In vivo imaging of advanced glycation end products (AGEs) of albumin: first observations of significantly reduced clearance and liver deposition properties in mice

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Advanced glycation end products (AGEs) are chemical substances that are non-enzymatically produced by the reaction of amino acids with the reducing end of saccharides.1–7 Whether introduced exogeneously (e.g. ingested from certain heated foods) and/or endogenously generated in the body, AGEs are associated with various diseases, such as atherosclerosis, nephropathy, retinopathy, and neurodegenerative disorders, and also the development of diabetes and uremia. To better understand these biological processes, investigation of the in vivo kinetics of AGEs, i.e., analysis of trafficking and clearance properties, was carried out by molecular imaging. Following the preparation of Cy7.5-labeled AGE-albumin and intravenous injection in BALB/cA-nu/nu mice, noninvasive fluorescence kinetics analysis was performed. In vivo imaging and fluorescence microscopy analysis revealed that non-enzymatic AGEs were smoothly captured by scavenger cells in the liver, i.e., Kupffer and other sinusoidal cells, but were unable to be properly cleared from the body. Overall, these results highlight an important link between AGEs and various disorders associated with them, which may serve as a platform for future research to better understand the processes and mechanisms of these disorders.

Introduction

Advanced glycation end products (AGEs) are chemical substances that are non-enzymatically produced by the reaction of amino acids with the reducing end of saccharides.1–7 Whether introduced exogeneously (e.g. ingested from certain heated foods) and/or endogenously generated in the body, AGEs are associated with various diseases, such as atherosclerosis, nephropathy, retinopathy, and neurodegenerative disorders, and also the development of long-term complications of diabetes.8–12 Investigating the in vivo kinetics of AGEs, i.e., analysis of trafficking and clearance properties by biodistribution studies, is therefore vital to understanding the mechanisms and processes of these AGE-induced disorders.13,14 With recent advances in molecular imaging techniques,15–17 time-dependent biodistribution, clearance and gastrointestinal absorption of AGE-albumin following intravenous injection can now be noninvasively analyzed with higher sensitivity in various small animal models.

Only a few molecular imaging studies, such as by Positron Emission Tomography (PET), have been reported for AGEs in the current literature.18–22 These studies have largely focused on using Nε-carboxymethyllysine (CML) as the major amino acid fragment of AGEs.18–20 Not surprisingly, however, the small size of CML allows rapid clearance through the kidney and any significant deposition, such as liver or gastrointestinal absorption, is not clearly observed. Alternatively, in vivo imaging of AGE-modified albumins, which are the main serum products prior to digestion into AGE peptide fragments, has not been reported.

Horiuchi and co-workers have previously reported the biodistribution studies of 125I-labeled glucose-albumin AGEs in rat models. Following intravenous administration, dissection experiments after 60 min showed that radioactivity localized preferentially in the liver.23,24 The authors found that liver accumulation was due to the receptor for AGEs (RAGE) expressed on the Kupffer and liver endothelial cells, which could contribute to scavenging and excreting the exogenous...
and endogenous AGEs from the liver. Recently, it was also found that 13C-labeled glucose-albumin AGEs in chickens, a good diabetology model organism for investigating albumin AGEs, were similarly accumulated in the liver within 30 min, presumably being captured by Kupffer cells.²⁵

In this paper, we would like to report the first noninvasive imaging of albumin AGEs over a long period of time by means of near-infrared fluorescence detection. We prepared Cy7.5-labeled AGE-albumin and performed noninvasive kinetics analysis in BALB/c-a nu/nu mice after intravenous injection. Consistent with previous reports, this research showed that AGE-albumins were rapidly captured by non-parenchymal cells, which resulted in liver accumulation. However, our noninvasive in vivo imaging also revealed that non-enzymatic AGE modification notably retarded clearance through the liver, and instead induced non-specific accumulation even after 24 h. As a result, it can be hypothesized that liver accumulation effects induced by AGE modifications on albumin are closely associated with the process and mechanism of various liver disorders.

