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Research paper

Delivery of *all trans*-retinoic acid (RA) to hepatocyte cell line from RA/galactosyl α-cyclodextrin inclusion complex

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Abstract

All trans-retinoic acid (RA) plays a role in regulation of P450RAI gene expression. In this study, hepatocyte cell line (HepG2) was used to study an effect of RA released from RA/galactosyl α-cyclodextrin (GCD) inclusion complex on regulation of P450RAI gene expression. A delivery system composed of RA/GCD inclusion complex was applied because RA is poorly water soluble, and organic solvents used to dissolve it often interfere with cytotoxicity. Solubility of RA in water was increased by forming complex with GCD. Inclusion complex between GCD and RA was checked by ^{1}H-nuclear magnetic resonance, Fourier transformation infrared (FT-IR) spectroscopy and X-ray diffraction (XRD). The chemical shifts of the interior and exterior GCD protons in the presence of RA indicated that the RA was included within the GCD macrocycle cavity. The carbonyl band of RA and crystalline peak of RA in RA/GCD inclusion complex disappeared from FT-IR and XRD measurements, respectively, indication of inclusion complex between RA and GCD. From the observation of fluorescence micrograph of hepatocytes and flow cytometry measurement of HepG2, the internalization of fluorescein isothiocyanate-GCD by the hepatocyte occurred. Gene expression of P450RAI in HepG2 by delivery of RA from RA/GCD complex was observed.

Keywords: *All trans*-retinoic acid; P450RAI; Galactosyl α-cyclodextrin; Inclusion complex

1. Introduction

Systemic drug administration has several problems such as biodistribution of drugs throughout the body, the lack of drug-specific affinity toward a pathological site, non-specific toxicity and other side effects resulting from high doses. An attractive strategy to overcome the problems is to specifically deliver the drugs to the defined target cells [1–5]. Galactose was recognized by asialoglycoprotein receptor (ASGPR) on hepatocytes [6–8]. Galactose bearing soluble polymeric drug-carriers has been reported as a ligand for hepatocyte-targeting carriers in vivo [9,10]. These polymer-based delivery systems, functioning through receptor-mediated endocytosis show potentials as specific and target-oriented delivery systems.

Cyclodextrins (CDs) are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose containing a relatively hydrophobic cavity, a wide variety of guest molecules forming inclusion complexes. The inclusion complexation of drugs with CDs has been extensively studied for improving definite characteristics, such as solubility in water, dissolution rate, stability and bioavailability. CD derivatives were used to improve stability and dissolution of drugs by forming inclusion compounds [11,12]. Also, dexamethasone/galactose-branched-β-cyclodextrin complex was distributed to liver tissue significantly more than the dexamethasone/glucose-branched-β-cyclodextrin [13]. Furthermore, β-cyclodextrin (βCD) with galactose end arms induced better recognition by a cell-linked galactose-specific lectin [14].

All trans-retinoic acid (RA), an endogenous metabolite of vitamin A, plays an important role in many physiological processes [15]. An RA-inducible P450 cytochrome, termed P450RAI, with specificity for RA metabolism was defined as CYP26 and became the first representative of a novel P450 family [16]. CYP26 is now characterized as an RA-inducible hydroxylase that specifically catalyzes hydroxylation of *all-trans* RA, but does not recognize the 13-*cis*

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and 9-*cis* isomers as substrates [17,18]. In the adult mouse, CYP26 appears to be expressed in liver and brain; interestingly, it was induced several fold by RA in the liver but not in the brain [19].

In this study, inclusion complex between galactosyl α-cyclodextrin (GCD) and RA was checked by ^{1}H-nuclear magnetic resonance (^{1}H-NMR), Fourier transformation infrared (FT-IR), and X-ray diffraction (XRD) studies. Specific interaction between galactose ligands of GCDs and ASGPR of hepatocytes [or hepatocyte cell line (HepG2)] was checked by fluorescence microscopy and flow cytometry. Also, gene expression of P450RAI in HepG2 by RA from RA/GCD complex was checked in an in vitro assay by reverse transcriptase-polymerase chain reaction (RT-PCR) to distinguish RA/α-cyclodextrin (αCD), not reported elsewhere.

