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Bilirubin photoisomers in rhesus monkey serum

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ABSTRACT

As rhesus monkeys exhibit physiological jaundice during the neonatal period, we used rhesus monkey serum to examine changes in bilirubin photoisomers. Bilirubin-rhesus monkey serum solution was irradiated with blue light-emitting diode, and changes in the absorbance and bilirubin fraction were compared with those in bilirubin- human serum albumin (HSA) and bilirubin-rat albumin solutions. The λ_{max} decreased with light irradiation. The mean production rate of cyclobilirubin IXα was 1.98, 199 and 0.76 × 10^{-2}/min in rhesus monkey serum, HSA and rat albumin, respectively. There was no significant difference between rhesus monkey serum and HSA. The *(ZE)*-bilirubin IXα/*(ZZ)*-bilirubin IXα ratio was 0.33, 0.45, and 0.10, respectively, differing significantly among the groups. The *(EZ)*-bilirubin IXα/*(ZZ)*-bilirubin IXα ratio was 0.020, 0.010, and 0.062, respectively, with no significant difference between rhesus monkey serum and HSA. The production rate of *(EZ)*-cyclobilirubin XIIIα(= (ZE)-cyclobilirubin XIIIα) was 0.73, 1.60, and 0.51 × 10^{-2}/min, respectively, with differing significantly among the groups. The *(EZ)*-bilirubin IIIα/*(ZZ)*-bilirubin IIIα ratio was significantly different among the groups at 0.20, 0.38, and 0.15, respectively.

This is the first report demonstrating the photoisomerization of bilirubin in rhesus monkey serum and the animal with the same cyclobilirubin production rate as HSA.Rhesus monkeys may be used as an animal model for neonatal hyperbilirubinemia in humans to evaluate the efficacy of phototherapy.

1. Introduction

Phototherapy is used to treat neonatal hyperbilirubinemia. It reduces the level of serum bilirubin by non-enzymatic photochemical reactions of bilirubin. The underlying mechanism of phototherapy is the production and excretion of photoisomers by configurational and structural photoisomerization of bilirubin. The production of bilirubin configurational isomers depends on the wavelength characteristics of a light source, and occurs under the photoequilibrium with *(ZZ)*-bilirubin [1,2]. Upon excretion into the bile, some of the bilirubin configurational isomers return to *(ZZ)*-bilirubin in bile and are reabsorbed by enterohepatic circulation [3]. The amount of bilirubin structural isomers increases with the duration and intensity of light irradiation at an affective wavelength [2,4], and the photoisomers are mainly excreted into the bile [3]. As bilirubin is hydrophobic, it is mainly bound to HSA in human serum. Thus, albumin plays a critical role in the production of bilirubin photoisomers [5–7]. A study demonstrated that configurational and structural photoisomerization of bilirubin in a mixture of non-primate albumin-bilirubin solution differs significantly from that of HSA-bilirubin solution [8]. However, this study examined animal models that do not exhibit physiological jaundice. On the other hand, rhesus monkeys are known to exhibit physiological jaundice [9], but bilirubin photoisomerization has not been studied in this model. In the present study, we analyzed bilirubin photoisomerization in rhesus monkeys in comparison with that in humans and rats using a blue LED, which is currently used for treatment of human neonatal hyperbilirubinemia. This is the first detailed report of bilirubin photoisomerization in rhesus monkeys.

2. Materials and Methods

2.1. Materials and Preparation of Bilirubin-Rhesus Monkey, -Human Serum Albumin and -Rat Albumin Solution

Bilirubin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), HSA (Sigma Aldrich Inc., St Louis, USA), rat albumin fraction V (Sigma

Abbreviations: HSA, Human serum albumin; HPLC, High-performance liquid chromatography; *(ZZ)*-bilirubin, *4Z, 15Z*-bilirubin; *(ZE)*-bilirubin, *4Z, 15E*-bilirubin; *(EZ)*-bilirubin, *4E, 15Z*-bilirubin; *(EE)*-bilirubin, *4E, 15E*-bilirubin; *(EZ)*-cyclobilirubin ((*Z*)-lumirubin, photobilirubin IIA), *4E, 15Z*-cyclobilirubin; *(EE)*-cyclobilirubin ((*E*)-lumirubin), *4E, 15E*-cyclobilirubin

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Chemical Company, St. Louis, USA), HPLC-grade distilled water, and HPLC-grade acetonitrile were used as reagents. Rhesus monkey serum (Lot 234) is purchased on Nippon bio-test Laboratories Inc. (Saitama, Japan). Albumin concentration measured by bilirubin oxidase method, and in rhesus monkey serum was 4.2 g/dL (650 μM).

