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Human NRAGE disrupts E-cadherin/β-catenin regulated homotypic cell–cell adhesion

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Abstract

Human NRAGE, a neurotrophin receptor p75 interaction MAGE homologue, confers NGF-dependent apoptosis of neuronal cells by inducing caspase activation through the JNK-c-jun-dependent pathway and arrests cell growth through the p53-dependent pathway. Our findings showed that human NRAGE could significantly alter the cell skeleton and inhibit homotypic cell–cell adhesion in U2OS cells. With further experiments, we revealed that human NRAGE disrupts colocalization of the E-cadherin/β-catenin complex and translocates β-catenin from the cell membrane into the cytoplasm and nucleus. Synchronously, NRAGE also decreases the total protein level of β-catenin, especially when NRAGE expresses for a long time. More importantly, knock down of NRAGE by RNA interference in PANC-1 cell significantly reinforces E-cadherin/β-catenin homotypic cell adhesion. The data demonstrate the importance of human NRAGE in homotypic cell-to-cell adhesion and illuminate the mechanism of human NRAGE in the process of inhibition of cell adhesion, which suggests that human NRGAE plays a potential negative role in cancer metastasis.

Keywords: NRAGE; E-cadherin; β-Catenin; Cell adhesion

NRAGE (Dlxin-1, MAGE-D1), a nuerotrophin receptor p75 interacting MAGE homologue, belongs to the MAGE (melanoma antigen) protein family. There are over 25 MAGE proteins in humans, but the normal physiological function of this family remains obscure. Recent studies show that NRAGE can bind with the nerve growth factor receptor p75NTR, block cell cycle progression, and promote p75NTR-mediated apoptosis [1,2]. In addition, NRAGE has been shown to associate with the homo-domain proteins Msx2 and Dlx5, and regulate the transcriptional function of Dlx5 [3,4]. In a previous study, we demonstrated that human NRAGE could inhibit hepatocellular carcinoma cell proliferation and arrest cell cycle through a p53-dependent pathway. Based on our data and previous reports [5], we hypothesized that human NRAGE would be a very important mediator of apoptosis and cell proliferation. By accident, we found that human NRAGE could significantly inhibit adhesion of U2OS, a human osteosarcoma cell, which indicates that human NRAGE may be involved in cell adhesion.

Cell adhesion is a key physiological event, tightly coupled to four other major cellular processes: proliferation, migration, differentiation, and death [6,7]. E-cadherin is a member of the transmembrane glycoprotein cadherin family that mediates homotypic calcium-dependent cell–cell adhesion to ensure the maintenance of a normal phenotype of epithelial cells [8,9]. The specific cytoplasmic domain of E-cadherin interacts with catenin molecules, which establishes an intracellular linkage with the actin cytoskeleton. The role of E-cadherin in metastasis has become topical in the past few years due to its apparent promise as a prognostic indicator, as its loss or reduction of expression correlates with enhanced aggressiveness and dedifferentiation of many carcinomas [10–13].

In this paper, we explore the effect of human NRAGE on cell adhesion for the first time. Our results reveal that

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human NRAGE can inhibit U2OS cell-to-cell adhesion by disrupting the E-cadherin/β-catenin complex. The data not only demonstrate that human NRAGE induces a reduction of homotypic cell adhesion but also illuminate the primary mechanism of human NRAGE in the cell adhesion process.

Materials and methods

Cell culture and adenovirus construction and infection. The 293A, COS7, and PANC-1 cell lines were cultured in Dulbecco's modified Eagle's medium. U2OS, a human osteosarcoma cell line, was cultured in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum at 37 °C in humidified air with 5% CO_2. Cells were grown to approximately 80% confluence prior to infection with adenovirus carrying human NRAGE (Ad-NRG) or AdGFP for 24 h. Infection efficiency was monitored with the expression of GFP protein. The construction of Ad-NRG was reported previously [5]. The small RNA interference adenovirus targeting NRAGE (Ad-NRG/RNAi) was constructed by inserting a 19-nt fragment into the pSupper-H1 vector under the control of the H1 promoter. The sequence of the inserting fragment is GATGAAAGTGCTGAGATTC.