2. Materials and methods

2.1. Chemicals

RA was purchased from Sigma Chemicals (St Louis, MO). αCD and GCD were kindly provided by Bio Research Corporation of Yokohama (Yokohama, Japan). The chemical structures of GCD and RA are shown in Fig. 1. All other chemicals were reagent-grade and were used without further purification.

2.2. Solubility studies

The solubility test was performed according to the method of Higuchi et al. [20]. Briefly, an excess amount (1 mg) of RA dissolved in 1 ml ethanol sheltered from light

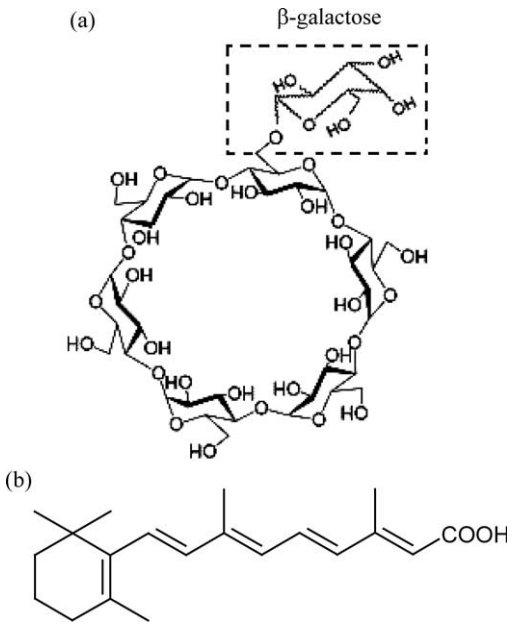

Fig. 1. Molecular structures of GCD (a) and RA (b).

was added to the screw capped vials containing the aqueous solutions of the different CDs (αCD or GCD) (5 ml) with various concentrations (2.0×10^{-3} to 1.4×10^{-2} M) at pH 7.4. The vials were stirred at room temperature until equilibrium solubility was reached (about 1 week). The contents were filtered through an Advantec MFS. 0.45 μm-filter, and then the solution was freeze-dried.

The stability constants of higher order complexes ($K_{1:1}$ and $K_{1:2}$) were calculated according to the optimization technique [21]. After thermodynamic equilibrium was reached, changes in RA solubility (S_0) were plotted as a function of the CD concentration.

2.3. ^{1}H-NMR spectroscopy measurement

GCD and RA/GCD complex were dissolved in D_2O whereas RA and physical mixture of RA and GCD were dissolved in dimethylsulfoxide-d_6 (DMSO-d_6) for NMR measurement. ^{1}H-NMR spectra were measured at 25 °C with AVANCE 600 spectrometer operating at 600 MHz.

2.4. FT-IR spectroscopy measurement

Samples were prepared as KBr disks using a hydrostatic press at a force of 5.2 T/cm^2 for 3 min. IR spectra were recorded using a Nicolet Magna 550 series II FT-IR spectrometer.

2.5. Wide angle X-ray diffractometry (WAXD) measurement

XRD spectra were measured as powder using a D5005 powder X-ray diffractometry with 45 mA power and 40 kV (Bruker Siemens, Germany). The samples were analyzed in the 2θ angle range of 5–35°.

2.6. Culture of HepG2

HepG2 was grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with glucose (4.5 g/l), 10% fetal bovine serum, and antibiotics (50 μg penicillin/ml and 100 μg streptomycin/ml). For general maintenance, cells were split every 3 days, seeded into tissue culture plates, and incubated in a humidified, 37 °C atmosphere of 5% CO_2. For treatment with samples, the number of cells per tissue culture 6-well plates was 3×10^5, grown for 24 h, and treated with RA, RA/αCD, RA/GCD complexes or DMSO (0.1%: v/v) prior to extraction of total RNA.

