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Visualization of transfection of hepatocytes by galactosylated chitosan-graft-poly(ethylene glycol)/DNA complexes by confocal laser scanning microscopy

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Abstract

Dual-labeled galactosylated chitosan-graft-poly(ethylene glycol) (PEG) (GCP)/DNA complexes were prepared and their hepatocyte-specific delivery and cellular distribution were investigated by confocal laser scanning microscopy (CLSM). The complexes were transfected into hepatocyte through specific interaction of galactose moiety of the GCP and asialoglycoprotein receptors (ASGPR) of the hepatocytes. The GCP/DNA complexes taken up by the hepatocytes were rapidly released into the cytoplasm, but nuclear trafficking of the released complexes was slow and rate-limiting process. The more efficient transfection of the complex occurred in the human-derived HepG2 cells than in primary hepatocytes.

Keywords: Chitosan; Galactose; PEG; Nuclear trafficking; Confocal laser scanning microscopy; Hepatocyte

1. Introduction

The gene delivery using non-viral vectors has already gained increased interest (Remy et al., 1995; Kukowska-Latallo et al., 1996; Chan et al., 2000). Various ligands for cellular receptors, including transferrin, viral proteins, asialoorosomucoid, have been used for targeting moiety of gene carriers (Wu and Wu, 1987; Wagner et al., 1990). The gene delivery via the asialoglycoprotein receptors (ASGPR) is known as one of the effective gene delivery systems into the liver (Plank et al., 1992; Niidome et al., 2000; Sagara and Kim, 2002). It has been widely thought that non-viral gene therapy can overcome several problems inherent to currently used viral vector-mediated therapy, including immune response, toxicity, restricted targeting of specific cell types, limited DNA carrying capacity, recombination and high cost (Crystal, 1995; Luo and Saltzman, 2000). Non-viral vector as an alternative to viral vector, however, still has some problems such as non-specificity, cell toxicity, biodegradability, stability and especially, low transfection efficiency. Many researchers are currently dedicated to optimizing non-viral vectors.

In previous studies (Park et al., 2000; Park et al., 2001), galactosylated chitosan (GC)-*graft*-dextran or poly(ethylene glycol) (PEG) were synthesized and characterized as hepatocyte-targeting gene carriers. The complexes were stable and non-toxic. Also, the transfection of the complexes only occurred in the

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cells with ASGPR, indication of receptor-mediated delivery of the DNA into the hepatocyte. But transfection efficiency of the carriers is still low compared with that of lipofection.

In this study, intracellular trafficking of the GC-*graft*-PEG/DNA complex (abbreviated as GCP/DNA complex) was visualized by confocal laser scanning microscopy (CLSM) for the mechanism of the transfection after rhodamine was labelled into the chitosan and fluorescein isothiocyanate (FITC) was labelled into the plasmid.

2. Materials and methods

2.1. Materials

Chitosan of low molecular weight (10 kDa and degree of deacetylation: 90.8%) was kindly donated by JAKWANG Co., Ltd. (Ansung, Korea). Rhodamine-β-isothiocyanate (R-ITC) was obtained from ICN Biomaterials Inc. (Ohio, USA). Fluorescein isothiocyanate was purchased from Dojindo Laboratory (Tokyo, Japan). All other reagents were used without further purification. pEGFP-N2 (4.7 kb) encoding green fluorescent protein driven by immediate early promoter of CMV was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The 5.3-kb pair expression vector pGL3-control (Promega, Madison, USA) contains luciferase gene driven by SV40 promoter and enhancer. The plasmids were propagated in *Escherichia coli* and purified by chromatography (Megaprep Kits, Qiagen, Chatsworth, CA).

2.2. Synthesis and R-ITC-labeling GCP

The GC, chitosan-*graft*-PEG (CP) and GCP were synthesized as previously described (Park et al., 2001). The composition of galactose group and PEG in the GCP determined by NMR spectroscopy (Bruker, 600 MHz, Germany) was 7.4 and 3.6 mol%, respectively.