Immunofluorescence analysis. U2OS cells were seeded onto 18 mm coverslips coated with 2 μg/ml fibronectin and infected with adenovirus for 24 h. Cells were fixed with 4% formaldehyde for 15 min at room temperature, washed three times with phosphate-buffered saline (PBS), and permeabilized with 0.2% Triton X-100 at room temperature. They were stained with polyclonal anti-β-catenin (1:100) for 1 h at room temperature. After washing three times with PBS, the bound primary antibody was detected using rhodamine-conjugated anti-rabbit IgG (1:500) antibodies. For analysis of the cytoskeleton, cells were permeabilized with 0.2% Triton X-100 and incubated for 1 h with 0.5 mM rhodamine-conjugated phalloidin (1:50). Images of stained cells were captured using an immunofluorescence microscope and a CCD camera. The polyclonal anti-β-catenin (H-102), polyclonal anti-E-cadherin (H-108), and rhodamine-conjugated anti-mouse IgG were purchased from Santa Cruz. Rhodamine–phalloidin and fibronectin were purchased from Sigma.

Dissociation assay. Cells were seeded equally and grown to confluence. Cells were trypsinized in 0.1% trypsin containing either 1 mM EDTA (TE) or 1 mM $CaCl_2$ (TC) and incubated at 37 °C for 30 min. Cells were pipetted five times gently in 10 ml PBS and counted in a Coulter counter. Each sample was done in triplicate. The degree of adhesion was expressed as the ratio of TC:TE [14].

Immunoprecipitation and Western blotting. Cells were washed twice with PBS and then lysed by using ice-cold 1% NP-40 buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycol, and 0.5 mM DTT). Immunoprecipitations were carried out by incubating cell lysates with appropriate antibodies for 2 h at 4 °C, followed by incubation overnight with protein G–agarose. After washing, immunocomplexes were subjected to SDS–PAGE. For subcellular fractionation, cells were scraped into 1 ml PBS containing 1 mM PMSF, 20 μg/ml leupeptin, and 20 μg/ml aprotinin. Then, cells were centrifuged at 3000 rpm for 2 min at 4 °C. Pellet samples were incubated in low infiltration buffer (25 mM Tris–HCl, pH 7.4; 1 mM EDTA, and 1 mM dithiothreitol) for 20 min with vortexing fiercely and then centrifuged at 12,000 rpm for 2 min at 4 °C. The supernatant represented the soluble fraction. The pellet samples washed twice by PBS represented the insoluble fraction. Western blotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody and the Roche ECL system for detection. Anti-cMyc (9E-10) was purchased from Santa Cruz.

Result

Human NRAGE decreases homotypic cell adhesion

Previous studies suggest that NRAGE is involved in cell cycle arrest and apoptotic response to nerve growth factor (NGF) binding to p75NTR in neural cells [1,2]. Interestingly, when we infected U2OS cells with Ad-NRAGE and AdGFP for 48 h, we found that overexpression of human NRAGE causes the cells to turn round and detach from the layer (Fig. 1A), and significantly arrest cell cycle but this does not lead to cell apoptosis (Fig. 1C). Using rhodamine-conjugated phalloidin, we found that actin filament shrunk considerably when the cells were infected with Ad-NRAGE (Fig. 1B). These data indicate that human NRAGE alters the cell skeleton and decreases the ability of cell adhesion. To further investigate whether human NRAGE affects the homotypic adhesion characteristics of cells, dissociation assays were performed. Cadherins are inactive on cell removal of calcium during trypsinization of cells. When calcium is added to the trypsin solution, adhesion molecules requiring calcium for function are preserved [14]. As shown in Fig. 2, in U2OS cell and COS7 cell, calcium-dependent homotypic adhesion assays demonstrated that human NRAGE

