Establishment and Application of Engineered NIH 3T3 Cell Line with Stable Human RAGE Expression

Xinyi Xiao[1]   Hui Chen[1]   Pameila Paerhati[1]   Meiqi Zhou[1]   Zhuoyi Yang[1]   Siyi Bai[1]   Yunsheng Yuan[1]

[1] Engineering Research Center of Cell & Therapeutic Antibody, Shanghai Jiao Tong University College of Pharmacy, Ministry of Education, Shanghai, People's Republic of China

Address for correspondence Yunsheng Yuan, PhD, Engineering Research Center of Cell & Therapeutic Antibody, Shanghai Jiao Tong University College of Pharmacy, Ministry of Education, 800 Dongchuan Road, Shanghai 200240, People's Republic of China (e-mail: yunsheng@sjtu.edu.cn).

Abstract

Aim   NIH3T3 cell line with expression of human receptor for advanced glycation end-products (hRAGE) transduced with lentivirus vectors was used to analyze affinity, biological activity, and/or molecular mechanisms of molecules targeting the hRAGE pathway.

Method   The DNA fragment coding for hRAGE gene was integrated into the genome of NIH3T3 cells using lentivirus transduction. Cells expressing hRAGE were selected with puromycin, and the level of hRAGE expression was analyzed by Western blot. To establish a stable cell line, colonies of hRAGE-expressing cells were generated, and the level of RAGE expression in each engineered cell line was analyzed within 20 generations. Flow cytometry assay was used to verify affinity of anti-hRAGE antibody binding to hRAGE on the surface of engineered cells. The engineered NIH3T3 cell line was applied to assess effects of anti-hRAGE blocking antibody on amyloid β-induced cells apoptosis by CCK-8 assay.

Results   The engineered NIH3T3 cell line (hRAGE-NIH3T3) could stably express human RAGE. Commercial anti-RAGE polyclonal antibody could recognize and bind to human RAGE on the surface of hRAGE-NIH3T3 but not original NIH3T3 cells. In addition, hRAGE-NIH3T3 was more sensitive to RAGE pathway-dependent stimulation. Our data show that the hRAGE-NIH3T3 cell line established is an excellent tool in the study of RAGE-targeting molecules based on the cellular level, biological function, and RAGE-mediated molecular mechanisms.

Keywords
► RAGE
► engineered cell line
► lentiviral
► transduction
► flow cytometry

Introduction

Receptor for advanced glycation end-products (RAGE) is one of the members of the immunoglobulin superfamily and a multiligand cell surface receptor with structural homology to other immunoglobulin-like receptors.[1] RAGE consists of an extracellular domain, a transmembrane domain, and a cytoplasmic domain.[2] The extracellular domain of RAGE includes a variable domain (V1) and two constant domains (C1 and C2), which are responsible for binding by different ligands.[3] V1 and C1 domains are generally regarded as the main interaction motifs with various ligands. Although the cytoplasmic tail is very short, it is critical for intracellular signal transduction.[4] RAGE has a variety of ligands, such as advanced glycation end-products (AGEs),[5] high mobility group protein B1 (HMGB1),[6] amyloid β protein (Aβ),[7] and S100 family proteins.[8] All these ligands interact with the extracellular domain of RAGE and activate downstream signaling pathways, including activation of extracellular regulated protein kinases (ERK 1/2) and phosphatidylinositol 3-kinase (PI3K).[9–13] Activated RAGE promotes the production of reactive oxygen species and proinflammatory cytokines, and it also inhibits the expression of tumor suppressor genes.[14,15] Overactivation of RAGE is associated with many diseases, including diabetes complications, atherosclerosis, neurodegenerative diseases, liver fibrosis, and tumors.[9,16–20]

Blockade of ligand/RAGE receptor interaction is a potential strategy for the treatment of these diseases, and RAGE has become a new target for drug discovery.

