Research Article

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The specificity and accuracy of ^{111}In-hexavalent lactoside in estimating liver reserve and its threshold value for mortality in mice

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Background & Aims: The asialoglycoprotein receptor on hepatocyte membranes recognizes the galactose residues of glycoproteins. We investigated the specificity, accuracy and threshold value of asialoglycoprotein receptor imaging for estimating liver reserve via scintigraphy using ^{111}In-hexavalent lactoside in mouse models.

Methods: ^{111}In-hexavalent lactoside scintigraphy for asialoglycoprotein receptor imaging was performed on groups of normal mice, orthotopic SK-HEP-1-bearing mice, subcutaneous HepG2-bearing mice, mice with 20–80% partial hepatectomy and mice with acute hepatitis induced by acetaminophen. Liver reserve was measured by relative liver uptake and compared with normal mice. Asialoglycoprotein receptor blockade was performed via an *in vivo* asialofetuin competitive binding assay.

Results: A total of 73.64 ± 7.11% of the injection dose accumulated in the normal liver tissue region, and radioactivity was barely detected in the hepatoma region. When asialoglycoprotein receptor was blocked using asialofetuin, less than 0.41 ± 0.04% of the injection dose was detected as background in the liver. Asialoglycoprotein receptor imaging data revealed a linear correlation between ^{111}In-hexavalent lactoside binding and residual liver mass (R^2 = 0.8548) in 20–80% of partially hepatectomized mice, demonstrating the accuracy of ^{111}In-hexavalent lactoside imaging for measuring the functional liver mass. Asialoglycoprotein receptor imaging data in mice with liver failure induced using 600 mg/kg acetaminophen revealed 19–45% liver reserve relative to normal mice and a fatal threshold value of 25% liver reserve.

Conclusion: The ^{111}In-hexavalent lactoside imaging method appears to be a good, specific, visual and quantitative predictor of functional liver reserve. The diagnostic threshold for survival was at 25% liver reserve in mice.

Keywords: Asialoglycoprotein receptor; Liver reserve; ^{111}In-hexavalent lactoside scintigraphy.

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Abbreviations: ASGPR, Asialoglycoprotein receptor; HL, Hexavalent lactoside; ICG15, Indocyanine green clearance test; ICG, Indocyanine green; GalNAc, N-acetylgalactosamine; DTPA, Diethylene triamine pentaacetic acid; GSA, Galactosyl-human serum albumin; NGA, Neogalactosylalbumin; NOD-SCID, Non-obese diabetic-severe combined immunodeficiency; SPF, Specific pathogen-free; ATCC, American type culture collection; AST, Aspartate aminotransferase; INER, Institute of Nuclear Energy Research; NTA, Nitrile triacetic acid; ITLC, Instant thin layer chromatography; SG, Silica gel; Rf, Retention factor; SPECT, Single photon emission computer tomography; CT, Computed tomography; ROI, Region of interest; %ID, Percent of injection dose; PHx, Partial hepatectomy; APACHE II, Acute Physiology and Chronic Health Evaluation; MELD, Models of End-Stage Liver Disease; CMC, carboxymethyl cellulose.

Introduction

More than 0.5 billion people worldwide suffering from chronic hepatitis are likely to experience liver fibrosis, cirrhosis, liver cancer or even liver failure [1,2]. When liver reserve falls below a critical threshold due to tumor growth, surgical resection or acute hepatitis, liver failure ensues. The experience of many physicians has indicated that recovery is possible after 70–80% resection of normal livers [3]; however, it is difficult to predict the resection size for the liver cancer and cirrhosis populations because their uncertain and low liver function will likely result in postoperative mortality with similar resection volumes compared to the healthy population [3]. Given the lack of good liver function tests, our knowledge about the functional liver is incomplete. Thus, visual, specific and quantitative measurements of remaining liver functional reserve are of primary importance in treatment planning. In addition, these measurements are also important for following hepatocyte regeneration after hepatectomy or living transplantation [4]. Furthermore, in clinical practice, measurement of the actual remaining liver function and determination of the cut-off value indicative of the need for liver transplantation

requires the development of more accurate measurement tools and methods [3].