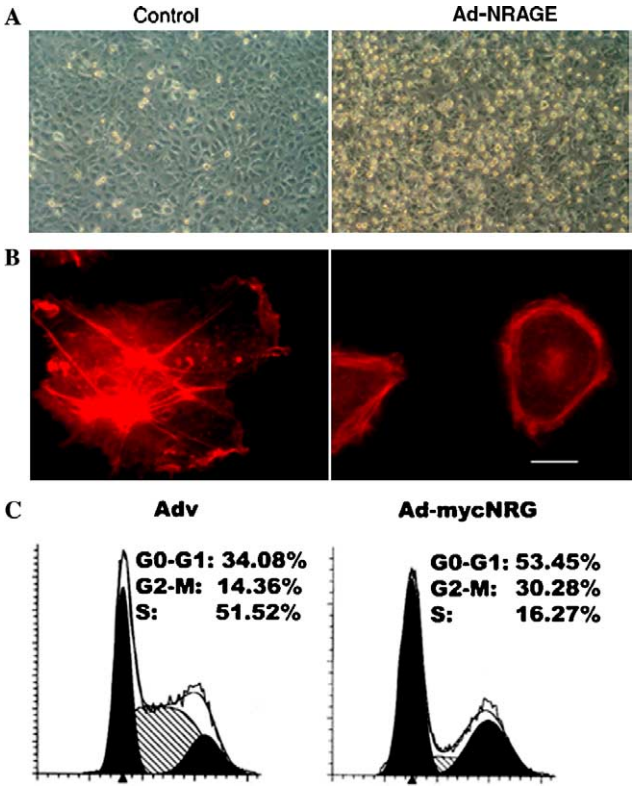

Fig. 1. Cytoskeletal changes upon expression of human NRAGE. (A) U2OS cells infected with AdGFP (left) or Ad-NRAGE (right) 48 h. The cells turned round and detached from the layer, which indicated that the cell adhesion was inhibited by NRAGE. (B) U2OS cells were dyed with rhodamine-conjugated phalloidin. The elongation of actin was altered sharply by human NRAGE, which showed that the cytoskeleton was changed by human NRAGE. Scale bar, 20 μm. (C) U2OS cells were infected with adenovirus carrying human NRAGE or adenovirus vector. Forty-eight hours later, cells were collected and fixed with cold ethanol and then resuspended in 1 ml solution containing 50 mg/ml RNase A and 50 mg/ml propidium iodide. At least 15,000 cells were collected at each treatment by FACScan and analyzed with the CellQuest program (Becton–Dickinson). NRAGE arrested the cell cycle mainly at G1/S and G2/M, whereas no apoptosis was observed.

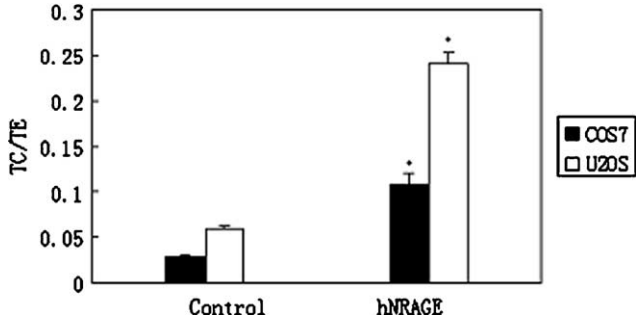

Fig. 2. Human NRAGE inhibits cell–cell adhesion. Calcium-dependent dissociation assay in U2OS and COS7 cells demonstrates that human NRAGE (Ad-NRAGE infection) reduces intercellular adhesion by up to fourfold over control (Ad-GFP infection). Results are expressed as NTC: NTE, comparing the number of particles in TE-treated cells, virtually a single-cell suspension, to the number of particles present in TC-treated samples. Each assay was performed twice in triplicate. Results represent mean ± SD ($^{*}P < 0.01$).

displayed up to a fourfold reduction in homotypic adhesion compared with control. Altogether, these data show that overexpression of human NRAGE could inhibit homotypic cell–cell adhesion.

