APA Hamster Model for Diabetic Atherosclerosis.

2. Analysis of Lipids and Lipoproteins

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Abstract: Syrian hamsters of the APA strain (APA hamsters) have recently been shown to have atheromatous lesions in the aortic arches under diabetic condition induced by a single injection of streptozotocin (SZ). In that model, fatty streaks, which are the initial lesions of atherogenesis, develop by 6 weeks after the injection (WAI). In this study, we evaluated plasma lipid concentrations and lipoprotein profiles in diabetic APA hamsters at 6 WAI to reveal the early stage of atherogenesis clinicopathologically. As a result, by biochemical analysis, hyperglycemic APA hamsters showed signs of hypercholesterolemia and hypertriglyceridemia. Low-density lipoprotein (LDL) cholesterol significantly increased, but high-density lipoprotein (HDL) cholesterol significantly decreased. Agarose gel electrophoresis showed an obvious increase in the fractions of chylomicron, LDL and abnormal lipoprotein. Plasma LDL in diabetic animals was in a state more susceptible to oxidation. In addition, a significant increase in glycated LDL was also found in the diabetic animals by enzyme linked immunosorbent assay (ELISA). Moreover, lipid peroxidation product (4-hydroxynonenal (4 HNE))-adducted proteins and advanced glycation end-products (AGE) were immunohistochemically detected in the foam cells of the fatty streaks. These results revealed that diabetic APA hamsters had hyperlipidemia characterized by increases in chylomicron, LDL and abnormal lipoprotein, and suggested that oxidized LDL and/or glycated LDL might be actively uptaken by macrophages and play an important role in the initial stage of atherogenesis.

Key words: APA hamster, diabetic atherosclerosis, lipoprotein, modified LDL

Introduction

Diabetes is known to be often accompanied by hyperlipidemia, which is most closely associated with atherosclerosis. In many clinical studies on patients with atherosclerosis, a high level of blood cholesterol

or triglyceride, a high level of low-density lipoprotein (LDL) or triglyceride-rich lipoproteins (very low-density lipoproteins (VLDL) and chylomicron), and a low level of high density lipoprotein (HDL) are described as risk markers for atherosclerosis [14, 15, 26, 27, 37, 54].

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Among various risk factors involved in atherogenesis, LDL has especially been studied and reported to play a crucial role in the formation of foam cells (FCs). The accumulation of FCs characterizes fatty streaks which are the initial atheromatous lesions. FC formation results from uptake of modified forms of LDL but not native LDL mainly by macrophages (M ϕ s). Most well known modified LDL is oxidized LDL (ox-LDL) [17, 28, 38], which has been detected in blood and atheromatous lesions of humans and laboratory animals [8, 22, 60, 64].

In addition to ox-LDL, glycated or glyco-oxidized LDL in diabetic patients has been considered to explain rapid and severe progression of atherosclerosis [56, 59]. In this connection, advanced glycation end-products (AGE), which are the irreversible last-stage products in non-enzymatic glycation, have drawn much attention recently. A significant increase in AGE has been detected in the blood of diabetic patients [31, 63] and AGE deposition in atherosclerotic lesions has been detected in patients with diabetes [25, 31, 34].

In Syrian hamsters, apolipoprotein (apo) B-48 containing lipoproteins is exclusively of intestinal origin and apoB-100 is exclusively of hepatic origin, which is analogous to the condition in humans but not in mice or rats [4, 13]. Syrian hamsters of the APA strain (APA hamsters) have recently been shown to have atheromatous lesions in the aortic arches under diabetic conditions induced by a single injection of streptozotocin (SZ) [62]. In that model, fatty streaks, which are the initial lesions in atherogenesis, develop until 6 weeks after the injection (WAI). In this study, we therefore evaluated plasma lipid concentrations and lipoprotein profiles in diabetic APA hamsters at 6 WAI to reveal the early stage of atherogenesis clinicopathologically. We also examined depositions of oxidized deoxynucleic acids (DNA), lipid peroxidation product-adducted proteins and AGE in the aortic walls to show some evidence of oxidation and glycation of lipoproteins.

