A Simplified Colorimetric Assay of Nonenzymatic Glycosylation of Human Low Density Lipoproteins in Normal and Diabetes Mellitus

Kyung-soo Hahm, Kwang Kyun Park and Yoon Soo Kim

A simplified colorimetric method for measurement of the levels of glycosylation of proteins was developed by a modification of an existing method. Employing this method, the extent of nonenzymatic glycosylation of apolipoprotein B subspecies (B-100, B-74; B-26), LDL, VLDL, and total serum proteins in human plasma obtained from patients with diabetes mellitus and control subjects was compared. Plasma LDL (1.019<d<1.063) and VLDL (d<1.006) were separated using the sequential ultracentrifugation method, and the subspecies of apolipoprotein B were isolated by extracting them from polyacrylamide gels after they were separated by preparative SDS-polyacrylamide gel electrophoresis. Increases in the level of glycosylation of serum proteins, LDL, VLDL, and apo B subspecies obtained from diabetic patients were observed. Among them, the increases of glycosylated LDL and apo B-26 were most significant (p<0.001). Also, good correlations were found between glycosylations of apo B-26 and LDL (r=0.88), and glycosylation of LDL and LDL cholesterol level (r=0.79). The results also showed an excellent correlation between levels of HbA1c and glycosylated apo B-26 (r=0.93).

Key Words: Nonenzymatic glycosylation, atherosclerosis, LDL, apo B subspecies, diabetes mellitus.

Nonenzymatic glycosylation of proteins involves the condensation of carbohydrate and free amino groups of a protein (Mohammad et al. 1949; Feeney et al. 1964). The reaction occurs nonenzymatically through the formation of covalent linkage with glucose (Gottschalk 1972). That similar reactions can also occur under physiological conditions was brought to light more recently, when it was shown that hemoglobin undergoes nonenzymatic glycosylation in human blood, in vivo, forming the so-called minor hemoglobin factions (HbA1a-c) (Bunn et al. 1975; Cerami and Koenig 1978; Dolhofer et al. 1977).

It has been suggested that the nonenzymatic glycosylation could occur at enhanced rates during hyperglycemia, and this may contribute to the progressive and accelerated atherosclerosis in chronic complications of diabetes (Crall and Roberts 1978; Christy and Clements 1982). One proposed mechanism contributing to the development of atherosclerosis involves disorders of low density lipoprotein (LDL) metabolism. Goldstein and Brown (1974, 1977) have suggested that the cellular LDL receptor pathway may play an important role in atherosclerosis and LDL is the source of most of the cholesterol present in vascular regions and cellular elements of the vessel wall. Recently it has been reported that the modification of lysine residues of LDL alters its catabolism in vivo (Weisgraber et al. 1978; Mahley et al. 1979; Brown et al. 1979, 1980; Fogelman et al. 1980; Gonen et al. 1983) and in vitro (Gonen et al. 1981; Kim and Kurup 1982; Sasaki and Cottam 1982; Witztum et al. 1982). It has also been demonstrated that glycosylation of LDL occurs at lysyl residues both in vitro and in vivo (Gonen et al. 1981; Kim and Kurup 1982). Glycosylated LDL has been reported to be elevated in diabetes (Schleicher et al. 1981; Witztum et al. 1982).

Various procedures have been used for the determination of the degree of glycosylation in proteins. These procedures include HPLC (Schleicher and Wieland 1981; Bisse et al. 1982; Monnier and Cerami 1983), affinity chromatography (Bannon et al. 1984;
Fluckiger et al. 1984; Agarwal et al. 1985), isoelectric focusing and electrophoresis (Spicer et al. 1978; Allen et al. 1980), and colorimetric assay (Dolhofer and Wieland 1980; Ney et al. 1981). However, the procedures using HPLC or affinity chromatography require either expensive equipment or reagents, and electrophoretic procedures are not well established, whereas the colorimetric assay method is time consuming. The first aim of the present study has been therefore, to modify the colorimetric assay method so that the determination of glycosylated level in proteins can be made with ease, less cost, and within a shorter length of time.

