Structural and functional changes in high-density lipoprotein induced by chemical modification†

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Reconstituted high-density lipoprotein (rHDL), a natural nanoparticle consisting of apolipoprotein A-I and phospholipids, was modified with a hydrophobic fluorescent dye before (pre-rHDL) and after (post-rHDL) reconstitution. Pre-rHDL particles had a similar size to unmodified rHDL, but post-rHDL particles were significantly larger and their avidity for a HDL receptor was 2.6 times of that shown by pre-rHDL.

High-density lipoprotein (HDL) mediates reverse cholesterol transport from peripheral tissues to the liver. As HDL can capture drugs administered intravenously and control or influence their biodistribution, it can be used as a natural carrier for targeted drug delivery. A nascent form of HDL can be reconstituted from phospholipids and apolipoprotein A-I (apoA-I), and the structure of this nascent form (rHDL) is believed to be a discoidal phospholipid bilayer circumscribed by apoA-I (Fig. 1). Since lysine, arginine, and tyrosine residues can be the sites of chemical modification, multiple chemical engineering strategies have been applied to modify apoA-I for the development of natural, biomaterial-based drug delivery systems. The pioneering studies by van Berkel’s group showed that apoA-I chemical modification enhanced the uptake of intravenously injected lactosylated native HDL and rHDL by rat parenchymal liver cells. Subsequently, folate and arginylglycylaspartic acid (RGD peptide) have been used with rHDL for tumor targeting. The targeting ligands have been successfully associated with the apoA-I moiety after mixing with rHDL. However, there is a possibility that hydrophobic ligands such as folate may also become entrapped in the rHDL lipid bilayer, even after repeated dialysis cycles (Fig. 1).

Tetramethylrhodamine isothiocyanate (TRITC) is a primary amine-reactive hydrophobic fluorescent dye, which, if used in rHDL labeling, could be incorporated into the rHDL lipid bilayer and could covalently bind to the apoA-I moiety. Although biomedical applications of functionalized rHDL nanoparticles have recently attracted much attention, to our knowledge, there has been no comparative report on the structural and functional effects of apoA-I conjugation with a hydrophobic molecule prior to and after HDL reconstitution (pre- and post-modification, respectively). Here, we used TRITC as a representative hydrophobic molecule to gain fundamental insights into rHDL chemical modifications, and unexpectedly found that the timing of TRITC-apoA-I conjugation significantly influenced the structural and functional properties of rHDL particles.

The reaction conditions for post-modification with fluorescein isothiocyanate were applied for TRITC modification of wild-type apoA-I. Fig. 1 summarizes the modification procedures of rHDL. In pre-modification,
m-apoA-I was first reacted with TRITC, then unincorporated TRITC was removed by gel chromatography, and the product was freeze-dried and mixed with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) for rHDL preparation. In post-modification preparations, freeze-dried m-apoA-I was first mixed with POPC and then reacted with TRITC before purification by gel chromatography and dialysis (molecular weight cut-off, 50 kDa). In these two schemes, rHDL preparation conditions were identical. Fig. S1a† shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of TRITC–rHDL complexes (600 ng protein per lane) obtained under various conditions. The fluorescence intensity of TRITC-conjugated m-apoA-I bands was proportional to the amount of added TRITC and was comparable between pre-rHDL and post-rHDL particles obtained using the same TRITC/protein (w/w) ratio. When TRITC was mixed with the protein at a ratio of 0.1 (w/w), calculated based on the number of primary amine groups (20) available in m-apoA-I for the reaction with TRITC, the TRITC/protein molar ratio in pre- and post-rHDL was estimated to be 5.8 at most. However, the band intensity for free TRITC trapped in rHDL lipid bilayers was higher for post-rHDL than for pre-rHDL samples (Fig. S1b†). A small amount of free TRITC detected in pre-rHDL samples may suggest relatively strong physical adsorption of TRITC to m-apoA-I during modification, which could hamper its complete removal. In contrast, higher TRITC incorporation into post-rHDL lipids was inevitable since the hydrophobic TRITC applied to already reconstituted rHDL particles could be retained in the lipid bilayers. Therefore, it was concluded that pre-modification under these conditions had the advantage of producing rHDL particles labeled with TRITC through its specific binding to m-apoA-I.

We next measured the size of rHDL–TRITC conjugates by dynamic light scattering (Fig. 2). Without modification, the mean rHDL diameter under the preparation conditions was 15.0 ± 0.4 nm, which was slightly larger than the 10 nm diameter previously reported for rHDL. The difference may be due to the method used for size analysis (i.e., volume-based dynamic light scattering) and/or the reconstitution procedure (application of urea for m-apoA-I solubilization; see the Experimental section in ESI†). In the pre-rHDL preparations, the mean diameter barely changed, regardless of the protein/TRITC molar ratio; however, a significant increase in diameter (more than two- or three-fold) was observed for post-rHDL particles after conjugation with TRITC (Table 1).

