BPB Reports 🎲

Report

Fiber-Knob Region of Adenovirus Type 5 Vector Promotes Migration of A549 Cells

Junpei Kano,^a Naoya Koizumi,^{*,a} Airi Terada,^a Ayana Matsuoka,^a Takamasa Hirai,^{a,b} Tetsuya Nomura,^c Hiroyuki Mizuguchi,^{c,d,e,f} and Naoki Utoguchi^a

^aDepartment of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University; 3–3165 Higashitamagawagakuen, Machida, Tokyo 194–8543, Japan; ^bDivision of Cell-Based Therapeutic Products, National Institute of Health Sciences; 3–25–26 Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210–9501, Japan; ^cLaboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan; ^dLaboratory of Hepatocyte Regulation, National Institutes of Biomedical Innovation, Health and Nutrition; Osaka 567–0085, Japan; ^eGlobal Center for Medical Engineering and Informatics, Osaka University; Osaka 565–0871, Japan; ^fIntegrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives (OTRI), Osaka University; Osaka, Japan

Received November 5, 2020; Accepted January 11, 2021

Adenoviral vectors based on adenovirus type 5 (Ad5) are commonly used for gene therapy. The Ad5 fiberknob region primarily interacts with the coxsackievirus and adenovirus receptor (CAR). Reportedly, when stimulated, this receptor participates in the regulation of cell-to-cell adhesion and cell migration. In oncogene therapy, cell migration can have adverse effects by promoting metastasis and infiltration. Alternatively, cell migration may enhance the therapeutic effect of gene therapy by promoting the healing of injured tissues. However, the effect of binding of the Ad fiber-knob region to CAR of target cells has not been investigated in detail. Therefore, the aim of the present study was to investigate the effects of the Ad5 vectors on cell migration with the use of wound healing and migration assays. The results showed that infection with the Ad5 vectors promoted the migration of A549 cells, as determined quantifiably. Furthermore, when the Ad5 fiber-knob protein was applied to A549 cells, the same results were obtained. Together, the results revealed that binding of the Ad fiber-knob protein to CAR causes cell migration as a functional change in target cells. Studying the effect of the Ad fiberknob protein will lead to the development of a gene transfer vector with greater safety and therapeutic effects.

Key words adenoviral vector, fiber-knob, CAR, cell migration

INTRODUCTION

Adenoviruses are characterized by the presence of an icosahedral capsid and the lack of an envelope. Currently, 57 serotypes of human adenovirus have been identified.¹) The Ad type 5 (Ad5) vector is widely used as a vector in gene therapy and as an oncolytic virus vector for the treatment of cancer.^{1,2})

The Ad5 vector primarily interacts with the coxsackievirus and adenovirus receptor (CAR), which is expressed in many tissues, including the liver, heart, brain, kidney, and lung.³⁾ In addition, the fiber-knob region of Ad5 is known to bind to CAR.⁴⁾ CAR is generally expressed at tight junctions (TJs) and is involved in regulating cell-to-cell adhesion.⁵⁾ In addition, the oncolytic Ad vectors, which are proliferative in cancer cells, produce several fiber proteins as the virus proliferates and distribute these fiber proteins to neighboring cells.^{6,7)} Hence, regarding the stimulation of CAR in a wide range of cells, including target cells, infection using an Ad vector is warranted. Therefore, when the knob region of the Ad vector stimulates CAR, there may also be some effect on the target cells as well as neighboring cells. To ensure the safety of gene therapy and oncolytic virus therapy with the use of Ad vectors, it is necessary to consider the effects of infection with the Ad vector itself.

Therefore, the aim of the present study was to investigate the effects of the interaction of the Ad fiber-knob region with CAR on cell migration. The results suggest that the interaction of CAR with the Ad knob region promotes cell migration.

MATERIALS AND METHODS

Cell Lines and Culture A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Merck; Germany) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin solution, and 1% non-essential amino acid solution (Nacalai Tesque, Japan) under a humidified atmosphere of 5% $CO_2/95\%$ air at 37°C. For the wound healing and migration assays, cells were cultured in DMEM containing 1% FBS, 1% penicillin–streptomycin solution, and 1% non-essential amino acid solution.