Human NRAGE affects subcellular localization and stability of β-catenin

Cadherin adhesion molecules are major determinants of tissue patterning which function in cooperation with the actin cytoskeleton. Therefore, we explored the possibility that the reduction in cell-to-cell adhesion by NRAGE is a result of alteration in the E-cadherin/β-catenin complex. By fluorescence microscopy, we found that, in U2OS cells overexpressing human NRAGE, β-catenin was translocated primarily into the cytoplasmic region and partially into the nucleus, while very little was detected at the cell surface (Figs. 3A and B). However, the distribution of E-cadherin remained intact in the cell membrane (data not shown). To confirm the changes of β-catenin distribution, we examined the β-catenin level in soluble and insoluble fractions. As shown in Fig. 3C, β-catenin in U2OS cells expressing NRAGE increased in the soluble fraction, and decreased sharply in the insoluble fraction (cytoskeleton matrix), which was in accordance with the results of relocalization of β-catenin by immunofluorescence. Interestingly, NRAGE also decreases the total protein level of β-catenin, especially when NRAGE expresses for a long time (Fig. 3D). Our present data indicate that overexpression of human NRAGE can alter colocalization of E-cadherin/β-catenin as well as β-catenin stability, so as to inhibit cell adhesion. In addition, human NRAGE may be partly responsible for the translocation of β-catenin from the cell membrane to the nucleus.

Human NRAGE disrupts the association of E-cadherin and β-catenin

Previous studies suggest that many factors appear to exert their effects through actions on β-catenin, either by stabilizing its bond with E-cadherin, promoting cell adhesion, or by dissolving its bonds, resulting in a break up of the E-cadherin/catenin complex, which causes downregulation of cell adhesion [15,16]. To investigate whether the human NRAGE disrupts the binding of E-cadherin and β-catenin, U2OS cell extracts infected with the Ad-NRAGE or AdGFP for 24 h were immunoprecipitated by the β-catenin antibody, followed by Western blotting with the antibody anti-E-cadherin. As shown in Fig. 4A, β-catenin was coprecipitated with E-cadherin in cells infected with AdGFP (left panel), but very little β-catenin was associated with E-cadherin in NRAGE overexpressing cells, which is consistent

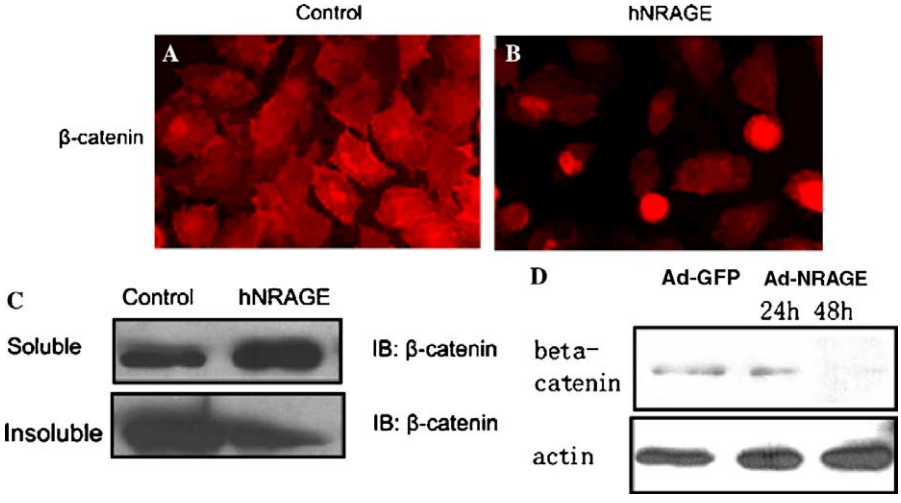

Fig. 3. Subcellular localization of β-catenin was changed upon expression of NRAGE. (A,B) U2OS cells infected with Ad-NRAGE or AdGFP for 24 h were stained by β-catenin antibody. In NRAGE overexpressing cells, β-catenin dissociated from the cell membrane and redistributed in cytoplasm and was partially translocated into the nucleus. (C) Western blotting showed that human NRAGE increases the β-catenin level in soluble fraction in comparison with a decrease sharply in the insoluble fraction. (D) U2OS cells infected with Ad-NRAGE or AdGFP for indicated time. Whole cell lysate was subjected to Western blotting of β-catenin and actin.