Several cell lines have been used in studies of RAGE ligand activities or biological function in vitro, such as neuroblastoma cells (SH-SY5Y), breast cancer cells (MCF-7), and human embryonic kidney cells (HEK293). However, RAGE expression is low in these cell lines, which were not suitable for investigation of the affinity of RAGE ligands or anti-RAGE antibodies.[21] The transgenic cell lines play a critical role in processes of new drug development, such as drug screening, activity assay, and expression of recombinant protein or antibody, etc. Establishment of an engineered cell line for expression of human RAGE (hRAGE) was necessary to support new drug development for targeting RAGE. Mouse embryonic fibroblast NIH3T3 was widely used in cellular signal transduction, protein–protein interaction, and molecular activity assays because it was easy to maintain, transfect, and grow.[22–24] In this study, NIH3T3 cells were used to generate transgenic cell lines for stable expression of hRAGE.

Materials and Methods

Instrument
A super clean bench (Suzhou Purification Equipment Co., Ltd., China), a cell counter (Ruiyun Biotechnology Co., Ltd.), a chemiluminescence imager (Tanon, China), a multifunctional microplate reader (Tecan, Switzerland), a flow cytometer (Beckman Coulter, United States), and protein electrophoresis gel equipment (Bio-Rad, United States).

Materials
NIH3T3 and HEK293T were purchased from Chinese Academy of Sciences Cell Bank (ACSCB), anti-hRAGE rabbit polyclonal antibody and rabbit immunoglobulin G (IgG) from Shanghai Kaijing Biotechnology Company, China, pLVX-puro-human-RAGE-HA from Universal Biological System Co., Ltd., China, Aβ1–42 from Chutai Biotechnology Company, China, mouse anti-HA tag antibody from Proteintech, United States, mouse anti-GAPDH antibody from Proteintech, United States, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) and HRP-conjugated rabbit anti-mouse IgG (H+L) from Jackson, United States; CCK8 Kit from Dojindo, Japan, and substrate of enhanced chemiluminescence (ECL) from Merck Millipore, United States.

Cell Culture
All cells were maintained in high-glucose DMEM (Gibco, United States) supplemented with 10% fetal bovine serum (FBS; Gibco) and incubated at 37 °C with 5% CO_2 in a humidified cell incubator. For passaging, cells were dissociated using 0.25% trypsin-EDTA at 80 to 90% confluence and replanted at 1:3 dilutions.

Package of Lentivirus and Transduction of Cells
The procedure of lentivirus packaging and cell transducing were performed as previously described.[25] The mixture of pLVX-puro-human-RAGE-HA plasmid (►**Fig. 1**), pVSV-G,
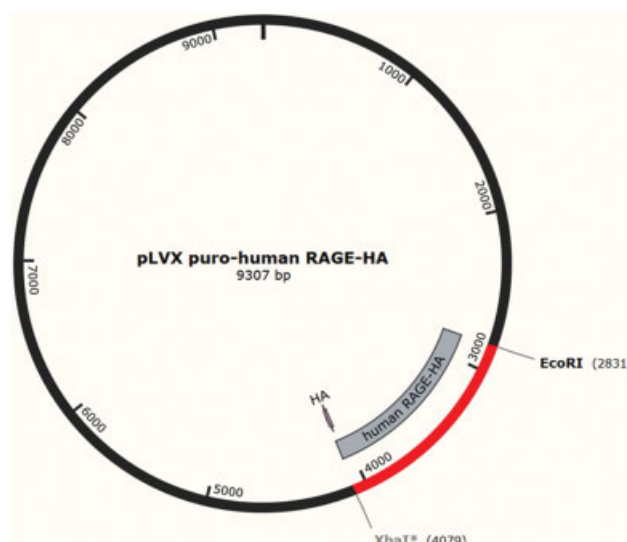

Fig. 1 The vector of pLVX-puro-human-RAGE-HA.

pRev, pTat, and pGag-pol plasmids was transfected into 293T cells by lipofectamine 2000. Lentiviral particles were harvested after 2 days of transfection. The NIH3T3 cells were seeded to a six-well plate and incubated overnight. The cells were fed with fresh medium containing lentiviral particles and polybrene whose final concentration was 10 μg/mL. The transduced cells were selected with 10 μmol/L puromycin.