LDL contains apolipoprotein B (apo B) as its major protein constituent (Blue et al. 1980) and apo B is now known to consist of subspecies, apo B-100, B-74, and B-26 according to their apparent molecular weights (Kane et al. 1980). Apo B-100 is also a protein component of very low density lipoproteins (VLDL) (Kane et al. 1980; Tikkanen et al. 1984). Apo B is also known to be the recognition marker on lipoproteins for the cellular LDL receptors, although the roles of various apo B subspecies are unknown. Therefore, the second aim of the present study has been to measure and compare the degree of glycosylation in LDL, VLDL, and various apo B subspecies obtained from the plasmas of normal and diabetic subjects, and find meaningful correlations between various factors including cholesterol levels and HbA1c in order to locate the most significant apo B subspecies.

**MATERIALS AND METHODS**

**Separation of Plasma Lipoproteins**

Blood was obtained from 13 apparently normal, healthy subjects (mean age: 33) and 20 diabetic patients (type II) after an overnight fasting using 1 mM EDTA as an anticoagulant. The patients were admitted to Severance Hospital at Yonsei University in Seoul, Korea, from December 1984 to February 1985. After erythrocytes were spun down, each plasma was dialyzed against 1.006 EDTA-saline (1 mM Na_{2}EDTA-165 mM NaCl, d=1.006) containing chloramphenicol (50 mg/l), gentamycin sulfate (50 mg/l), and sodium azide (0.02%) (Kane et al. 1980). Apo B containing lipoproteins, VLDL (d<1.006) and LDL (1.019<d<1.063) were isolated by the density-adjusted sequential ultracentrifugation method (Schenfeld et al. 1974) using a Beckman L2-75B Ultracentrifuge. Density was adjusted with solid KBr and two ultracentrifugations were carried out at each density. After isolation, each lipoprotein fraction was dialyzed exhaustively against 1 mM EDTA-water, pH 8.0, containing the above antibiotics, passed through 0.45 µm membrane filter (Gelman, Ann Arbor, Mich., USA) and stored at 4°C.

**Chemical Analysis**

Plasma and LDL cholesterol levels were determined by the method of Leffler (1959) and the triglyceride contents were measured by the method of Biggs et al. (1975). Protein concentrations in separated apo B subspecies were measured according to the modified micro method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard, and protein concentrations of lipoprotein fractions and whole plasma were determined in the same way except that each sample was delipitated by extracting it with water-saturated ether before measuring the absorbance at 750 nm. Levels of fasting blood sugar were measured using YSI (model 23A) glucose analyzer.

**SDS-Polyacrylamide Gel Electrophoresis**

Analysis of apo B subspecies in lipoprotein fractions was carried out by 3.5% polyacrylamide gel electrophoresis containing 0.1% SDS (SDS-PAGE) (Hahm et al. 1983). The electrode buffer was 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS, and the sample buffer contained 20% sucrose, 2.5% SDS, 5% 2-mercaptoethanol, and 0.0075% bromophenol blue. For electrophoresis, each lipoprotein fraction (20 µg) was mixed with 40 µl of the sample buffer and treated at 100°C for 2 min. Electrophoresis was carried out at the constant current of 30 mA for 20 hrs at room temperature in an LKB 2001 vertical electrophoresis unit. The gels were stained with 0.1% Coomassie brilliant blue R-250 in 12.5% trichloroacetic acid and 50% methanol for 18 hrs at room temperature and destained in 10% acetic acid-30% metanol for 8 hrs. Cross-linked BSA (Davies and Stark 1970) was used as a molecular weight standard.

**Isolation of Apo B Subspecies**

For isolating various apo B subspecies of VLDL and LDL from normal and diabetic subjects, preparative 3.5% SDS-PAGE was carried out as described above except using 3 mm thickness slabs rather than 1 mm thickness slabs. On each slab, 10 sample wells were prepared in order to apply as much as 170 µl of sample in each well. After electrophoresis, gels were stained briefly (1 hr) with Coomassie blue and destained for 2 hrs. The apoprotein bands were then cut from the gel, homogenized in 2 ml of cold 10% trichloroacetic acid-30% ethanol using Teflon
Glycosylated LDL Apo B Subspecies in Diabetes

homogenizer, and centrifuged at 7,500 × g for 10 min. This washing procedure was repeated 4 more times. To the final pellet, 0.1 N NaOH (1 ml) was added, incubated at 37°C for 2 hrs with gentle shaking, and then centrifuged at 10,500 × g for 10 min. The clear supernatant was used for further analysis.

