〈Original Article〉

Effect of methylglyoxal-mediated apolipoprotein A-I modification on ABCA1-dependent cholesterol efflux from cells

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Summary Apolipoprotein A-I (apo A-I) in HDL plays an important role in lipid metabolism. Recently, it has been reported that apo A-I is modified by methylglyoxal (MG), which is generated in chronic hyperglycemic state, and cross-linked. The purpose of this study is to confirm how the changes in the apo A-I structure by MG modification in vitro affect ABCA1-dependent cholesterol efflux from cells.

MG-modified apo A-I (MG-A-I) was confirmed to be cross-linking. Then fibroblasts were cultured with normal apo A-I or MG-A-I, and cholesterol in the medium was determined. Compared to the control, the cholesterol efflux to the medium added MG-A-I was decreased. Furthermore, MG-A-I did not increase ABCA1 protein levels in fibroblasts, but normal apo A-I increased.

Conclusively, MG-mediated apo A-I modification resulted in change of the apo A-I structure and loss of ABCA1-mediated cholesterol efflux-inducing capacity.

Key words: Apolipoprotein A-I, HDL, Methylglyoxal, ABCA1, Cholesterol efflux

1. Introduction

Diabetes is associated with atherosclerotic vascular disease[1)]. It is known that HDL and apolipoprotein A-I (apo A-I), a major protein in HDL, are significantly decreased in diabetes patients, which elevates cardiovascular risk[2)]. HDL plays an important role in the reverse cholesterol transport (RCT) pathway, which is due to the function of apo A-I. Apo A-I is involved to the RCT pathway in three stages, 1) recovering free cholesterol (FC) in peripheral tissues via ATP-binding cassette, subfamily A, member 1 (ABCA1)[3)], 2) esterification of FC by activating lecithin cholesterol acyltransferase (LCAT)[4)], and 3) liver uptake of esterified cholesterol via the scavenger receptor class B type I (SR-BI)[5)].

Because cholesterol in peripheral cells is not catabolized, cholesterol efflux transport from peripheral cells is an especially important mechanism in the RCT pathway. There are four known mechanisms of Cholesterol efflux from peripheral cells[6, 7)]: (1) aqueous diffusion, (2) SR-BI-mediated FC flux, (3)

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ABCA1-mediated efflux, and (4) ATP-binding cassette, subfamily G, member 1(ABCG1)-mediated efflux. In contrast to SR-BI and ABCG1-mediated FC efflux, the preferred cholesterol acceptor for ABCA1 is lipid-free or lipid-poor apo A-I[8, 9]. Lipid-free or lipid-poor apo A-I can interact with the ABCA1 on the surface of the cell, promoting efflux of FC and phospholipids. This results in the formation of nascent HDL particles that are further modified by LCAT, generating esterified cholesterol and forming mature HDL[10]. SR-BI[11] and ABCG1[12] preferred the mature HDL as cholesterol acceptor. Tangier disease, an inherited HDL deficiency, is reportedly due to the mutation of ABCA1 gene[13-15]. Francis et al. have reported that the fibroblasts of patients with Tangier disease are unresponsive to lipid-free apo A-I, while the cholesterol efflux of nonspecific diffusion to mature HDL is similar to that in normal fibroblasts[16]. These reports indicate that cholesterol efflux via ABCA1 interaction is the first stage in RCT, and is especially important in HDL regeneration in serum.

Chronic hyperglycemia generates highly reactive and toxic alpha-oxoaldehydes, such as methylglyoxal (MG), glyoxal, and 3-deoxyglucosone. It has recently been reported that these oxoaldehydes affect the apo A-I structure and its function[17]. In that report, apo A-I modified by MG was aggregated and showed decreased LCAT activating ability. Oxoaldehyde binds to the N-terminals of amino groups and reactive lysine and arginine residues in plasma proteins, generating early glycation adducts and advanced glycation endproducts[18-21]. These modifications are associated with protein cross-linking and aggregation[22].

There is some evidence showing the effect of glycation of HDL on cholesterol removal from cells. In those studies, recombinant HDL[23, 24] or HDL_3[25] fractionated from serum is glycated, and that glycated HDL is evaluated for its cholesterol removal from cells and its binding capacity to macrophages and fibroblasts[26]. However, it remains to be fully elucidated how the conformation change of lipid-free apo A-I affects the cholesterol efflux from cells by the interaction between lipid-free apo A-I and ABCA1. Additionally, while fibroblasts containing ABCA1 are cultured with glyoxal or MG, treating cells with glyoxal strongly inhibited the ABCA1-dependent cholesterol efflux, while MG had little effect[27]. However, when fibroblasts containing ABCA1 are cultured with glyoxal or MG, cholesterol efflux may be affected not only by the modification of apo A-I but also by that of ABCA-1 and other proteins. The purpose of this study is to confirm how the changes in the lipid-free apo A-I structure by MG-modification affect the ABCA1-dependent cholesterol efflux.

