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Receptor-mediated delivery of all-*trans*-retinoic acid (ATRA) to hepatocytes from ATRA-loaded poly(*N*-*p*-vinylbenzyl-4-*o*-β-D-galactopyranosyl-D-gluconamide) nanoparticles

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Abstract

All-*trans*-retinoic acid (ATRA) plays a role in regulating CYP26 gene expression in hepatocytes. Poly(*N*-*p*-vinylbenzyl-4-*o*-β-D-galactopyranosyl-D-gluconamide) (PVLA) nanoparticles have been used as hepatocyte-specific targeting candidates. The objective of this study was to investigate receptor-mediated delivery of ATRA using PVLA nanoparticles. ATRA-loaded PVLA nanoparticles were confirmed by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and powder X-ray diffraction (XRD). In the ^{1}H-NMR study, the proton signals of ATRA disappeared in the spectrum of ATRA-loaded PVLA nanoparticles in D_2O, whereas in dimethylsulfoxide-d_6, the spectrum seemed like an addition of the respective spectrum of each of the pure components. The crystalline peaks of ATRA disappeared in the XRD pattern of ATRA-loaded PVLA nanoparticles after ATRA was loaded into PVLA nanoparticles. In the measurement of size distribution, diameter of PVLA and ATRA-loaded PVLA nanoparticles in aqueous solution was 6.9 and 61.2 nm in number average, respectively. Flow cytometric analysis showed that the internalization of FITC-PVLA nanoparticles by hepatocytes in the absence of a competitive inhibitor was larger than preincubated with galactose. In reverse transcription–polymerase chain reaction (RT-PCR) analysis, ATRA-loaded PVLA nanoparticles induced CYP26A1 gene in hepatocytes in the absence of a competitive inhibitor but not preincubated with galactose. The results indicate that the ATRA-loaded PVLA nanoparticle can induce CYP26A1 gene in aqueous phase by an asialoglycoprotein receptor (ASGPR)-mediated delivery system.

Keywords: Poly (*N*-*p*-vinylbenzyl-4-*o*-β-D-galactopyranosyl-D-gluconamide); All-*trans*-retinoic acid; Nanoparticle; Galactose; Hepatocyte; CYP26A1; Asialoglycoprotein receptor

1. Introduction

All-*trans*-retinoic acid (ATRA), an active metabolic of vitamin A, plays an important role in a number of biological process, such as cell growth, differentiation of various organs through modulations of the transcription of various genes and apoptosis [1]. ATRA is the natural ligand for the retinoic acid receptors, and 9-*cis*-RA for the rexinoid receptors, although the latter compound binds to both receptor families. The specificity of interactions would also suggest the possibility that various retinoids, both natural and synthetic, may specifically be useful as drugs to combat diverse diseases [2]. The major pathway for ATRA inactivation is mediated by the microsomal cytochrome *P*450 enzyme system, which converts ATRA to 4-hydroxy-RA, 18-OH-RA, 4-oxo-RA, and other polar metabolites [3,4]. Recently, a novel cytochrome *P*450 enzyme, CYP26, with specific RA 4-hydroxylase activity was cloned [5–7]. CYP26 was found to be highly specific for the hydroxylation of ATRA but not for the hydroxylation of 9-*cis*-RA and 13-*cis*-RA [7,8]. The liver is a principal site of retinoid metabolism. Dietary

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vitamin A is transported from the intestine to liver and cleared rapidly into hepatocytes [9].

Naturally existing cell surface receptors provide convenient systems for transporting substances from the extracellular environment to the interior of cells. When such receptors are highly selective for certain cell types, the possibility exists for targeting biological agents of interest to specific tissues [10]. Parenchymal liver cells, hepatocytes, are the only cells that possess large numbers of high-affinity cell-surface receptors that can bind asialoglycoproteins (ASGP). ASGP possess clustered galactose residues for recognition and binding by ASGP receptors (ASGPR) [11].