Quantification of Glycosylated Proteins

The degree of glycosylation in total plasma proteins, lipoprotein fractions, and apo B subspecies were determined according to the procedure of Ney et al. (1981) with the following modifications. To 1.0 ml sample, 0.5 ml of 1.0 M oxalic acid was added to make final concentration of 0.33 M oxalic acid and pH 1.0. The solution was hydrolyzed by autoclaving the samples in Teflon-capped tubes at 125°C for 60 min. After cooling the solutions to room temperature, 50% trichloroacetic acid (0.5 ml) was added and centrifuged at 1,000 × g for 10 min. To the supernatant (1.5 ml), 0.5 ml of 0.05 M thiobarbituric acid (TBA) was added, incubated at 40°C for 30 min and the amount of 5-hydroxymethylfurfural (5-HMF) produced by the reaction was measured from the absorbance readings at 443 nm using fructose as a standard (Pecoraro et al. 1979). The level of glycosylation was expressed as pmoles of 5-HMF/mg of protein for total serum proteins and nmols of 5-HMF/mg of protein for lipoprotein fractions and apo B subspecies.

RESULTS

In Table 1 are shown the blood chemical analysis data for control subjects and patients with diabetes. All the patients were diagnosed as type II diabetics with a mean HDL-cholesterol of 29 mg/dl and plasma triglycerides of 207 mg/dl. The result in Table 1 further shows that total plasma cholesterol level and LDL cholesterol level as well as fasting blood sugar were greatly elevated in diabetics. LDL cholesterol level was expressed as mg/100 mg protein instead of unit volume because the concentrations of LDL in whole blood volume varied greatly with different preparations.

LDL and VLDL were separated by the sequential ultracentrifugation method from plasmas obtained from normal and diabetic subjects and were analyzed by 3.5% SDS-PAGE in order to identify and compare the apo B subspecies in lipoprotein fractions. As the result (Fig. 1) shows, all of the LDL samples showed apol B-100, B-74, and B-26, while all of the VLDL samples showed only the apo B-100. No apo B-48 was observed in any of the VLDL samples tested and there were no differences in apo B compositions between normal and diabetic lipoproteins. For the isolation of apo B subspecies, electrophoreses were run that were identical except for the fact that thicker (3 mm) gel was used with larger sample wells so that up to 170 µl of sample containing 250 µg proteins could be applied in each well. A total of 500 µg of lipoprotein was applied routinely for separating apo B subspecies. From VLDL, an average of 44 µg of apo B-100 was obtained, and from LDL (200 µg), an average of 33 µg of apol B-100 and B-74, and 23 µg of apo B-26 were obtained. Apol B-100 and B-74 were extracted together because the resolution between these two apo B subspecies was such that it was almost impossible to completely separate and isolate apo B-100 and B-74 from each other to the extent that they would be rendered free from contamination with each other.

Fig. 2 compares the glycosylated total serum proteins and total apo B containing lipoproteins between normal and diabetic subjects. In diabetics, both the glycosylated total serum proteins and total apo B containing lipoproteins were increased, but the increase in glycosylated total serum proteins (0.373±0.139 vs 0.339±0.100 pmols 5-HMF/mg protein) was not statistically significant, while the increase in glycosylated total apo B containing lipoproteins (0.196±0.026 vs 0.080±0.004 pmols 5-HMF/mg pro-

| Table 1. Chemical analysis of control subjects and patients with diabetes mellitus |
|---------------------------------|--------------------------------|------------|
|                                | Control                       | Diabetes   |
|                                | (mg/dl)                       | Mellitus   |
| Fasting Blood Sugar            | 116.56±2.87 (8)               | 224.32±14.77 (22) |
| Total Cholesterol              | 159.54±6.18 (8)               | 235.60±14.32 (20) |
| LDL Cholesterol                | 41.79±6.00 (9)                | 129.86±17.51 (13) |

Each value represents mean ± SE with the number of samples indicated in parenthesis.

Number 1
tein) was highly significant (p<.001). Since we found that the total apo B containing lipoproteins were glycosylated significantly more in diabetics as compared to normal subjects, we then measured the level of glycosylation in apo B containing lipoproteins separately. The result (Fig. 3) showed that the levels of glycosylation were increased in diabetics for both VLDL and LDL. These increases in both VLDL (0.102±0.018 vs 0.050±0.004 nmoles 5-HMF/mg protein) and LDL (0.094±0.013 vs 0.031±0.003 nmoles 5-HMF/mg protein) were statistically significant, the increase in LDL glycosylation being more significant (p<.001 vs p<.02).

