A Comparative Study of Cultured Smooth Muscle Cell Proliferation and Injury, Utilizing Glycated Low Density Lipoproteins with Slight Oxidation, Auto-oxidation, or Extensive Oxidation

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We investigated the influence of glycated low density lipoprotein (LDL) for vascular smooth muscle cell (SMC) proliferation or injury. We utilized glycated, slightly oxidized LDL (GLDL-LOX), glycated, auto-oxidized LDL (GLDL) and glycated, metal-induced extensively oxidized LDL (GLDL-OX) to examine the effect of glycation itself or combined glycation and oxidation on SMC. GLDL-LOX induced SMC proliferation and migration, and increased the number of platelet-derived growth factor receptor, β subunits, (PDGF-R) positive SMC. Also, GLDL-LOX promoted protease activity, compared with the other groups including native LDL (control). GLDL and GLDL-OX demonstrated SMC injury with apoptosis and Bax protein expression, compared with native LDL and GLDL-LOX. These results suggested that LDL glycation contributed to the progression of atherosclerosis by promoting SMC migration and proliferation, with little dependence on oxidative modification. Secondary auto-oxidation adding to glycation induced SMC apoptosis, and SMC injury occurred in the state of strong oxidation with glycation. We concluded that LDL glycation might play a key role in the progression of atherosclerosis in diabetes, and glycated LDL promoted atherosclerosis, even with little assistance from oxidation. *J Atheroscler Thromb*, 2000 ; 7 : 132-137.

Key words : Glycated LDL, Oxidative stress, Smooth muscle cells, Atherosclerosis

Introduction

Diabetes mellitus is well-known to accelerate atherosclerotic lesions as well as microvascular injury (1). Even in young people, high glycohemoglobin group (diabetic) raises the atherosclerotic lesions more extensively than normal subjects (non-diabetic) (2). It is still unclear how hyperglycemia affects the atherosclerosis progression.

The modified low density lipoprotein (LDL) is unanimously recognized as an important factor for the progression of atherosclerosis (3, 4). LDL particles in diabetic patients might be altered in a number of ways that may affect their metabolism and atherogenicity (5, 6). These mainly include alteration in composition, enhanced

nonenzymatic glycosylation (5) and increased susceptibility to oxidative modification (7, 8). Glycated LDL is increased in diabetic patients, where disease also proceeds under euglycemic conditions (9). The function of glycated LDL, especially glycated and slightly oxidized LDL, has neither been explained well nor distinguished from secondarily oxidized LDL in diabetes.

In this study, we utilized three glycated LDLs ; 1. glycated and slightly-oxidized LDL 2. glycated and auto-oxidized LDL, and 3. glycated and metal-induced extensively-oxidized LDL. The effect of these glycated LDLs for vascular smooth muscle cell (SMC) was investigated in order to clarify the influence of glycated LDL on atherosclerosis. Our study was focused on the proliferation, migration, cell injury, programmed cell death (apoptosis) and protease activity of SMC influenced by three different types of glycated LDL addition.

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Received November 5, 1999.

Accepted for publication June 30, 2000.

Materials and Methods

Low density lipoprotein (LDL)

LDL, derived from human plasma, was purchased from Sigma Co., St. Louis, USA. LDL was used immediately after purchase as native LDL (nLDL). This purchased LDL was lyophilized from 1 mM of LDL solution in 0.15 M NaCl and 0.01% EDTA at pH 7.4 and refrigerated at 4°C.

