Apolipoprotein H Levels in Diabetic Subjects: Correlation With Cholesterol Levels

Maurizio Cassader, Gianluca Ruiu, Roberto Gambino, Fabrizio Veglia, and Gianfranco Pagano

To assess the relationship between apolipoprotein H (apo H) plasma levels and lipid metabolism in diabetes mellitus, we have examined the correlation between apo H plasma concentration and the main plasma lipid levels in 127 non–insulin-dependent (NIDDM) and 118 insulin-dependent (IDDM) diabetes mellitus patients. The data are compared with those in 286 nondiabetics. Our data show a significant increase in plasma apo H in diabetic as opposed to nondiabetic subjects (NIDDM, 29.9 ± 10.8 mg/dL; IDDM, 31.3 ± 9.9; controls, 22.5 ± 7.7; F = 53.3, $P = .0001$). The relation between plasma lipids and apo H was simultaneously evaluated in the three groups with inclusion of diabetes, sex, body mass index (BMI), and age as covariates in the model. This analysis showed a strong positive correlation ($P = .0009$) between apo H and total cholesterol, and a weaker positive correlation with triglycerides ([TGs] $P = .016$). The correlation between apo H and hemoglobin A_{1c} (HbA_{1c}) levels in diabetics ($P = .03$) highlights the importance of glycemic control for plasma levels of this apoprotein, which is highly glycated. Although the role of apo H in lipid metabolism is still uncertain, recent investigations on the possible relation between plasma apo H levels and increased plasma lipids and thrombotic risk could explain the increased atherosclerotic risk in diabetic patients.

ONE OF THE MOST COMMON alterations in non–insulin-dependent diabetes mellitus (NIDDM) involves the triglyceride (TG)-rich lipoproteins.[1] Quantitative and qualitative apolipoprotein variations appear to figure among the several factors responsible. Of the several apolipoproteins that modulate TG metabolism, apolipoprotein H (apo H) may be involved.[2,3]

Apo H, also known as β_2-glycoprotein I,[4,5] is a single, approximately 50-kd chain glycoprotein synthesized by liver cells.[6] It is present in human plasma in a mature form composed of 326 amino acids.[7] Its physiological role has not been fully established. In vitro studies suggest that it is involved in coagulation[8-12] and could be the mandatory cofactor for binding anionic phospholipids to some groups of antibodies that may be present in patients in autoimmune diseases.[13-15] The role of these antibodies is being investigated, as recently reviewed.[16]

Plasma apo H is partly free and partly (~35%) associated with plasma lipoproteins.[17] Its ability to activate lipoprotein lipase both in vitro and in vivo points to its modulation of TG metabolism.[2,18] It also seems to variably influence lipid metabolism. In this regard, one reason may be its genetically based structural polymorphism, although the data are conflicting.[19-22] Previous investigations of the possible relation between plasma apo H and thrombosis or changes in plasma lipid levels have not produced clear results, although enhanced apo H has recently been described in subjects with primary hyperlipidemia.[23]

To assess the plasma levels of apo H and the relationship between these levels and lipid parameters, we studied a group of nondiabetics (n = 286) and both NIDDM (n = 127) and insulin-dependent diabetes mellitus ([IDDM] n = 118) patients.

SUBJECTS AND METHODS

Subjects

Two hundred eighty-six unrelated nondiabetics were enrolled from the staff of the University of Turin Institute of Internal Medicine and Blood Bank donors in accordance with the following criteria: freedom from liver and kidney diseases and thyroid function alterations and no treatment with drugs acting on lipid metabolism. Diabetics were subjects attending the Institute's diabetology outpatient section. Since diabetes is treated without charge in Italy, they can be regarded as sufficiently representative of Piedmont's diabetic population. Their clinical and metabolic characteristics are illustrated in Table 1. Diabetes was diagnosed on the basis of World Health Organization criteria. Hemoglobin A_{1c} (HbA_{1c}) was determined by high-performance liquid chromatography (Bio-Rad Laboratories, Milan, Italy). Body mass index ([BMI] kg/m^2) was also evaluated. All patients with IDDM and 32 with NIDDM were treated with insulin; the remaining NIDDM patients were treated with oral hypoglycemic agents.

Plasma Samples

Blood samples (1 mg/mL EDTA-Na_2) were centrifuged and stored at −20°C until processed. Cholesterol and TG levels were measured enzymatically (Poli Diagnostici, Milan, Italy), high-density lipoprotein cholesterol (HDL-C) was determined after precipitation of apo B–containing lipoproteins with heparin and manganese chloride by automated enzymatic methods with a Shimadzu CL-7000 (Shimadzu Instruments, Kyoto, Japan).