2.7. Isolation of primary hepatocyte

Hepatocytes were prepared by non-circulation perfusion of male ICR mouse liver with the two-step collagenase perfusion technique of Seglen [22]. Briefly, the male ICR mice (International Genetic Standard) employed in this study were purchased from Charles River Japan,

Inc. (Kanagawa, Japan). The liver was perfused with 1.25×10^{-2} wt% collagenase dissolved in Hanks' balanced salt solution. After the liver had been excised, parenchymal cells were separated from non-parenchymal cells by differential centrifugation at $50 \times g$ for 120 s. The dead parenchymal cells were removed by density gradient centrifugation on Percoll (Pharmacia, Piscataway, NJ). The viable parenchymal cells were suspended in a serum free Williams' E (WE) medium containing antibiotics (50 μg penicillin/ml, 50 μg streptomycin/ml, and 100 μg neomycin/ml).

2.8. Observation of fluorescence microscopy

Hepatocytes (5×10^4 cells/ml) were placed into a 24 well non-tissue plate precoated with collagen type I solution (10 μg/ml, pH 3.0 in HCl) in a humidified atmosphere of 5% CO_2 and 95% air for 3 h at 37 °C. Then, fluorescein isothiocyanate (FITC)-GCD or FITC-αCD was added to the above plate and incubated at 37 °C. Hepatocytes were observed by fluorescence microscopy (Olympus, Japan).

2.9. Measurement of flow cytometry

HepG2 (2×10^6 cells/ml) was incubated with 1 mg/ml of FITC-GCD (or FITC-αCD) in DMEM containing 0.1% BSA and 0.1 μg/ml of propodium iodide (PI) for 20 min on ice with tapping every 5 min. All cells were resuspended in 1 ml of buffer (0.1% BSA and 1 mM $CaCl_2$ in PBS) on ice to be measured by a flow cytometry (FACSCalibur; Becton Dickinson) after washing with the buffer three times. HepG2 region was selected by adjusting the forward scatter (FS) and the side scatter (SS). Surface-labeled HepG2 cells with FITC-GCD were selected from the region of PI-unlabeled HepG2 to remove cytoplasm-labeled damaged HepG2 cells.

2.10. RT-PCR analysis

Total cellular RNA was extracted from HepG2 attached onto tissue culture plates after dissolving in TRIzol Reagent (Life Technologies, Inc.) according to the manufacturer's instruction. RT-PCR was performed under standard condition using moloney murine leukemia virus reverse transcriptase (M-MLV RT) and *Taq* polymerase with 2 μg of RNA and 50 pmol of each primer. For RT-PCR detection of human P450RAI mRNAs a pair of oligonucleotide primers was designed in the corresponding cDNA sequences as follows: sense primers; 5′-TCTCTGATCACTTACCTGGG-3′ and antisense primers; 5′-CAGCATGAATCGGTCAGGAT-3′ (expected size: 332 bp) [23]. For the detection of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, 5′-AACAGCCTCAAGATCATCAGC-3′, and 5′-ATGGTACATGACAAGGTGCG-3′ (expected size: 760 bp) were used as sense primer and antisense primer, respectively. Thirty PCR cycles were performed at a temperature for primer annealing of 56 °C.

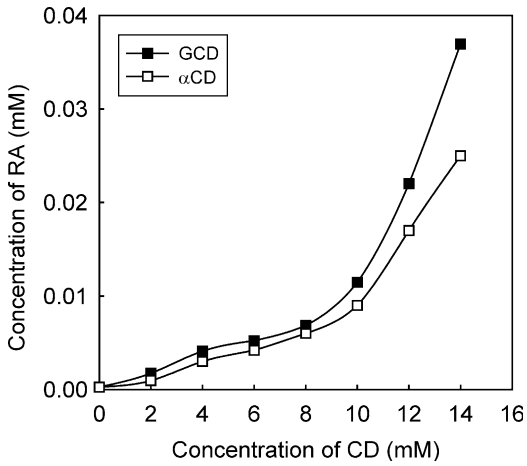

Fig. 2. Phase solubility diagram of RA with αCD (□) and GCD (■) at pH 7.4 and at room temperature. Each point is the mean of three experiments.

The amplified band was electrophoresed on a 1% agarose gel containing 0.1 μg/ml ethidium bromide. The gel was viewed and pictured under ultraviolet light.