Flow Cytometry A549 cells were labeled with anti-His-tag (dilution, 1:1000; clone OGHis, Medical & Biological Laboratories, Nagoya, Aichi, Japan) primary antibodies at 4°C for 30 min to detect the binding of the recombinant knob protein fused with a His-tag. Subsequently, the cells were washed with phosphate-buffered saline and incubated with allophycocyanin-conjugated secondary antibodies (dilution, 1:2000, Invitrogen, Carlsbad, CA, USA). The stained cells were washed thoroughly and subjected to flow cytometry with FACSCaliburTM cell analyzer (BD Biosciences, San Jose, CA, USA) and CellQuestTM software (BD Biosciences).

Preparation of Recombinant Ad5knob Protein (rAd-5knob) DNA fragment of Ad5 fiber-knob was cloned into a pET16b vector for production of rAd5knob expression plasmids, which were used to transfect Escherichia coli BL21 (D3) cells. The synthesis of rAd5knob was stimulated by the addition of isopropyl-D-thiogalactopyranoside. Cells were harvested, resuspended in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.1 mM [p-amidinophenyl] methanesulfonyl fluoride hydrochloride, and 1 mM β -mercaptoethanol), and then lysed by sonication. The lysates were applied to a Ni-column, and rAdknob bound to the Niresin was eluted with a 0-400 mM imidazole gradient in buffer A. The solvent for rAd5knob was changed to phosphate-buffered saline and the proteins were subjected to gel filtration with the use of a PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The concentrations of rAd5knob were estimated using a protein assay kit with bovine serum albumin as a standard.

Western Blot Analysis After boiling for 3 min, protein samples in 1× sample buffer with 4% β -mercaptoethanol were separated by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis and then electrotransferred to polyvinylidene fluoride membranes. After blocking with 1% Block Ace® reagent (Megmilk Snow Brand, Japan)/TBS-T (10 mM Tris– HCl, 0.1 M NaCl, and 0.05% Tween 20), the membranes were incubated with anti-His-tag monoclonal antibodies (dilution, 1:10000, QIAGEN, Germany), followed horseradish peroxidase-labeled goat anti-mouse secondary antibodies (dilution, 1:2000, AMERICAN QUALEX Manufactures, USA). The membranes were developed using electrochemiluminescence western blotting detection system, and signals were detected using a LAS- 4000 mini (Fujifilm Co., Japan).

Wound Healing Assay A549 cells were seeded onto a 12-well plate and cultured at humidified conditions of 5% CO₂ at 37°C for 24 h. After 24 h, culture medium from the wells was removed, and a regular and defined wound was created within the cell monolayer. Then, the cells were washed with phosphate-buffered saline, and culture medium was added to the wells again. Cells were treated either with epidermal growth factor (EGF) (200 ng/mL) (Sigma-Aldrich, Saint Louis, USA) or rAd5knob (20 µg/mL) and cultured further. After 24 or 48 h, wound area closure was observed under a microscope. Experiments using Ad5 vector (20 VP/cell) were also performed in a similar manner. Previous report have shown that EGF stimulates migration but is not associated with cell proliferation in non-small lung cancer.8) The rAd5knob amount to be added was determined using concentration-dependent experiments (data not shown).

Migration Assay A549 cells were seeded on the apical side of Transwell[®] Permeable Supports (8.0 μ m polycarbonate membrane, 6.5 mm insert, 24-well plate, Corning, NY, USA), and EGF (200 ng/mL) or rAd5knob (20 μ g/mL) was added to the basal side of the transwell. Cells were cultured at humidified conditions of 5% CO₂ at 37°C for 48 h. After 48 h, the well insert was removed, and the cells that had migrated to the basal side were observed under a microscope. Furthermore, 300 μ L of AlamarBlueTM cell viability reagent (Invitrogen) was added to the migrated cells and further cultured for 24 h. After 24 h, 100 μ L/well of the culture medium containing alamarBlue reagent was collected, and the fluorescence intensity was measured using Flioroskan (Fluoroskan Asent FL.,Thermo Scientific, Rockford, IL, USA). Experiments using Ad5 vector (20 VP/cell) were also performed in a similar manner.

RESULTS

Adenocarcinomic human alveolar basal epithelial A549 cells were used to elucidate the mechanism of Ad infection. First, expression levels of CAR were detected in A549 cells, which were confirmed as $91\% \pm 0.37\%$ compared with antibody-untreated A549 cells (data not shown).