Although the increase in glycosylated VLDL in diabetics was significant, there was no significant difference in the level of glycosylation of isolated VLDL apo B-100 between normal subjects and diabetics (Fig. 4). On the other hand, the result (Fig. 4) showed that the level of glycosylation in LDL apo B-100 + B-74 and in LDL apo B-26 was increased significantly in diabetics (1.418±0.104 and 1.242±0.035 nmoles 5-HMF/mg protein compared to 0.873±0.041 and 0.605±0.034 nmoles 5-HMF/mg protein, respectively). It was also noted from the scattergram (Fig. 4) that the increase in glycosylated LDL apo B-26 in diabetics was even more significant, judging from dense population of dots.

With the above information we then analyzed the results to correlate various factors related to the state of hyperglycemia. Fig. 5 shows the result of regression analysis which shows a good positive correlation (r=.79) between LDL cholesterol and glycosylated LDL. When we analyzed the relationship between glycosylated LDL and various apo B subtypes, we found that there is a very good linear correlation (r=.88) between glycosylated LDL and LDL apo B-26 (Fig. 6), which in turn showed an excellent positive correlation (r=.93) with HbA1c (Fig. 7). The results showed that the level of glycosylated apo B-26 could be a sensitive indicator of a hyperglycemic state as
Fig. 3. Glycosylated lipoprotein levels in normal (○; n=10) and patients with diabetes mellitus (♦; n=13). Apo B containing lipoproteins, VLDL and LDL, were isolated by density adjusted sequential ultracentrifugation method and the degree of glycosylation was measured by the modified TBA assay as described under Materials and Methods.

Fig. 4. Glycosylated levels of apolipoprotein B subspecies in normal (○; n=11) and patients with diabetes mellitus (♦; n=14). Apolipoprotein B subspecies were separated by preparative SDS-PAGE, extracted from the gel, and the degree of glycosylation was measured by the modified TBA assay as described under Materials and Methods.
Fig. 5. The relationship between LDL cholesterol and glycosylated LDL.

Fig. 6. The relationship between glycosylated LDL apo B-26 and glycosylated LDL.
Glycosylated LDL Apo B Subspecies in Diabetes

![Graph showing the relationship between glycosylated apo B-26 and hemoglobin A1c](image)

**Fig. 7.** The relationship between glycosylated apo B-26 and hemoglobin A1c.

HbA1c. The results showing a good correlation between LDL cholesterol, glycosylated LDL, and glycosylated LDL apo B-26 also indicate that the measurement of LDL cholesterol could be used in place of glycosylated LDL apo B-26 for indices of a hyperglycemic state.

**DISCUSSION**

Soon after the first description of the nonenzymatic glycosylation of serum proteins in vivo appeared in a publication (Bunn et al. 1975), the increased level of glycosylation was reported in patients with diabetes mellitus (Gabbey et al. 1977), and since then there have been many studies devoted to gaining an understanding of the functions of glycosylated proteins in diabetic complications. Several methods for measuring the degree of glycosylation have been reported. Although HPLC (Schleicher and Wieland 1981; Bisse et al. 1982; Monnier and Cerami 1983) and affinity chromatography (Bannon et al. 1984; Fluckiger et al. 1984; Agarwal et al. 1985) are efficient methods, they require expensive equipment and have drawbacks in the sense that only a few samples or one sample can be analyzed at a time. Electrophoretic methods (Spicer et al. 1978; Allen et al. 1980), on the other hand, are time consuming and also require specialized equipment. The colorimetric method using TBA (Fluckiger and Winterhalter 1976; Dolhofer and Wieland 1980; Ney et al. 1981) can be performed with ease requiring a minimum amount of equipment, but the latest known procedure (Ney et al. 1981) required at least 8 hrs for analysis. The central step of the TBA assay is the release of added glucose from the protein as 5-HMF by weak acid hydrolysis, a reaction which is time- and temperature-dependent. In the latest known procedure (Ney et al. 1981), the hydrolysis requires 6 to 8 hrs at 115°C for obtaining optimum results, and, at first in using it, we frequently lost samples by evaporating during the relatively long period of hydrolysis. However, we were able to avoid the sample loss completely by hydrolyzing samples at 125°C for 1 hr in an autoclave. As a suitable and accurate alternative standard for 5-HMF, fructose has been reported to be used as it is coverted to 5-HMF by weak acid hydrolysis (Pecoraro et al. 1979). By introducing these modifications (weak acid hydrolysis for 1 hr in an autoclave and fructose standard), we were able to quantitate the level of glycosylation more rapidly. We were able to compare on two occasions the reproducibility of the present method and the unmodified method. The mean±SD for 4 separate LDL apo B-26 samples was 1.43±0.512 when the unmodified method was used and 1.36±0.155 when the
present method was used, and the mean±SD for 10 separate VLDL apo B-100 samples was 1.057±0.622 and 1.042±0.343, respectively, which shows a considerable improvement over the unmodified method.