2. Materials and Methods

1. Isolation of apolipoprotein A-I and preparation of MG-modified apo A-I

HDL was isolated from pooled healthy volunteer plasmas by ultracentrifugation ($1.063 < d < 1.21$ g/ml). The HDL was delipidated and apo A-I was isolated using DEAE Sepharose CL-6B (GE Health Care Co. Ltd.). Eluted fractions of apo A-I were dialyzed against NH_4HCO_3 (50 mmol/l, pH 8.2), and the concentration was adjusted to 1 mg/ml. The apo A-I appeared as a single band when subjected to SDS-PAGE and Coomassie staining.

Lipid-free apo A-I（1 mg/ml）was added to 0-6 mmol/l MG (final concentration), and the mixtures were incubated at 37℃ for 12 h[17]. Apo A-I cross-linking was confirmed by 5-20% SDS-PAGE and immunoblotting.

2. Cell culture and cellular cholesterol release assay

MRC-5 and WI-38 were subcultured at a density of 1.0×10^5 cells/well in 10% fetal bovine serum containing Dulbecco's modified Eagle's medium (Sigma D6429). Cellular lipid release was induced by normal apo A-I or MG-modified apo A-I at various concentrations for 24 h in each medium containing 0.1% fatty acid-free bovine serum albumin. Lipid was extracted from the medium with chloroform/methanol (2:1). After dissolving the extracted lipid with isopropanol, the total cholesterol was determined by a colorimetric enzymatic assay kit[28].

3. ABCA1 protein levels

To measure ABCA1 protein levels, total cellular

protein was solubilized in SDS buffer and resolved by SDS-PAGE. ABCA1 protein was detected by immunoblotting analysis[29]. Equal amounts of cellular protein (20 μg) were added per gel lane. ABCA1 was visualized by the reaction with mouse anti-human ABC1 (A00121.01) monoclonal antibody (Santa Cruz, diluted to 1/200 in skim milk-PBS), peroxidase conjugated goat anti-mouse IgG antibody (Calbiochem, diluted to 1/1000 in skim milk-PBS), and 3, 3', 5, 5'-tetramethylbenzidine (Ez West Blue, Atto Co., Ltd., Tokyo, Japan), which is as sensitive as enhanced-chemiluminescence[30]. For the amount of ABCA1 protein levels, the WI-38 cells cultured in the absence of apo A-I were taken to be 100, and the relative concentrations were shown using the intensity of the band obtained with densitograph software, CS Analyzer ver. 2.0 (ATTO Co. Ltd., Tokyo, Japan).

4. Statistical analyses

Significant differences were determined by one-way ANOVA with Tukey's multiple comparison post-test analysis. Ky Plot 5.0 (Kyens Lab Inc., Tokyo, Japan) was used for all analyses. Significance was set at $p < 0.05$.

3. Result

1. Modification of apo A-I by MG

Lipid-free apo A-I modified by 0-6 mmol/l MG, the reactive dicarbonyl derivative of glucose, for 37℃ for 0-24 h were migrated on 5-20% SDS-PAGE and detected by immunoblotting. MG-modified apo A-I multimerized pattern up to tetrameric band in a MG-concentration-dependent manner and a time-dependent manner (Fig. 1). It was confirmed with coomassie blue stain that MG-mediated apo A-I modification decreased the concentration of monomeric apo A-I in association with a concomitant increase in dimer-trimer-tetramer formation.