LDL preparations

LDL was incubated with 200 mM glucose at 37°C for 1 weeks (10). This LDL was indicated as glycated LDL (GLDL), which seemed to be naturally and minimally oxidized LDL. Butyl-hydroxytoluene (BHT: 25 μ g/l) and EDTA were added to LDL at the starting point of incubation with glucose, in order to stop LDL oxidation. The LDL produced by this method was called glycated LDL with slight oxidation (GLDL-LOX). LDL incubated with 200mM glucose for 1 week with 8 μ mol CuSO₄ for 48 hours to produce extensively-oxidized, glycated LDL, was indicated as GLDL-OX. The thiobarbituric acid-reactive substances (TBARS) values of each utilized LDL were follows; nLDL: 0.11 ± 0.08 , GLDL-LOX: 0.16 ± 0.01 , GLDL: 0.22 ± 0.04 , GLDL-OX: 0.4 ± 0.22 (nmol/100 μ g protein). There was a significant difference between the TBARS value of nLDL and that of GLDL-OX ($p < 0.05$). The lag time for conjugated diene formation was 100 ± 20 minutes in GLDL-OX. In the other three groups, the lag time exceeded 180 minutes.

Cell culture

SMC was purchased from Kurabou Co. (Kurashiki, Japan), derived from media of human aorta, twice-passaged (5×10^5 /ml). The conditions of cell culture were 37°C, 5% CO₂. These cells were divided and seeded in the slide chamber (Nunc Co. Naperville, USA), and filled with medium without any serum. The cells were incubated with (a) nLDL: $n=5$, (b) GLDL-LOX: $n=5$, (c) GLDL: $n=5$, and (d) GLDL-OX: $n=5$, in each chamber space for 24 hours.

Cell number count

After incubation in a slide chamber, each slide was fixed in neutral buffered formalin, and stained with hematoxylin solution. The cell number of each chamber was counted. Each datum was expressed as the percentage of cell number before incubation.

SMC migration rate

SMC were divided and seeded into a chemotaxis chamber (Kurabo Co., Japan). Each chemotaxis was put into 24 wells filled with medium. The cells which migrated to the lower surface of chemotaxis were counted. Each datum was expressed as the percentage of preincubation SMC number.

Apoptosis

Apoptotic cells were examined by the TUNEL method, using an ApopTag (Oncor, Gaithersburg, USA) kit for probing DNA fragmentation. Briefly, each slide was fixed with 10% neutral buffered-formalin. After quenching endogenous peroxidase, an equilibration buffer was applied. Then, terminal deoxynucleotidyl transferase (TdT) was applied, followed by prewarmed Stop/Wash buffer at 37°C for 1 hour. Finally, anti-digoxigenin-peroxidase was added. Diaminobenzidine tetrahydrochloride (DAB) was utilized for color development. Slides were counter-stained and mounted. Data were expressed as the percentage of total cell number.

Immunohistochemistry for platelet-derived growth factor receptor (PDGF-R) and Bax protein

Each slide was fixed with 10% neutral buffered formalin. Briefly, each slide was incubated with mouse antihuman PDGF-R, β subunit, monoclonal antibody (Genzyme Co., Cambridge, USA; dilution 1:100) and Bax protein monoclonal antibody (MBL Co., Nagoya, Japan; dilution 1:100) at 4°C overnight after blocking by normal serum. After washing, the slide was incubated with the second antibody at room temperature, followed by incubation with avidin-biotin complex at room temperature. Immunoreactivity of PDGF-R and Bax was developed by DAB. The positive cells were counted, and data were expressed as the percentage of total cell number.

Protease activity

The gel was prepared as follows; 1% agarose melted in 50 mmol/l Tris-HCl, pH 7.4, containing 10 mmol/l calcium chloride and 0.05% Brij 35 was mixed 1:1 with 1 mg/ml casein (11). The supernatant of each group was mixed with this gel. After reaction for 1 month, the weight of each gel was calculated. Each datum was expressed as follows; $100 - 100 \times \text{Gel weight after reaction} / \text{Gel weight before reaction} (\%)$.

Statistical evaluation

Results are expressed as mean $\pm$ S.D. of the overall data. The statistical significance of the difference between various parameters was calculated using the students *t* test. The significant difference was taken as $p < 0.05$.