Western Blotting
hRAGE-NIH3T3 and NIH3T3 cells were lysed in protein lysis buffer and β-mercaptoethanol, followed by electrophoretic separation on a 10% tris-polyacrylamide gel, and then transferred onto a polyvinylidene fluoride (PVDF) membrane at 200 mA for 120 minutes. The membrane was blocked with 5% nonfat dry milk in TBST for 1 hour. Membranes were incubated in primary antibody (mouse anti-HA antibody, mouse anti-GAPDH antibody) at 4 °C overnight and subsequently with the corresponding horseradish peroxidase-labeled secondary antibody diluted in 5% nonfat dry milk in TBST for 45 minutes at room temperature. The signal of blot was detected by ECL substrate and captured by a Tanon Image system.

Flow Cytometry Assay
hRAGE-NIH3T3 and NIH3T3 cells were harvested and suspended by DMEM with 10% FBS. In total, 2.5×10^5 cells for each sample were centrifuged and washed three times using FACS (PBS, pH 7.2, 2% FBS). Various concentrations of anti-hRAGE antibody (1, 2, and 5 μg) were added to each sample, using PBS as the control. All samples were incubated for 30 minutes at 4°C and then washed three times with FACS. FITC-conjugated goat anti-rabbit IgG was added to the samples which were then incubated at 4°C in the dark. After 30 minutes, cells were washed, suspended, and analyzed by a flow cytometer.

CCK8 Cell Viability Assay
hRAGE-NIH3T3 or NIH3T3 cells were seeded in 96-well plates (5,000 cells/well) and incubated overnight in 100 μL DMEM

with 10% FBS. Then, the medium was changed and fed with DMEM with 1% FBS for starvation. After 24 hours of starvation, the cells were fed a complete medium (DMEM + 10% FBS) and treated with Aβ (8 μmol/L) with different concentrations of anti-hRAGE antibody (3.125, 6.25, 12.50, 25.00, and 50.00 μg/mL) or PBS as control. After 48 hours of treatment, cell viability was assessed by a CCK8 kit according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were performed using Prism 7.0 (GraphPad Software, San Diego, California, United States). Significant differences ($p < 0.05$) were calculated by one-way analysis of variance followed by Tukey's multiple-comparison test.

Results

Selection and Identification of hRAGE-NIH3T3 Cell Colonies

Lentivirus is a useful tool in gene delivery and has high efficiency to integrate exogenous gene in the genome of host cells. In this study, pLVX-Puro lentivirus vector was used as a backbone and the insert was a hRAGE-fused HA tag DNA fragment. The expected molecular weight of recombinant hRAGE-HA expressed by NIH3T3 should be approximately 43 kDa and it could be recognized by the anti-HA tag antibody in Western blotting. Interestingly, there were two bands on the membrane detected by the anti-HA antibody with molecular weights of approximately 45 and 55 kDa, respectively (►**Fig. 2**). The migration pattern of hRAGE-HA was in accordance with literature data.[26]

Colony selection played a critical role in the construction of transgenic cells for stable expression of exogenous genes because protein levels of every cell are distinctive in the cell pool. Cell clones expressing the highest level of hRAGE-HA could be chosen and used in subsequent research studies. We isolated 12 hRAGE cell clones because of their growth capacity in culture. The selected clones were plated on six-well plates and samples of the cells were collected for Western blotting analysis. Our data showed that three clones have higher hRAGE levels than others (►**Fig. 3**). These three cell clones, named RAGE-1, RAGE-7, and RAGE-10, were
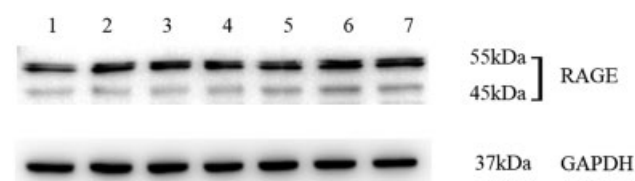
Fig. 3 Expression of hRAGE-HA in different colony samples from colony 1 to 12. The anti-HA tag antibody or anti-GAPDH antibody was used to probe the membrane.

selected for cell-line establishment, in vitro antibody binding, and ligand bioactivity assays.

hRAGE-NIH3T3 Cell Stability Assay

The exogenous genes in host cells could be inactive due to genome epigenetic modifications (reference: doi 10.1074/jbc. M109.092007). To investigate the stability of hRAGE-HA integrating in NIH3T3 cells, hRAGE-HA expression in selected clones was analyzed from the 1st to the 20th generation. We found that the levels of hRAGE-HA were not significantly different during 20 generation passages. This indicated that the hRAGE-HA was stably expressed in hRAGE-NIH3T3 cells (►**Fig. 4**).