Very large nanoparticles (≥100 nm in diameter) could represent not only rHDL aggregates but also POPC liposomes and/or cholate/POPC micelles, possibly formed by lipid impurities contaminating rHDL prepared by the cholate dialysis method. To check this possibility, we performed gel chromatography analysis, which clearly excluded the presence of POPC liposomes, because their retention time (11.3 min for 116 nm liposomes) was significantly shorter than that of post-rHDL particles (13.3 min) (Fig. S2†).

We also examined cholate/POPC micelles prepared according to the cholate dialysis method without protein addition (see the experimental section in ESI†). The micelles had a mean size of ~28 nm, which did not change even after mixing with TRITC (Fig. S3†), suggesting that the enlarged nanoparticles in post-rHDL samples were not cholate/POPC micelles. Taken together, these results indicate that the fraction of enlarged post-rHDL particles is represented by rHDL aggregates.

Cellular dynamics of rHDL–TRITC conjugates were investigated in Chinese hamster ovary (CHO) cells overexpressing the HDL receptor SR-BI. CHO-SR-BI cells were treated with pre-rHDL and post-rHDL particles prepared at the TRITC/protein (w/w) ratios of 0.01, 0.04, and 0.1, as indicated in each panel. Unconjugated rHDL (designated as rHDL) is shown as a reference (upper panel). Size distribution and mean size of pre-rHDL particles appeared unchanged from those of rHDL. However, for post-rHDL samples, size distribution broadening towards larger particles was clearly observed. The data for each rHDL sample represent the mean of three independent preparations.

Table 1 Mean size and yield of pre-rHDL and post-rHDL particles

| TRITC/protein ratio | Mean sizea (nm) | Yielda (% w/w protein) |
|---------------------|-----------------|------------------------|
| rHDL                |                 |                        |
| 0.01                | 15 ± 0.4        | 59 ± 5.4               |
| 0.04                | 11 ± 2.9        | 57 ± 3.6               |
| 0.1                 | 12 ± 3.1        | 64 ± 5.0               |
| Post-rHDL           |                 |                        |
| 0.01                | 46 ± 11         | 56 ± 1.6               |
| 0.04                | 27 ± 4.5        | 64 ± 2.4               |
| 0.1                 | 52 ± 8.6        | 55 ± 3.6               |

a Average ± standard deviation of three preparations.

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TRITC in post-rHDL lipid bilayers (Fig. S1) and CHO-SR-BI cells treated with pre-rHDL (15 µg protein mL⁻¹) or post-rHDL (10 µg protein mL⁻¹) particles (TRITC/protein ratio, 0.1/1) in the presence or absence of non-labeled rHDL (rHDL) at 37 °C for 1 h. The concentration of pre-rHDL and post-rHDL particles was adjusted to TRITC equivalent, and that of non-labeled rHDL was adjusted to protein equivalent. The total binding efficiency of post-rHDL particles was 2.6 times that of pre-rHDL particles, while SR-BI-dependency of their binding was similar. The analysis was performed in triplicate.

Finally, we examined the dynamics of rHDL enlargement by post-reconstitution TRITC modification. Decreasing the reaction time from 24 h to 4 h resulted in smaller particle sizes and lower protein labeling efficiency, whereas TRITC embedded in the lipid bilayer was unchanged (Fig. S8†).
Surprisingly, when TRITC freshly prepared in dimethylsulfoxide (DMSO; see the experimental section in ESI†) was used, there was no particle enlargement, even after 24 h reaction. On the other hand, the protein labeling efficiency was significantly higher than that of the samples prepared with TRITC stored at −30 °C, whereas lipid embedding was similar for both types of TRITC (Fig. S8†). These findings could be attributed to the decreased reactivity of the TRITC isothiocyanate group.

In summary, we compared the effects of chemical conjugation with TRITC, a hydrophobic fluorescent dye, on the structure and function of rHDL particles assembled before and after TRITC modification. Compared with pre-rHDL particles, post-rHDL particles showed a significantly larger mean size, and incorporated more TRITC in the lipid bilayer. In HDL receptor-overexpressing cells, the two types of rHDL–TRITC conjugates showed similar intracellular distribution; however, post-rHDL particles had a significantly higher binding efficiency, most probably due to their increased size. This study identified a previously unknown phenomenon that should be considered in rHDL chemical modifications and could be applied to the development of novel strategies in the design of rHDL-based drug delivery systems.

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