2. Effect of MG-modification on apo A-I ability to induce ABCA1-mediated cholesterol efflux from cells

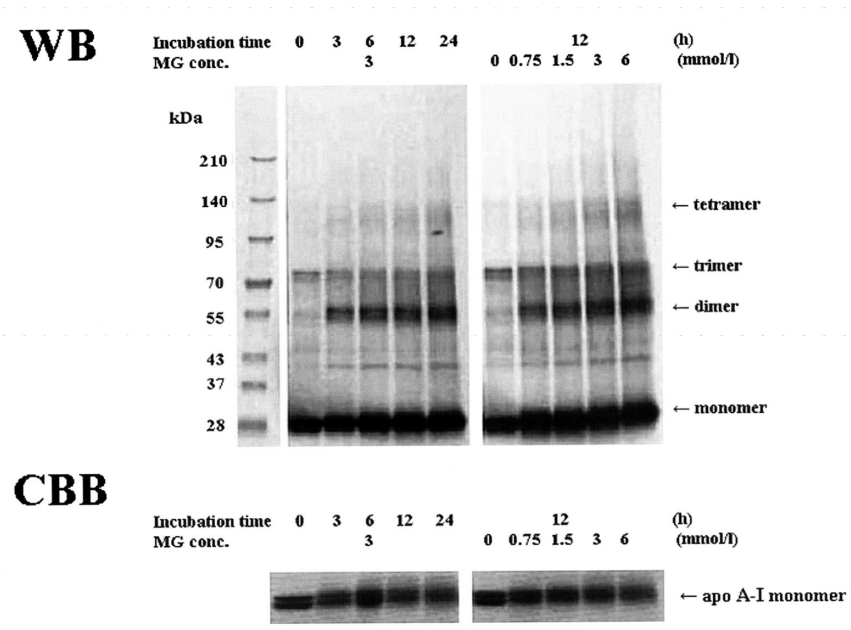

Fig. 1 Characterization of MG-modified apo A-I by electrophoresis.
Purified apo A-I (final concentration 1 mg/ml) were incubated at 37℃ in the absence or presence of 0-6 mmol/l MG for 0-24 h. After incubation, MG-modified apo A-I was migrated on 5-20% SDS-PAGE and visualized by westernblot (WB) or Coomassie stain (CBB).

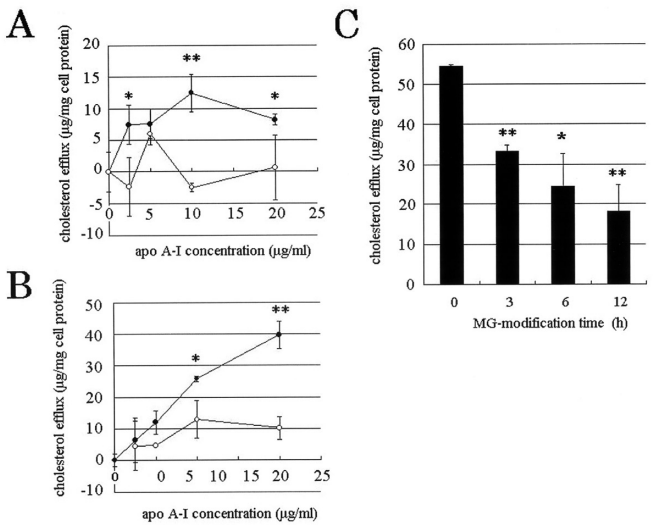

Fig. 2 Effect of apo A-I modification by MG on apo A-I-induced cholesterol efflux from fibroblasts.
MRC-5 (A) and WI-38 (B) were cultured for 24 h at 37℃ in the presence of normal apo A-I (black) or MG-modified apo A-I (white) (0-20 μg/ml). Apo A-I modified by 3 mmol/l MG for 12 h was used as MG-modified apo A-I. Cholesterol in the medium was determined as described in Materials and methods. Apo A-I of 20 μg/ml modified by 3 mmol/l MG for 0-12 h was added to WI-38, and the cholesterol concentration in the medium was determined by similar methods (C). Results are expressed as the mean ± SEM.
*: $p<0.05$ versus MG-modified apo A-I (A,B) or 0 h (C), **; $p<0.01$ versus MG-modified apo A-I (A, B) or 0 h (C).

In MRC-5 and WI-38, normal apo A-I induced cholesterol efflux in an apo A-I concentration-dependent manner. On the other hand, the MG-modification of apo A-I decreased the cholesterol efflux (Fig. 2 A, B), and at various times decreased the cholesterol efflux in a modified time-dependent manner (Fig. 2 C). Compared with the control, the cholesterol efflux by apo A-I modified for 12 h decreased by 67% (18.1 ± 6.67 μg/mg cell protein vs. control; 54.4 ± 0.41 μg/mg cell protein).

3. Determination of ABCA1 protein in cells examined

It is reported that ABCA1 is involved in apolipoprotein-mediated cholesterol release. The ABCA1 level in WI-38 used in the present study was determined by immunoblotting (Fig. 3). ABCA1 proteins were increased depending on the apo A-I concentration in WI-38. On the other hand, the increase of ABCA1 protein levels in WI-38 was not detected in WI-38 with added MG-modified apo A-I.

