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Inhibition of discoidin domain receptor 2-mediated lung cancer cells progression by gold nanoparticle-aptamer-assisted delivery of peptides containing transmembrane-juxtamembrane 1/2 domain

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ABSTRACT

The delivery of biologically functional peptides into mammalian cells can be a direct and effective method for cancer therapy and treatment of other diseases. Discoidin domain receptor 2 (DDR2) is a collagen-induced receptor tyrosine kinase recently identified as a novel therapeutic target in lung cancer. In this study, we report that peptides containing the functional domain of DDR2 can be efficiently delivered into lung malignant cancer cells via a gold nanoparticle-DNA aptamer conjugate (AuNP-Apt)-based system. Peptide delivery resulted in the abrogation of DDR2 activation triggered by collagen. Moreover, the peptide delivered by the AuNP-Apt system inhibited cancer cell proliferation and invasion mediated by DDR2 activation. Thus, these results suggest that peptide loaded onto AuNP-Apt conjugates can be used for the development of peptide-based biomedical applications for the treatment of DDR2-positive cancer.

1. Introduction

The discoidin domain receptors (DDRs) are tyrosine kinase receptors composed of two subfamilies: DDR1 and DDR2 [1,2]. DDRs have unique structural features and distinctive functions in collagen-mediated signaling or tyrosine phosphorylation, thereby modulating cell functions. Collagen within the extracellular matrix can activate DDRs as part of normal development and tissue homeostasis, while abnormal expression and activation of these receptors causes injury or disease [3]. Therefore, regulation of DDR activation is closely associated with various diseases such as cancer, atherosclerosis, lung and liver fibrosis, and osteoarthritis [3–5].

Recently, several studies have reported that inhibition of DDR activity can reduce DDR-mediated cancer progression [5]. Therefore, blocking the interaction of DDRs with collagen to decrease its receptor activity is a possible strategy for developing efficient therapeutics for DDR-mediated diseases [3,6]. For example, it has been shown that excess expression of the juxtamembrane 2 (JM2) domain blocked DDR2 activation, thereby reducing proliferation and invasiveness of cancer cells *in vitro* [7]. Despite its importance, delivery of the peptide-containing JM2 domain to cancer cells is inefficient, leading to difficulty using this peptide in cancer treatment.

Many different methods have been developed to transport peptides into mammalian cells [8,9]. However, their therapeutic use has been hampered by the complex process of linking a target protein to a carrier; these protein-carrier complexes are susceptible to endosomal internalization, instability, and extensive modifications of the protein [9]. To solve these problems, gold nanoparticles-DNA aptamer (AuNP-Apt) composites were recently developed as a universal carrier for *in vivo* delivery of recombinant proteins [10]. This system allows any recombinant protein to be loaded without additional modifications and efficiently delivered into mammalian living systems, without any evidence of cytotoxicity [10].

In this study, we utilized an AuNP-Apt-based protein delivery system to transport peptides containing the hexahistidine (His)- or glutathione S-transferase (GST)-tagged transmembrane-juxtamembrane 1/2 (TM-JM1/2) domain of DDR2 into a lung cancer cell line. We show that intracellular delivery of TM-JM1/2

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peptides via this AuNP-Apt-based system can efficiently inhibit DDR2 activation induced by collagen, resulting in decreased cell proliferation and invasion. Thus, our results show that TM-JM1/2 peptides loaded onto AuNP-Apt conjugates can be a useful strategy for DDR2-positive cancer therapy.

2. Materials and methods

2.1. Cell culture

Non-small cell lung carcinoma H1299 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). H1299 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). All cell cultures were maintained at 37°C/5% CO_2.

2.2. Plasmid generation

Constructs were prepared by polymerase chain reaction as reported previously [7]. The His-tagged TM-JM1/2 and GST-tagged TM-JM1/2 used in this study were produced in BL21(DE3) cells and purified using Protein Refolding Kit (Novagen, Madison, WI) according to the manufacturer's instructions.