HSA and rat albumin were each dissolved into phosphate buffer (pH 7.4), and the concentration was adjusted to 4.0 g/dL (600 and 620 μM, respectively). Bilirubin was dissolved into 0.1 M sodium hydroxide solution, and then added into HSA, rat albumin, or rhesus monkey serum to a final concentration of 10 mg/dL (171 μM). One hundred microliters of the bilirubin mixture was suspended in a glass tube and irradiated with a blue light-emitting diode (LED; Bili-Therapy Pad, ATOM Medical, Tokyo, Japan) for 1, 2, 3, 10, or 15 min to make the measurements described below. The blue LED had a peak wavelength between 450 and 480 nm and irradiation energy of 40 μW/cm^2/nm (Minolta Photometer M451 (Minolta Air-Shields)). This experiment was performed in triplicate.

2.2. Absorption Spectroscopy

The absorption spectrum of bilirubin-rhesus monkey serum mixture was measured within 400–550 nm at each time point using a UV-visible spectrophotometer (UV-2450, Shimadzu Co. Kyoto, Japan). A 1-cm quartz cuvette was used for measurements, and distilled water was used as a control.

2.3. Bilirubin Photoisomers

The bilirubin fraction was measured at each time point using HPLC as described previously [10]. A gradient elution reversed phase HPLC system (Shimadzu LC-9A, Shimadzu Co., Kyoto Japan) with UV/Visible detector (SPD-6AV detector, Shimadzu Co., Kyoto Japan) was employed.

2.4. Statistical Analysis

One-way ANOVA was used to compare 3 groups, and the Tukey post-hoc test was used to compare each group. Changes in the bilirubin fraction over time in rhesus monkey serum was compared to those in other groups using MANOVA, and the Tukey honestly significant difference test was used to compare each group. The analyses were performed using the JMP ver. 13.00.

3. Results

3.1. Absorption Spectroscopy

Fig. 1 shows the time course of changes in the light absorption spectrum of the bilirubin- rhesus monkey serum mixture within the range of 400–550 nm. The λ_{max} of bilirubin-rhesus monkey serum (bilirubin:albumin molar ratio = 0.26) was 460 nm. The absorption spectra had a shoulder in the 430–450 nm range, and λmax decreased with a longer duration of light irradiation.

3.2. Bilirubin Photoisomers

The chromatogram in Fig. 1 shows changes in bilirubin photoisomers over time following irradiation of bilirubin-rhesus monkey serum with light. The bilirubin reagent used in the study contained bilirubin XIIIα and IIIα in addition to bilirubin IXα; therefore, these photoisomers were also detected upon light irradiation. The following peaks were identified: *(EE)*-cyclobilirubin IXα, *(EZ)*- [= *(ZE)*]-cyclobilirubin XIIIα, *(EZ)*-cyclobilirubin IXα, *(ZE)*-bilirubin IXα, *(ZE)*- [= *(EZ)*]-bilirubin IIIα, *(EZ)*-bilirubin IXα, *(ZZ)*-bilirubin XIIIα, *(ZZ)*-bilirubin IXα, *(ZZ)*-bilirubin IIIα. All photoisomers were detected with light irradiation. *(ZE)*-bilirubin IXα and *(ZZ)*-bilirubin IXα changed significantly 1 min after irradiation, and *(EE)* − /*(EZ)*-cyclobilirubin IXα and *(EZ)*-cyclobilirubin XIIIα increased gradually with a longer duration of light irradiation.