The effect of infection of Ad vectors on the migratory capacity of A549 cells was investigated using the wound healing and migration assays. EGF was used as a positive control for evaluating the migration of A549 cells. Cell migration to the wounded area was promoted in the experimental groups, but not the control group. In addition, infection with the Ad5 vectors promoted cell migration to the same extent as the positive control group with EGF (Fig. 1). The results of the migration assay confirmed the same effect of the Ad5 vectors on the migration of A549 cells (Fig. 2A). In this study, the ability of the Ad vector to enhance cell invasion was also assessed. The results of the alamarBlue (cell viability) assay showed that the addition of EGF and Ad5 vector significantly increased fluorescence intensity relative to the control (Fig. 2B). Thus, infection with the Ad5 vector may activate cell migration and invasion.

Therefore, the region of the Ad vector involved in cell migration and invasion was investigated by focusing on the interaction between the viral fiber-knob protein and the primary receptors CAR, which is suspected to increase the migrator capabilities of the target cells. The presence of rAd5knob was confirmed by staining with Coomassie brilliant blue (Fig. 3A) and western blot analysis (Fig. 3B). The rAd5knob was used as a sample for western blotting. Furthermore, the fluorescence intensity of the A549 cells was increased following the addition of rAd5knob (Fig. 3C), which confirmed the binding of A549 cells to rAd5knob. Hence, these proteins were further used in this study. In addition, the effect of the Ad fiberknob region interacting with CAR on the proliferation of A549 cells was investigated. When the cells were cultured in medium with rAd5knob, there was no increase in cell number relative to the control group (data not shown).

Addition of rAd5knob promoted cell migration to the wounded areas (Fig. 4). Furthermore, the results of the migration assay confirmed that addition of rAd5knob promoted the migration of A549 cells (Fig. 5). Collectively, these results indicate that the fiber-knob region of the Ad5 vector promoted the migration of A549 cells.

DISCUSSION

Infection with the Ad vector promotes cell migration.⁹ CAR regulates cell-to-cell adhesion and promotes cell migration.^{5,10} Therefore, the use of an Ad vector in gene therapy may con-





Fig. 1. Effect of the Ad5 Vector on Wound Healing

A549 cells were cultured in the wells of a 12-well plate at 3.0×10^5 cells/well. After 24 h, the culture medium in the well was removed and a straight wound to the cell monolayer was created. Wells were either left treated with EGF (200 ng/mL), Ad5 vector (20 VP/cell) or further cultured. Control cells were incubated with 1% FBS in DMEM only. After 24 or 48 h, closure of wounded areas was observed under a microscope.



Fig. 2. Effect of the Ad5 Vector on the Migration of A549 Cells

A549 cells were cultured on the apical side of a transwell at 3.0×10^5 cells/well. Then, EGF (200 ng/mL), the Ad5 vector (20 VP/cell) was added to the apical side of the transwell. After 48 h, the insert in the well was removed, and the cells that had migrated to the basal side were observed under a microscope (A). Furthermore, the migrated cells were incubated with AlamarBlue reagent for 24 h. Then, the culture medium was collected, and the fluorescence intensity was measured (B). The results are presented as the mean \pm standard deviation (SD); n = 3. **p < 0.005.

sequently lead to functional changes in target cells. Therefore, we investigated the effect of the Ad fiber-knob region on cell migration. The wound healing assay and migration assay results showed that infection with either the Ad5 vector (Figs. 1, 2) or the addition of rAd5knob (Figs. 4, 5) similarly promoted the migration of A549 cell.

CAR is localized in epithelial TJs and plays a key role in the junctional barrier function.⁵⁾ Infection with the Ad5 vector reportedly reduced the barrier function and diminished the cell-to-cell junction integrity.¹¹⁾ Furthermore, in response to an infection with the Ad vector, the PI3K/Akt pathway was activated in epithelial cells,¹²⁾ which mainly involved cell growth, invasion, and migration, while inhibiting apoptosis.¹³⁾ In fact, the Ad vector also stimulated the growth and migration of human lung microvascular endothelial cells.⁹⁾ In the present study, rAd5knob addition promoted cell migration. Therefore, cell migration in response to rAd5knob addition may be mediated by the PI3K/Akt pathway activation, as reported in previous studies.¹²⁾ In addition, cell migration occurred to the same extent following infection with the Ad vector and after the addition of the rAd5knob. Hence, cell migration does not necessarily occur due to the influence of the Ad vector alone but rather due to the interaction between the Ad fiber-knob region and CAR.

The fiber-knob region may activate specific signal transduction systems that regulate cell-to-cell adhesion and promote cell migration by interacting with CAR. Therefore, the use of the Ad5 vector may cause functional changes to infected cells and promote the migration of cancer cells to the same extent. Hence, gene therapy with the use of an Ad vector may lead to metastasis or invasion of cancer cells.