2.3. Preparation of AuNP-Apt conjugates

Citrate-stabilized gold nanoparticles (15 nm diameter) were functionalized with anti-His aptamers or anti-GST aptamers, as previously described [10].

2.4. Production of AuNP-Apt-protein complex

The AuNP-Apt-protein complex was formed as previously described [10]. Aptamers of AuNP-Apt conjugates were denatured at 80 °C for 5 min and then cooled at room temperature for 10 min to allow the formation of correct secondary structures. AuNP-His/GST-Apt (1 nM) and purified His/GST-tagged proteins were then incubated at room temperature in 1 × PBS with 5 mM $MgCl_2$ (pH 7.2) for 10 min. H1299 cells were seeded on 6-well culture dishes and cultured for 24 h, after which the cells were incubated with AuNP-Apt-protein complexes in the culture media for an additional 24 h. Cells were then harvested and used for further experiments.

2.5. Fractionation of plasma membrane and cytosolic proteins

After 24-h incubation with AuNP-Apt-protein complex, cells were washed with ice-cold PBS. Next, the cells were lysed in lysis buffer (20 mM Tris-Cl [pH 7.4], 100 mM NaCl, 2 mM MgOAc, 5 mM KCl, 10 μM GTP, 1 mM PMSF) for 10 min and homogenized with a Dounce homogenizer 45 times. After centrifugation of the cell lysate at 2000 × *g* for 10 min, the supernatant was centrifuged at 14,000 × *g* for a further 30 min. The supernatant was then transferred and used as the cytosolic fraction. After washing with lysis buffer, the pellet was lysed with lysis buffer containing 1% NP40 and used as the plasma membrane fraction. Both cytosolic and plasma membrane fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting.

2.6. Western blot analysis

Western blot analysis of protein extracts was performed as described previously [7]. Prepared samples were subjected to SDS-PAGE and transferred to membranes. The membranes were immunoblotted with anti-His (05-949; Millipore, Bedford, MA), anti-GST (sc-138; Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin (T9026; Sigma–Aldrich, St. Louis, MO), anti-β-catenin (PA5-16762; Thermo Scientific, San Jose, CA), anti-pTyr (sc-508, Santa Cruz Biotechnology), and anti-DDR2 (AF2538, R&D Systems, Minneapolis, MN) antibodies. The signal was developed with ECL reagent. Band intensity was measured by Quantity One (Bio-Rad, Richmond, CA).

2.7. Cell proliferation assay

Cells were seeded on a 35-mm dish and cultured for 6 days. At the indicated time points, cells were harvested and counted using a hemocytometer. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a WST-1 cell proliferation assay system (Takara, Tokyo, Japan) was used according to the manufacturer's instructions.

2.8. Cell invasion through Transwells

Transwell inserts with an 8-mm pore size (Corning, Corning, NY) were coated with 10 μg type I collagen at 37 °C overnight. H1299 cells treated with AuNP-Apt-protein complexes (1 μM) were placed in the upper chamber. RPMI 1640 supplemented with 10% FBS was added to the lower chamber as the chemoattractant. Non-migrated or non-invaded cells were removed with a cotton swab. Cells were fixed and stained with 0.1% crystal violet and counted in all areas.

2.9. Statistical analysis

Differences between controls and treatments were analyzed by Student's *t*-test using Microsoft Excel. Data were expressed as the mean ± standard deviation (SD) of at least two independent experiments. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Delivery of TM-JM1/2 domain-containing peptides by AuNP-Apt conjugates