R-ITC was labeled to GCP. One hundred microliters of GCP was dissolved in 9 ml of sodium bicarbonate buffer (0.2 M, pH 9.0). Ten miligrams of R-ITC dissolved in 1 ml of *N*,*N*-dimethylformamide (DMF) was slowly dropped to GCP solution with stirring, and the reaction mixture was incubated for 4 h at room temperature with continuous stirring. R-ITC-conjugated GCP (R-GCP) was separated from unreacted R-ITC using gel filtration chromatography (Sephadex G-75, Sigma) and lyophilized. The amino group of GCP was determined by ninhydrin assay and the amount of R-ITC was determined according to free R-ITC calibration curve. The ratio of R-ITC conjugated to GCP was 1.2 μmol% per one amino group.

2.3. FITC labeling of plasmid

FITC-labeled plasmid was prepared by Ishii's method (Ishii et al., 2000). Briefly, FITC (0.257 mmol) was reacted with 2-(4-aminophenyl)-ethylamine (0.257 mmol) in 1.5 ml of DMF overnight at 25 °C under stirring, resulting in FITC-aniline. FITC-diazonium salt was prepared by reacting FITC-aniline (25.7 μmol in 150 μl of DMF) with sodium nitrite (110 μmol) in 2 ml of 0.5 M HCl for 5 min at 0 °C under stirring. The reaction was quenched by adding 1 ml of 1 M NaOH. Then, the solution of FITC-diazonium salt (25 μmol) was mixed with the solution of plasmid (2 mg) in 15 ml of 0.1 M borate buffer (pH 9.0). The reaction was carried out for 15 min at 25 °C under stirring. The FITC-labeled plasmid (F-plasmid) was isolated by ethanol precipitation and gel-exclusion chromatography (Sephacryl S-200, Amersham Pharmacia Biotech). The average number of FITC linked to one plasmid determined by absorbance at 260 nm and fluorescence intensity (Ex = 495 nm and Em = 520 nm) was 6.9. The ordered structure of the F-plasmid confirmed by 1% agarose gel electrophoresis was the same as non-labeled plasmid.

2.4. Complex formation of R-GCP/F-plasmid

Complexes were induced to self-assemble in 10 mM phosphate buffered saline (PBS, pH 7.4) containing 150 mM NaCl by mixing plasmid (30 μg/ml) with appropriate polymer solution at fixed charge ratio 5. DNA concentration was adjusted to 10 μg/ml with PBS and left standing at room temperature before use. Complex formation was confirmed by electrophoresis on a 1.0% agarose gel with Tris-acetate (TAE) running buffer at 100 V for 30 min.

2.5. Dynamic light scattering (DLS) measurement

Particle size and distribution of the complex were determined using an electrophoretic light scattering spectrophotometer (ELS 8000, Otsuka Electronics Ltd., Osaka, Japan) with a 90° scattering angle at 25 °C.

2.6. Hepatocyte-specific delivery of GCP/plasmid complex

Hepatocyte-specific delivery of the complex was evaluated using HepG2 cells which express ASGPR and HeLa and CT-26 cells which do not express ASGPR. HepG2 and HeLa were grown in media containing 10% fetal bovine serum at 37 °C under 5% CO_2 atmosphere. HeLa, CT-26 and HepG2 cells were seeded at a density of 5×10^4/ml in 22 mm culture plate and grown to 60–70% confluency. GCP/pEGFP complexes diluted with serum-free media were added to culture plate and incubated for 6 h at 37 °C under a 5% CO_2 atmosphere. The serum-free media were then replaced with fresh media containing serum and incubated for additional 3 days at 37 °C under 5% CO_2 atmosphere. After incubation, the cells transfected with pEGFP were observed under inverted fluorescent and phase-contrast microscopes.

The plasmid DNA (1 μg) was diluted in 200 μl of Dulbecco's modified Eagle Medium (DMEM). The 1,2-bis(oleoyloxy)-3-(trimethylammonium)propane (DOTAP) (10 μg) was diluted in 200 μl of DMEM. The diluted DNA and DOTAP were combined and incubated at room temperature for 15–20 min. Cells with transfection reagents were incubated for 4 h according to the manufacturer's instructions. Transfection medium was then replaced with growth medium containing 10% FBS. Cells were cultured for an additional 24 h before gene expression was determined.