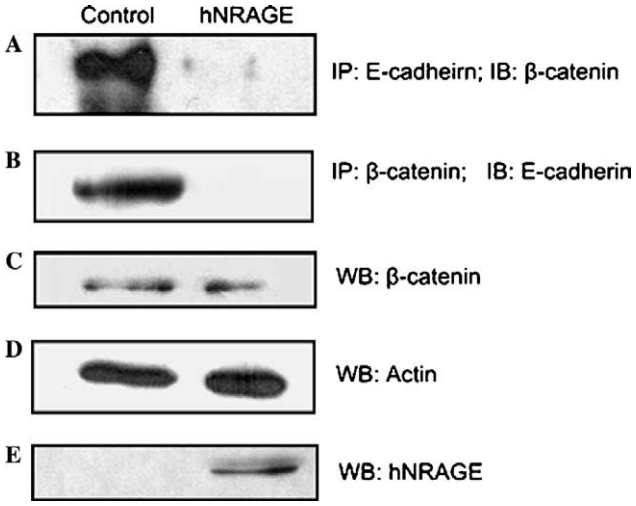

Fig. 4. Disruption of E-cadherin/β-catenin complex by human NRAGE. (A) Immunoprecipitation of U2OS cell extracts with E-cadherin, Western blotting of β-catenin showed the disruption of E-cadherin/β-catenin complex by human NRAGE. (B) Immunoprecipitation of U2OS cell extracts with β-catenin, Western blotting of E-cadherin confirmed the result. (C) Western blotting of total β-catenin showed that the endogenous expression of β-catenin does not decrease significantly by human NRAGE. (D) Western blotting of actin as control. (E) Additional expression of human NRAGE was blotted with c-myc antibody.

with the result of an additional experiment after involving immunoprecipitation of U2OS cell extracts with the E-cadherin antibody and Western blotted with β-catenin (Fig. 4B). These results are also consistent with the observations shown in Fig. 3 that E-cadherin and β-catenin appear to be minimally colocalized in the cellular membrane of U2OS cells overexpressed NRAGE. Western blotting of the cell lysate indicated that the total level of β-catenin was still stable after Ad-NRAGE infection for 24 h, eliminating the possibility of altered catenin levels causing lower association with cadherin. These data suggest that the human NRAGE is able to disrupt the cadherin/catenin complex and that the disruption of this complex is possibly responsible for the decrease in calcium-dependent adhesion.

Knock down of NRAGE by RNA interference reinforces E-cadherin/β-catenin homotypic cell adhesion

To further address the importance of NRAGE in homotypic cell adhesion. We tested whether silencing endogenous NRAGE by RNAi enhances cell–cell adhesion. We did not find significant alteration in cell–cell adhesion in U2OS and COS7 cells infected with Ad-NRG/RNAi, probably because of the low level of NRAGE in these cells (in fact, we cannot detect endogenous NRAGE by total cell lysate Western blotting in U2OS and COS7 cells, data not shown). Fortunately, we found that PANC-1, a human pancreatic cancer cell line, expresses considerable NRAGE, which can be silenced efficiently by Ad-NRG/RNAi (inserted). Consistent with the overexpression experiments, knock down of endogenous of NRAGE in PANC-1 cell clearly enhances homotypic cell adhesion (Fig. 5).