To evaluate the intracellular delivery of TM-JM1/2 peptides by the aptamer-conjugated AuNP delivery system (Fig. 1A), we loaded purified His- or GST-tagged TM-JM1/2 peptides onto AuNPs conjugated with a His- or GST-tagged aptamer (AuNP-His- or GST-Apt) by simple mixing and incubation. Approximately 40% of the tagged peptides in the reaction mixture were loaded onto the AuNP-Apt (data not shown). Next, H1299 non-small cell lung carcinoma cells were treated with the purified His- or GST-tagged TM-JM1/2 peptides loaded onto AuNP-(His or GST)-Apt conjugates (AuNP-Apt-TM-JM1/2). We evaluated transfection efficiency and cellular localization by Western blot analysis. As shown in Fig. 1B, His- or GST-tagged TM-JM1/2 peptides were efficiently delivered by AuNP-(His- or GST)-Apt. Furthermore, almost all peptides were localized at the plasma membrane, in agreement with the biological function of DDR2 as a transmembrane receptor protein [11]. Thus, these results suggest that AuNP-Apt conjugates can deliver TM-JM1/2 peptides into H1299 cells efficiently, and that delivered peptides were subsequently released from the AuNP-Apt, allowing for normal localization.

3.2. Inhibition of DDR2 activation by AuNP-Apt-TM-JM1/2 composites

Previously, it has been shown that overexpression of the TM-JM1/2 domain inhibits DDR2 dimerization, which is essential for

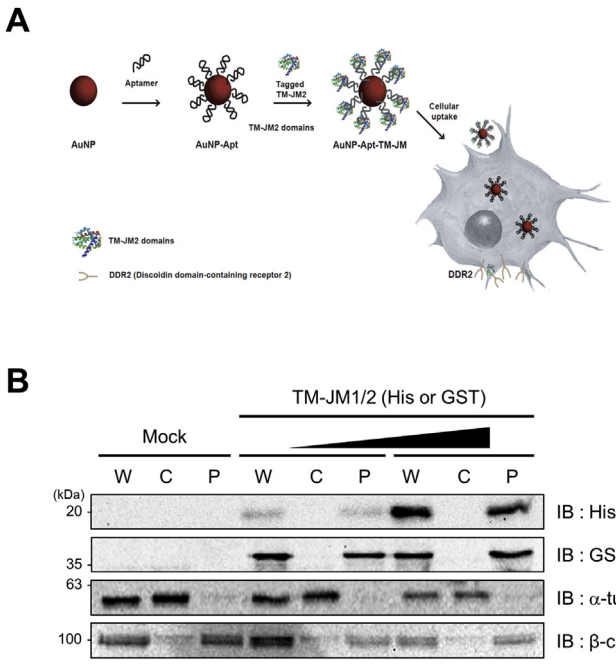

Fig. 1. Intracellular delivery of the His/GST-tagged transmembrane-juxtamembrane 1/2 (TM-JM1/2) peptide by gold nanoparticle-hexahistidine- or glutathione S-transferase (GST)-tagged DNA aptamer conjugates (AuNP-His/GST-Apt). (A) Schematic diagram of the generation of TM-JM1/2 peptides containing the aptamer-conjugated AuNP delivery system. (B) H1299 cells transfected with increasing amount of His or GST-tagged TM-JM1/2 loaded onto AuNP-His/GST-Apt (1 and 2 μM) were lysed, and then whole cell lysates (W) were separated into the cytosol (C) and plasma membrane (P) fractions. Almost all TM-JM1/2 peptides were localized at the plasma membrane. α-tubulin and β-catenin were used as positive controls for the cytosolic and plasma membrane fraction, respectively.

DDR2 activation in response to collagen stimulation [7]. To investigate the intracellular effect of AuNP-Apt-TM-JM1/2 conjugates, we examined DDR2 activation triggered by collagen. As shown in Fig. 2, activation of DDR2 by collagen was significantly decreased by both His- or GST-tagged TM-JM1/2 peptides compared with mock cells treated with AuNP-Apt only.