Reagents

Horseradish peroxidase (HRP), salts for making phosphate-buffered saline (PBS), Tween 20, bovine serum albumin (BSA) fraction V, *o*-phenylenediamine dihydrochloride (OPD), and flat-bottomed polystyrene microtiter plates were all obtained from Sigma Chemical (St Louis, MO). Rabbit anti–apo H was obtained from Istituto Behring (Scoppito, Italy).

Determination of Apo H

Our method of isolation and purification of apo H was previously described.[24] Briefly, rabbit anti–human apo H antibodies were immobilized on a cyanogen bromide–activated Sepharose 6B column (Pharma-

From the Department of Internal Medicine, University of Turin, Turin; the Central Laboratory "Baldi e Riberi," Hospital S. Giovanni Battista, Turin; and the Epidemiology Unit, Scientific Institute H.S. Raffaele, Milan, Italy.

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Address reprint requests to Maurizio Cassader, PhD, Dipartimento di Medicina Interna, Corso A.M. Dogliotti 14, 10126 Torino, Italy.

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Table 1. Anthropometric Characteristics and Plasma Lipid and HbA_{1c} Levels in Controls and Diabetic Patients

Parameter	Controls	NIDDM	IDDM
No. of subjects	286	127	118
Age (yr)	48.7 ± 20.1	63.2 ± 10.2	35.5 ± 16.7
Sex (M/F)	144/142	75/52	61/57
BMI (kg/m²)	24.3 ± 3.1	27.9 ± 4.4	23.9 ± 4.1
HbA_{1c} (%)	—	8.3 ± 1.6	9.1 ± 1.9
Duration of diabetes (yr)	—	13.7 ± 11.1	11.5 ± 8.8
TG (mmol/L)	1.50 ± 1.15	1.63 ± 1.04	1.04 ± 0.59
Cholesterol (mmol/L)	5.74 ± 1.11	5.28 ± 1.06	4.93 ± 1.11
HDL-C (mmol/L)	1.49 ± 0.33	1.14 ± 0.33	1.49 ± 0.39
Apo H (mg/dL)	22.5 ± 7.7	29.9 ± 10.8	31.3 ± 9.9
Adjusted mean*	22.1	30.1	31.9
Range	8.0-48.0	10.0-75.0	11.0-58.0

NOTE. Data are the mean ± SD.
*Adjusted for age, sex, BMI, total cholesterol, and TGs.

cia, Uppsala, Sweden). Normal plasma samples were applied. Apo H that bound to the column was eluted with 0.1 mol/L glycine, 0.05% NaN_3, pH 2.5. To achieve maximum purification of apo H, the concentrated sample was then subjected to continuous-elution electrophoresis in a Prep-Cell (Bio-Rad Laboratories, Milan, Italy). Following identification of the fractions containing the pure protein (purity, 98% to 100%), apo H was used both for HRP labeling and as the standard for the enzyme-linked immunosorbent assay (ELISA) after measuring apo H protein concentration with the Bio-Rad protein assay. The standard solution (3 μg/mL apo H) was diluted to provide a calibration curve.

For HRP labeling, the two-step glutaraldehyde method was used. Briefly, glutaraldehyde was diluted in 0.1 mol/L phosphate buffer, pH 6.8, to a final concentration of 1.25%; 5 mg HRP was dissolved in 0.1 mL glutaraldehyde solution and allowed to incubate overnight at room temperature. The HRP-glutaraldehyde mix was added to an apo H solution (400 μg in 200 μL 0.1-mol/L carbonate/bicarbonate buffer, pH 9.5) and incubated overnight at room temperature. The remaining sites were blocked with 0.2 mol/L ethanolamine, pH 7, for 2 hours at 4°C. Labeled apo H was dialyzed in 0.05 mol/L Tris, 0.15 mol/L NaCl, pH 7.4, overnight at 4°C. Unconjugated HRP molecules were removed by gel filtration.

Rabbit anti–apo H antibodies were diluted 1:8,000 with 0.05 mol/L carbonate//bicarbonate buffer, pH 9.5, and 100 μL was pipetted into each well of a 96-well microtiter plate. This was then sealed and left at 37°C for 1 hour. Washing with 0.02 mol/L sodium phosphate, pH 7.8, 0.15 mol/L NaCl (PBS) to remove unbound antibodies was followed by blockade of the remaining sites by incubation with 350 μL 4% BSA in PBS for 1 hour at 37°C.

