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A gene-delivery system specific for hepatoma cells and an intracellular kinase signal based on human liver-specific bionanocapsules and signal-responsive artificial polymer

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ABSTRACT

Recently, our group has proposed a novel gene-regulation system responding to cAMP-dependent protein kinase (PKA) that has been applied to living cells. In this study, human liver-specific bionanocapsules (BNCs) are used as a gene-delivery system to increase transfection efficiency and to target specific cell types. BNCs can efficiently deliver a target gene to human hepatocytes and hepatoma cells *in vitro* or *in vivo*. The combination of a signal-responsive gene-delivery system with BNCs led to an increase in the transfection efficiency and selectivity for hepatoma cells. Expression from the delivered gene was identified from PKA-activated hepatoma cells (HepG2), but not from colon tumor cells (WiDr). These results show that the combination of a gene-regulation system responding to an intracellular signal with BNC can be used for the selective treatment of human hepatoma cells.

1. Introduction

Gene therapy is one of the most promising approaches to treat various heritable diseases and malignant tumors (Wolkowicz and Nolan, 2005). However, there remains the serious problem of the delivered gene being expressed not only in abnormal target cells but also in normal cells. To overcome this issue, many targeting strategies have been investigated using molecular markers on the target disease cell surface (Mignet et al., 2008; Pan et al., 2008; Zheng et al., 2009). Recently, we have proposed a novel strategy for gene therapy using drug- or gene-delivery systems responding to cellular signals (D-RECS) (Katayama et al., 2002; Oishi et al., 2006a,b; Kang et al., 2008). D-RECS exploits the abnormally activated intracellular signals of target cells as a trigger to release the gene from the carrier. This system reacts with cells containing over-expressed intracellular signals, such as tumor cells, but shows no reaction with normal cells, in which the intracellular signal is normally controlled.

Phosphorylation of various proteins by protein kinases plays an essential role in signal transduction pathways and is a general mechanism for the control of intracellular processing of information. It is known that extraordinary and continuous activation of protein kinase is seen in many cancers (Cohen, 2002). One such kinase is the cyclic AMP-dependent protein kinase (protein kinase A/PKA; here after referred to as PKA), which plays an important role in various cellular responses, such as hormone secretion, cell proliferation or differentiation (Cho-Chung et al., 1995). We have previously designed an artificial polymer possessing a cationic peptide, which is the substrate of PKA, as a pendant chain (Katayama et al., 2002; Oishi et al., 2006a,b).

Hepatoma is the most common type of malignant tumor originating in the liver and shows highly resistant to conventional chemotherapy and radiotherapy (Zheng et al., 2008; Fulda, 2009). Hepatoma-targeted gene therapy may increase therapeutic efficiency and avoid side effects. Recently, Kuroda's group has described a novel gene-delivery approach based on hepatotropic nanoparticles (Yamada et al., 2003; Jung et al., 2008). These nanocapsules are engineered to display the hepatitis B virus surface L antigen (HBsAg) on their surface but lack the viral genome. Bionanocapsule (BNC) is a gene-delivery system and can efficiently

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deliver target gene to human hepatocytes and hepatoma cells *in vitro* or *in vivo* (Yamada et al., 2003; Jung et al., 2008).

In this study, human liver-specific BNC is used to increase transfection efficiency to hepatoma cells. The combination of a signal-responsive gene-delivery system with BNCs led to an increase of transfection efficiency and selectivity for hepatoma cells.

2. Materials and methods

2.1. Preparation of N-isopropylacrylamide (NIPAM)–Kemptide–PEG

For the synthesis of NIPAM–Kemptide–PEG, a methacryloyl peptide (methacryloyl-ALRRASLGW-NH_2) (6.2 mg, 6.7 μmol), in which the methacryloyl group was attached at the amino-terminus of the peptide, N-isopropylacrylamide (40 mg, 0.35 mmol), and N-methacryloyl-PEG (8.8 mg MW 12,000) were dissolved in degassed water and allowed to stand at room temperature for 1 h after the addition of ammonium persulfate (3.1 mg, 2.9 mmol) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (4 μL, 5.8 mmol) as the redox initiator couple. The product was then purified by overnight dialysis against water using a semipermeable membrane bag with a molecular weight cutoff of 100,000, followed by lyophilization to obtain a white powder. The concentration of peptide and of PEG in NIPAM–Kemptide–PEG was estimated by H NMR and absorbance at 280 nm derived from Tryptophan.