Materials and Methods

Animals

We examined 4 hyperglycemic male APA hamsters which were once injected at the age of 10 weeks intraperitoneally with 40 mg/kg body weight of SZ (Sigma

Chemical Co., St Louis, USA) dissolved in 2 ml of 0.1M citrate buffer (CB) (pH 4.5), and the same number of animals injected with the same volume of CB only were used as controls. The former were called the SZ group and the latter the CB group. The animals were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained under controlled conditions (temperature: 24 $\pm$ 2°C; dark-light cycle: 12–12 hr) in plastic cages with sterilized wood shavings for bedding, and fed a commercial diet, CMF (Oriental Yeast Co. Ltd., Tokyo, Japan) with tap water *ad libitum*. This experiment was approved by the Animal Care and Use Committee of Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Sampling

At 6 WAI, each animal was weighed and killed by collecting blood from the vena cava under ether anaesthesia after overnight (14–16 hr) fasting. Immediately after exsanguination, the aorta was taken from each animal and fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS). Fresh plasma samples were separated from 0.1% ethylenediaminetetraacetic acid (EDTA) containing blood samples and added to the mixture including 0.02% sodium azide (NaN₃), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.03% benzamidine, 10 μ g/ml gentamycin and 5 mM butylated hydroxytoluene (BHT) for storage at 4°C.

Plasma biochemistry

Plasma samples were subjected to the measurement of glucose, triglyceride, total cholesterol, and HDL cholesterol with commercial test kits (Wako Pure Chemical Industries Inc., Osaka, Japan). HDL cholesterol was measured after removing apolipoprotein B-containing particles by the magnesium/phosphotungstic acid precipitation method [57, 58]. LDL cholesterol levels were quantified with LDL Cholesterol Kit (Sigma Diagnostic, Inc., St Louis, USA) and the above mentioned test kit for total cholesterol [45]; after the removal of HDL and VLDL by incubating with latex beads coated with goat polyclonal antibodies to human apolipoproteins and filtrating, LDL cholesterol in the filtrate was determined. Since plasma samples in the SZ group were milky white, the absorbance of plasma in each reaction buffer without an enzyme was measured as a background, except for measuring HDL and LDL cholesterol. Plasma

samples in the SZ group were also 2 or 4 fold diluted with saline so that values might be within the limits in the manufacturer's guide. Student's *t*-test was used for statistical analysis.

Lipoprotein electrophoresis

Fractions of plasma lipoproteins were analyzed by agarose gel electrophoresis [58]. Aliquots of 1 μ l plasma in the CB group and 1 μ l twice-diluted plasma with saline in the SZ group were used. Electrophoresis was carried out on 1% agarose gel films (Agasheet-L, Wako) with a horizontal apparatus (Submerge-Agarose-System, Atto Corporation, Tokyo, Japan) at 90 volts for 45 min, followed by staining with Fat Red 7B, on the day of sampling and after storage of the plasma at 4°C for 2 days. Electrophoresis patterns of lipoproteins were also scanned by means of a computerized densitometry system.

Enzyme-linked immunosorbent assay (ELISA)

Glycated LDL levels in plasma samples were measured with the ELISA kit (Exocell, Inc., Philadelphia, USA), according to the manufacturer's guide. Twenty μ l of plasma was mixed with 800 μ l of a diluent reagent and added to a microplate pre-coated with glycated LDL. The plate was then incubated with a mouse monoclonal antibody to human glycated LDL for 1 hr, washed, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G goat antibody for 1 hr. Color developing was performed with tetramethylbenzidine (TMB) for 20 min. The standard curve was drawn by plotting 5 points: 1, 2, 4, 8 and 16-fold diluted standard glycated LDL. Statistical analysis was done with Student's *t*-test.

Immunohistochemistry

Four μ m thick paraffin sections of the longitudinal aortic arches were stained by the indirect immunohistochemical method for 8-hydroxydeoxyguanosine (8-OHdG), 4-hydroxynonenal (4 HNE) and AGE. 8-OHdG is an oxidized product of 2-deoxyguanosine and is a marker for oxidative damage to DNA. 4-HNE is an oxidized product from polyunsaturated fatty acids (n-6), such as arachidonic acid, and reacts with amino acid residues of proteins, leading to the formation of stable Michael adducts. Both these oxidized markers can be confidentially detected on the usual paraffin sections