Anti-hRAGE Antibody Recognition of hRAGE-HA in Engineered Cells

Recently, over 30 therapeutic antibodies have been used in the clinic and target receptor-overproducing cell lines were involved in all stages of therapeutic antibody development, for example, cell-based binding and biological function assays.[27,28] Anti-hRAGE rabbit polyclonal antibody was used to verify whether hRAGE-HA localized on the cell surface could be specifically recognized by anti-hRAGE antibody. Based on the assay of FACS, we found that signals from both anti-hRAGE antibody and control IgG were very weak on NIH3T3 cells. The data showed that anti-hRAGE antibody had very low affinity in binding to mouse RAGE on the surface of NIH3T3 cells (►**Fig. 5A**). Meanwhile, in hRAGE-NIH3T3 cells, signals of anti-hRAGE were stronger than that of control IgG (►**Fig. 5B**).

Anti-hRAGE Antibody Protection of hRAGE-NIH3T3 Cells against Aβ-Induced Apoptosis

It is well known that therapeutic antibody works as a receptor inhibitor depending on distribution of ligands bound to receptor. We investigated hRAGE-HA biological function induced by Aβ, which is one of the RAGE ligands and could induce cell apoptosis.[29] To confirm the availability
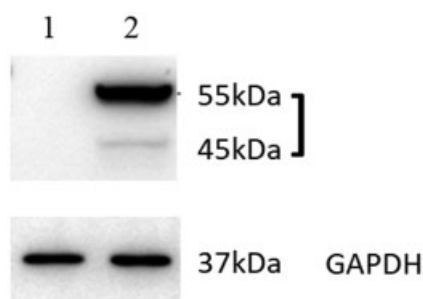

Fig. 2 hRAGE-HA expression in hRAGE-HA-transduced NIH3T3 cell lysate. The cell lysate was analyzed with Western blotting. The anti-HA tag antibody or anti-GAPDH antibody was used to probe the membrane. Lane 1: NIH3T3 cell lysate; lane 2: hRAGE-HA-transduced NIH3T3 cell lysate.
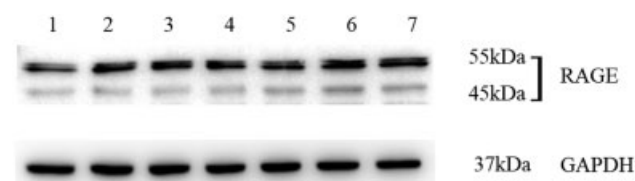