2.2. Gel permeation chromatography (GPC) analysis

GPC of NIPAM–Kemptide–PEG was performed using a Viscotek model 300 TDA system equipped with refractive index, viscosity, and light-scattering detectors operating at 25 °C. TSKgel R-M columns (TOSOH Co. Ltd., Tokyo, Japan) were used with DMF containing 10 mM LiBr as the eluent and the flow rate was 0.8 mL/min.

2.3. Dynamic light-scattering and ξ-potential measurement

Dynamic light-scattering and ξ-potential measurement were carried out in a disposable cuvette made by Zetasizer (Malvern Inc., Worcestershire, UK). A He/Ne laser (λ_0 = 633 nm) was used as the incident beam.

2.4. Purification of the L protein from the yeast

HBsAg L nanoparticles were prepared from yeast cells, *Saccharomyces cerevisiae* AH22R$^-$, carrying the HBsAg L protein expression plasmid pGLDLIIP39-RcT. The whole cell extract of the recombinant yeast was fractionated with PEG 6000 and separated twice by CsCl isopycnic ultracentrifugation and once by sucrose density gradient ultracentrifugation, as described previously (Yamada et al., 2003; Jung et al., 2008). The level of the HBsAg L protein in the yeast cell extracts was determined by silver-stained SDS-PAGE. The S-antigenic activity in the cell extracts was determined by the IMx HBsAg assay kit (Abbott Japan Co., Tokyo, Japan), in conjunction with a microparticle enzyme immunoassay (MEIA) in accordance with the manufacturer's instructions.

2.5. Agarose gel electrophoresis

pEGFP-C1 (clontech, 0.25 μg) and NIPAM–Kemptide–PEG were mixed in 10 μL phosphorylation buffer [1.0 mM $MgCl_2$, 0.2 mM ATP, and 10 mM HEPES (pH 7.2)] at cation/anion (C/A) ratios of 0.5, 1.0, and 2.0 and allowed to stand for 15 min. For the disintegration of complex, PKA C-subunit (10 U, Promega Co., Madison, WI, USA) was added to each solution and incubated at 37 °C for 2 h. The formulation and the disintegration of complex were assayed by 1% agarose gel electrophoresis using Tris–EDTA buffer.

2.6. Cell-free expression of DNA

All experiments were performed using a cell-free expression system (T7 S30 extract system for circular DNA, Promega) containing T7 S30 extract and an amino acid mixture. Luciferase was expressed for 40 min at 37 °C using luciferase-encoding DNA. Reaction mixture was added to the luciferase assay solution (Promega), and the chemiluminescence was measured using a multilabel counter, ARVO (WALLAC Inc., Turku, Finland). Other experimental conditions were as described under agarose gel electrophoresis.