One of the known complications of diabetes mellitus is accelerated atherosclerosis. The cholesterol of atherosclerotic plaques is derived from LDL that circulates in the blood stream. The more LDL there is in blood the more rapidly atherosclerosis develops (Goldstein and Brown 1977). Plasma LDL is known to be catabolized mostly (~80%) by the high affinity LDL receptor pathway (Brown and Goldstein 1984) specifically recognizing apo B of LDL on the cell surface. LDL contains apol B-100, B-74, and B-26, and VLDL usually contains only apo B-100 as its apo B components (Hahm et al. 1983; Tikkanen et al. 1984), whereas apo B-48 is reported to be derived from the intestine and thus associated with chylomicrons (Lock et al. 1983; Hahm et al. 1985). LDL undergoes nonenzymatic glycosylation in vivo, and in vitro with glucose to yield glycosylated derivatives on its ε-lysine groups (Gonen et al. 1981; Witzum et al. 1982), and the level of glycosylation was reported to be increased in patients with diabetes mellitus (Schleicher et al. 1981; Witzum et al. 1982). However, there have been no reports on quantitative relationships either between the degree of glycosylation of LDL and the degree of severity of diabetes, or between the degree of glycosylation of LDL and various apo B subtypes. In the present study, we have measured and compared the level of glycosylation in apo B containing lipoproteins, VLDL and LDL, and various apo B subtypes as it is found to occur in normal subjects and in patients with diabetes mellitus, and found that the amounts of glycosylated LDL, glycosylated LDL apo B-100 + B-74, and apo B-26 were increased significantly in diabetic patients when compared to those found in normal subjects. Furthermore, we found a good linear relationship between glycosylated LDL apo B-26 and glycosylated LDL. The results also showed that glycosylated LDL is linearly correlated with the level of LDL cholesterol suggesting that the level of LDL cholesterol in type II diabetics reflects the degree of glycosylated LDL apo B-26. At present, the measurement of HbA1c is being used in many clinics to assess diabetic control and diabetic severity. From the results showing that there is a very good correlation between HbA1c and glycosylated LDL apo B-26, we have shown that LDL apo B-26 or LDL cholesterol could also be used as an alternative to using HbA1c values.

CONCLUSIONS

A reliable and rapid method for measuring the level of glycosylation of proteins was developed by employing an existing method. Employing this method, the degree of glycosylation of VLDL, LDL, and apo B subtypes were compared between normal subjects and patients with diabetes mellitus. The levels of glycosylation of LDL, LDL apo B-100 + B-74, and LDL apo B-26 were found to be increased significantly in the diabetics. There was a good linear correlation between glycosylated LDL apo B-26 and glycosylated LDL, which in turn correlated well with the level of LDL cholesterol. Glycosylated LDL apo B-26 was also found to be in excellent linear relationship with hemoglobin A1c and was most significant among the glycosylated apo B subtypes.

REFERENCES

Bunn HF, Haney DN, Gabbay KH, Gallop PM: Further iden-
Glycosylated LDL Apo B Subspecies in Diabetes

tification of the nature and linkage of the carbohydrate in hemoglobin A1c. Biochem Biophys Res Commun 67: 103-109, 1975
Ney KA, Colley KH, Pizzo SV: The standardization of the thiobarbituric acid assay for nonenzymatic glucosylation of human serum albumin. Anal Biochem 118: 294-300, 1984
Schonfeld G, Lees RS, George PK, Pfleger B: Assay of total