3. Results

3.1. Physicochemical characterization

The phase solubility diagram for the complex formation between RA and the CDs (αCD or GCD) at pH 7.4 is presented in Fig. 2. The phase solubility diagram of RA with αCD and GCD showed the A_p-type according to Higuchi's method [21], indicating formation of 1:1 and 1:2 RA/CD complexes. These curves were similar to the results of Lin et al. [24]. For the 1:1 and 1:2 complexes, the calculated stability constants are listed in Table 1. The intrinsic solubility of RA was almost nil ($\approx 2.67 \times 10^{-7}$ M) [25]. However, the aqueous solubility of RA for RA/αCD and RA/GCD complexes was increased more than 93-, and 262-fold, respectively.

Fig. 3 shows ^{1}H-NMR spectra of RA (a), GCD (b) and RA/GCD complex (c). In Fig. 3(a), the peak corresponding to C20−H_3 of RA was observed at 2.3 ppm. As shown in Fig. 3(c), the peak of C20−H_3 of RA was still present although the C3−H and C5−H protons inside the cavity of GCD were slightly shifted upfield. The peaks [$\delta = 3.934$ (C3−H), 3.816 (C5−H), and 3.837 (C6−H)] of GCD in RA/GCD complex were not much displaced (average displacement: 0.011 ppm) in comparison with those of GCD itself [$\delta = 3.952$ (C3−H), 3.829 (C5−H), and 3.840

Table 1
The stability constants of RA/αCD and RA/GCD complexes

	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
RA/αCD	2757	558
RA/GCD	3899	739

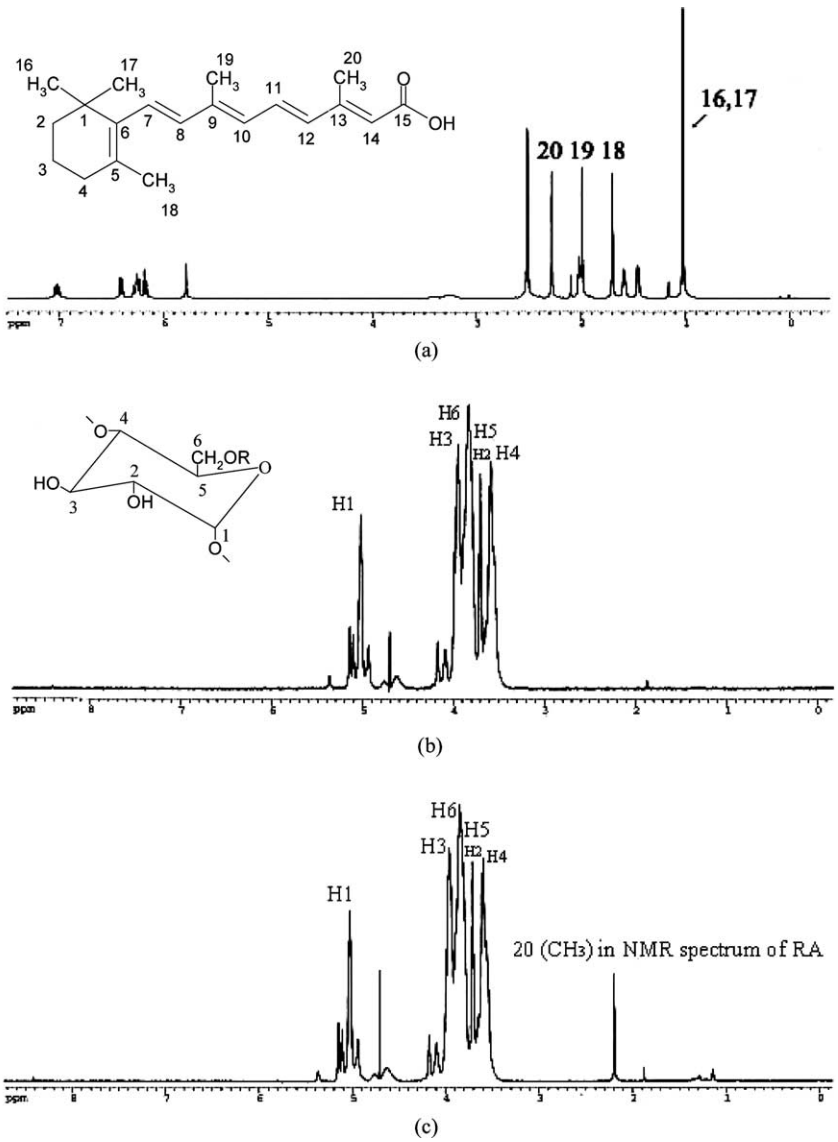

Fig. 3. ^{1}H NMR spectra of RA (a), GCD (b) and RA/GCD inclusion complex (c).

