Geiger[a,*]

[a] *Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel*
[b] *Adherex Technologies Inc. Ottawa, Ontario, Canada*
[c] *Division of Urology, Department of Surgery, McGill University, Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1*

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Abstract

Cadherins are a family of transmembrane glycoproteins mediating calcium-dependent, homophilic cell–cell adhesion. In addition, these molecules are involved in signaling events, regulating such processes as cell motility, proliferation, and apoptosis. Members of the cadherin subfamily, called either classical or type I cadherins, contain a highly conserved sequence at their homophilic binding site consisting of the three amino acids—histidine-alanine-valine (HAV). Previous studies have shown that peptides containing the HAV motif inhibit cadherin-dependent events such as cell aggregation, compaction, and neurite outgrowth. We report here that a cyclic peptide, N-Ac-*CHAVC*-NH_2 can perturb cadherin-mediated endothelial cell interactions, resulting in a progressive apoptotic cell death. This effect depends on cell density, as it is only observed when dense cultures are treated with the peptide. Adherens junction (AJ)-associated cadherin and catenins are differentially affected by the N-Ac-*CHAVC*-NH_2 treatment, as judged by double immunofluorescence labeling followed by immunofluorescence-ratio imaging. However, cell–cell adhesions are largely retained during the first few hours after addition of the peptide. It was also observed that following treatment, actin filaments partially lose their plasma membrane anchorage at AJs and translocate towards the cell center. Interestingly, addition of basic fibroblast growth factor to confluent, peptide-treated, endothelial cell cultures, completely blocks apoptosis and the inhibitory peptide reduce the phosphorylation of the FGF receptor target protein FRS2, suggesting that the peptide exerts its effect by inhibiting cadherin-mediated activation of fibroblast growth factor receptor signaling. We propose that cadherin-mediated signaling is essential for maintaining viability of confluent endothelial cells, and that its perturbation by N-Ac-*CHAVC*-NH_2 drives these cells to apoptosis.
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Keywords: N-cadherin; Apoptosis; FGFR; Endothelial cells

Introduction

Classical cadherins are a family of calcium-binding, transmembrane receptors that mediate homophilic cell–cell adhesion [1,2]. The various members of the cadherin family are expressed in a cell-type specific manner, and play key roles in the regulation of tissue formation and embryonic development [3,4]. These effects are attributed both to their capacity to directly mediate cell–cell adhesion, and to their transmembrane signaling activity [5]. Different cadherins share a high degree of sequence homology and they consist of a large extracellular domain, a single membrane-spanning region and a highly conserved cytoplasmic tail. Cadherins bind catenins and other anchor, cytoskeletal and signaling molecules through their intracellular domain. The N-terminal extracellular domains of classical cadherins harbor the homophilic cell adhesion recognition sequence, His-Ala-Val (HAV) [6,7]. It has been established that linear or cyclic synthetic peptides containing this sequence, as well as antibodies directed to it, can disrupt cadherin-mediated cell adhesion. HAV motif is also present in other proteins, including the extracellular domain of the fibroblast growth factor receptor (FGFR) and the influenza virus hemagglutinin [6,8–10].

Recent studies have indicated that beyond its involvement in the physical interaction between cells at AJs,

* Corresponding author. Fax: +972-8-9465607.
E-mail address: benny.geiger@weizmann.ac.il (B. Geiger).

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cadherin-mediated adhesion can also induce a variety of transmembrane signals. These include growth inhibitory signals [11–13], differentiation-promoting signals [14], activation of signaling systems such as the Mek/Erk [15], Akt/PKB [16] and fibroblast growth factor (FGF) receptor [17,18] pathways, and the small GTPases Rac1 and Cdc42 [19–21]. Classical cadherins can also regulate apoptotic processes. For example, N-cadherin-mediated cell adhesion plays a pivotal role in follicular and luteal cell survival and its disruption induces apoptosis of these cells in culture [9,22,23]. Similarly, it was demonstrated that disruption of cadherin-mediated adhesion in mouse intestinal epithelial cells leads to cell death [24].

In the present study, we have investigated the involvement of cadherin-mediated signaling in apoptosis of cultured endothelial cells. These cells express N-cadherin (which possesses the HAV motif), and VE-cadherin (which is a Type II or atypical cadherin, and does not posses the HAV motif) [25,26]. Antibodies against either N-cadherin or VE-cadherin perturb the structural and functional integrity of endothelial cell intercellular junctions [8,27]. We show here that treatment of endothelial cells with the classical cadherin antagonist, N-Ac-*CHAVC*-NH_2 [10], leads to cell density-dependent apoptosis. This treatment has differential affects on the distribution of different junctional components including β-catenin, plakoglobin, and actin, yet it does not induce dissociation of AJs. Interestingly, addition of bFGF to N-Ac-*CHAVC*-NH_2-treated cells completely rescues the cells from apoptosis, suggesting that activation of FGF receptor might be involved in cadherin-mediated signaling in endothelial cells. This is further supported by the fact that N-Ac-*CHAVC*-NH_2 suppresses the tyrosine phosphorylation of the FGFR target FRS2 in dense cultures of endothelial cells.

Materials and methods

Cell culture and immunofluorescence staining

Bovine Capillary Endothelial Cells (BCAP) [28] were provided by Nitzan Resnick (Technion, Haifa Israel) and were cultured in low-glucose (1 mg/ml) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Biological Industries, Israel). All cells used here were from passage 10 to 20.

Murine capillary cells (H5V) [29] were provided by Elizabetta Dejana (Mario Negri institute, Milano, Italy). These cells were cultured in high glucose (4.5 mg/ml) DMEM supplemented with 10% fetal calf serum.

For immunofluorescence, labeling cells were plated on 13-mm round glass coverslips (Marienfeld, Germany) in a 24-well plate (Falcon, USA). Following the different treatments, cells were simultaneously fixed and permeabilized with 0.5% Triton X-100, 3% paraformaldehyde (PFA) solution in phosphate buffer saline (PBS) for 2 min followed by fixation with 3% PFA in PBS for additional 20 min.

The fixed cells were then rinsed with PBS, and the coverslips were incubated for 45 min with the primary antibodies. After washing with PBS, cells were incubated with the appropriate fluorescent secondary antibodies for 30 min. After additional washes with PBS, the coverslips were mounted in Elvanol (Mowiol, 4-88, Hoechst, Germany) on glass slides.

Immunochemical reagents

The primary antibodies used in this study included: rabbit anti-FRS2 antibody kindly provided by Joseph Schlessinger and Irit Lax (Yale University, New Haven, CT), rabbit anti-β-catenin, monoclonal anti-Pan-cadherin antibody were from Sigma Immunochemical (MO, USA). The pan-cadherin antibody used here reacts with N-cadherin, as well as with E-cadherin, but not with VE-cadherin (Erez, unpublished results). Monoclonal anti-phosphotyrosine was from Upstate Group Inc., USA and monoclonal antibody to plakoglobin was from Transduction Labs (California, USA).

As secondary antibody reagents, we have used Cy3-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson Labs, ME, USA) and Alexa488-conjugated goat anti-mouse or goat anti-rabbit antibodies (Molecular Probes, OR, USA). Actin filaments were labeled with FITC-labeled Phalloidin (Sigma Immunochemical).