Receptor-mediated gene delivery of GCP/plasmid complexes was confirmed by the assay of enzyme-dependent light production using a luciferase assay kit (Promega, Madison, WI) for checking involvement of ASGPR. GCP/pGL3 complex, CP/pGL3 complex, and GCP/pGL3 complex with free galactose (10 μM) were transfected into HepG2 cells. The cells were washed twice with PBS and incubated at room temperature for 10 min in the presence of 100 μl of lysis buffer (Promega) and then centrifuged at 12,000 × g. Twenty microliters of each sample was placed in a 5-ml polystyrene test tube and the tubes were then loaded into an automated luminometer Autolumat LB953 (EG & G Derthold, Germany). At the time of measurement, 100 μl of luciferase substrate was automatically injected into each sample, and total luminescence was measured over a 10-s time interval. Output is quantitated as relative light units (RLU). Protein concentration in the supernatant was determined by BCA protein assay reagent (Pierce, Rockford, IL). Luminescence detected was standardized per mg protein present in the supernatant and the luminescence data were expressed as relative values (%) compared with the luminescence value of GCP/pGL3 complex.

2.7. Primary hepatocyte isolation and culture

The female ICR mice employed in this study were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The liver was perfused with 1.25×10^{-2} wt.% collagenase solution. After the liver had been excised, parenchymal hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50 × g for 90 s. The dead parenchymal hepatocytes were removed by density gradient centrifugation on Percoll (Pharmacia, Piscataway, NJ). The viable parenchymal hepatocytes were suspended in Williams' E (WE) medium containing antibiotics.

2.8. Intracellular localization of R-GCP/F-plasmid complexes

The parenchymal hepatocytes were plated on collagen-coated glass coverslips in six-well plates (Iwaki Glass Co., Tokyo, Japan) at 2×10^5 cells per well. The hepatocytes were incubated at 37 °C for 3 h. Then, the old medium was removed and serum-free WE medium containing R-GCP/F-plasmid complexes was added to cells. Cells were rinsed and treated with 1 ml of ethanol for 30 min at −20 °C. After being rinsed twice with 0.1 M PBS, the coverslips were enclosed in 1 ml of glycerol and visualized by confocal laser scanning microscope (Micro Systems LSM 410, Carl Zeiss, Germany). Gallery mode of optical sections was used for checking internalization of complexes into cells (Aubele et al., 1997; Cho et al., 2001). Primary hepatocytes were treated immediately after attachment of the cells to plates

in order to perform the parenchymal hepatocytes uptake study of R-GCP/F-plasmid complexes in better cellular conditions because proliferation of the hepatocytes in vitro is extremely limited although hepatocytes have tremendous capacity to proliferate in vivo (Michalopoulos and Pitot, 1975).

In case of CP/F-plasmid complex or F-plasmid control, the similar transfection procedures were performed except insertion of propidium iodide (PI) (1 μg/ml) treatment as fluorescent stain for chromosome.

HepG2 (human hepatocellular carcinoma cells) was also used for evaluation of cell type dependence and was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 wt.% FBS. The cells were plated on the collagen-coated coverslips at a density of 2.5×10^4 cells/cm^2. One day after incubation, the old medium was removed and serum-free DMEM containing R-GCP/F-plasmid complexes was added to cells. The cells were treated in the similar way with primary hepatocytes and visualized by confocal laser scanning microscope.

3. Results and discussion

The efficiency of DNA transfection by non-viral vector is dependent on several steps including adsorption of the transfection complex to the cellular surface, uptake by the cell, escape from endosome and/or lysosome, nuclear translocation, and nucleic acid expression (Davis, 2002). Several factors affecting each step in these cellular uptake and trafficking as well as improved vectors with rational chemical design require a better knowledge of the multi-stage processes by which non-viral vectors promote transgene expression (Brown et al., 2001). The study of intracellular trafficking of chitosan-based gene therapy is a prerequisite for improved transfection. Indeed, the mechanism has not been clear yet, although transfection efficiencies have been mentioned in many papers (Mao et al., 2001).

The particle sizes and their distributions of GCP/plasmid complexes with various charge ratios were measured. Unlabeled plasmid was used in this study. The particle sizes of GCP/plasmid complexes had uniform distribution and decreased with increasing charge ratio of GCP to DNA and had a minimum value around 27 nm at the charge ratio of 5 as shown in Fig. 1, which means that GCP/plasmid complexes have the size distribution suitable for receptor-mediated endocytosis of hepatocytes.