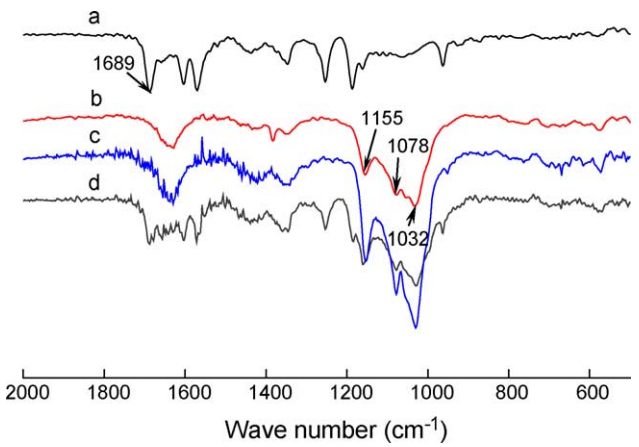

Fig. 4. IR spectra of RA (a), GCD (b), RA/GCD inclusion complex (c), and physical mixture of RA and GCD (d).

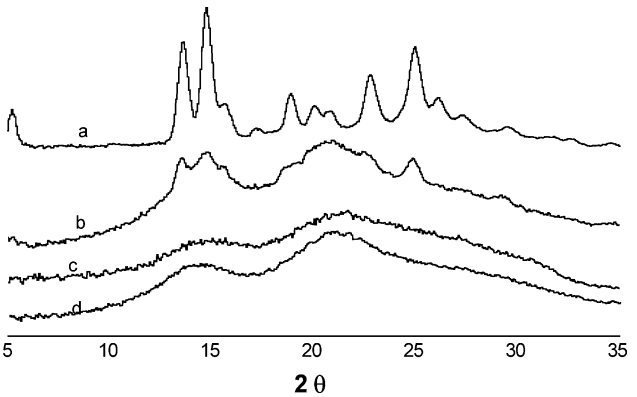

Fig. 5. XRD profiles of RA (a), physical mixture of RA and GCD (b), GCD (c), and RA/GCD inclusion complex (d).

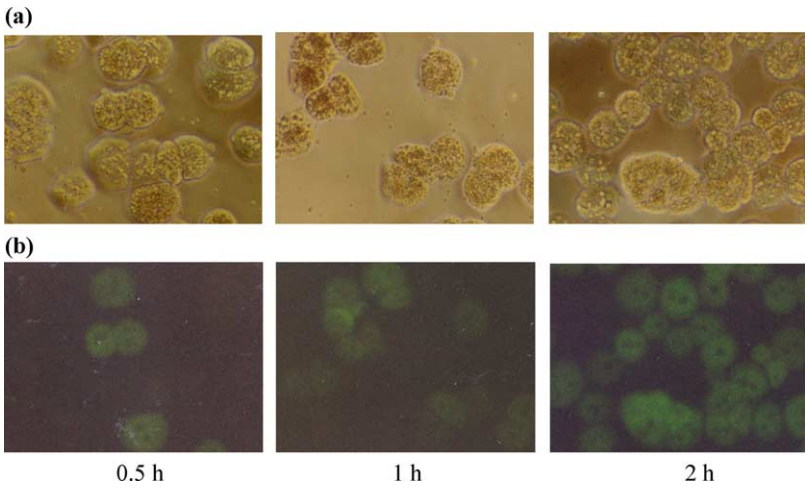

Fig. 6. Phase contrast (a) and fluorescence (b) micrographs of hepatocytes (magnification 100 ×). Hepatocytes (5×10^4 cells/ml) were cultured in 24-well plates coated with collagen type I and incubated with FITC-GCD according to the time.