Treatment with cyclic peptides

The two cyclic peptides used in this study, N-Ac-*CHAVC*-NH_2 and N-Ac-*CHGVC*-NH_2, were kindly provided by Adherex Technologies Incorporated (Ottawa, ON, Canada). They were synthesized as previously described by Williams et al. [10] and dissolved in water (10 mg/ml). The cyclic peptide N-Ac-*CHAVC*-NH_2 was shown to inhibit N-cadherin-mediated cell adhesion, whereas N-Ac-*CHGVC*-NH_2 had no effect on cadherin-mediated interaction and was thus used here as a control peptide. Cells were cultured until reaching confluence, and then the medium was replaced with fresh medium, containing either N-Ac-*CHAVC*-NH_2 or N-Ac-*CHGVC*-NH_2 at a final concentration of 0.5 mg/ml. The cyclic N-Ac-*CHAVC*-NH_2 peptide has a half-life in culture of 12 h (Adherex Technologies Inc., unpublished data), and in longer experiments, the medium containing the peptide was replaced after 24 h.

Image acquisition and processing

Immunofluorescence microscopy was carried out using the DeltaVision digital microscopy system (Applied Precision, Issaquah, WA) consisting of a Zeiss Axiovert S100 TV microscope (Zeiss, Oberkochen, Germany) and a cooled

CCD camera (Photometrics, Tucson, AZ, USA). Fluorescence ratio imaging between two different components using double-labeled cells was carried out as previously described [30]. In short, Cy3 or Alexa-488 images were subjected to high-pass filtration and ratio values were calculated pixel by pixel and displayed using a spectrum-scale look-up table.

Monitoring of cell cycle and apoptosis

Cells were cultured until reaching confluence. After an additional 24 h, the medium was removed and the cells were incubated for 24–36 h with peptide-containing medium. Ten-micromolar BrdU was then added and the cells were permeabilized and fixed 45 min later, as described above. The nuclear membrane was further permeabilized by 20 min treatment with 2 M HCl + 0.5% Triton X-100 and the cells were washed with PBS and stained with monoclonal antibody against BrdU (Becton-Dickinson, California, USA) diluted in 0.5% Tween-20 in PBS. After washing with PBS, the cells were stained with Cy3-conjugated goat anti-mouse IgG, diluted in 0.5% Tween-20 in PBS. The cells were washed again with PBS, stained with 5 μg/ml of 4′,6-Diamidino-2-phenylindole (DAPI) and mounted in Elvanol. For identification of nuclei and measurement of BrdU and DAPI intensities, we used the ‘‘Water’’ algorithm software as previously described [30]. To distinguish between G1, S, G2, and sub-G1 cells (which are mostly apoptotic), we plotted the BrdU intensity against that of DAPI. Apoptotic nuclei were also monitored using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) kit (Roche Applied Science, Germany). To determine the progression of the apoptotic process at different cell densities, cultures of H5V cells on 35-mm dishes with a patterned glass coverslip (MatTek Corporation, MA) were treated with the inhibitory peptide. Following different incubation periods with the peptides, cells were examined by phase contrast microscopy.

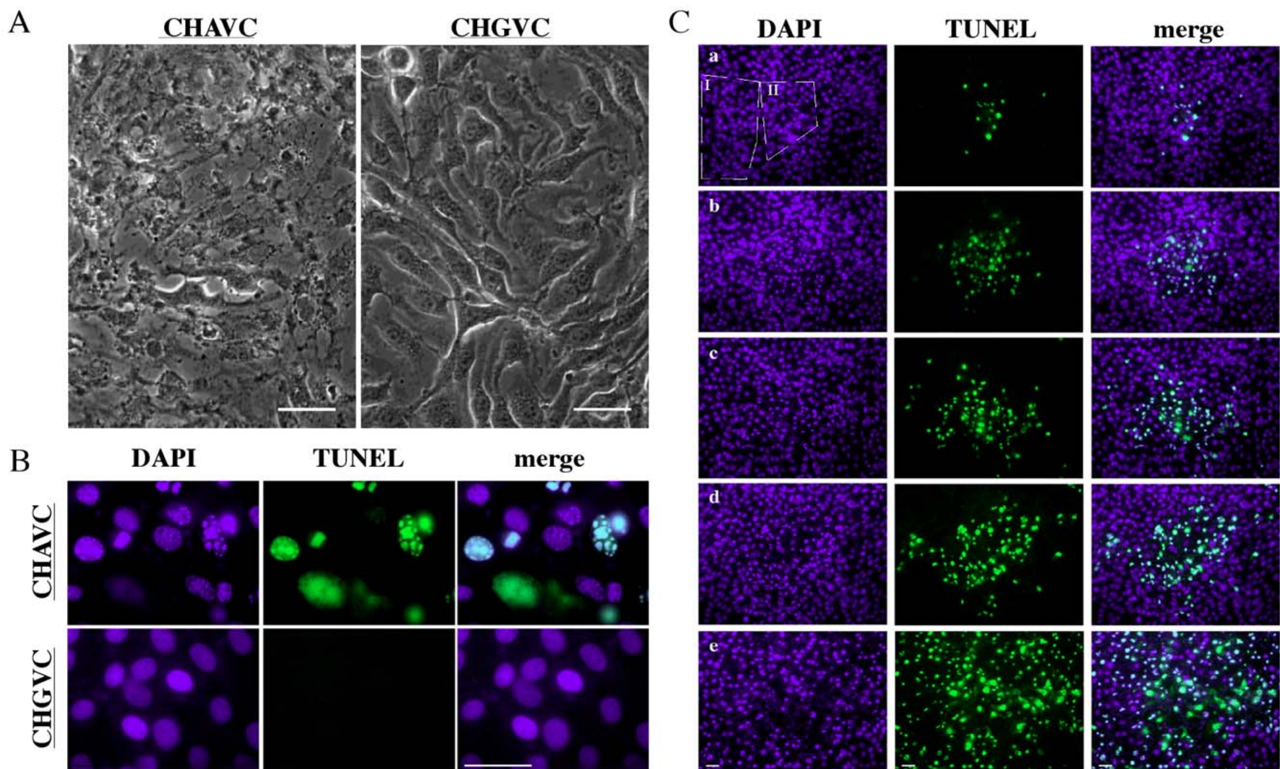


Fig. 1. Apoptosis of endothelial cells, induced by N-Ac-*CHAVC*-NH_2. (A) H5V cells were seeded on a 35-mm dish and incubated until reaching confluence. 24 h later, the cadherin inhibitory peptide, N-Ac-*CHAVC*-NH_2 or the control peptide, N-Ac-*CHGVC*-NH_2, were added to the medium and the cells were incubated for additional 24 h. Massive apoptosis was apparent by phase contrast microscopy in the N-Ac-*CHAVC*-NH_2-treated culture but not in the cells treated with the control peptide. (B) N-Ac-*CHAVC*-NH_2 treatment induces DNA and nuclear fragmentation. BCAP cells were treated as indicated above and stained with DAPI and TUNEL. Apoptotic cells were positive for TUNEL staining and exhibited picnotic or fragmented nuclei. No DNA fragmentation and nuclear abnormalities were observed in cells treated with the control peptide. (C) N-Ac-*CHAVC*-NH_2-induced apoptosis is cell-density dependent. Apoptotic foci were apparent after approximately 24 h of treatment with N-Ac-*CHAVC*-NH_2, in circular foci where cell density was particularly high (a). These sites grew radially after longer incubation with the active peptide (b–c) until apoptosis was apparent throughout the entire culture (d–e). To determine cell density in non-apoptotic (aI) and TUNEL-positive (aII) areas, polygons were drown corresponding to the two areas and density of DAPI-stained Nuclei was determined. This calculation showed that cell density in apoptotic areas was approximately 40% higher than in non-apoptotic regions. Scale bars = 30 μm.