Unknown serum samples and standards were diluted in assay buffer solution before assay. The optimum ELISA conditions were as follows: 50 μL standards or serum diluted 400-fold and 50 μL purified apo H labeled with HRP diluted 1,000-fold. Samples were pipetted into the wells of a precoated microtiter plate, which was then covered and incubated for 2.5 hours at 37°C. The plate was washed six times with assay buffer solution. One hundred microliters of a freshly prepared enzyme substrate solution made by dissolving an OPD tablet in 0.05 mol/L phosphate-citrated buffer, pH 5.0, to a final concentration of 1 mg/mL was added to the wells. The plate was briefly agitated, covered, and left in the dark at room temperature for 20 minutes. The reaction was then stopped by adding 50 μL 3 mol/L HCl to each well, and the plate was agitated to ensure thorough mixing. Well absorbance was measured with a Bio-Rad 3550 reader at 490 nm. Serum concentrations were expressed in milligrams per deciliter. Standard curves were constructed with apo H isolated from human plasma. Standard solutions of 0.005 to 0.3 mg/dL were made by diluting the primary standard serially with the ELISA washing buffer. Considering the dilution factor of the sample calibration curve allows determination of apo H in the plasma ranging from 2 to 120 mg/dL. The intraassay coefficient of variation (CV) was 4.8% to 6.9%; the interassay CV was 8.5% to 10.1%.

Statistical Analysis

Apo H serum levels were compared in three groups of subjects (NIDDM, IDDM, and nondiabetic subjects) by one-way ANOVA. Differences between individual groups were analyzed by Scheffe's test to adjust for multiple comparisons. The relation between apo H and plasma lipid or HbA_{1c} values was assessed by analysis of covariance (ANCOVA) using gender, age, BMI, and group as covariates. To test whether the relation between serum lipids and apo H was heterogeneous with respect to presence or type of diabetes, we also performed an ANCOVA including the interaction term of group and lipid values. TG levels were logarithmically transformed before analysis to account for their skewed distribution.

RESULTS

The mean apo H serum level in control, NIDDM, and IDDM subjects was 22.5, 29.9, and 31.3 mg/dL. Figure 1 shows the distribution of apo H levels in the three groups. The distributions show a slight asymmetry with skewness of 0.46, 1.19, and 0.47, respectively, in normal, NIDDM, and IDDM subjects. To correct such asymmetry, logarithmic (log) transformation or square-root transformation are usually used. Log transformation of apo H levels resulted in an overcorrection, yielding a negative skewness in all groups except NIDDM, whereas square-root transformation, which is usually milder, resulted in a good correction of asymmetry. To check if this modest departure from normality would affect the results of the parametric test, we also analyzed the data after log and square-root transformation. The one-way ANOVA for the difference in the three groups was highly significant ($F = 53.3$, $P = .0001$). After adjusting for gender, BMI, age, total cholesterol, and TG, apo H mean values were virtually unchanged (Table 1), as was the statistical significance of the test ($F = 60.12$, $P < .0001$). This result suggests that the observed differences are not attributable to a differential distribution of cholesterol or TG among the three groups. We also performed covariance analysis on log- and square-root–transformed data, and the results in both cases were almost identical to those obtained from raw data ($F = 59.0$ for the effect of diabetes after log transformation and $F = 61.2$ after square-root transformation v $F = 60.1$ with raw data, $P < .0001$ in all cases). Pairwise comparisons between groups as assessed by Scheffe's test were significant for IDDM versus control and NIDDM versus control, but not for IDDM versus NIDDM.

The relation between plasma lipids and apo H was simultaneously evaluated in the three groups by ANCOVA with inclusion of diabetes, sex, age, and BMI as covariates in the model (Table 2). This analysis showed a strong positive correlation ($P = .0009$) between apo H and total cholesterol, with a mean increase of 0.04 mg apo H/mg cholesterol. A weaker positive correlation was also present between apo H and TG ($P = .016$), with a mean increase of 1.71 mg/log unit TG, and between apo H and HbA_{1c} ($P = .03$), with a mean increase of 0.78 mg/1% increase of HbA_{1c}. No significant correlation was found with HDL-C (Table 2).