without special antioxidative treatment [9, 21, 24, 52, 53]. After treated with 0.1% trypsin only for 8-OHdG, with 0.3% hydrogen peroxide in methanol, and with 1% bovine serum albumin, the sections were treated with mouse monoclonal antibodies to 8-OHdG (NOF Corporation, Tokyo, Japan; 1:100 dilution), 4-HNE (NOF Corporation, 1:1000 dilution) or AGE (Kumamoto Immunochemical Laboratory Co., Ltd., Kumamoto, Japan, 1:300 dilution), followed by biotinylated rabbit antibody to mouse immunoglobulins (DAKO A/S, Glostrup, Denmark) and streptavidin-biotin peroxidase complex (StreptABComplex/HRP kit, DAKO). Visualization was performed with 3,3' diaminobenzidine (DAB).

Results

Plasma biochemistry (Table 1)

Plasma glucose levels in the SZ group were 3-fold higher than those in the CB group (statistical significance: $p < 0.001$). Triglyceride levels were about 10-fold higher and total cholesterol levels were about 4.5-fold higher in the SZ group than in the CB group (statistical significance: $p < 0.01$ and $p < 0.001$, respectively). LDL cholesterol levels in the SZ group were about 3-fold higher than those in the CB group (statistical significance: $p < 0.01$). In contrast, HDL cholesterol levels in the SZ group were significantly lower than in the CB group (statistical significance: $p < 0.01$).

Lipoprotein electrophoresis

The fractions of chylomicron and LDL significantly increased in the SZ group, as compared with the CB group (Figs. 1a, b). The fractions of VLDL in the SZ group had broad bands but no clear peaks and (Figs. 1a, b), and they increased a little, as compared to the CB group (Fig. 1b). Abnormal fractions also appeared between the chylomicron and the LDL fractions in the SZ group (Figs. 1a, b). The HDL fractions were not detected in either the CB or SZ group.

Moreover, the relative mobility of the LDL fractions in the SZ group increased as compared to that in the CB group after storage of the plasma samples for 2 days at 4°C (Figs. 1c, d), suggesting that oxidation of LDL in the SZ group progressed in spite of the addition of antioxidants.

Fig. 1. Lipoprotein electrophoresis on agarose gels on the day of sampling (a, b) and after storage at 4°C for 2 days (c, d). Lanes 1–4: the CB group; lanes 5–8: the SZ group. CHYL: chylomicron; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; ABN: abnormal lipoprotein. In b and d, dark-blue line: lane 1; blue line: lane 2; violet line: lane 3; green line: lane 4; yellow line: lane 5; pink line: lane 6; magenta line: lane 7; red line: lane 8. Note that the samples in the SZ group were twice-diluted with saline (a, c) and scanned data in the SZ group were twice-multiplied data (b, d).

Glycated LDL levels

A significant increase in plasma glycated LDL levels was seen in the SZ group, as compared with in the CB group (Fig. 2).

Immunohistochemical findings

In the SZ group, 8-OHdG was clearly detected in the nuclei of FCs of the fatty streaks (Fig. 3a) and in some endothelial cells (ECs), and weakly detected in smooth muscle cells. 4-HNE-adducted proteins (Fig. 3b) and AGE (Fig. 3c) are seen mainly in FCs of the fatty streaks and sparsely in the media. In the CB group, 8-OHdG, 4-HNE and AGE were very rarely found only in the media (data not shown).

Fig. 2. Plasma glycated LDL levels in the CB and SZ group. Values are expressed as means $\pm$ SDs [mg/dl]. Statistical analysis was performed with Student's *t*-test; $p < 0.01$.

Fig. 3. Immunohistochemical detection of 8-OHdG (a), 4-HNE (b) and AGE (c) on the aortic arches of APA hamsters in the SZ group. L: lumen; M: media. 8-OHdG (a) is clearly found in the nuclei of foam cells (FCs) and some endothelial cells in a fatty streak, and is weakly detected in the nuclei of smooth muscle cells. 4-HNE-adducted proteins (b) and AGE (c) are seen mainly in FCs in the fatty streaks and sparsely in the media. $\times 220$.