**Results and discussion**

AGEs of albumin were prepared according to the reported procedure. The selection of human serum albumin (HSA) in our studies, of which in vivo kinetics in mice has been well documented,²⁶–²⁸ was made so that the effects of AGE formation could easily be assessed. An aqueous solution of Cy7.5-labeled HSA (Cy7.5-HSA: two Cy7.5 labels on albumin, 400 μM) was incubated with excess amounts of glucose (30 M), which was chosen as the reactive saccharide, at 37 °C in water for 20 and 41 days (Fig. 1A).²⁵ After small molecules were simply filtered off by Amicon® centrifugation, the products were analyzed by MALDI-TOF mass spectroscopy (Fig. 1B). An approximate increase of 500 and 1000 molecular weight units was observed for the incubated albumins Cy7.5-AGE-HSA (20d) and Cy7.5-AGE-HSA (41d), respectively. Although the Cy7.5 on albumin (prepared as shown in Fig. 1) disturbed the clear observation of the AGE formation by fluorescence analysis, we detected the fluorescence at around 440–460 nm (excitation at 370 nm) of AGE-albumin without Cy7.5 or model aliphatic amines under the identical conditions shown in Fig. 1 as control experiments. Furthermore, we treated the AGEs of albumin with diluted hydrochloric acid during the MALDI-TOF-MS analysis, but no change in MS was observed; hence the modification of albumin was not due to the reversible imine or aminoacetal formation with lysines, but due to the successful AGE-albumin formation.

AGE-albumin prepared in this manner, which are labeled with the near-infrared fluorescent dye Cy7.5, was injected into BALB/c-a nu/nu mice via the tail vein, and noninvasive fluorescence imaging was performed using IVIS® under anesthesia. Fluorescence signals from the abdominal and dorsal sides at 1, 2, 3, 4, 6, and 24 h after injection are shown in Fig. 2.

According to the literature, native HSA proteins (without any AGE-modifications) following injection are known to be immediately distributed over the whole body through the capillary vessels on the skin, and then gradually excreted through the kidney/urinary bladder pathway over 3 h.²⁶–²⁸ In contrast, HSA modified with AGEs exhibited kinetics notably different from that of the control albumin. Both Cy7.5-AGE-HSA (20 d) and Cy7.5-AGE-HSA (41 d) rapidly trafficked to the liver within 1 h (Fig. 2A–D), and surprisingly, did not show observed clearance from the liver and urinary bladder even after 6 h (which is the typical clearance time of proteins in vivo). The fluorescence signals around the liver, calculated within an arbitrarily defined region of interest (ROI), were still significantly high even 24 h after injection (Fig. 2E).

One interesting observation which should be noted is that following 24 h, Cy7.5 fluorescence was once again found to be distributed over the whole body (see the dorsal sides of images in Fig. 2), hence suggesting that fluorescence trafficked to the liver may potentially circulate back through the capillary vessels on the skin.

As we and other research groups have experienced, the stability of fluorescent albumin in serum is not simply assessed as those of peptides by HPLC, as albumin interacts with other serum proteins and small hydrophobic molecules in serum, or presumably AGEs themselves are metabolized. But the labeled glycoalbumins or their metabolites (which should be large enough to prevent excretion through kidney filtration) are generally stably circulated in the bloodstream. If the albumin become trapped by scavenger cells in the serum and/or digested by proteases, the resulting small peptide

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**Fig. 1** Preparation of AGES of albumin. (A) Incubation of Cy7.5 fluorescence labeled human serum albumin (Cy7.5-HSA) with glucose at 37 °C for 20 and 41 days. (B) MALDI-TOF-MS of AGE-albumin.

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**Fig. 2** Fluorescence signals on the skin and in the liver after intravenous injection of Cy7.5-labeled albumins.
fragments or even the detached NIR dye can rapidly be excreted through the kidneys (renal clearance) and are not taken up by specific organs or circulated in vivo, as observed in this study.26–28

To complement the in vivo imaging results, the mice were sacrificed following 24 h and fluorescence from the dissected organs was quantified and compared (Fig. 3). Confirming the selective liver accumulation of AGE-albumin, fluorescence levels in other organs (e.g., brain, kidney, spleen, or pancreas) were shown to be negligible compared to the liver. While the mass of the AGEs of albumin, hence the amount of AGEs, increased due to the prolonged reaction time with