4. Discussion

Highly reactive alpha-oxoaldehyde is generated in chronic hyperglycemia. Since oxoaldehyde is reported to affect apo A-I functions[17], it has been suggested that alpha-oxoaldehydes contribute to intercurrent hyperlipidemia in diabetes patients. Apo A-I plays important roles in the RCT pathway. One of the functions of apo A-I is as a cholesterol efflux via ABCA1 on peripheral cells. Previous studies have shown the relationship between the HDL modification with nonenzymatic glycation and the cholesterol removal from cells. On the other hand, the effect of oxoaldehydes-modification on lipid-free apo A-I-induced cholesterol efflux from cells has yet to be thoroughly examined. In this study, we determined how the conformation change of lipid-free apo A-I by MG-modification affects the apo A-I/ABCA1 -induced cholesterol efflux from cells.

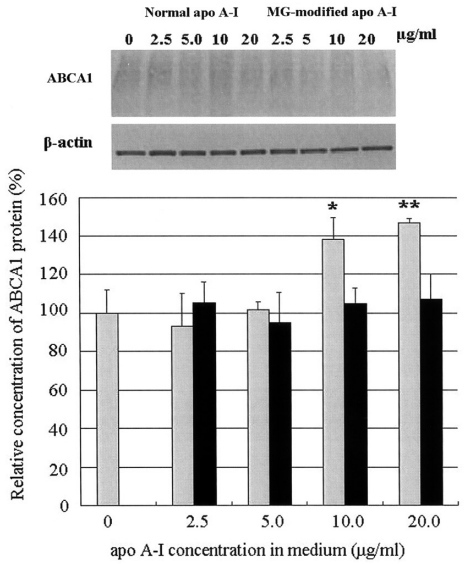

Fig. 3 Effect of MG modification of apo A-I on ABCA1 levels in WI-38.
WI-38 were cultured for 24 h at 37℃ in the presence of normal apo A-I (gray bar) or MG-modified apo A-I (black bar) (0-20 μg/ml). Total cellular protein (20 μg each) was subjected to SDS-PAGE for ABCA1 detection. After separation in polyacrylamide gel, proteins were transferred electrophoretically onto polyvinylidene difluoride membrane for probing with anti-ABCA1 antibody. Results are expressed as the mean ± SEM.
*: $p<0.05$ versus 0 μg/ml apo A-I; **; $p<0.01$ versus 0 μg/ml apo A-I.

It was confirmed that purified lipid-free apo A-I was cross-linked by incubation with MG, which agrees with the report by Nobecourt[17]. Cholesterol efflux was determined in the medium when the MG-modified apo A-I was added to the fibroblasts. Compared to normal apo A-I, adding MG-modified apo A-I decreased the cholesterol efflux in the medium. This indicates that cholesterol removal by apo A-I from the peripheral cells was reduced by the MG-modification of apo A-I. It has been reported that glycation of HDL_3 obtained from healthy serum by glucose decreases the binding capacity of HDL_3 to human fibroblast[26]. It was suggested that the binding capacity of apo A-I to cells or ABCA1 was reduced by MG-modification, resulting in a decrease in cholesterol removal.

ABCA1 protein levels were increased in fibroblasts added to normal apo A-I, while they were not increased in fibroblasts added to MG-modified apo A-I. It was reported that helical proteins inhibited ABCA1 degradation by calpain, resulting in the stabilization of ABCA1[31]. The apo A-I structure consists of a number of helical domains. The helical domain in N-terminal is particularly important for binding to ABCA1[32]. It was suggested that MG-modification affected the helix structure in apo A-I. The ABCA1 levels in cells were reported to correlate with a phospholipids release rather than with a cholesterol release[33]. Therefore, the MG-modification of apo A-I may affect the phospholipid efflux from cells more strongly than the cholesterol efflux.

Hypoalphalipoproteinemia, which is a major risk factor of arteriosclerosis, is frequently associated with diabetic patients, and the decrease of apo A-I expression in the liver in those patients has been confirmed as the cause[34]. However, the alpha-oxoaldehyde generated in hyperglycemia may also be due to the reduction of HDL cholesterol in the serum. Oxidation by reactive oxygen species is also related to apo A-I cross-linking[35, 36]. It is important to verify not only the relationship between conformation and function of apo A-I, but also the mechanism of cross-linked apo A-I formation in vivo for a better understanding of abnormal lipid metabolism as a risk factor of arteriosclerosis.

In this study, MG-mediated apo A-I modification resulted in change of the apo A-I structure and loss of cholesterol efflux-inducing capacity. Furthermore, MG-modified apo A-I showed no increase of ABCA1 protein levels in fibroblasts. These indicated that MG-modified apo A-I lose ABCA1-mediated cholesterol efflux-inducing capacity.

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