2.7. Preparation of NIPAM–Kemptide–PEG/DNA/BNC complex and real-time PCR analysis

For the preparation of NIPAM–Kemptide–PEG/DNA complex, NIPAM–Kemptide–PEG was mixed with 1.0 μg of DNA (C/A = 1.0) for 10 min at room temperature and then for 5 min at 37 °C. Next, a 3 μL aliquot of liposome solution, prepared according to the recommended procedure (COATSOME EL-01-D, NOF corporation, Tokyo, Japan), was mixed with the polymer/DNA solution for 15 min. The NIPAM–Kemptide–PEG/DNA/liposome solution was mixed with 2.5 μg of BNCs for 15 min. This solution (NIPAM–Kemptide–PEG/DNA/BNC complex) was added to cells. 24 h after the addition of complex, medium was removed and cells were washed 3 times with 300 μL PBS. Extraction of DNA was performed as described in protocols of the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The levels of pCMV-luc in HepG2 cells were estimated by quantitative real-time PCR (LightCycler 1.5, Roche Diagnostics, Swiss) in which a part of the luciferase region of pCMV-luc in extracted DNA samples was amplified by PCR. Sequences of PCR primers were as follows: forward, 5′-aagatggaaccgctgaga-3′ and reverse, 3′-cttctgccaaccgacg-5′. Each reaction mixture (20 μL) for PCR consisted of 2 μL DNA template (1 ng/μL), 0.8 μL primers (10 μM stock solutions), and 10 μL SYBR Premix Ex Taq™ (TaKaRa Bio, Tokyo, Japan). The levels of pCMV-luc were standardized against total DNA amounts extracted from HepG2 cells.

2.8. GFP expression assay in HepG2 and WiDr cells

HepG2 and WiDr cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco Invitrogen Co., Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. The cells were incubated in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

NIPAM–Kemptide–PEG/DNA (1.0 μg)/BNC complexes were prepared by mixing as described above and the complex solution was poured onto the cells. After incubation for 24 h at 37 °C, the medium was changed. To stimulate intracellular PKA, the cell sample was treated with forskolin (Fsk, 10 μM) and isobutylmethylxanthine (IBMX, 100 μM). 48 h after transfection, fluorescence micrographs of the cells were obtained using confocal laser microscopy.

2.9. Western blot

After PKA stimulation by Fsk (10 μM) + IBMX (100 μM) for 15 min, cells were lysed in lysis buffer containing 20 mM Tris–HCl, pH 7.4, 0.05% Triton-X 100, and 2 mM EDTA, supplemented with Complete™ protease inhibitor cocktail (Roche Diagnostics) on ice for 15 min. The lysates were immunoblotted with anti-phosphoPAK

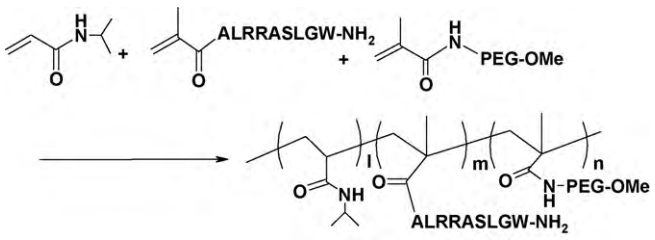

Fig. 1. Synthetic scheme and chemical structures of NIPAM–Kemptide–PEG. NIPAM–Kemptide–PEG contained the peptide side chain at a concentration of 1.2 mol% and the PEG side chain at a concentration of 0.5 mol%, as estimated by H NMR and elemental analysis.

(Thr197) serum (Cell Signaling, Beverly, MA, USA) and the reacting proteins were visualized by chemiluminescence.

3. Results and discussion

3.1. Synthesis and physiochemical evaluation of NIPAM–Kemptide–PEG

In our previous study, the diameter of the polymer (PAK)/DNA complex was >200 nm and showed a wide distribution of particle size (Oishi et al., 2006b). For the stability and size reduction of the complex, the polymer was redesigned by the changing two details as follows: (1) the polyacrylamide main chain was changed to poly-N-isopropylacrylamide, which has greater hydrophobicity and (2) a PEG chain was grafted to enhance the stability of the complex. The new polymer (NIPAM–Kemptide–PEG) was synthesized by means of radical polymerization using N-isopropylacrylamide (NIPAM), a peptide monomer and PEG macromonomer, in which the methacryloyl group was attached at the amino-terminus of the peptide (Fig. 1). The resulting NIPAM–Kemptide–PEG contained a peptide side chain (1.2 mol%) and a PEG side chain (0.5 mol%). NIPAM–Kemptide–PEG had a weight-average molecular mass of 53.0 kDa with a polydispersity of 1.9, as measured by gel permeation chromatography.