Results

SMC number

The number of SMC incubated with GLDL-LOX was significantly increased ($p < 0.05$) compared with the nLDL. This revealed that GLDL-LOX might promote SMC proliferation. On the other hand, the SMC number incubated with GLDL-OX was significantly decreased ($p < 0.05$) compared with the nLDL. This indicated that GLDL-OX induced SMC cytotoxicity. There was no significant

Fig. 1. This graph showing the change of SMC number between before incubation and 24 hours after incubation. Cell number before incubation is 5×10^5 /ml, each group. The value of GLDL-LOX group is higher, and the value of GLDL-OX is lower than that of nLDL (control) group, after 24 hours.
◇: nLDL group, □: GLDL-LOX group, △: GLDL group, ○: GLDL-OX group. Each group: $n=5$, *: significant difference ($p < 0.05$)

difference of SMC number between the groups of nLDL and GLDL (Fig. 1).

SMC migration rate

The migrated SMC number in the GLDL-LOX group was significantly increased compared with the nLDL, GLDL and GLDL-OX groups. This suggested that GLDL-LOX might promote SMC migration. There was no significant alteration by the addition of GLDL-OX, compared with the nLDL group (Fig. 2).

PDGF-R immunoreactivity

SMC in the nLDL and GLDL-LOX groups showed immunoreactivity of PDGF-R in the cell surface and cytoplasm (Fig. 3). The GLDL-LOX group revealed a higher number of PDGF-R positive cells than the nLDL group ($p < 0.05$). PDGF-R expression was not induced by the procedure of oxidation of glycated LDLs in cultured SMC (Fig. 4).

Apoptosis and Bax protein

Apoptotic cells, detected by the TUNEL method, and Bax protein-positive cells were demonstrated in SMC of

Fig. 2. This bar graph showing the rate of migrated SMC after incubation. The value of GLDL-LOX group is higher than nLDL (control) group. Each group: $n=5$, *: significant difference ($p < 0.05$)

Fig. 3. This photograph revealing PDGF-R positive SMC of GLDL-LOX group. Intense immunoreactivity is demonstrated in the SMC cytoplasm, left side of this photograph (original magnification $\times 200$).

the GLDL and GLDL-OX groups (Fig. 5). The number of apoptotic cells of GLDL was significantly higher than that of the GLDL-OX group. No notable reactivity was found in SMC of the nLDL and GLDL-LOX groups, for either apoptosis or Bax protein (Figs. 6, 7).

Protease activity

In the GLDL-LOX group, protease activity, calculated by the formula described above, was significantly higher than in the nLDL, GLDL and GLDL-OX groups (Fig. 8). This

Fig. 4. This bar graph showing the rate of positive SMC for PDGF-R on immunohistochemistry. The value of GLDL-LOX group is higher than nLDL (control) group. In contrast, the values of GLDL and GLDL-OX are lower than control. Each group: $n=5$, *: significant difference ($p<0.05$)

Fig. 5. This photograph revealing apoptotic SMC of GLDL group, detected by TUNEL method. The nuclei also show shrinking appearance (original magnification $\times 200$).

suggested that GLDL-LOX evoked the secretion of protease from SMC, leading to the casenolytic activity.

Discussion

There was the evidence that the biochemical characteristics of glycated LDL are similar to those of oxidized LDL. Actually, superoxide anions were produced in a hyperglycemic state, followed by the formation of oxidized LDL (12). By lipid analyses of glycated LDL, LDL incubat-

Fig. 6. This bar graph showing the rate of apoptotic SMC detected by TUNEL method. Only GLDL and GLDL-OX groups reveal apoptotic SMC. The value of GLDL group is higher than GLDL-OX group. *: no expression of apoptosis, *: significant difference ($p<0.05$)

Fig. 7. This bar graph showing the rate of positive SMC for Bax protein. Only GLDL and GLDL-OX groups reveal expression. Each group: $n=5$, No significant difference is noted. *: no expression of Bax protein.