In clinical practice, the ICG15 (indocyanine green) clearance test is one of the most commonly used methods to assess residual liver function [5,6]. ICG dye binds to albumin and is transported to hepatocytes and excreted completely via the bile without enterohepatic circulation. ICG15 is the percent of ICG retained at 15 min after intravenous (i.v.) injection. However, the test has known limitations in predicting liver disease severity [7–10], such as lack of correlation with patient prognosis and mortality rate [11], large intra-examiner variation [8,12], and lack of imaging information. Hepatic shunts can also influence ICG clearance from hepatocytes [8,13].

The strategy we used for liver reserve measurement was specific receptor imaging-mediated endocytosis. In 1968, Ashwell and Morell observed that asialoglycoprotein receptors (ASGPR) residing on hepatocyte membranes exhibit a high specificity for glycans with Gal or GalNAc termini [14]. ASGPRs maintain the balance of physiological proteins by removing old sialic-acid-free glycoproteins or those with exposed GalNAc via receptor-mediated endocytosis [14,15]. Therefore, radiolabeled asialoglycoprotein or glycopeptides with Gal/GalNAc termini are candidate ligands of ASGPRs that may enable the evaluation of residual functional liver mass [16,17].

In 1991, Ha-Kawa introduced technetium-99m-diethylene triamine pentaacetic acid-galactosyl-human serum albumin (^{99m}Tc-GSA, Nihon Medi-Physics, Japan) for clinical hepatic imaging. ^{99m}Tc-GSA is commercially available and has been used in Japan as a liver receptor agent to evaluate the success of liver surgery following liver injury due to ischemia/reperfusion. Imaging data appear to indicate a statistically significant difference between normal volunteers and patients with liver cirrhosis [18]. Yang (2010) used free GSA to block the binding of ^{99m}Tc-dimercaptopropionyl neogalactosylalbumin (^{99m}Tc-NGA) to ASGPRs. Unfortunately, when the receptors are blocked completely by free GSA, the liver retains more than 30% of the radioactive background. The unfavorable uptake of proteins by Kupffer cells may lead to high background in the liver when using protein-based radio-asialoglycoproteins [19]. To address this limitation, we used the peptide-based compound ^{111}In-hexavalent lactoside (^{111}In-HL) as an imaging marker of ASGPR. Its physical and chemical properties have been published previously [20,21]. In this study, we further characterized its specificity and accuracy for ASGPR imaging and revealed its diagnostic threshold of liver reserve for maintaining life.

Materials and methods

Animals and cells

Male Balb/c mice, aged 6–8 weeks and weighing 24 ± 2 g, were purchased from the National Animal Center, Taipei, Taiwan. NOD-SCID male mice, aged 5–6 weeks and weighing 20 ± 3 g, were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). All animals were bred in a specific pathogen-free (SPF) animal room maintained at a constant temperature (23 ± 3 °C) and relative humidity (50 ± 20%), with periodic air changes and 12 h of light illumination per day. Food and tap water were supplied *ad libitum* during the experimental periods. The human cell lines HepG2 and SK-HEP-1 (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured using Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and maintained in a 5% CO_2 incubator at 37 °C.