A dynamic light-scattering (DLS) measurement was carried out to evaluate the size of the NIPAM–Kemptide–PEG/DNA complex. The diameter and polydispersity of NIPAM–Kemptide–PEG/DNA complex were 50 nm and 0.062 in saline at 37 °C, respectively.

3.2. Gel retardation assay

We investigated whether NIPAM–Kemptide–PEG can form a stable complex with DNA. When the NIPAM–Kemptide–PEG was added to the DNA solution, the migration of DNA was suppressed during gel electrophoresis. This result means that NIPAM–Kemptide–PEG formed a stable complex with DNA through electrostatic interaction. On the other hand, when activated PKA was added, the band of the original DNA was completely recovered (Fig. 2). This result strongly suggests that the NIPAM–Kemptide–PEG is phosphorylated by activated PKA, resulting in DNA release.

3.3. Cell-free protein expression responding to PKA activity

We investigated whether the NIPAM–Kemptide–PEG/DNA complex can regulate gene expression a using cell-free expression system in the presence or absence of PKA. Luciferase expression was suppressed up to approximately 5% by the addition of NIPAM–Kemptide–PEG/DNA complex (C/A = 1.0 and 3.0) and in the absence of PKA. This result indicates that NIPAM–Kemptide–PEG/DNA complex can regulate gene expression completely. On the other hand, the expression level at C/A = 1.0

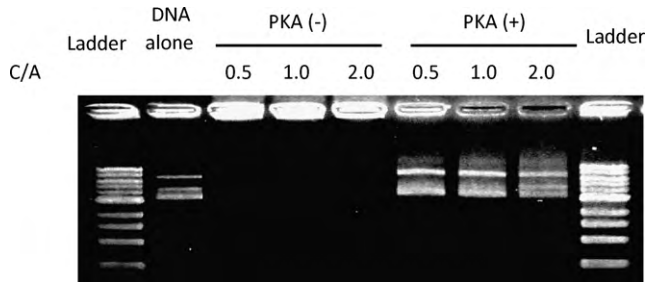

Fig. 2. Formation of the NIPAM–Kemptide–PEG/DNA complex and its disintegration with PKA signaling. The phosphorylation reaction was carried out in 50 μL of buffer [10 U PKA, 1.0 mM $MgCl_2$, 0.2 mM ATP, and 10 mM HEPES (pH 7.2)]. After incubation for 2 h at 37 °C, agarose gel electrophoresis was performed.

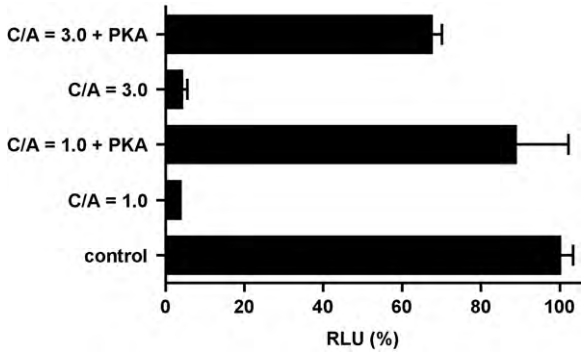

Fig. 3. Suppression of luciferase expression with NIPAM–Kemptide–PEG and amelioration of suppression in response to PKA in a cell-free expression assay. After phosphorylation for 2 h at 37 °C, the cell-free luciferase expression assay was performed. In the control experiment, luciferase was expressed using the solution containing 0.25 μg DNA without polymer and PKA. Data represent mean ± standard errors ($n = 3$). RLU, relative luminescence units.

and 3.0 was recovered to 90 and 70%, respectively, in the presence of PKA (Fig. 3). These results suggest that phosphorylation of complex by PKA renders the complex unstable, leading to transcription and gene expression.