The efficacies of GCP/plasmid complex in gene transformation of HepG2 cells bearing ASGR on cells and HeLa and CT-26 cells without ASGRs are shown in Fig. 2. HepG2 cells, which were transfected

(a)

Charge Ratio (C/D)	Average Sizes (nm)
C/D = 1	91.9 ±18.9
C/D = 3	57.3 ± 9.6
C/D = 5	27.0 ± 2.8
C/D = 7	61.4 ± 9.5
C/D = 10	87.7 ± 14.7

(b)

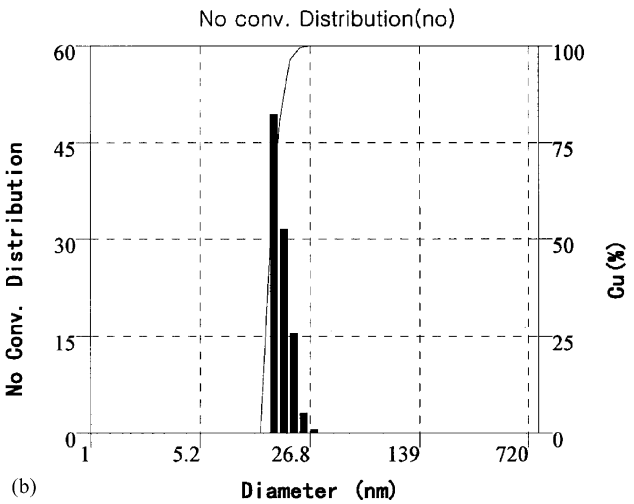

Fig. 1. The particle sizes (a) of GCP/DNA complexes with various charge ratios and particle size distribution profile (b) of representative GCP/DNA complex (charge ratio: 5).

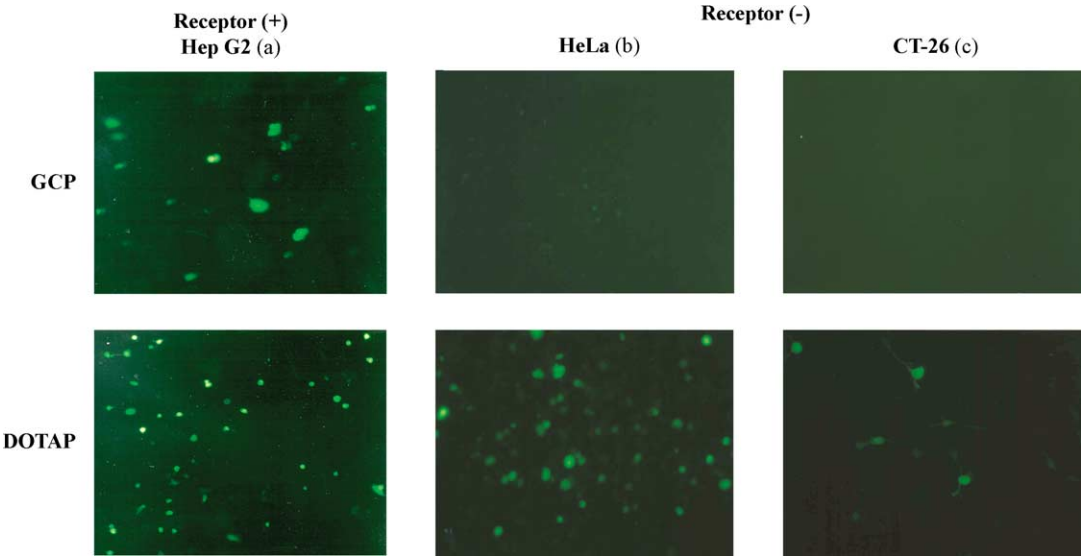

Fig. 2. Fluorescence micrographs of HepG2 (a), HeLa (b), and CT-26 (c) transfected with GCP/DNA or DOTAP/DNA complexes.

with GCP/DNA complex and DOTAP/DNA one as a control, efficiently produced green fluorescence under UV irradiation, while HeLa and CT-26 cells were transfected with DOTAP/DNA but not with GCP/DNA complex, suggesting that GCP/DNA complex transfects the ASGPR-bearing cells selectively through the ASGPR.