3.3. Anti-tumor activity of delivered TM-JM1/2 in lung cancer cells

Next, we investigated whether the delivered TM-JM1/2 peptides are able to inhibit DDR2-mediated cancer cell proliferation, invasion, and motility. We examined the effect of delivered TM-JM1/2 peptides on cell proliferation, by cell counting and MTT assays. As shown in Fig. 3A, H1299 cells treated with AuNP-His/GST-Apt-TM-JM1/2 showed a significant decrease in proliferation rate compared with mock-transfected cells. We also found that viable cell ratios were considerably decreased for cells treated with AuNP-His/GST-Apt-TM-JM1/2 compared with mock-transfected cells (Fig. 3B). We further examined the invasion and motility of TM-JM1/2-transfected H1299 cells by Transwell assay. The invasion capacity of TM-JM1/2-transfected H1299 cells through collagen-coated membrane was significantly reduced compared with mock-transfected cells, without significant differences in cell motility through non-coated membrane (Fig. 3C). These results show that the TM-JM1/2 delivered by this aptamer-based AuNP system can effectively inhibit proliferation and invasion of DDR2-expressing cancer cells.

4. Discussion

The use of tyrosine kinase inhibitors (TKIs) to treat various diseases including cancers is becoming increasingly widespread.

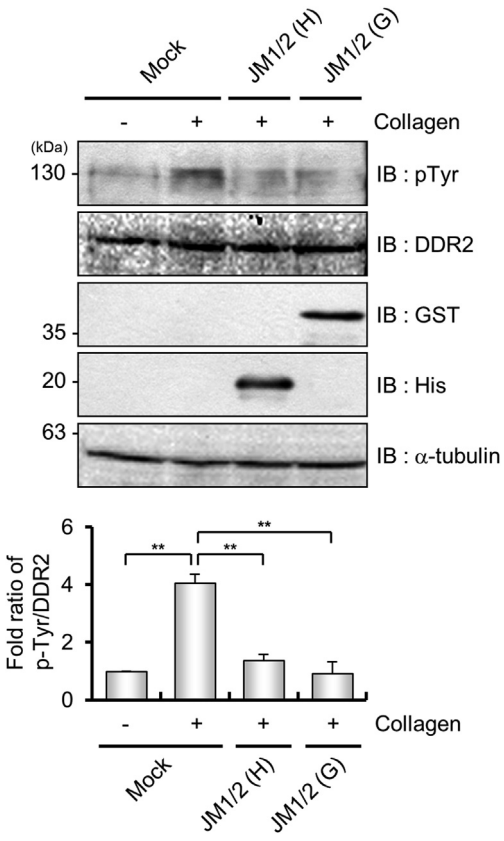

Fig. 2. Inhibition of DDR2 activation by gold nanoparticle-DNA aptamer-transmembrane-juxtamembrane 1/2 (AuNP-Apt-TM-JM1/2) composites. H1299 cells were treated with AuNP-His-Apt only (Mock) or AuNP-His/GST-Apt-His- or GST-tagged TM-JM1/2 (labeled JM1/2 (H) or JM1/2 (G)) composites for 24 h, followed by stimulation with type I collagen (50 μg/ml). Induced tyrosine phosphorylation was dramatically decreased by addition of AuNP-His/GST-Apt-His or GST-TM-JM1/2. $^{**}p < 0.01$, Student's *t*-test.

However, many TKIs inhibit several receptor tyrosine kinases simultaneously [5]. Whilst these multi-target TKIs might be convenient to treat patients, they are associated with unacceptably high toxicity and unwanted side effects [12,13]. These facts imply that inhibitors that target specific molecules or small peptides need to be developed. Cell-penetrating peptides (CPPs), such as Tat peptide or penetratin, have been widely used to transport specific targets such as proteins or chemical molecules efficiently into cells; CPPs are the shortest amino acid sequences that can be used for efficient cell uptake [14–16]. Although they have delivery efficiency, CPPs could have negative effects such as poor specificity, unwanted cytotoxic effects, and cell type dependency [17,18]. Because cell specificity is highly important for cancer therapy to minimize side effects on normal cells, development of cancer cell specificity is required through the rational design of CPPs. In this regard, our aptamer-based AuNP system can provide not only high delivery efficiency of therapeutic peptides, but also target specificity for cancer treatment when co-loaded with a ligand that interact with cancer-specific surface receptors, as previously shown for epidermal growth factor receptor-overexpressing human epidermoid carcinoma cells [10]. Thus, we suggest that this effective and simple method of delivering functional peptides into mammalian living systems can be used in biomedical applications including peptide therapeutics and bio-imaging technologies.