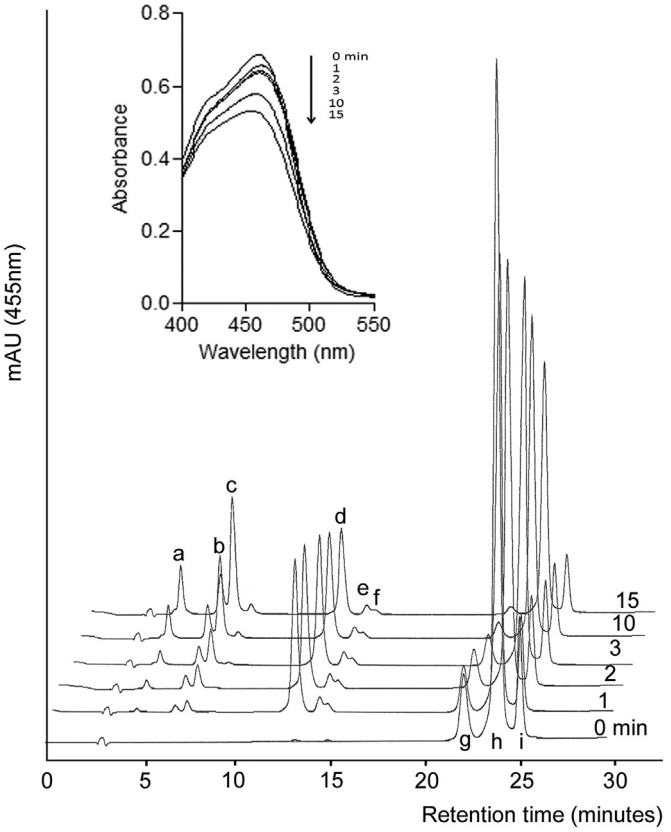

Fig. 1. Absorption spectra and HPLC chromatogram of bilirubin-rhesus monkey serum upon light irradiation over time.
The absorption spectra are shown on the upper left side. The HPLC Chromatogram is shown in the center. Irradiation time (minutes) is indicated next to the absorption spectra. Numbers on the right side indicate the duration of light irradiation. The following peaks were identified: a: *(EE)*-cyclobilirubin IXα, b: *(EZ)*- [= *(ZE)*]-cyclobilirubin XIIIα, c: *(EZ)*-cyclobilirubin IXα, d: *(ZE)*-bilirubin IXα, e: *(ZE)*- [= *(EZ)*]-bilirubin IIIα, f: *(EZ)*-bilirubin IXα, g: *(ZZ)*-bilirubin XIIIα, h: *(ZZ)*-bilirubin IXα, i: *(ZZ)*-bilirubin IIIα.

3.3. Changes in the Bilirubin Fraction

The ratio of bilirubin fraction divided the area under the curve (AUC) of each bilirubin fraction by the total AUC. Fig. 2 shows changes in the fraction of bilirubin IXα in bilirubin-rhesus monkey serum, bilirubin-HSA, and bilirubin-rat albumin mixtures following light irradiation. The ratio of *(EE)*-cyclobilirubin IXα and *(EZ)*-cyclobilirubin IXα increased over time (Fig. 2A, B), and the ratio of *(EE)*-cyclobilirubin IXα was significantly higher in rhesus monkey serum than in HSA with 3, 10 or 15-minute irradiation ($p < .01$). The ratio of *(EE)*-cyclobilirubin IXα and *(EZ)*-cyclobilirubin IXα were significantly higher in rhesus monkey serum than in rat albumin under all irradiation conditions ($p < .01$). The ratio of *(EZ)*-cyclobilirubin IXα in rhesus monkey serum was no significant difference to that in HSA ($p > .05$). The mean (SD) production rate of cyclobilirubin IXα, defined as the sum of *(EE)*-cyclobilirubin IXα and *(EZ)*-cyclobilirubin IXα, during the first 3 min of irradiation was 1.98 (0.16), 1.99 (0.10), and 0.76 (0.06) $\times 10^{-2}$/min for rhesus monkey serum, HSA, and rat albumin, respectively (Table). The difference was significant between rhesus monkey serum and rat albumin ($p < .01$), but not between rhesus monkey serum and HSA ($p = .96$). The ratio of *(ZE)*-bilirubin IXα increased immediately after irradiation and decreased gradually in rhesus