ASGPR-mediated gene delivery of GCP/pGL3 complex was also confirmed by competition assay (Fig. 3). Luciferase activity of CP/pGL3 complex

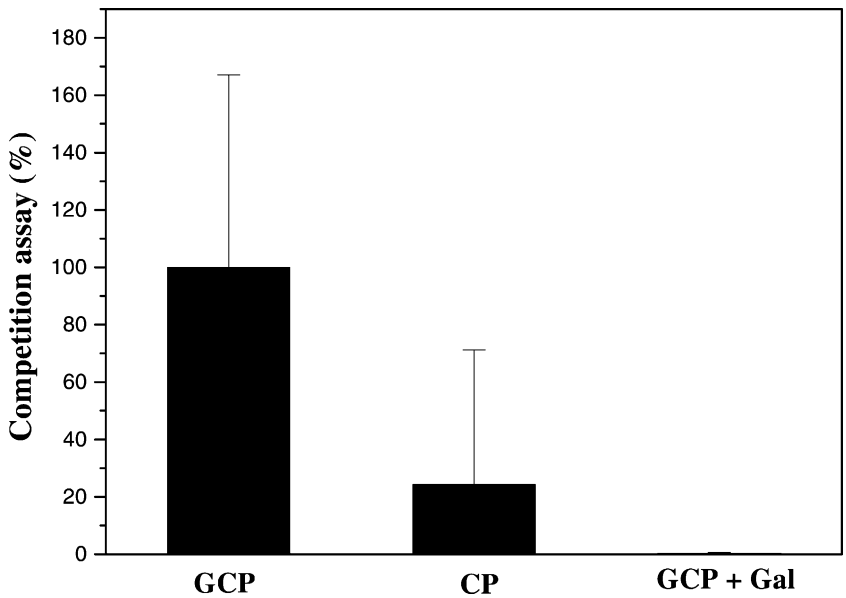

Fig. 3. Luciferase gene delivery to HepG2 cells using GCP, CP, and GCP with free galactose (10 μM). The data were represented as the mean values ± S.D. ($n = 3$) of values measured.