(C6–H)] as shown in Fig. 3(b) and (c) owing to the weak interaction between RA and GCD.

The carbonyl band (1689 cm^{-1}) of RA [Fig. 4(a)] is characterized by peaks appearing between 1700 and 1680 cm^{-1}, which does not overlap with GCD peaks (1032, 1078, and 1155 cm^{-1}) [Fig. 4(b)] around 1200–1000 cm^{-1}. The FT-IR spectrum of the physical mixture in Fig. 4(d) appears like an addition of the respective spectra of each of the pure components as seen in Fig. 4(a) and (b). On the contrary, disappearance of the spectrum of RA could be seen in the FT-IR spectrum of the inclusion complex as shown in Fig. 4(c).

For further evidence of complex formation between RA and GCD, the XRD patterns of RA, GCD and the corresponding binary systems are represented in Fig. 5. The X-ray powder diffraction patterns of pure RA and GCD are shown in Fig. 5(a) and (c), respectively. The diffraction pattern of the physical mixture in Fig. 5(b) was found to correspond exactly to the simple sum of the raw materials diffractograms. On the other hand, the crystalline patterns of RA in the RA/GCD inclusion complex disappeared as shown in Fig. 5(d), indicating the formation of an inclusion complex between RA and GCD.

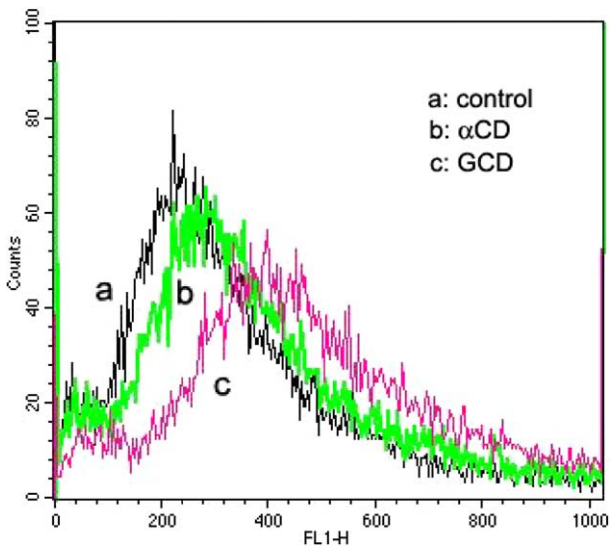

Fig. 7. Flow cytometry of HepG2 (2×10^6 cells/ml) incubated with FITC-αCD or FITC-GCD for 20 min on ice.

3.2. Interaction between GCD and hepatocyte

Phase contrast (a) and fluorescence (b) micrographs of hepatocytes against time after incubation with FITC-GCD are shown in Fig. 6. From the fluorescence micrograph of the hepatocytes, the internalization of FITC-GCD by the hepatocytes was increased with time. From the results of flow cytometry, fluorescence intensity of FITC-GCD was stronger than that of FITC-αCD as shown in Fig. 7.

3.3. Gene expression of P450RAI in HepG2

Human P450RAI gene in HepG2 was detected by RT-PCR after HepG2 was incubated with samples (RA, RA/αCD, or RA/GCD) in the vehicles (DMSO, αCD or GCD) for 6 h as shown in Fig. 8. Not only RA itself dissolved in DMSO but also the RA/CD-complexes were capable of

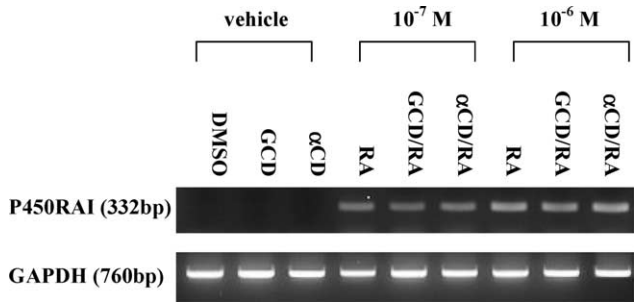

Fig. 8. RT-PCR analysis of human P450RAI mRNA expression in HepG2 cells (30 cycles). HepG2 (3×10^5 cells/ml) was grown for 24 h, and treated with RA itself or RA/CD complexes at 10^{-7} or 10^{-6} M for 6 h. Total RNA was extracted and then RT-PCR was performed using the primers for human P450RAI gene or human GAPDH gene.

inducing P450RAI gene expression in HepG2. RA/GCD induced P450RAI gene expression at almost the same level as RA itself although the difference of P450RAI gene expression between RA/αCD and RA/GCD was not found.