Inhibition of N-Ac-CHAVC-NH_2-mediated apoptosis by bFGF

To study the involvement of FGF receptor activation in the anti-apoptotic, cadherin-mediated signaling, 2 ng/ml of bFGF (kindly provided by Israel Vlodavsky, Hebrew University, Jerusalem) was added to the culture medium during treatment with either N-Ac-*CHAVC*-NH_2 or N-Ac-*CHGVC*-NH_2 cyclic peptides. The effect of bFGF addition on cell viability was monitored microscopically.

Immunoprecipitation and immunoblotting

Confluent BCAP cells were subjected to N-Ac-*CHAVC*-NH_2 or bFGF for 3 h and lysed in lysis buffer (20 mM Tris–HCl, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 10 μg/ml aprotinin and 2 mM PMSF). The lysates were clarified by centrifugation at 14,000 × *g* for 10 min at 4°C and aliquots containing equal amounts of protein (determined by Bradford assay) were incubated with antibodies to FRS2 for 60 min at 4°C. Protein A-Sepharose was then added to the samples and incubated for an additional 2 h at 4°C. The beads were sedimented by brief centrifugation and washed extensively with 20 mM Tris buffer, 150 mM NaCl, 0.5% NP-40. The samples were boiled in Laemmli sample buffer with 1 mM sodium orthovanadate and subjected to 9% SDS-PAGE. The proteins were then transferred to Hybond-C nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). The nitrocellulose membrane was blocked with 1% BSA in TBST buffer containing 10 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.6, incubated with anti-phosphotyrosine antibodies at 4°C for 16 h, washed extensively with TBST, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Life Science) for 60 min. The immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Life Science), and exposed to X-ray film. The bands were quantified by densitometry using an imaging densi-

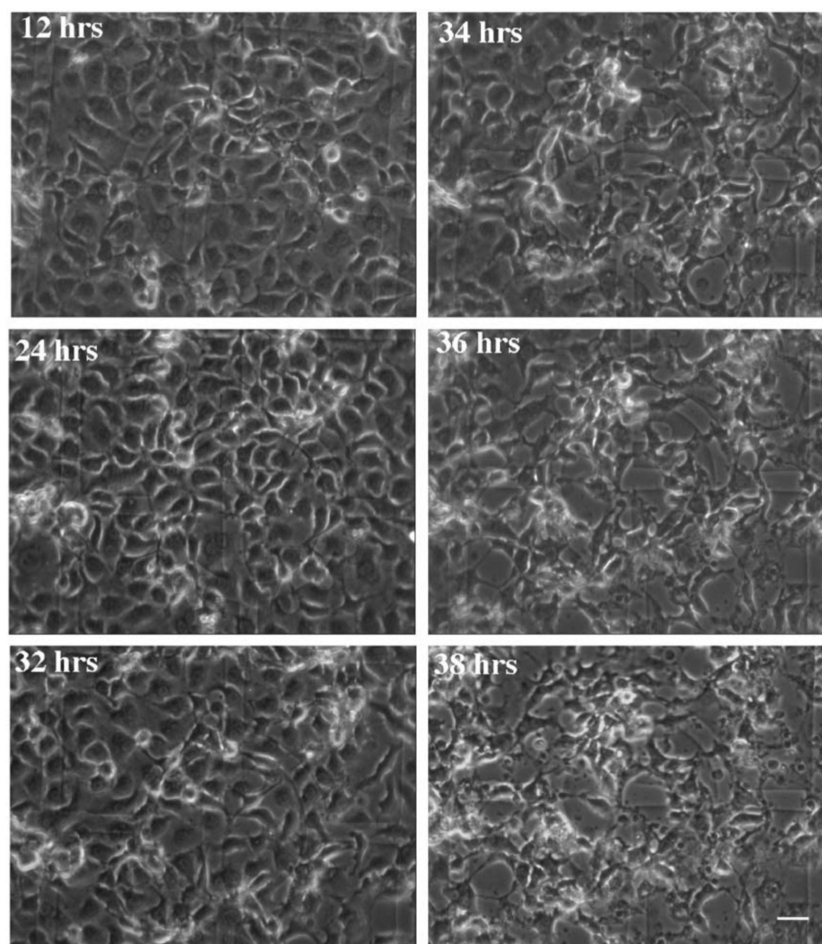


Fig. 2. Time-lapse examination of the progression of N-Ac-*CHAVC*-NH_2-mediated cell death. H5V cells were cultured on a patterned coverslip in the presence of N-Ac-*CHAVC*-NH_2. The same fields were monitored by phase contrast microscopy and recorded at different time point ranging from 12 to 38 h after addition of the peptides. The progress in cell death induced by the peptide is manifested by cell separation and contraction (32 and 34 h) followed by massive cell death and detachment (36 and 38 h, respectively). Scale bar = 30 μm.

tometer, model GS-700 (BioRad Laboratories, Hercules, CA) and analyzed by NIH Image software.

Results

N-Ac-CHAVC-NH_2 induces apoptosis of cultured endothelial cells

Incubation of densely plated BCAP or H5V endothelial cells for 24 h with N-Ac-*CHAVC*-NH_2 resulted in progressive and massive cell death, readily observed by phase contrast microscopy (Fig. 1A). Control peptide, N-Ac-*CHGVC*-NH_2, did not affect cell viability. The two peptides, N-Ac-*CHAVC*-NH_2 and N-Ac-*CHGVC*-NH_2, were tested at different concentrations ranging from 1 μg/ml to 1 mg/ml. However, apoptotic effect induced by the N-Ac-*CHAVC*-NH_2 peptide was observed only at concentrations higher than 250 μg/ml. Similar concentrations were previously shown to be effective in inhibition of neurite-outgrowth [10]. The N-Ac-*CHAVC*-NH_2 peptide also induced massive nuclear fragmentation, which is typical of apoptotic processes, manifested by DAPI and TUNEL staining (Fig. 1B). The superimposed image shows correlation between frag-