Discussion

We have recently demonstrated that the fatty streaks, which are the initial atheromatous lesions, developed in diabetic APA hamsters until 6 WAI [62]. In this

Fig. 4. Changes in cholesterol levels of lipoproteins at in the CB and SZ groups. Black bars: high-density lipoprotein; line bars: low-density lipoprotein; dot bars: other lipoproteins.

study, we evaluated plasma lipid concentrations and lipoprotein profiles in diabetic APA hamsters at 6 WAI to reveal the early stage of atherogenesis clinicopathologically. As a result, diabetic APA hamsters showed signs of hypercholesterolemia and hypertriglyceridemia, which are usual complications in diabetic patients. Biochemical analysis of the cholesterol of lipoproteins revealed a significant increase in LDL and a decrease in HDL, which are general markers for atherosclerosis in humans [25, 37, 54]. Nevertheless, an increase in cholesterol levels of non-LDL and non-HDL lipoproteins was more conspicuous after subtracting LDL and HDL from total cholesterol levels (Fig. 4). In fact, lipoprotein electrophoresis revealed obvious increases in chylomicron and abnormal fractions as well as the LDL fraction. It is noted that apo B-48 containing lipoproteins are exclusively of intestinal origin in Syrian hamsters [4, 13] and the chylomicron level was high even though blood was collected during overnight fasting in this model. The lipoprotein changes in this model could not simply be classified as hyperlipidemia in human patients, and further studies are needed to clarify the mechanisms.

The abnormal fraction which appeared between the chylomicron and LDL fractions in lipoprotein electrophoresis might be suspected of being lipoprotein X

(Lp-X) [47], which has been reported in patients with cholestatic liver diseases [11, 33, 44], patients with a deficiency of lecithin/cholesterol acyltransferase (LCAT) [16, 36, 48] and during intravenous infusion of fat emulsions [2, 50, 51]. Lp-X is a bilayer vesicle of phospholipids (51–66%) and free cholesterol (22–30%) and contains a small amount of proteins essentially including albumin and apoCs but also including apoE and apoAI [2, 16, 33, 35, 36, 39, 44, 48]. Lp-X is postulated to be related to biliary lipid vesicles, but the mechanism of Lp-X generation remains unclear. The role of Lp-X in atherogenesis has also not been clarified; Lynn *et al.* [29] have only reported that Lp-X enhanced FC formation in rat M ϕ s, whereas other reports have demonstrated that Lp-X did not enhance [42, 43] but rather suppressed [1, 2] FC formation. More studies are needed to reveal whether the abnormal lipoprotein seen in this study is Lp-X, and its pathological significance in this model.

Agarose gel electrophoresis revealed advanced oxidation of LDL in the SZ group by increasing the relative mobility of the LDL fraction [6, 18, 19, 41, 61], though a clear difference between the plasma ox-LDL concentrations in the SZ group and those in the CB group could not be detected by dot blot analysis (data not shown). The susceptibility of LDL to oxidation has been reported to have a significant relation to progression of atherosclerosis in humans [3, 40], and more attention has been paid to the oxidizability of LDL from the aspect of local oxidation in the arterial walls [5, 46, 49, 54]. Therefore, when there would be excessive LDL in blood, LDL might stay longer in the arterial wall, where it could be oxidatively modified because of its high susceptibility to oxidation. Moreover, ox-LDL in the arterial wall would actively be taken up by invading M ϕ s, enhancing FC formation. In fact, lipid peroxidation product (4-HNE)-adducted proteins and oxidized DNA (8-OHdG) were immunohistochemically detected in the fatty streaks in diabetic APA hamsters, giving evidence of oxidative stress in the aortic walls of those animals.

Moreover, glycated LDL, whose levels significantly increased in the SZ group, has also been shown to be very sensitive to oxidation [7, 23, 32]. It has been suggested that glyco-oxidized LDL is more rapidly taken up by M ϕ s than merely oxidized LDL. In fact, AGE deposition in the atheromatous lesions was im-

munochemically detected in FCs in this study. In diabetic complications, AGE is especially important and has been supposed to be associated with more rapid and severe development of atheromatous lesions in diabetic patients than in non-diabetic people [10, 12, 20, 30].

In diabetic APA hamsters, hyperlipidemia and hyperlipoproteinemia are characterized by increases in chylomicron, LDL and abnormal lipoprotein. Glycated and/or oxidized LDL is considered to be actively taken up by M ϕ s and to play an important role in the initial stage of atherogenesis.

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