In conclusion, we demonstrated the ability of the DNA aptamer-based AuNP system to deliver peptides with biological function into mammalian living systems. AuNP-Apt was successfully able to

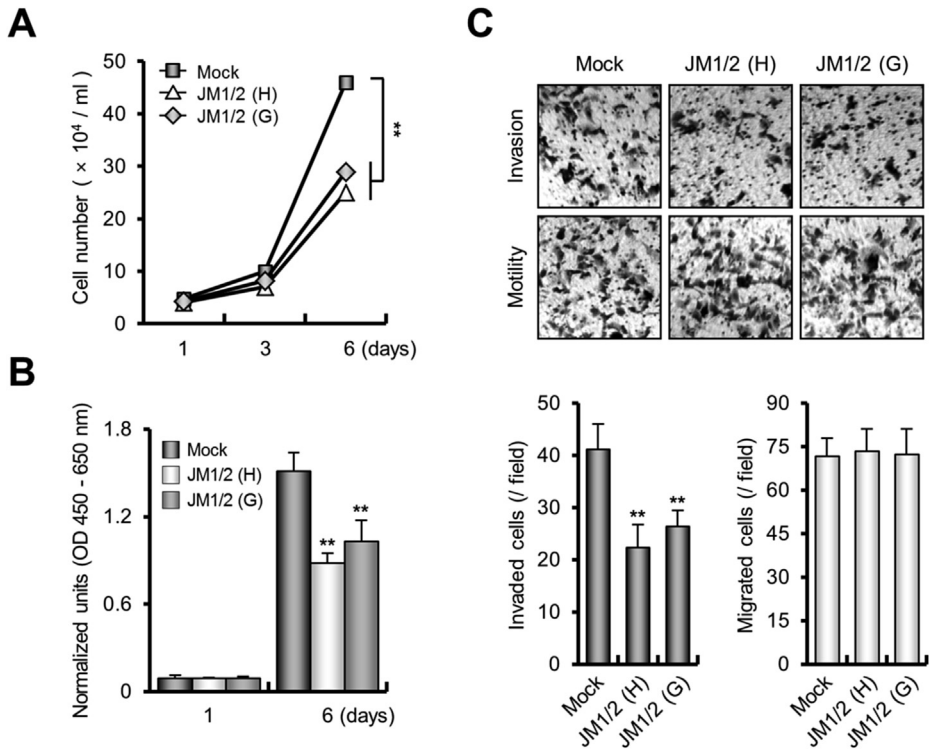

Fig. 3. Inhibition of tumor cell proliferation and invasion by gold nanoparticle-DNA aptamer-transmembrane-juxtamembrane 1/2 (AuNP-Apt-TM-JM1/2) composites. (A, B) Proliferation of H1299 cells transfected with His/GST-TM-JM1/2 by AuNP-His/GST-Apt (labeled JM1/2 (H) or JM1/2 (G)) was assessed by cell counting (A) and MTT assay (B). Cell proliferation and viability were markedly inhibited by delivery of His/GST-TM-JM1/2. (C) Transwell assay was performed with TM-JM1/2-treated H1299 cells. Invasion was considerably suppressed by TM-JM1/2 peptides in collagen-coated inserts, whereas the number of migrated cells in non-coated inserts was not significantly changed. $^{**}p < 0.01$, Student's *t*-test.

deliver His- or GST-tagged TM-JM1/2 peptides into H1299 lung cancer cells by a simple mixing procedure. Furthermore, the delivered peptides efficiently inhibited DDR2 activation, resulting in decreased cell proliferation and invasion. Therefore, AuNP-Apt-based TM-JM1/2 peptides can be useful tools for treatment of DDR2-mediated carcinogenesis.

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