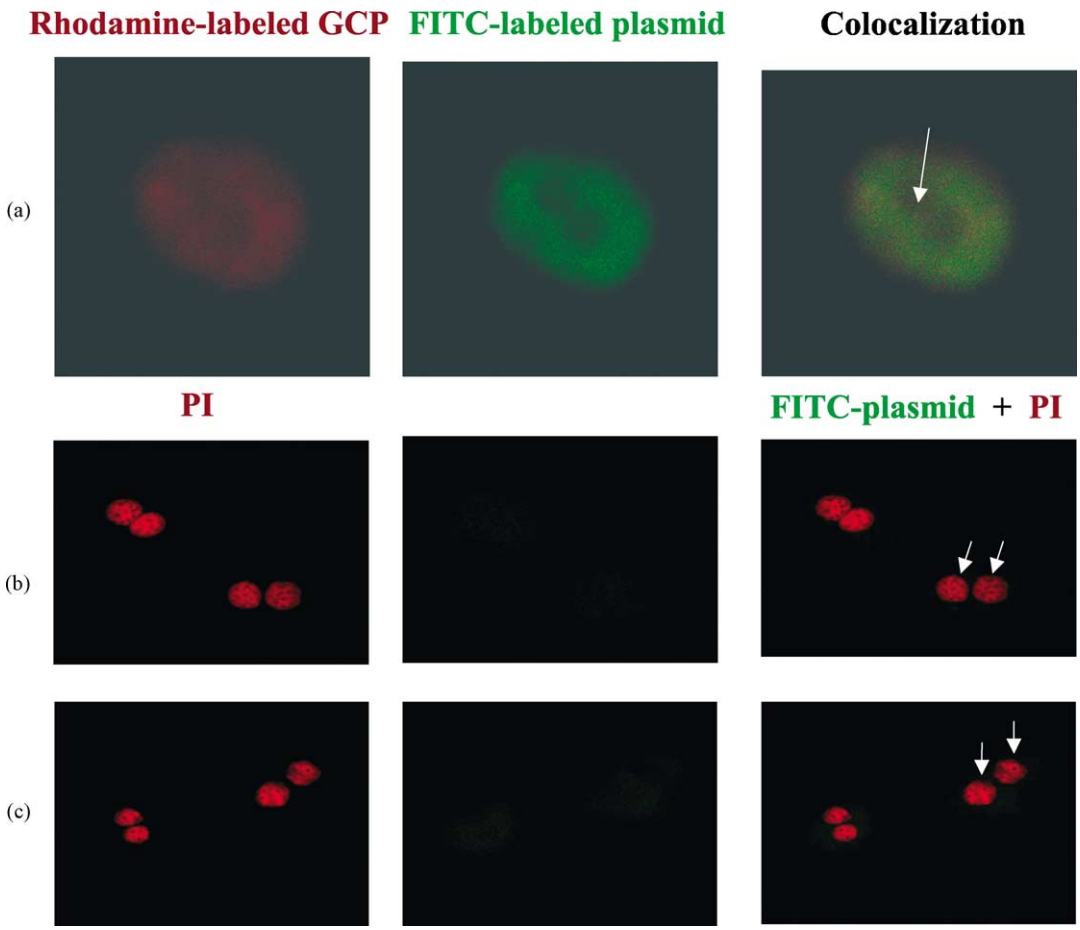

Fig. 4. Confocal laser scanning microscopy of R-GCP/F-plasmid complex after 1 h (a), CP/F-plasmid complex after 3 h (b), and F-plasmid control after 3 h (c) transfected into primary hepatocytes (charge ratio: 5 and concentration of plasmid: 10 μg/ml). The arrow indicated nucleus of the cell.

without galactose ligand showed significantly reduced value (about 25%) compared with the original value of GCP/pGL3 complex. Also, co-incubation of excess free galactose blocked luciferase gene delivery of GCP to HepG2 cells, indicating that gene delivery using GCP occurred by binding to ASGPR.

The hepatocyte-specific delivery of rhodamine-labeled GCP (R-GCP)/fluorescein-labeled plasmid (F-plasmid) complex was compared with those of CP/F-DNA one or F-plasmid using CLSM (Fig. 4). Cellular uptake of R-GCP/F-plasmid complexes occurred rapidly after 1 h, while that of CP/F-plasmid one or F-plasmid alone almost never occurred, indicating that receptor-mediated endocytosis by interaction between galactose ligands of the GCP and ASGPR of the hepatocytes is the major route. Also, this ASGPR-mediated endocytosis took place considerably even at 30 min after addition of the complex to the cell and continued to accumulate mainly in the cytoplasm by 3 h post-incubation (data not shown), suggesting that uptake of galactose-carrying complexes by ASGPR is both high-affinity and high-capacity processes. The complexes did not concentrate in multiple spots corresponding to intracellular vesicles and were diffused uniformly into the cytoplasm. This may partly result from rapid release of the complexes from endosomal compartments by the fusogenic property of PEG in the complex (Reid and Rand, 1997). In some places, the colocalization of R-GCP and F-plasmid was detected, indication of F-plasmid still bound to