3.4. Complex of NIPAM–Kemptide–PEG/DNA with BNCs

Complexes can be combined with BNCs using a liposome that is composed of DOPE/cholesterol/DC-6-14. The size of BNC and liposome are approximately 30 and 100 nm, respectively (Jung et al., 2008). The BNC is a nanoparticle containing the human hepatocyte L protein embedded in a yeast endoplasmic reticu-

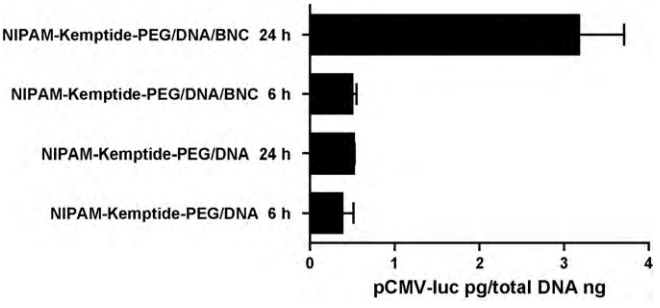

Fig. 4. DNA binding and uptake into cells mediated by NIPAM–Kemptide–PEG/DNA/BNC complex. Cells were incubated with NIPAM–Kemptide–PEG/DNA/BNC or with NIPAM–Kemptide–PEG/DNA at a C/A ratio of 1.0 for 6 or 24 h at 37 °C. The levels of plasmid DNA in HepG2 cells were estimated by quantitative real-time PCR. Amounts of plasmid DNA were standardized against total DNA amounts in HepG2 cells. Data represent mean ± standard errors ($n = 3$).