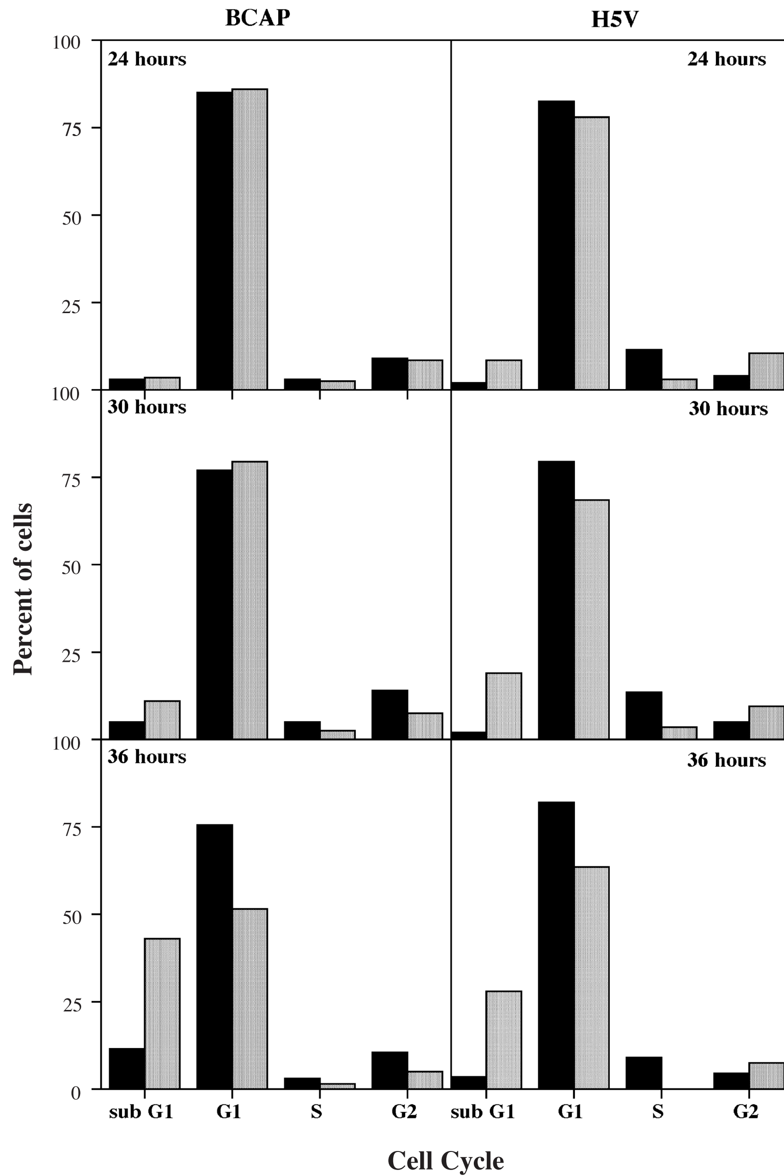


Fig. 3. Changes in DNA content in H5V and BCAP cells treated with the cadherin inhibitory peptide N-Ac-*CHAVC*-NH_2 or the control peptide N-Ac-*CHGVC*-NH_2 (▒ and ■, respectively). Cell death was monitored by quantitative, microscope-based cell cycle staging of more than 6000 cells in each experiment. In H5V cells, a progressive and specific increase in sub-G1 cells was noted as early as 24 h after incubation with N-Ac-*CHAVC*-NH_2. This percentage of sub-G1 cells increased with time and by 36 h about 25% of the nuclei were apoptotic. In BCAP cells, this process was somewhat slower and reached high apoptotic level of 30–40% only after 36 h of incubation.

mented, TUNEL-positive cells and fragmented nuclei (observed by DAPI staining). No fragmentation was observed in N-Ac-*CHGVC*-NH_2-treated cells and TUNEL assay for these cells was negative. Interestingly, apoptotic nuclei were first detected following 24 h of treatment, in small foci where cell density was particularly high. Typically, cell density in areas where early apoptotic process was apparent was nearly 40% higher than in non-apoptotic areas (over 5000 cells per mm^2, compared with about 3500 cells/mm^2; for BCAP cells; data not shown). Once formed, these foci expanded radially and within additional 12 h, apoptotic cells were detected throughout the entire culture (Fig. 1C). It is noteworthy that at late stages, even though the entire culture is TUNEL positive, the apparent density of cells is decreasing (compare panel e and c) because dead cells tend to detach from the substrate. The control peptide, N-Ac-*CHGVC*-NH_2, had no effect on cell morphology or survival even after long incubation. The apoptotic processes observed in the two endothelial lines, H5V and BCAP, were similar except that the onset of apoptosis was usually somewhat delayed in BCAP cells, compared to H5V (data not shown).

To follow the progression of the apoptotic process in live cells, we plated H5V cells on patterned coverslips and treated them with the peptides as described above. The phase contrast images show the progression of cell death in the same field at different time points following addition of the inhibitory peptide (Fig. 2). Examination of the cells revealed first apparent effects of the peptide after about 24 h of treatment, manifested by mild rounding of the cells, followed by their contraction and separation from their neighbours (Fig. 2, 32–34 h). This was followed by loss of matrix adhesions and massive detachment (Fig. 2, 36–38 h).

The apoptotic process was also characterized by a progressive increase in sub-G1 cells in the H5V and BCAP cultures. In the former cells, a progressive and consistent increase in sub-G1 cells was noted in N-Ac-*CHAVC*-NH_2-treated cells starting at 24 h after addition of the peptide. This process increased with time and by 36 h about 25% of the nuclei were apoptotic. In BCAP cells, this process was somewhat delayed, reaching an apoptotic level of 30–40% only after 36–48 h of incubation (Fig. 3). Cells that were treated with the control peptide N-Ac-*CHGVC*-NH_2 exhibited a very small percentage of sub-G1 cells (approximately 4% in H5V cells and approximately 10% in BCAP cells) similar to cells incubated with fresh medium without inhibitory or control peptides.

Apoptosis induced by N-Ac-CHAVC-NH_2 is cell density-dependent

As mentioned above, the apoptotic process induced by N-Ac-*CHAVC*-NH_2 was first detected in discrete foci corresponding to particularly dense areas in the culture. This observation suggested that N-cadherin-mediated signaling is essential for the viability of these cells. To test this hypothesis, we plated H5V endothelial cells at different initial densities and then added N-Ac-*CHAVC*-NH_2 for up to 36 h and determined the number of viable cells per unit area. As shown in Fig. 4, cells in sparse or sub-confluent cultures were not affected by the presence of the cyclic peptide and continued to proliferate. In contrast, when the cyclic peptide was added to confluent cultures, there was a sharp decline in the number of cells due to apoptosis. As indicated above, cell density in apoptotic areas was approximately 40% higher than the rest of the culture, confirming that the apoptotic response induced by N-Ac-*CHAVC*-NH_2 is cell density dependent. It is important to note that the BCAP and H5V cells differ in their maximal densities in culture. BCAP cells can reach a maximal density, which is five times higher than that of H5V cells. Thus, the critical density for induction of apoptosis, due to N-Ac-*CHAVC*-NH_2 treatment, is higher in BCAP cells in comparison to H5V cells. We would like to mention that apoptosis induced by the N-Ac-*CHAVC*-NH_2 peptide was reversible only when the incubation with the peptide was shorter than 12 h. After longer incubations, cells were already committed to apoptosis.

N-Ac-CHAVC-NH_2-induced apoptosis is blocked by bFGF

Previous studies have suggested that N-cadherin stimulates neurite outgrowth by activating the FGF receptor-mediated signaling pathway [31]. It was further shown that N-cadherin forms a complex with FGFR1 [32]. N-Ac-*CHAVC*-NH_2 blocks neurite outgrowth [10], suggesting that

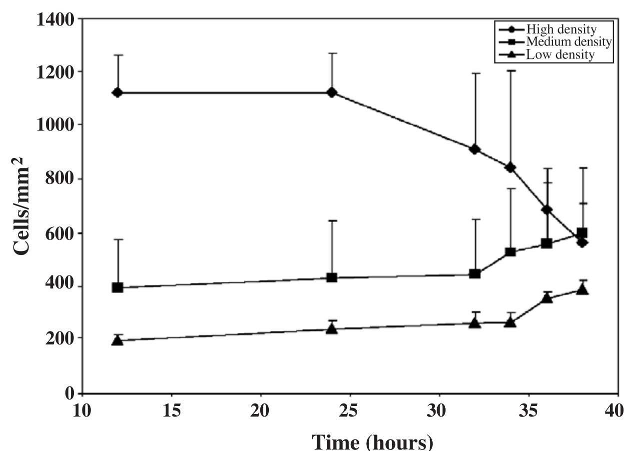


Fig. 4. Apoptosis induced by N-Ac-*CHAVC*-NH_2 is cell density dependent. H5V endothelial cells were plated at different initial densities and incubated with N-Ac-*CHAVC*-NH_2 for up to 38 h. At low and medium densities (approximately 200–400 cells/mm^2), cells proliferated in the presence or absence of N-Ac-*CHAVC*-NH_2 without displaying any sign of apoptosis. When the inhibitory peptide was added to a confluent culture (>1000 cells/mm^2), a sharp decline in cell number was noted, within 24–36 h of treatment. Each value is the mean (±SD) of 6 different experiments.