Fig. 2 In vivo fluorescence imaging of AGE-albumin: Effects of AGE formation on excretion and accumulation. 1.5 nmol per 30 µL of AGE-albumin, Cy7.5-AGE-HSA (20 d) and Cy7.5-AGE-HSA (41 d), were diluted in 70 µL saline, and injected into 8 to 10-week-old BALB/cA-nu/nu mice via the tail vein (N = 3). The mice were then anesthetized with isoflurane and placed in a fluorescence imager. Abdominal images were taken at 1 hour intervals. (A–D): Fluorescence in vivo images of Cy7.5-AGE-HSA (20 d) from (A) abdominal (B) dorsal sides, and of Cy7.5-AGE-HSA (41 d) from (C) abdominal (D) dorsal sides. The liver is denoted by arrows. (E) Semi-quantitative analysis of fluorescence signal in the liver. Fluorescence at 745 nm was initially measured, and then the background fluorescence was subtracted at 675 nm.
glucose (from 20 d to 41 d incubation), the fluorescence intensity in live mice (Fig. 2) and in dissected tissues (Fig. 3) was almost identical between Cy7.5-AGE-HSA (20 d) and Cy7.5-AGE-HSA (41 d). The results showed that even fewer AGEs might alter the normal metabolism properties of albumin.

To further analyze the mechanism that drives AGE-albumin liver accumulation, sliced liver sections were prepared and target cells were identified by immunostaining with specific antibodies (Fig. 4). The near-infrared dye, Cy7.5, which can be detected with light at 780 nm, can be clearly detected using the Keyence All-in-one Fluorescence Microscope®. Thus, the trafficking and accumulation of fluorescence injected into the mice can be directly traced at the cellular level.

Observations indicated that AGE-albumins were not captured by parenchymal liver cells, i.e., through the asialoglycoprotein receptor (AGCR), but rather by non-parenchymal cells (Fig. 4). Tissues stained with green-colored anti-F4/80 (for Kupffer cells) extensively co-localized with infrared fluorescence derived from Cy7.5-AGE-HSA (Fig. 4A), and to a lesser extent the anti-LYVE1 antibodies (for sinusoidal endothelial cells, Fig. 4B). Cy7.5-AGE-HSA, on the other hand, did not overlap with anti-desmin (for stellate cells, Fig. 4C), therefore Kupffer and endothelial cells are mainly responsible for the liver-specific accumulation of these AGE-modified albumins.

Our in vivo imaging (Fig. 2 and 3) and microscopy analysis (Fig. 4) are in good agreement with those reported previously. Namely, AGEs of albumin are captured by the scavenger cells, i.e., preferentially by the Kupffer cells, which contributes to the clearance of the AGE albumin from the serum and accumulation in the liver. Our observation through noninvasive fluorescence imaging also indicated that the AGEs cannot smoothly be excreted through the liver. AGE proteins and/or their disintegrated fragments still remain in the liver over 24 h without a significant decrease. Moreover, we observed that fluorescence once trafficked into the liver can return to the circulatory system non-specifically. Overall, the

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**Fig. 3** Fluorescence intensities of the dissected organs. After 24 hours of in vivo imaging experiment as shown in Fig. 2, the mice were sacrificed and perfused with 4% paraformaldehyde solution, and fluorescence intensities in the brain, kidney, spleen, pancreas, and liver were measured.

**Fig. 4** Fluorescence intensities accumulated at the dissected liver after 24 hours. Co-staining of liver tissues treated with Cy7.5-AGE-HSA (41 d) with (A) anti-F4/80 antibodies (to detect Kupffer cells and macrophages), (B) anti-LYVE1 (to detect sinusoidal endothelial cells), and (C) anti-desmin (to detect stellate cells); red: AGE albumin; green: indicated antibodies; blue: nucleus. Red fluorescence in the images might contain non-specific accumulation in the liver. Scale bars represent 20 nm.
collection of these imaging data suggests that even if AGEs are captured, endocytosed, and degraded through the scavenger receptors on Kupffer and endothelial cells in the liver, their removal from the body does not rely simply on ordinary excretion pathways. As a consequence of the observed strong liver deposition and reduced clearance properties, AGE modification on albumin can thus be associated with the process and mechanism of various age-related disorders.