Animal models

The orthotopic xenograft models of SK-HEP-1 hepatocellular carcinoma were established by slowly injecting 5×10^6 cells into the left lateral liver lobe of SCID mice (n = 3, 5–6 weeks). The subcutaneous xenograft model of HepG2 hepatocellular carcinoma was established by slowly injecting 5×10^6 cells into the right legs of SCID mice (n = 3, 5–6 weeks). The partial hepatectomized models were generated by performing consecutive liver lobe resections (left lateral lobe, median lobe, and right lower lobe) in male Balb/c mice to produce 20–80% remnant liver masses (remnant liver weight/total liver weight; n = 4 per group, 6–8 weeks) [22–24]. All removals were performed quickly and typically did not last longer than 15 min, and the weights of the resected lobes were measured. Following wound suturing, the hepatectomized mice were warmed with light and quickly underwent ^{111}In scintigraphy within 30 min to minimize the possibility of increasing liver mass due to regeneration. The remaining liver weight was measured after euthanasia when ^{111}In scintigraphy was performed (n = 4, 6–8 weeks). For monitoring purposes, following wound suturing, animals subjected to surgery were allowed to recover in cages in the animal room for liver regeneration and ^{111}In-HL imaging (n = 4, 6–8 weeks). Acute hepatitis was induced by administering 0, 100, 250, 300, 400, 500, 650, 700, or 800 mg/kg of acetaminophen via intraperitoneal injections into Balb/c mice (n = 4 or 6 in each group, 6–8 weeks). The acetaminophen was prepared as a 40 mg/ml saline solution in a 65 °C water bath. The dose of acetaminophen that caused half of the mice in the treatment group to die was used to induce the liver failure model (n = 4, 6–8 weeks). At 2, 4, 6, 8, and 24 h after acetaminophen induction, blood samples (200 μl) were collected in microcentrifuge tubes and centrifuged (1000 × g and 4 °C for 15 min) within one hour to obtain serum for the aspartate aminotransferase (AST) activity assay using a Fujifilm FDC3500 i/s analyzer (Fujifilm Corporation, Japan). After euthanasia, liver tissue was collected, fixed using 10% (v/v) buffered formalin solution, and embedded in paraffin wax for conventional histological examination. Briefly, 5–7 μm thick sections were cut, stained with hematoxylin & eosin, and observed under the microscope to assess morphological changes.

Synthesis of hexavalent lactoside (HL)

The abbreviation of HL (hexavalent lactoside) indicates that hexa-lactoses are bound onto the terminal site of the glycopeptide. The INER HL kit was synthesized by Institute of Nuclear Energy Research of Taiwan (INER, Taoyuan, Taiwan) using a patented formulation [25]. Briefly, ε-amino-protected Nα,Nα-bis(carboxymethyl)-L-lysine hydrate containing a "nitrilo triacetic acid" (NTA) group surrounding the original α-amino group was used to attach three lactose glycoside residues to each carboxyl group of NTA. The trivalent lactoside compound was dimerized by conjugating it to the amino-protected hexanoyl-L-aspartic acid, and the ε-amino group was unmasked and modified with DTPA dianhydride for ^{111}In chelating purposes. For convenience, the formulation for radiolabeling, which comprised 16 μg DTPA HL, 55 mg citric acid, 61 mg trisodium citrate, and 100 mg mannitol in 10 ml distilled water, was previously mixed in a clean room and then aliquoted into ten vials using a sterile 0.22-μm filter. Then, each aliquot was lyophilized for use in lyophilized kits.

Labeling of ^{111}In-HL

Radiolabeling was performed by adding 1 ml sterile and apyrogenic $^{111}InCl_3$ (1 mCi, i.e., 3.7×10^7 Bq, in sodium chloride, pH 2.0) to the lyophilized INER HL kit with a pH 4 buffer followed by a 10–15 min incubation at room temperature. The radiochemical purity of ^{111}In-HL was determined via radio-instant thin layer chromatography (ITLC) and was confirmed via radio-high performance liquid chromatography (HPLC) [21]. The radiochemical purity was up to 99%, and the specific radioactivity was 2.5×10^{10} Bq/mg. The radiochemical product was very stable with more than 95% radio-purity observed up to 120 h at 4 °C.

^{111}In-HL scintigraphy

The dose used for SPECT scintigraphy depends on the collimator sensitivity and resolution. The collimator is a photon filter with central holes that allow the passage of irradiated photons from tissues but block unwanted scattering photons from penetrating via the thick septa. The parallel-hole collimator used in microSPECT/CT (Gamma Medica, Inc., Salem, NH, USA) has large diameter holes and thus has higher sensitivity and moderate resolution, whereas the multiple-pinhole collimator used in nanoSPECT/CT (Mediso Ltd., Budapest, Hungary) produces magnified images but sacrifices sensitivity. For ^{111}In-HL scintigraphy, 4 μCi ^{111}In-HL (i.e., 0.148 MBq ^{111}In per 6 ng HL) and 0.5 mCi (i.e.,