it might interfere with the cadherin-mediated activation of FGF signaling. To determine whether such a mechanism might be involved in the apoptotic effect described here, we checked the ability of soluble bFGF to block the apoptotic process induced by N-Ac-*CHAVC*-NH_2 in BCAP cells. For that purpose, the cells were treated with N-Ac-*CHAVC*-

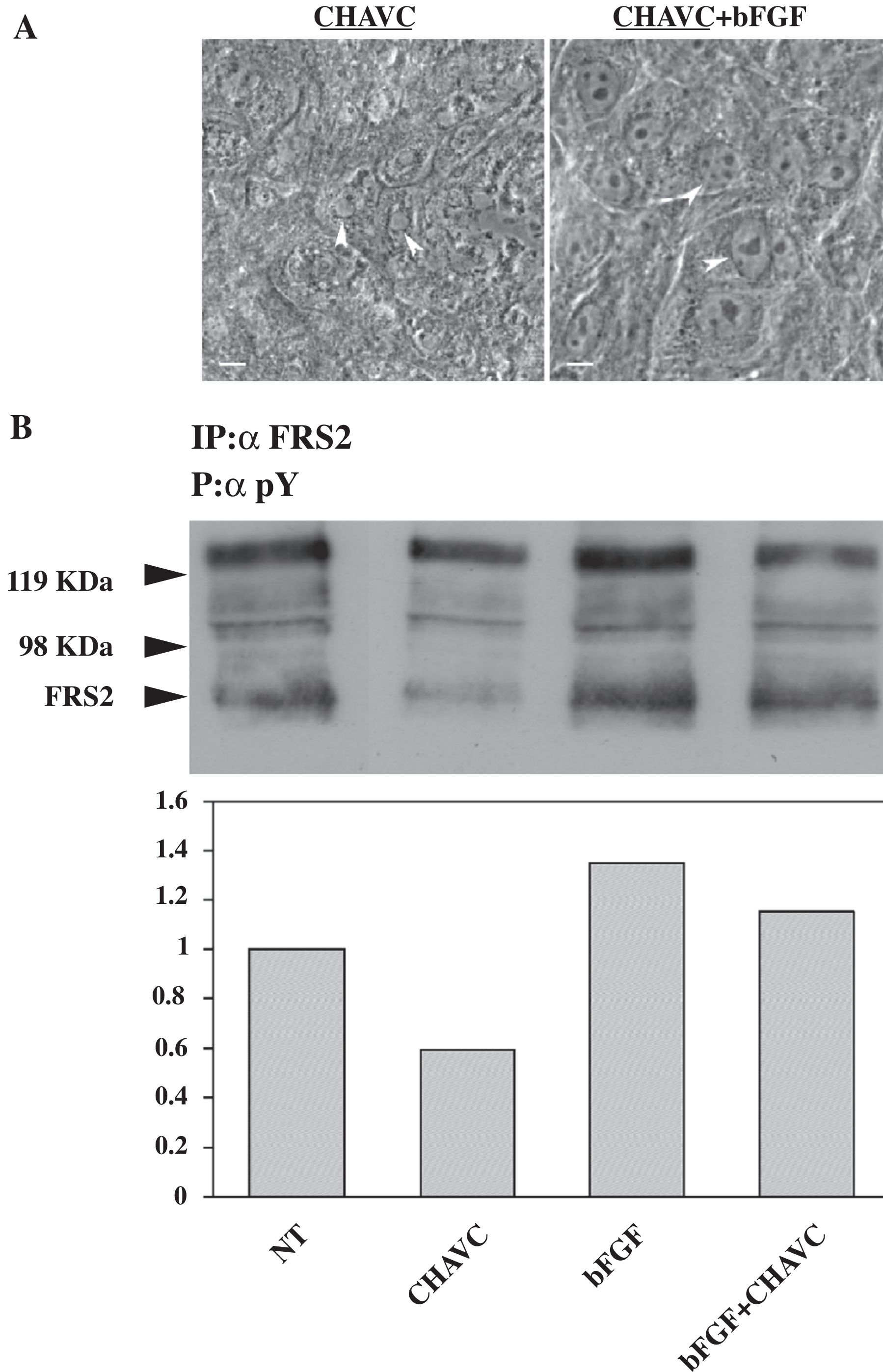


Fig. 5. N-Ac-*CHAVC*-NH_2 attenuates fibroblasts growth factor receptor mediated signaling. (A) Basic FGF blocks N-Ac-*CHAVC*-NH_2-induced apoptosis of cultured endothelial cells. BCAP cells were incubated for 24 h with the inhibitory peptide in the presence or absence of 2 ng/ml bFGF, and cell integrity was monitored by phase contrast microscopy. Cells that were treated with N-Ac-*CHAVC*-NH_2 only underwent massive cell death, which was manifested by condensation and fragmentation of nuclei as well as overall cell contraction (see arrowheads in left panel). Cells that were incubated with the inhibitory peptide in the presence of bFGF did not display any sign of apoptosis (see cells marked by arrowheads in the right panel). Scale bar = 10 μm. (B) BCAP cells were treated with either N-Ac-*CHAVC*-NH_2 ,bFGF, or the combination of the two for 3 h. Cells were then lysed and FRS2 was immunoprecipitated and its tyrosine phosphorylation level was determined by immunoblotting. Densities of the immunoblots (histograms in lower panel) indicate that tyrosine phosphorylation of FRS2 in the N-Ac-*CHAVC*-NH_2-treated cells decreased by approximately 40% in comparison with untreated cells. Addition of bFGF to naive or peptide-treated cells induced similarly high stimulation of FRS2 phosphorylation.

NH_2, as described above, in the presence or absence of bFGF. As shown in Fig. 5A, N-Ac-*CHAVC*-NH_2, in the absence of bFGF, induced massive cell death, manifested by condensed picnotic nuclei (marked by arrowheads) which was completely blocked by the addition of 2 ng/ml bFGF to the culture medium (arrowheads point at normal looking, intact nuclei. We further checked whether N-Ac-*CHAVC*-NH_2 treatment indeed interferes with FGFR-mediated signaling by testing the tyrosine phosphorylation status of FRS2. This protein is a major target of FGFR1, which undergoes tyrosine phosphorylation upon activation of FGFR1 [33]. Indeed, tyrosine phosphorylation level of FRS2 in BCAP cells was dramatically decreased following treatment with the inhibitory peptide compared to the basal phosphorylation level of untreated cells or to bFGF-treated cells (Fig. 5B). However, the N-Ac-*CHAVC*-NH_2 peptide was not able to suppress FRS2 phosphorylation, directly stimulated by bFGF (Fig. 5B). It is noteworthy that immunofluorescence labeling did not show any significant alteration in tyrosine–phosphorylation state of AJ in cells treated with either the inhibitory or control peptide. Furthermore, we did not detect tyrosine phosphorylation of cadherin or β-catenin in these cells, with or without peptide treatment (data not shown).