Conclusion

In conclusion, we have analyzed the in vivo kinetics and liver accumulation of AGEs of albumin in mice, by means of non-invasive fluorescence imaging and microscopy of dissected tissues. While AGE-modified albumins were shown to be rapidly captured by scavenger cells and accumulated within the liver, observations also indicate that these albumins and/or disintegrated fragments are not susceptible to liver excretion. As a result, the increased levels of AGE modification could alter the normal liver excretion pathways, which over a long-period of time could lead to various age-related disorders.

This work also presents how molecular imaging can be a useful tool in the investigation and diagnosis of various age-related diseases. Albumin AGEs as molecular probes have been extensively used to investigate the effects of oxidative stress, mitochondrial dysfunction, or apoptosis on neurotypic cells which are associated with neurodegeneration disorders. In particular, these research studies showed that the albumin AGEs, when derived from the reaction with d-ribose, produced highly toxic amyloid-like aggregations to trigger the cellular apoptotic signaling. Our albumin AGEs, which were prepared by using glucose and were mostly present in the monomeric state as judged by MALDI-TOF-MS in Fig. 1, is rather suited for in vivo kinetics studies by molecular imaging following intravenous administration, to avoid the nonspecific organ accumulation or blockage in the bloodstream. Further research directed in this field using various age-related disease models is now in progress.

Experimental

Preparation of AGEs of albumin

Cy7.5-labeled HSA (40 nmol) was incubated with d-glucose (16.5 μmol) in H2O (100 μL) at 37 °C for 20 or 41 days. The incubated samples were filtered with a 0.2 μm syringe-driven filter unit (Merck, USA). The filtrate was centrifuged with an Amicon-Ultra-3 K (Merck, USA). Protein concentrations were analyzed by using the Pierce BCA protein assay kit (Thermo Fisher Scientific, USA).

In vivo kinetics and distribution analysis

All procedures involving experiment animals were approved by the Ethics Committee of RIKEN (MAH21-19-17). The experiments were performed in accordance with the institutional and national guidelines.

1.5 nmol per 30 μL of AGEs of albumin were diluted in 70 μL saline, and injected into 8 to 10-week-old BALB/c-nu/nu mice (CLEA Japan, Inc.) via the tail vein (N = 3). The mice were then anesthetized with isoflurane (1.5%) and placed in a fluorescence imager, IVIS kinetics fluorescence imager® (Caliper Life Sciences, Inc., Hopkinton, Massachusetts, USA). Abdominal and dorsal side images were obtained at 1 hour intervals for 6 hours, and at 24 hours after the injection. For semi-quantitative analysis, the fluorescence around the liver was calculated within an arbitrarily defined region of interest (ROI). Fluorescence at 745 nm was initially measured, and then the background fluorescence was subtracted at 675 nm.

After the in vivo kinetics study described above, the mice were sacrificed and perfused with saline, followed by a 4% paraformaldehyde solution. Fluorescence intensities in the kidney, spleen, brain, pancreas and liver were then measured.

Immunohistochemistry

Livers were further postfixed with 4% PFA at 4 °C for 24 hours, transferred to 15% sucrose in PBS and kept at 4 °C for 24 hours, then to 30% sucrose in PBS likewise. The tissues were frozen in OCT compound® at −78 °C. The tissue blocks were cut into 6–8 μm sections. After blocking with 3% BSA/10% normal goat serum/0.1 M glycine/PBST for 30 min, the sections were incubated with anti-desmin (RB-9014, 1:300, rabbit; Thermo Fisher Scientific, Fremont, CA), anti-LYVE-1 (ab14917, 1:200, rat, AbD serotec, Oxford, UK), and anti-F4/80 (MCA497GA, 1:200, rat, AbD serotec, Oxford, UK) antibodies overnight at 4 °C, followed by incubation with both Alexa Fluor 555 anti-rat IgG (1:2000; green, Life Technologies, Carlsbad, CA) and Alexa Fluor 555 anti-rabbit IgG (1:200; green, Life Technologies, Carlsbad, CA) for 2 hours at room temperature. Thereafter, the sections were incubated with Hoechst 33258 (1:2000; blue, Dojindo Laboratories, Kumamoto, Japan) for 10 min at room temperature, and were mounted with Fluoromount® (Diagnostic BioSystems, Pleasanton, CA). The slides thus prepared were placed in a Keyence BZ-X710 All-in-one Fluorescence Microscope®, and observed at appropriate wavelengths.

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