Therefore, when using Ad5 vectors, which are most widely used in gene therapy and oncolytic cancer therapy for several diseases, it is necessary to consider functional changes and the influence on the migratory capabilities of target cells.



Fig. 3. Confirmation of Recombinant Knob Protein Binding to A549 Cells

The protein solution purified using Ni-column and buffer-substituted by PD-10 column was used as a sample for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following separation by gel electrophoresis, the rAd5knob were stained with Coomassie brilliant blue (A) or Western blot analysis (B). A549 cells were cultured in the wells of a 6-well plate at 3.0×10^5 cells/well. After 24 h, the rAd5knob ($20 \mu g/mL$) was added to the wells. After incubation at 4° C for 30 min, the cells were harvested and immunostained with anti-His-tag antibodies and anti-mouse allophycocyanin-conjugated antibodies against IgG, whereas the blank samples were antibody-untreated. The fluorescent intensity of the cells was determined by flow cytometry (C).



Fig. 4. Effect of rAd5knob on Wound Healing

A549 cells were cultured in the wells of a 12-well plate at 3.0×10^5 cells/well. After 24 h, the culture medium in the well was removed and a straight wound to the cell monolayer was created. Wells were either left treated or further cultured with EGF (200 ng/mL), rAd5knob (20 µg/mL). Control cells were incubated with 1% FBS in DMEM only. After 24 or 48 h, closure of the wounded areas was observed under a microscope.



Fig. 5. Effect of rAd5knob on the Migration of A549 Cells

A549 cells were cultured on the apical side of a transwell at 3.0×10^4 cells/well. Then EGF (200 ng/mL), rAd5knob (20 µg/mL) was added to the apical side of the transwell. After 48 h, the insert in the well was removed, and the cells that had migrated to the basal side were counted under a microscope (A). Furthermore, the migrated cells were incubated with AlamarBlue reagent for 24 h. Then, the culture medium was collected, and the fluorescence intensity was measured (B). The results are presented as the mean \pm SD; n = 3. ** p < 0.005.

Conflict of interest The authors declare no conflict of interest.

REFERENCES

- Alemany R. Oncolytic adenoviruses in cancer treatment. *Biomedicines*, 2, 36–49 (2014).
- 2) Gao J, Zhang W, Ehrhardt A. Expanding the spectrum of adenoviral vectors for cancer therapy. *Cancers (Basel)*, **12**, 1139 (2020).
- Wehbi A, Kremer EJ, Dopeso-Reyes IG. Location of the cell adhesion molecule "coxsackievirus and adenovirus receptor" in the adult mouse brain. *Front. Neuroanat.*, 14, 28 (2020).
- Bergelson JM. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science*, 275, 1320–1323 (1997).
- Cohen CJ, et al. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proc. Natl. Acad. Sci. USA, 98, 15191–15196 (2001).
- Kiyokawa J, Wakimoto H. Preclinical and clinical development of oncolytic adenovirus for the treatment of malignant glioma. *Oncolytic Virother.*, 8, 27–37 (2019).
- 7) Trotman LC, Achermann DP, Keller S, Straub M, Greber UF. Non-

classical export of an adenovirus structural protein: adenovirus penton base export. *Traffic*, **4**, 390–402 (2003).

- 8) Lauand C, Rezende-Teixeira P, Cortez BA, Niero EL. de O. Machado-Santelli, G. M. Independent of ErbB1 gene copy number, EGF stimulates migration but is not associated with cell proliferation in non-small cell lung cancer. *Cancer Cell Int.*, **13**, 38 (2013).
- Kornberg LJ, Grant MB. Adenoviruses increase endothelial cell proliferation, migration, and tube formation: partial reversal by the focal adhesion kinase inhibitor, FRNK. *Microvasc. Res.*, **73**, 157–162 (2007).
- Mirza M, Petersen C, Nordqvist K, Sollerbrant K. Coxsackievirus and adenovirus receptor is up-regulated in migratory germ cells during passage of the blood-testis barrier. *Endocrinology*, **148**, 5459–5469 (2007).
- 11) Walters RW, *et al.* Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell*, **110**, 789–799 (2002).
- 12) Tan PH, et al. Effect of vectors on human endothelial cell signal transduction: implications for cardiovascular gene therapy. Arterioscler: Thromb. Vasc. Biol., 26, 462–467 (2006).
- Xu W, Yang Z, Lu N. A new role for the PI3K/Akt signaling pathway in the epithelial-mesenchymal transition. *Cell Adhes. Migr.*, 9, 317– 324 (2015).