Fig. 8. This bar-graphical presentation shows the protease activity of each group after 1 month reaction. The value of GLDL-LOX group is higher than nLDL (control) group. Each group: $n=5$, *: significant difference ($p < 0.05$)

ed with glucose and 1 mM EDTA was described as primary surface oxidized LDL, characterized by phosphatidylcholine hydroperoxide production (13, 14). Thus, GLDL was described as glycated and auto-oxidized LDL (15). The GLDL-LOX utilized for this study was not determined exactly as "pure glycated, non-oxidized LDL" because it was unclear whether BHT and EDTA could inhibit oxidation completely, especially minimal oxidation. Minimal oxidation could not be evaluated by the TBARS method. Although further investigation for lipid analysis might be required, our GLDL-LOX could be described as slightly-oxidized LDL.

In our results, GLDL-LOX addition increased number of the SMC and migrated SMC. The cell number increasing could not conclusively indicate SMC proliferation, but Ki67 expression was demonstrated in a few SMC of GLDL-LOX (data not shown). Thus, these results suggest that glycated LDL evokes intimal thickening by migrated SMC proliferation in the early stage of diabetes mellitus; the state of little oxidative stress. PDGF receptor, already associated with the expression of SMC in thickened intima of atherosclerosis (16), could be related to this proliferation by our method of counting the positive cell number. In addition, GLDL-LOX could induce the phenotypic change of SMC, leading to SMC migration. In contrast, GLDL-OX and GLDL revealed strong cell injury or apoptosis induction. These results might imply the formation of a necrotic core in late or advanced stage atherosclerosis.

Two different types of glycation-reactive substance are

known. One is the amadori product, produced in the early stage during glycation, approximately one to three weeks after commencement (17). The other is advanced glycosylation end products (AGE), formed in the late stage (18). In this study, little attention was paid to AGE, because the time of glycation was almost 1 week. Thus, the amadori product might have played a major role in some of our results. The function of the amadori product has not been investigated, but its existence was established in experimental diabetic rat aorta (19).

Apoptosis, well-known as the morphosis of programmed cell death, plays a key role in atherosclerosis (20). Oxidative stress is a mediator of apoptosis (21). Our study suggested that LDL glycation itself caused neither apoptosis nor necrosis, with little oxidation.

In glycated LDL, apoB protein glycation, mainly on lysine residues, and the change of lipid composition were main alterations in addition to auto-oxidation (22). Glycated LDL enhances uptake of LDL by monocyte-derived macrophages. This is probably mediated by a low-affinity, high capacity surface receptor (called AGE receptor) (23) or scavenger receptor (24). Glycation and oxidation are closely related and could mutually accelerate each other (25). Oxidized LDL performs various functions (26), and the combined glycation and oxidation of LDL generated a product that was more atherogenic than either glycation or oxidized LDL alone (27).

The importance of atheromatous plaque rupture in the incidence of acute myocardial infarction has been identified (28). Protease, like matrix metalloprotease (MMP), took a key role of plaque rupture in the extracellular matrix degradation. The expression of MMP has been demonstrated on migrated SMC in the ruptured atheroma (29-31). Although we did not investigate the function of MMP in detail, the casenolytic activity we presented could suggest MMP-3 (stromelysin) activity (11). Thus, this result might suggest that diabetic patients are at risk of plaque rupture.

We emphasized on the clear difference among glycated slightly-oxidized, glycated, auto-oxidized, and glycated extensively-oxidized LDLs. Further investigation will be required for detail biochemical or molecular analyses, but our hypothesis was that LDL glycation itself promoted the early atherosclerosis and glycated LDL was concerned with the formation of a necrotic core under the additional oxidative stress. It was concluded that a high level of serum glucose was a definitive high risk factor for atherosclerosis, even with little oxidative stress.

Acknowledgments: The authors wish to appreciate Professor Isamu Sakurai and Professor Norimichi Nemoto, Department of Pathology, Nihon University School of Medicine, for their advice and revision of the manuscript in this research.

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