Effect of N-Ac-CHAVC-NH_2 on the molecular organization of adherens junctions

To assess the effect of N-Ac-*CHAVC*-NH_2 treatment on the structural integrity and molecular organization of AJs, BCAP cells were treated with N-Ac-*CHAVC*-NH_2 or the control peptide N-Ac-*CHGVC*-NH_2 for 24 h and then double-labeled for pairs of AJ components (cadherin/β-catenin, cadherin/actin and plakoglobin/β-catenin). The use of microscopy-based quantitative approach was essential, in view of the non-synchronized and non-uniform nature of the cellular response to the inhibitory peptide. At that time point, no typical signs of apoptosis were apparent.

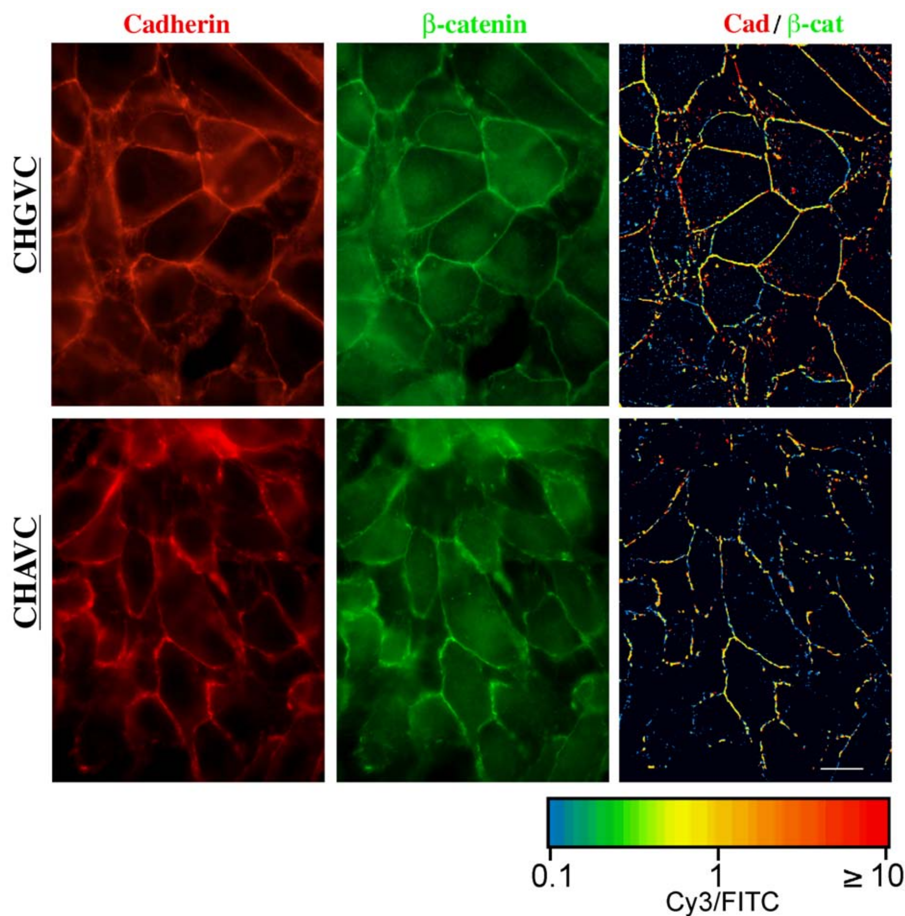


Fig. 6. The effect of N-Ac-*CHAVC*-NH_2 on the distributions of cadherin and β-catenin in BCAP cells. N-Ac-*CHAVC*-NH_2 or N-Ac-*CHGVC*-NH_2 (0.5 mg/ml) were added to confluent cells for 24 h followed by fixation and staining for cadherin and β-catenin. As seen in the ratio image (right column), cells treated with the control peptide display normal AJs containing both cadherin and β-catenin (indicated by the yellow color in the ratio image). Treatment with N-Ac-*CHAVC*-NH_2 induces partial loss of cadherin labeling from cell–cell junctions, manifested by "blue" regions along the AJs. The cells shown here were not associated with apoptotic foci, and did not display classical apoptotic morphology. Scale bar = 10 μm.

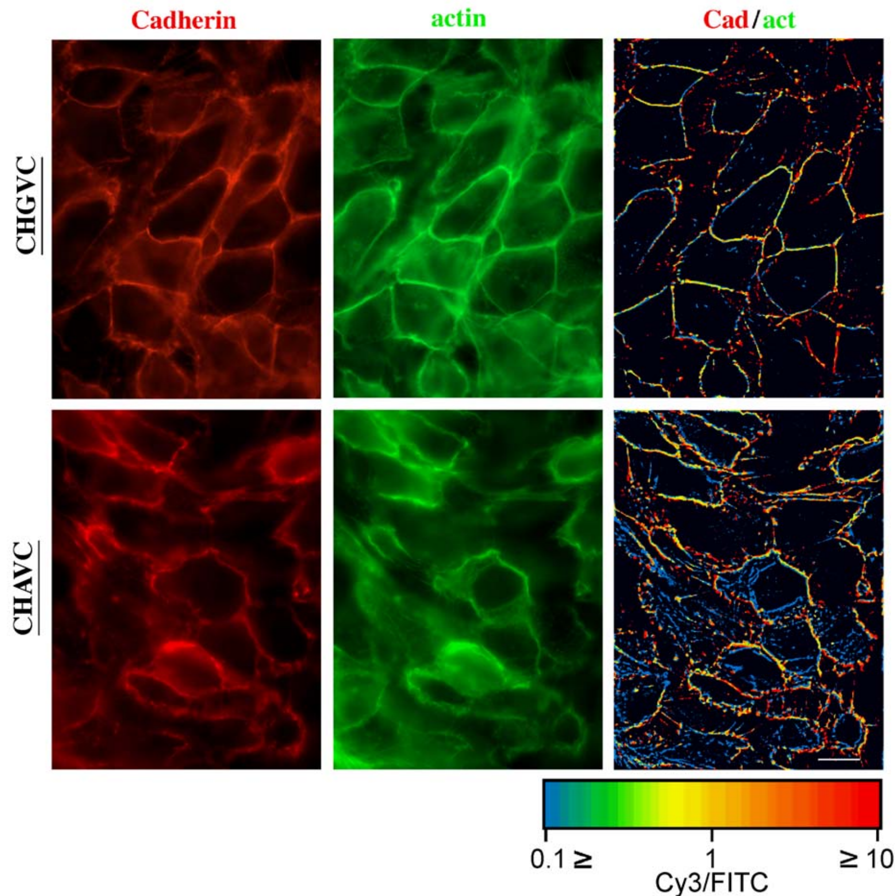
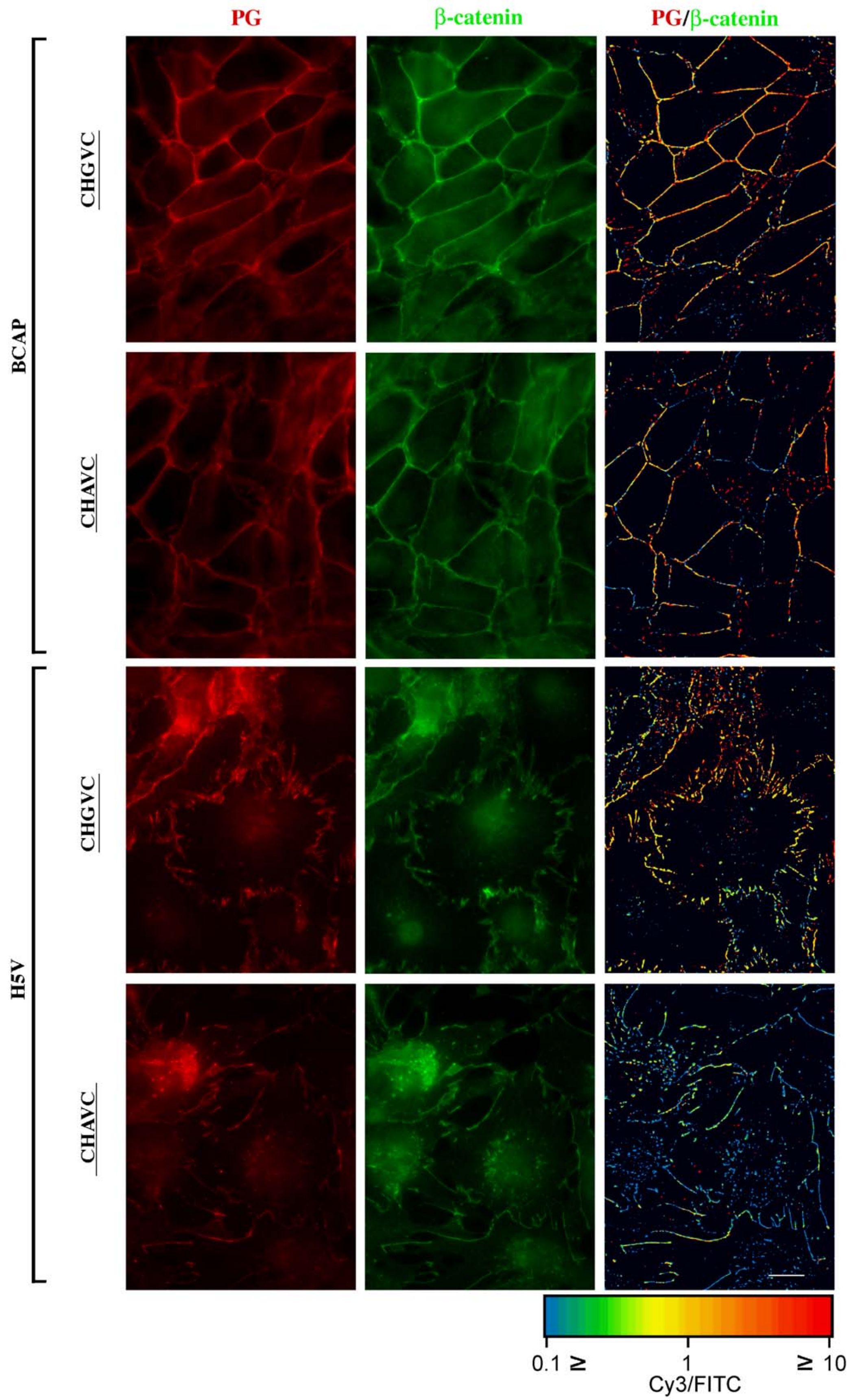