In this study, receptor-mediated delivery of ATRA to hepatocytes using poly(*N*-*p*-vinylbenzyl-4-*o*-β-D-galactopyranosyl-D-gluconamide) (PVLA) nanoparticles was investigated. ATRA-loaded PVLA nanoparticles were confirmed by physicochemical analysis such as ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and X-ray diffraction (XRD). Also, the internalization of PVLA nanoparticles by hepatocytes was investigated by flow cytometric analysis, and CYP26A1 gene expression in hepatocytes by ATRA released from the ATRA-loaded PVLA nanoparticles was checked by reverse transcription–polymerase chain reaction (RT-PCR). PVLA is known to interact with hepatocytes [12,13] because galactose residues in the side chain of PVLA are recognized by ASGPR on the surface of hepatocytes [14]. Also, PVLA formed nanoparticles based on polymeric micelle in water due to the presence of hydrophobic polystyrene (PS) backbone and hydrophilic galactose moieties [15]. Receptor-mediated ATRA-loaded polymeric nanoparticle systems based on the targeting of ASGP to highly selective receptors on hepatocytes will be expected to introduce CYP26 gene expression. Also, the solubility in water and stability in light of ATRA will be increased after loading it into polymeric nanoparticles because polymeric nanoparticles known collectively as colloids can be used for solubilization, stabilization and delivery of challenging agents.

2. Materials and methods

2.1. Materials

PVLA (MW = 5×10^4) was prepared as previously described [16]. ATRA was supplied by Sigma Chem Co. (St. Louis, MO, USA). The chemical structures of PVLA and ATRA are shown in Fig. 1. All other chemicals were reagent grade and were used without further purification.

2.2. Preparation of ATRA-loaded PVLA nanoparticles

ATRA-loaded PVLA nanoparticles were prepared by a dialysis method as described previously [17]. Briefly, 0.1 mg of ATRA was dissolved in 3 ml of dimethylsulfoxide (DMSO) in a 20-ml sample vial and subsequently PVLA aqueous solution (10 mg/ml in 5 ml) was added dropwise to the above solution with stirring by a magnetic stirrer at 400 rpm for 10 min at room temperature. To form nanoparticles, the solution was dialyzed against distilled water using dialysis membrane

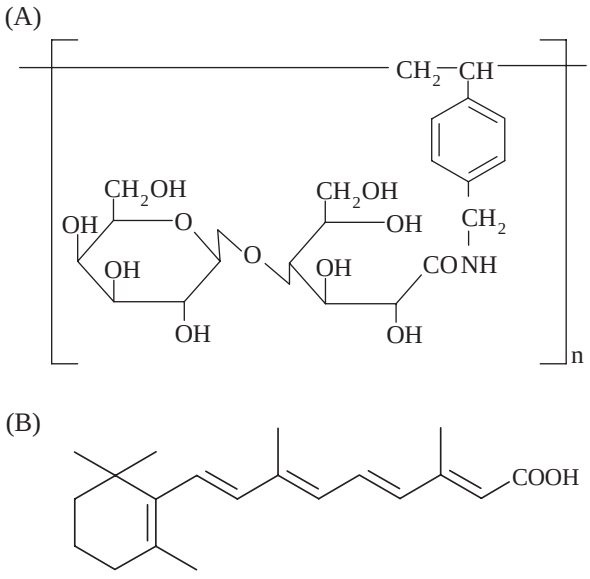

Fig. 1. Molecular structures of PVLA (A) and ATRA (B).

(MWCO: 6000) for 2 days at 4 °C. After dialysis, ATRA-loaded PVLA nanoparticles were filtered by through an Advantec MFS 0.2-μm filter, and then freeze-dried for the study of their characteristics. The loading concentration of ATRA was estimated by dissolving them in ethanol because only ATRA is soluble in ethanol. Their resuspension by ethanol was centrifuged at 10,000 rpm for 5 min and then spectrophotometrically assayed for ATRA concentration at 350 nm.

2.3. ^{1}H-NMR spectroscopy

To characterize ATRA-loaded PVLA nanoparticles, ^{1}H-NMR was measured using D_2O and DMSO-d_6. ^{1}H-NMR spectra were recorded at 25 °C using an Avance-600 NMR (Bruker, Germany) spectrometer operating at 600 MHz.

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

XRD patterns were measured as powder using a D5005 powder X-ray diffractometry with 45 mA power and 40 kV (Bruker, Germany). ATRA, PVLA and ATRA-loaded PVLA nanoparticles were analyzed in a 2θ angle range of 5–35°.

2.5. Measurement of particle size

The size distribution of ATRA-loaded PVLA nanoparticles prepared by a dialysis method was filtered through an Advantec MFS 0.2-μm filter and assessed using a dynamic light scattering spectrophotometer (DLS-7000, Otsuka Electronics, Japan) with a He–Ne (10 mW) and Ar (75 mW) laser beam at a scattering angle of 90°. The concentration of the samples was kept at 1.0 mg/ml.

2.6. Hepatocyte isolation and culture

Hepatocytes were prepared by non-circulation perfusion of male ICR mouse liver with a two-step collagenase perfusion