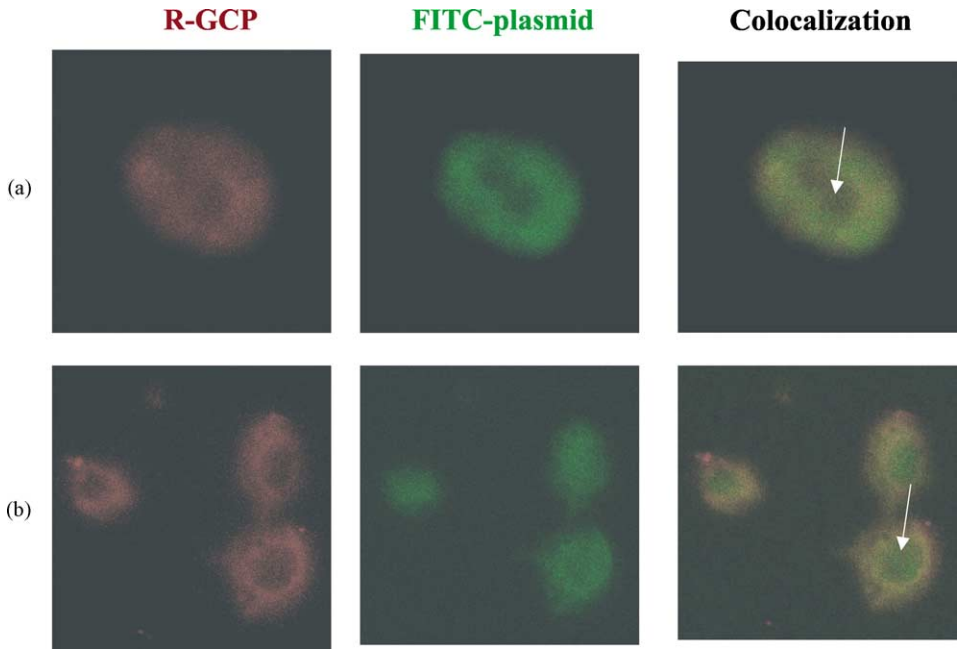

Fig. 5. Comparison of transfection of R-GCP/F-plasmid complex after 1 h between: (a) ICR primary hepatocytes and (b) HepG2 cells using CLSM. The arrow indicated the nucleus of the cell.

R-GCP. Therefore, the slow nuclear trafficking of plasmid followed by rapid escape from endosome is thought to be the rate-limiting step and may cause low transgene expression in GCP-mediated transfection.

Cell type dependence of transfection was evaluated using primary hepatocytes and HepG2 human hepatocarcinoma cell line (Fig. 5). Although the number of ASGPR in the primary hepatocytes are more than those of HepG2, the uptake and release into the cytoplasm of R-GCP/F-plasmid complexes for the hepatocytes took place very slowly compared with those of HepG2. Also, the complexes had poorer nuclear trafficking in primary hepatocytes than in HepG2. Furthermore, primary hepatocytes had lower luciferase expression compared to HepG2 cells, when transfected with GCP/pGL3 (Promega, Madison USA) complexes (data now shown). It is generally accepted that the transfection efficiency of the cell line is higher than that of primary cell due to mitosis of cell, indication of easy cell division of cell line.

It was checked whether the complex existed in the cytosol or was only attached to the plasma membrane of the cell (Fig. 6). The cell was sectioned by CLSM, and each fluorescence distribution was ob-

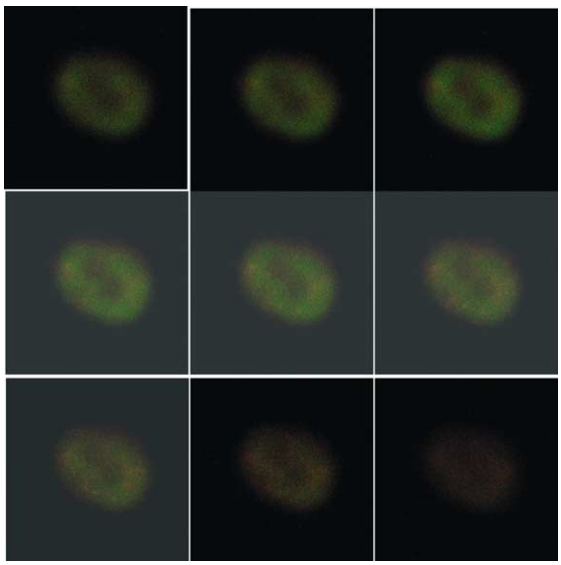

Fig. 6. Gallery-mode observation of R-GCP/F-plasmid complexes transfected into ICR primary hepatocytes.

served. The fluorescence of the complexes was uniformly distributed in the cytosol, rarely in the nucleus.

4. Conclusions

The development of successful gene delivery systems depends on the rational design of the carriers which requires better understanding of the transfection mechanism. We prepared dual-labeled GCP/DNA complexes. The GCP/DNA complexes were only transfected into hepatocytes through ASGPR-mediated endocytosis. Poor nuclear trafficking followed by rapid uptake of the complexes which resulted in low transfection efficiency must be overcome in our study.

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