Fig. 7. The effect of N-Ac-*CHAVC*-NH_2 on the distribution of cadherin and actin in BCAP cells. N-Ac-*CHAVC*-NH_2 or N-Ac-*CHGVC*-NH_2 (0.5 mg/ml) were added to confluent cells for 24 h followed by fixation and staining for cadherin and F-actin.N-Ac-*CHAVC*-NH_2-treated cells appear flat and cadherin labeling is perturbed, indicating partial loss from AJs. Actin filaments, in N-Ac-*CHGVC*-NH_2-treated cells are organized in a marginal belt, overlapping the cadherin-labeled junctions (yellow in the ratio image). Treatment of the cells with N-Ac-*CHAVC*-NH_2 leads to partial detachment of the actin belt from the membrane and its translocation towards the cell-center. Scale bar = 10 μm.

To depict differential effects of the peptides on the junctional components, we have calculated ‘‘ratio images’’ showing, pixel-by-pixel, the relative labeling intensities tested for the two antigens.

Comparison of cadherin and β-catenin labeling in BCAP cells, treated with the control peptide (N-Ac-*CHGVC*-NH_2), revealed well-organized AJs with nearly overlapping distributions of the two proteins (yellow color in Fig. 6) identical to those of untreated cells (not shown). Treatment with the inhibitory peptide, N-Ac-*CHAVC*-NH_2, resulted in the loss of cadherin labeling from AJs, accompanied by only mild reduction in β-catenin-labeling levels (Fig. 6). N-Ac-*CHAVC*-NH_2 induced also changes in actin distribution, manifested by an increase in cytoplasmic localization of actin and by partial fragmentation of the AJ-associated actin belt (Fig. 7). Similar changes were also noted with H5V cells (data not shown). To further characterize the molecular events induced by the peptide at AJs, we compared the effect of the inhibitory and control peptides on the junctional distribution of β-catenin and its homologue plakoglobin (PG). As shown in Fig. 8, BCAP cells, treated with the control peptide N-Ac-*CHGVC*-NH_2, displayed an extensive co-localization of the two catenins along cell–cell AJs. Incubation of these cells with N-Ac-*CHAVC*-NH_2 for 24 h significantly reduced the immunofluorescence staining intensities for both catenins. The ratio imaging shows,

Fig. 8. The effect of N-Ac-*CHAVC*-NH_2 on the distributions of plakoglobin and β-catenin in BCAP and H5V cells. N-Ac-*CHAVC*-NH_2 or N-Ac-*CHGVC*-NH_2 (0.5 mg/ml) were added to confluent cells for 24 h followed by fixation and staining for plakoglobin (PG) and β-catenin. Cells treated with N-Ac-*CHGVC*-NH_2 show co-localization of PG and β-catenin in AJs, represented by the yellow color in the ratio image. In BCAP cells treated with N-Ac-*CHAVC*-NH_2, there is a preferential loss of PG from cell–cell junctions (blue color in the ratio image). The effect on PG to β-catenin ratio in H5V cells is even more prominent showing a loss of PG from AJ and retention of β-catenin. This suggests that plakoglobin is rapidly removed from AJ due to N-Ac-*CHAVC*-NH_2 treatment, while β-catenin stays behind. Scale bar = 10 μm.


PG
β-catenin
PG/β-catenin
BCAP
CHGVC
CHAVC
H5V
CHGVC
CHAVC
0.1 ≥
1
≥ 10
Cy3/FITC

however, that PG is more susceptible to the peptide treatment than β-catenin. The selective effect of N-Ac-*CHAVC*-NH_2 treatment on the two catenins was also observed in H5V endothelial cells and was often even more pronounced than that described above for BCAP cells. Thus, the inhibitory peptide had a dramatic effect on the relative distributions of the two catenins, preferentially affecting PG distribution.

Discussion

In this study, we have investigated the effects of a cadherin antagonist (N-Ac-*CHAVC*-NH_2) on endothelial cell survival. This antagonist is a cyclic peptide that contains the classical cadherin cell adhesion recognition sequence, HAV. We show here that this cyclic peptide: (1) induces apoptosis in two endothelial cell lines, H5V and BCAP; (2) affects only dense endothelial monolayers; (3) interferes with FGFR signaling but cannot induce apoptosis in the presence of bFGF; (4) perturbs the molecular organization of AJs. Collectively, these observations support the notion that cadherin-mediated cell adhesion, beyond its obvious role in tissue formation, is involved in signaling processes that are essential for endothelial cell survival. Consequently, perturbation of this signaling by a classical cadherin antagonist can induce apoptosis. As previously shown, cadherin-mediated signaling can exert different affects on cells including growth arrest [12,34] and induction of differentiation [14]. Cadherins can activate or modulate specific signaling pathways activated by such growth factors as EGF, FGF, and VEGF [15,17,18,35] [32] and affect the activation of specific small G-proteins [16,20,21]. Inhibition of cadherin action, using specific antibodies to the extracellular domain of the molecule or inhibitory peptides containing the HAV motif, can block such signaling events. The signaling process studied here is distinct from other, previously described, cadherin signaling events in that it is primarily involved in the regulation of apoptosis of endothelial cells, and the effect induced by the inhibitory peptide strictly depends on the density of the target cell monolayer.