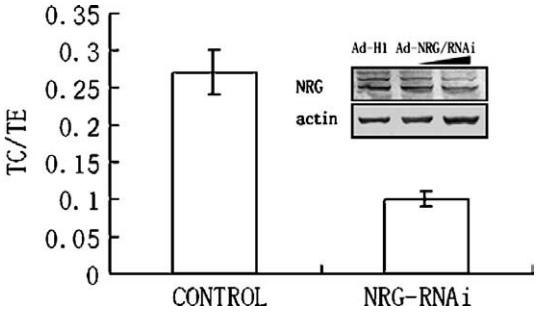

Fig. 5. Silencing of NRAGE by RNA interference reinforces E-cadherin/β-catenin homotypic cell adhesion. Calcium-dependent dissociation assay in PANC-1 cell demonstrates that silencing of NRAGE by RNA interference reinforces E-cadherin/β-catenin homotypic cell adhesion. Inserted panel, PANC-1 cell was infected with an indicated amount of Ad-NRG/RNAi or Ad-H1 as control. Thirty hours later, total cell lysed was subject to NRAGE detection by Western blotting. The same membrane was reblotted for actin as loading control.

Discussion

In this study, we explored the involvement of human NRAGE in homotypic cell-to-cell adhesion and β-catenin stability for the first time. We propose that cell-to-cell adhesion in U2OS is inhibited significantly by human NRAGE through disruption the E-cadherin/β-catenin complex. E-cadherin is a member of the cadherin family that mediates calcium-dependent cell–cell adhesion for the maintenance of a normal phenotype of epithelial cells [8,9].

The full adhesive function of E-cadherin depends on the integrity of the entire cadherin–catenin–actin network. Our data show that the network of E-cadherin–catenin–actin is altered significantly and the actin filament is shrunk sharply when overexpressing human NRAGE, which causes cells to turn round and detach from the layer. We further observed that the colocalization of E-cadherin/β-catenin complex is disrupted in cells expressing human NRAGE. Consequently, β-catenin dissociates from E-cadherin and appears to be translocated into the cytoplasmic region and partially into the nucleus. Although the total amount of β-catenin remained intact after 24 h infection by Ad-NRAGE (in fact, we found that the total amount of β-catenin was decreased after 48 h infection by Ad-NRAGE, Fig. 3D), the proportion of β-catenin present in the soluble fraction increased and that of β-catenin in the cytoskeletal fraction decreased after being infected with Ad-NRAGE, which confirms the redistribution of β-catenin induced by human NRAGE. Therefore, we propose that human NRAGE induces a redistribution of the E-cadherin/β-catenin complex and possibly disrupts the association between E-cadherin and β-catenin.

β-Catenin is a central factor, which functions both in cell adhesion as a link between cell surface cadherin molecules and the actin, and also as a signaling molecule for gene transcription in the Wnt signaling pathway. Upon stimulation by Wnt, β-catenin is translocated into the nucleus, where it binds to LEF/TCF transcription factor

that controls the transcription of genes involved in cell proliferation [17]. Wnt signaling acts as a positive regulator by inhibiting β-catenin degradation, which stabilizes β-catenin, and causes its accumulation. E-cadherin may act as a negative regulator of β-catenin signaling as it binds with β-catenin at the cell surface. In our experiments, NRAGE disrupts the E-cadherin/β-catenin interaction and translocates β-catenin from the cell membrane into the cytoplasm as well as partially to nucleus. Therefore, we hypothesize that human NRAGE may be involved in the Wnt pathway.

Another question is how NRAGE disrupts the E-cadherin/β-catenin complex. Because we do not find any alteration of β-catenin mRNA expression (data not shown), we suppose that β-catenin undergoes a certain kind of modification, which not only blocks its association with E-cadherin, but also induces its degradation. The structural and functional integrity of the cadherin/catenin complex is regulated by phosphorylation [18]. Serine/threonine phosphorylation facilitates degradation of β-catenin, but enhances the stability of the cadherin/catenin complex [19]. Actually, we did not observe any effect of NRAGE overexpression on the serine/threonine phosphorylation level of β-catenin (data not shown). Tyrosine phosphorylation of β-catenin disrupts the binding of β-catenin to cadherin, but increases β-catenin stability [20,21]. So, further experiments should be applied for identifying the type of β-catenin modification induced by NRAGE.

In conclusion, these data suggest the human NRAGE may be important in homotypic cell-to-cell adhesion. This process is linked to the disruption of the catenin/cadherin complex.

Acknowledgments

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