Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 247–251
BBRC
www.elsevier.com/locate/ybbrc

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

Bin Xue, Chuanjun Wen *, Yihuan Shi, Donghong Zhao, Chaojun Li

*Jiangsu Key Laboratory for Molecular and Medical Biotechnology, Nanjing Normal University, Nanjing 210097, PR China*

Received 8 August 2005
Available online 19 August 2005

**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

* Corresponding author. Fax: +86 25 83598812.
*E-mail address:* wenchuanjun@njnu.edu.cn (C. Wen).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.069

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

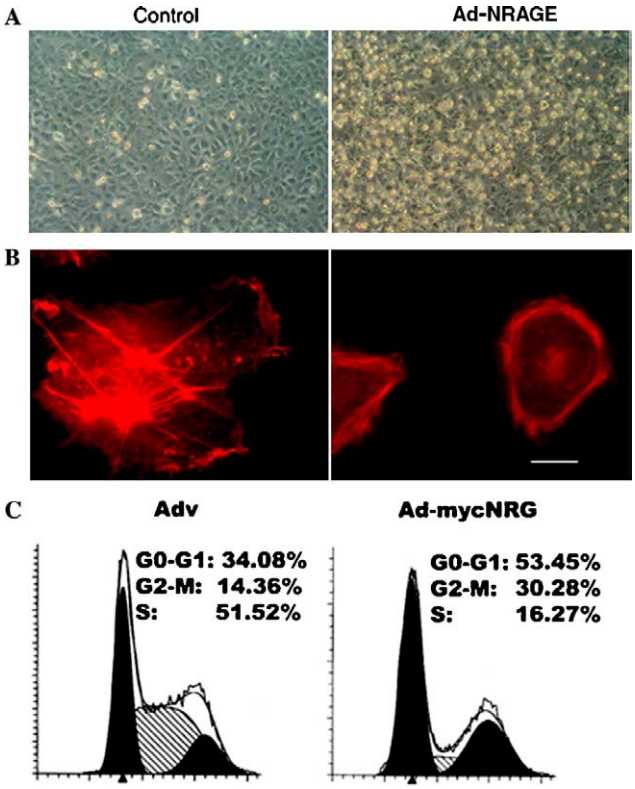


Fig. 1. Cytoskeletal changes upon expression of human NRAGE. (A) U2OS cells infected with AdGFP (left) or Ad-NRAGE (right) 48 h. The cells turned round and detached from the layer, which indicated that the cell adhesion was inhibited by NRAGE. (B) U2OS cells were dyed with rhodamine-conjugated phalloidin. 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.