To explore the specific role of cadherins in apoptosis, we have perturbed AJs using a cyclic peptide containing the HAV motif. Inhibitory peptides containing this sequence have been previously reported to affect a variety of cadherin-dependent processes including neurite outgrowth, myoblast fusion and calcium-dependent cell aggregation [36–38]. More recently, it was shown that HAV-containing peptides can induce epithelial cells apoptosis when cultured under anchorage independent conditions [39].

To define the involvement of AJs in the regulation of cell survival, we have examined their integrity and molecular structure following treatment with N-Ac-*CHAVC*-NH_2. As shown here, AJ integrity was grossly preserved though the molecular organization of the junctions was perturbed. The most conspicuous structural effects of the treatment were the reduction in the junctional levels of N-cadherin and plakoglobin, as well as perturbation of actin organization. β-catenin, on the other hand, was still associated with the cell membrane. The intriguing molecular reorganization of AJ following N-Ac-*CHAVC*-NH_2 treatment suggests that different junctional molecules can be selectively modulated by cadherin inhibitors and that cell–cell adhesion in peptide-treated cells can be mediated by other adhesion molecules such as VE-cadherin or CD31. While the mechanism underlying this selectivity is still unclear, it is in line with previous observations showing that during formation of AJ in endothelial cells, PG becomes associated with the membrane long after the incorporation of α- and β-catenins [40]. It is thus possible that survival signals in endothelial cells depend on the presence of mature, PG-containing AJs. The loss of cadherin and/or PG from the junctions, induced by N-Ac-*CHAVC*-NH_2, might thus perturb the integrity of the junctional plaque and consequently interfere with cadherin signaling. However, it should be borne in mind that while these changes in AJ structure were noted well before classical manifestations of apoptosis were apparent, one cannot exclude the possibility that they are, in fact, an early manifestation of apoptosis, rather than part of the apoptosis-inducing system.

As discussed above, the apparent integrity of AJs, in N-Ac-*CHAVC*-NH_2-treated cells, was preserved, although local intensity of N-cadherin decreased. Endothelial cells contain two major types of cadherins: N-cadherin, which contains the HAV motif, yet it is not strictly localized at AJ but is rather distributed all over the plasma membrane, and vascular endothelial cadherin (VE-cadherin) which does not contain the HAV motif and is restricted to AJs [41]. In the present study, we visualized cadherins using an antibody, which does not recognize VE-cadherin. Hence, the reduction we see in cadherin level (Fig. 6) due to N-Ac-*CHAVC*-NH_2 treatment can be attributed to reduction in N-cadherin only. Being insensitive to N-Ac-*CHAVC*-NH_2, VE-cadherin level may stay unchanged and together with other molecules such as PECAM-1 [42], may preserve the integrity of AJs.

Another possible explanation for the apoptotic effect of N-Ac-*CHAVC*-NH_2 on endothelial cells is indirectly suggested by the fact that the induced apoptosis was strictly cell density-dependent. Thus, sparsely plated cells, or even cells that just reached confluence were not affected by the peptide, while cells reaching high density (>5000 cells/mm^2 in the case of BCAP cells) were susceptible. This feature was demonstrated in two different ways. First, we noticed that early onset of apoptosis occurred at restricted foci or small "apoptotic islands" throughout the culture, corresponding to areas of relatively high cell density (Fig. 1C). In addition, it was shown that only endothelial cells plated at high densities were susceptible to N-Ac-*CHAVC*-

NH_2 (Fig. 4). This observation raises the possibility that cell–matrix and cell–cell adhesions are differentially involved in the generation of survival signals at low and high cell densities, respectively. Thus, in sparse or sub-confluent cells, there is substantial matrix adhesion, and consequently ECM-mediated signaling. Perturbation of cadherin-mediated signaling in such cells has small effect, if any, on cell survival. In dense cultures, on the other hand, cell–matrix focal adhesions become scarce [43–45] and cell survival may depend mainly on cadherin-mediated signaling. Perturbation of cadherin-mediated interactions may cause cell death. What is the nature of the specific signaling pathway, which is activated by cadherins in endothelial cells? One possibility, which was considered in this paper is that mature AJs with all their structural and signaling molecules are needed for cell survival. Another appealing possibility is that cadherins might be involved in maintaining cell survival by directly stimulating survival and signaling pathways in neighboring cells. Cadherins were shown to form supramolecular complexes with receptor tyrosine kinases, such as VEGFR2 [35] and FGFR [18,32]. Both receptors are key regulators of endothelial cell function and survival [17]. It was also shown that depletion of the corresponding growth factors from the growth medium can induce apoptosis in endothelial cells [46,47]. Furthermore, N-cadherin was shown to form a complex with FGFR1 and inhibits its internalization, thus potentiating its activation [32]. In epithelial MDCK cells, E-cadherin-mediated adhesion was shown to activate signaling pathways, which are involved in cell proliferation and anti-apoptotic events. These include the activation of MAPK by recruitment of the EGFR to cell–cell contacts and its ligand-independent activation [15]. It was also shown that formation of E-cadherin-mediated AJs leads to the recruitment of PI3-kinase followed by activation and translocation to the nucleus of Akt/PKB [16]. Taken together, these studies demonstrate that several important anti-apoptotic pathways might be triggered or regulated by cadherin-mediated interactions.

In this study, we have specifically addressed an attractive mechanism, which might account for the apoptotic effect of N-Ac-*CHAVC*-NH_2, namely the perturbation of N-cadherin-mediated activation of FGFR signaling. Beyond the published data on the cross talk between N-cadherin and FGFR, this notion is supported by the fact that addition of bFGF to the medium completely blocks the N-Ac-*CHAVC*-NH_2-induced formation of apoptotic foci. Moreover, we show here that the tyrosine phosphorylation level of a specific FGFR1 substrate, namely FRS2, is dramatically suppressed the inhibitory peptide. This is in line with previous studies that demonstrated that N-cadherin can directly interact with FGFR and activate it [18,31,32]. It was, however, noticed that the peptide was capable of blocking only cell-mediated activation of FGFR, but not stimulation by bFGF itself. The fact that inhibitory anti-N-cadherin antibodies did not exert an apoptotic effect like the N-Ac-*CHAVC*-NH_2 peptide (data not shown), suggests that it is not the direct perturbation of cadherin–cadherin interaction, but rather the modulation of other interactions, such as N-cadherin-FGFR1 binding, by the peptide, that push the cells to an apoptotic fate.

The exact mode of interaction of N-cadherin with FGFR1, and in particular the involvement of the HAV-containing domain in this interaction is rather controversial. Williams et al. [10] showed that the HAV motif is involved in FGFR activation. While Suyama et al. [32] claim that the interaction involves other regions of the cadherin extracellular domain. Our data support the notion that the HAV-motif is important for the N-cadherin-FGFR signaling, yet it remains to be determined whether the HAV-motifs of either FGFR or cadherin are directly involved in the molecular interaction between the two.

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