Fig. 4 The hRAGE-HA stably expresses through 20 generations in hRAGE-NIH3T3. The samples were sequentially collected at generations 1, 4, 7, 10, 13, 16, and 20. These sample were loaded on SDS-PAGE from lane 1 to lane 7, respectively. The anti-HA tag antibody or anti-GAPDH antibody was used to probe the membrane in Western blotting assay.
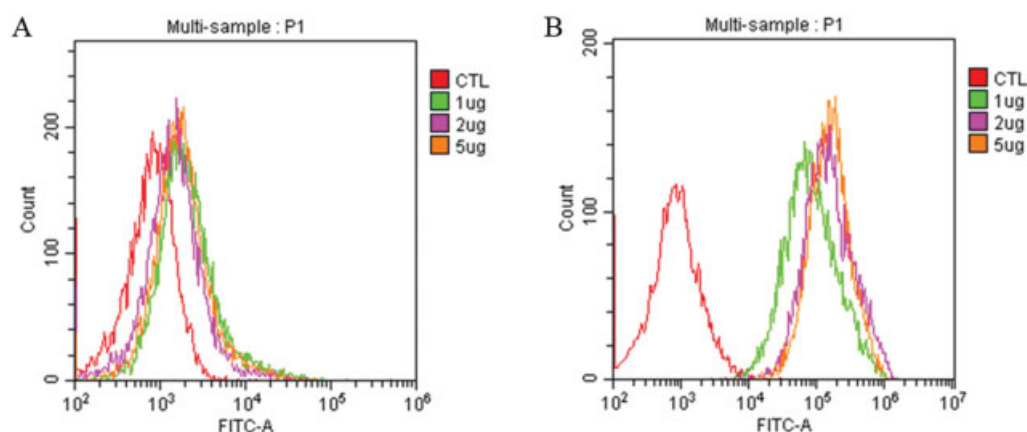
Fig. 5 Anti-hRAGE antibody had high affinity binding with hRAGE-NIH3T3 cells (A: NIH3T3 cells, B: hRAGE-NIH3T3 cells).
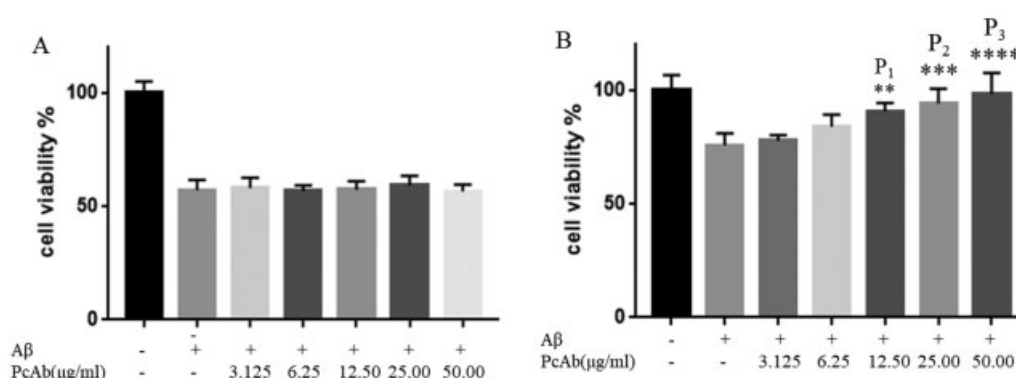

Fig. 6 Anti-hRAGE antibody improvement of hRAGE-NIH3T3 cell survival following Aβ treatment. The anti-hRAGE rabbit polyclonal antibody (PcAb) and amyloid β (Aβ) were used to treat NIH3T3 cells (**A**) or hRAGE-NIH3T3 cells (**B**). Data of cell viability were normalized by the control group. Experiments were repeated three times: p_1 value $= 0.0017$, p_2 value $= 0.0001$, p_3 value $= 0.0001$.

of hRAGE–NIH3T3 cells for anti-RAGE molecule screening and functional study, NIH3T3 cells or hRAGE-NIH3T3 cells were treated with Aβ or Aβ/anti-hRAGE antibodies for 48 hours. The data showed that anti-hRAGE antibodies significantly improved hRAGE-NIH3T3 survival under Aβ stimulation, but the antibody had no effects on protecting NIH3T3 cells (►**Fig. 6**). hRAGE-HA in hRAGE-NIH3T3 cells also had biological function and the engineered cell line could be used to access biological activities of anti-hRAGE antibodies or ligands in vitro.

Discussion

Pulmonary fibrosis, diabetes complications, and other diseases are closely associated with overactivation of the RAGE pathway. RAGE inhibitors have the potential to treat these diseases.[30,31] We have analyzed RAGE expression in several human cancer cell lines by Western blotting and FACS. The protein level of RAGE in those tumor cells was much lower than that in hRAGE-NIH3T3 cells (data not shown). The hRAGE-NIH3T3 cell line should have good sensitivity and high efficiency for screening antibodies or interaction partners of hRAGE. We employed a lentivirus gene delivery technique to construct engineered cell lines because the lentivirus system has high safety and efficiency in transfer-

ring exogenous genes into host cells.[32] It is also used to produce chimeric antigen T-cells for clinical treatment.[33]

In summary, the hRAGE-transgenic NIH3T3 cell line established in this study stably expresses hRAGE through 20 generations and it was successfully applied to analyze specific affinity and biological activity of anti-RAGE antibody. This cell line may be a powerful tool for the development of drugs targeting the RAGE pathway in the future. It could also be used to study the molecular mechanism of RAGE-mediated signal transduction in vitro.

Conflict of Interest
The authors declare no conflicts of interest.

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