The interaction term between total cholesterol and subject

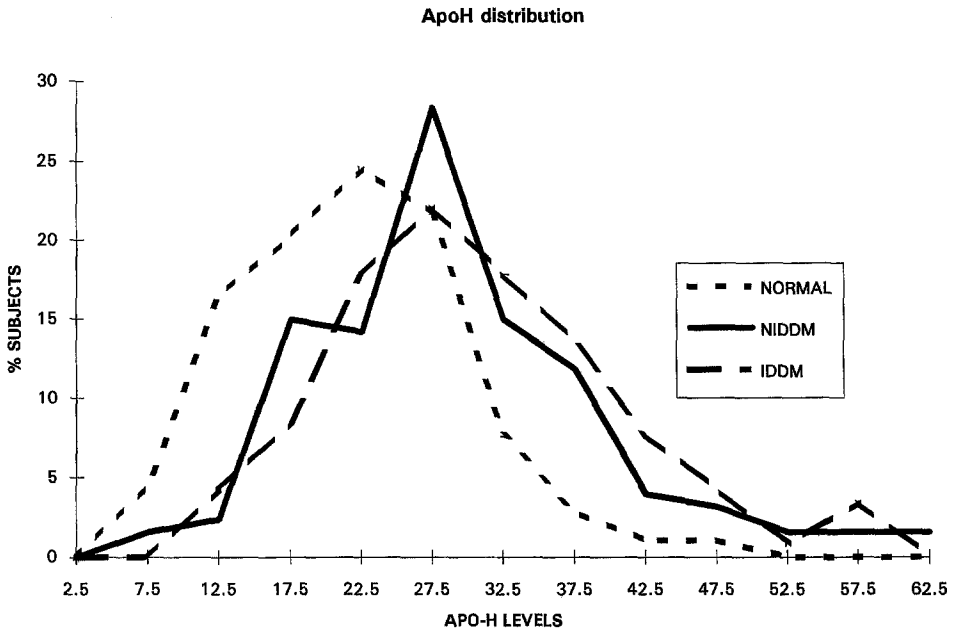

Fig 1. Serum apo H levels in the 3 groups.

group was not significant ($P = .73$), suggesting no difference between the regression coefficients in normal and diabetic subjects.

DISCUSSION

Apo H values obtained with our competitive ELISA method for the normal subjects are comparable to those obtained by others using radial immunodiffusion, immunoelectrophoresis, and noncompetitive ELISA.[25-27] Results obtained in this large sample of healthy controls are similar to those reported in the literature.

All studies that have assessed the association between apo H levels and the presence of metabolic disorders have so far failed to show conclusive results.[16] Our data show a significant increase in plasma apo H in diabetic as opposed to nondiabetic subjects, both in NIDDM and IDDM patients.

No ready explanation can be found for the enhancement of apo H levels in both types of diabetes. This markedly glycosylated protein may be further glycosylated in diabetes. As a matter of fact, apo H is rich in lysine residues[7] representing potential sites of nonenzymatic glycosylation when plasma glucose levels are too high. It is known that glycosylation also involves the apolipoproteins and may have some effect on their chemical, physical, and metabolic properties.[28] Glycation may thus have an effect on the residence times of circulating apo H, whereas diabetes itself may influence its levels, as reported for lipoprotein(a).[28] In any event, a relation between the apo H level and degree of glycation can be deduced from the association between apo H and HbA_{1c} levels shown by ANCOVA. Moreover, with apo H being partly associated with TG-rich lipoprotein and HDL, a possible explanation for the elevation of the plasma level in diabetes could be the increase of remnant particles: these lipid changes might contribute to the changes in apo H level.

Table 2. Covariance Analysis of Apo H, the Main Lipid Parameters, and HbA_{1c} Levels (adjusted for sex, age, BMI, and type of diabetes)

Factor	Regression Coefficient	Confidence Interval	P*
TG (log-transformed)	+1.71	0.32, 3.10	.016
Cholesterol	+0.03	0.012, 0.048	.0009
HDL-C	+0.004	−0.05, 0.06	.89
HbA_{1c}	+0.78	0.05, 1.51	.03

*For the *t* test of the difference of the regression coefficient *v* 0.

Furthermore, in view of the enhanced renal excretion of apo H reported in patients with tubule disorders,[29] a relation was sought between apo H levels and microalbuminuria in 20 NIDDM subjects; no significant association was found in this small population (data not shown). This question deserves further investigation.

The role of apo H in lipid metabolism is still uncertain. Its involvement in TG metabolism has been postulated.[3] Subsequent study suggests that it is more significantly associated with cholesterol.[23] Corroboration of this view can be found in the close association ($P = .0009$) between apo H and cholesterol levels in the whole population of the present study, independently of the presence of diabetes. Since our series is characterized by normal TG and HDL-C plasma levels in IDDM patients, an association between TG and apo H levels cannot be shown. The increase of apo H levels in these patients seems to depend on glycemic control, as measured by HbA_{1c} levels. A similar result is obtained for NIDDM patients, although with higher TG and lower HDL-C plasma levels.

The weaker association ($P = .016$) between apo H and TG could be due to the role that apo H seems to play in the regulation of metabolism of TG-rich lipoproteins. Many other factors may be involved in the increase of TG plasma levels, particularly in NIDDM patients, due to hyperinsulinemia and insulin resistance, considering the moderate metabolic control of our series. In this context, the role of apo H in TG metabolism regulation is difficult to evaluate. Further studies are necessary to clarify this point.

The results of this study are sufficiently unexpected to warrant an in-depth examination of the relations between apo H levels, lipid levels, and coagulative parameters in the diabetic population, since there is a high prevalence of atherosclerotic lesions in these patients. Apo H levels should be added to other macroangiopathic risk factors evaluated in diabetics.

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