4. Discussion

CDs are well known to improve solubility of hydrophobic drugs by forming inclusion complex [11,12]. The solubility test is useful for checking inclusion complexation of poorly water-soluble drugs with CDs in water, because it gives not only the solubility of host molecules but also the stability constant of the complexes by analyzing the solubility curve [21]. As shown in Fig. 2, the solubility of RA was increased by formation of inclusion complex between RA and CDs (αCD or GCD). The solubility of RA for the RA/GCD complex was increased more than 262-fold whereas that of RA for the RA/αCD complex was increased more than 93-fold. This result can be explained by the increased aqueous solubility of GCD more than that of αCD [26]. Also, the reason can be explained by intermolecular hydrogen bonds among GCDs which appear to be a dominant factor in the water solubility [27]. The results suggested that it is possible to develop a parenteral formation and/or an aqueous oral formation of RA by using GCD although the biopharmaceutical properties of such a formulation would be necessary before its use.

NMR spectroscopy is at present the most useful tool for the study of CD complexes; initially only in solution, but recently also in the solid state [28]. As shown in Fig. 3, the C3–H and C5–H protons inside cavity of GCD in the RA/GCD complex were slightly shifted upfield, whereas the C3–H and C5–H protons were much displaced by the β-blockers/β-CDs and flavonoids/β-CD inclusion complexes [29,30]. The results indicated that inclusion between RA and the cavity of GCD really occurred through weak interaction between RA and GCD.

The most commonly reported FT-IR spectroscopic studies of CD complexes are of complexes with a guest bearing a carbonyl group [28]. For the RA/GCD complex, the RA peaks mostly disappeared in FT-IR spectrum of the RA/GCD complex as the same tendency of the results reported by Montassier et al. [25].

From disappearance of crystalline patterns of RA in the RA/GCD inclusion complex by XRD measurement, it is thought that the formation of an inclusion complex between RA and GCD which is similar to the result of Montassier et al. [25]. From physicochemical characterization of RA/GCD complex using solubility test, NMR, FT-IR and XRD measurements, it is suggested that the formation of an inclusion complex between RA and GCD occurred.

It is already reported that the affinity of ASGPR of hepatocyte with natural and synthetic oligosaccharides having non-reducing galactose residues occurred [31,32]. From the results of fluorescence microscopy and flow cytometry, it can be said that specific interaction between ASGPR of hepatocyte (or HepG2) and galactose moiety of GCD occurred although the interaction seems to be weak owing to a small number of galactose moiety in GCD.

As shown in Fig. 8, P450RAI gene was detected concentration-dependently in HepG2 incubated with RA itself as well as RA released from the inclusion complexes for 6 h. Difference of gene expression of P450RAI in HepG2 between RA/αCD and RA/GCD complexes was not found although specific interaction between GCD and hepatocyte (or HepG2) was observed by fluorescence microscopy and flow cytometry. The results suggest that RA released from the RA/GCD complex can regulate P450RAI gene expression in HepG2 as similar to RA itself [16], although difference of gene expression of P450RAI in HepG2 between RA released from RA/GCD (or RA/αCD) complexes and RA itself was not found within the time scale and concentration of RA chosen in this experiment. Further experimental condition should be found to distinguish between the systems.

5. Conclusions

The results of solubility test, ^{1}H-NMR, FT-IR and XRD demonstrated the effective inclusion complex formation between RA and CDs. Specific interaction between galactose ligands of GCD and ASGPR of hepatocytes was confirmed from fluorescence micrograph and flow cytometry, although difference of P450RAI gene expression in HepG2 cells between RA/αCD and RA/GCD complexes was not found.

Acknowledgement

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