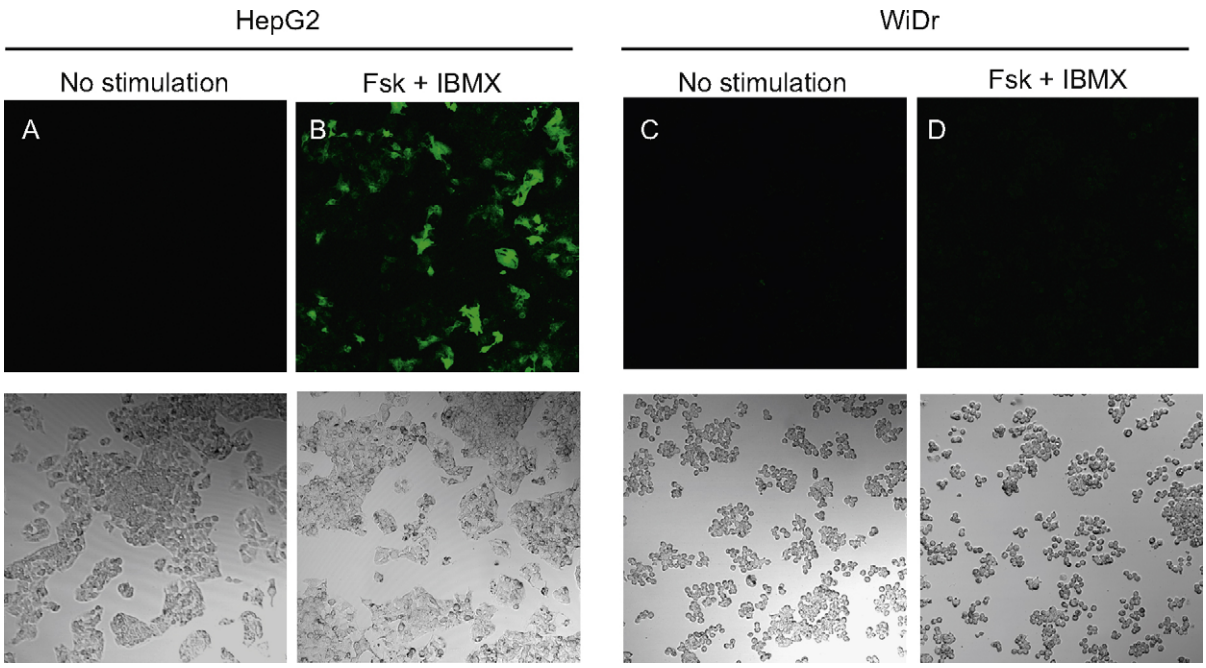

Fig. 5. Gene expression in HepG2 and WiDr cells after the transfection of NIPAM–Kemptide–PEG/EGFP-encoding DNA/BNC complex. (A) HepG2 cells were incubated with the complex for 24 h. The medium was changed and the fluorescence was observed 48 h after transfection. (B) The same experiment as (A) with PKA stimulation. (C) The same experiment as (A) was carried out in WiDr cells. (D) The same experiment as (C) with PKA stimulation. For PKA stimulation, the cells were treated with Fsk (10 μM) and IBMX (100 μM) for 24 h after transfection.

lum (ER) membrane-derived phospholipid vesicle. Therefore, the NIPAM–Kemptide–PEG/DNA complex coated with liposome can effectively combine with the phospholipid vesicle of the BNC. The diameter of complexes after combining polymer complex was measured by DLS. The diameter complex/liposome/BNC was 183 ± 57 nm.

3.5. Evaluation of DNA uptake efficacy in cells using real-time PCR

We evaluated whether the NIPAM–Kemptide–PEG/DNA/BNC complex is able to deliver DNA into specific target cells. The NIPAM–Kemptide–PEG/DNA/BNC complex prepared at a C/A ratio of 1.0 was incubated with HepG2 cells. After 6 or 24 h, cells were lysed and real-time PCR was performed. After 24 h, the amount of DNA mediated by BNCs was 6-fold higher compared to that in the control sample (Fig. 4). These results indicate that the NIPAM–Kemptide–PEG/DNA complex, combined with BNCs, is specifically taken up into HepG2 cells.

3.6. Delivery of NIPAM–Kemptide–PEG/DNA/BNC complex into cells

For the practical use of our system, the delivery of the polymer complex into target cells is very important. Thus, we applied the NIPAM–Kemptide–PEG/DNA/BNC system to living cells. To stimulate intracellular PKA, the cell sample was treated with Fsk (10 μM) and IBMX (100 μM). It is known that Fsk activates adenylate cyclase and produces cAMP and that IBMX inhibits cAMP-degrading phosphodiesterase, resulting in the activation of intracellular PKA. When the complex at a C/A ratio of 1.0 was added to unstimulated HepG2 cells, no EGFP-derived fluorescence was detected. In the HepG2 cells stimulated with Fsk and IBMX, however, EGFP-derived fluorescence was detected 48 h after transfection (Fig. 5). We examined whether PKA can be activated in HepG2 and WiDr cells by PKA stimulation. An increase in the PKA activity was identified from HepG2 and WiDr cells in the presence of PKA stimulation [Fsk (10 μM) + IBMX (100 μM)] by western blotting (Fig. 6).

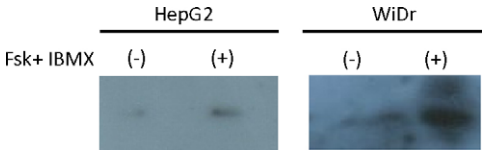

Fig. 6. Western blot analysis of lysate prepared from HepG2 and WiDr cells in the absence or presence of PKA stimulation [Fsk (10 μM) + IBMX (100 μM)]. The lysates were immunoblotted with anti-phosphoPAK (Thr197) serum and the reacting proteins were visualized by chemiluminescence.

These results indicate that NIPAM–Kemptide–PEG/DNA/BNC complexes are successfully delivered into HepG2 cells and that NIPAM–Kemptide–PEG/DNA actually regulates gene expression in living cells through PKA signals. On the other hand, no fluorescence in human colon cancer cells (WiDr) was detected from either PKA-stimulated cells or non-stimulated cells. Thus, combination of D-RECS with BNC may be a promising new strategy for gene therapy of hepatoma.

Acknowledgments

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