Contribution of Acute-Phase Proteins and Cardiovascular Risk Factors to Erythrocyte Aggregation in Normolipidemic and Hyperlipidemic Individuals

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Summary

Background. Numerous studies have demonstrated that elevated concentrations of acute-phase proteins affect red blood cell (RBC) aggregation. Plasma lipids and lipoproteins were also shown to be correlated with RBC aggregation in hypercholesterolemia. However, whether acute-phase proteins promote RBC hyperaggregation in hyperlipidemic patients is unknown. The main objective of the study was to identify the impact of acute-phase proteins such as fibrinogen (Fib), haptoglobin (Hp), ceruloplasmin (Cp), α_1-acid glycoprotein (AGP), α_1-antitrypsin (AT), immunoglobulin G (IgG), and albumin (Alb) on RBC aggregation in 35 hyperlipidemic patients. The influence of these proteins in 32 normolipidemic subjects was also determined.

Methods and Results. RBC aggregation parameters reflecting the kinetics of rouleau formation and the adhesive strength between RBCs were measured by laser reflectometry. Multivariate forward stepwise linear regression analyses were performed to study the relationship between RBC aggregation and these acute-phase proteins, total cholesterol (TC), triglycerides (TG), high (HDL-C) and low (LDL-C) density lipoprotein cholesterol, age, gender, body mass index (BMI), mean blood pressure ($M_{pressure}$), and smoking habit. The kinetics of rouleau formation was positively correlated with the linear combination of IgG and Hp ($r = 0.76$, $p < 0.0001$) in hyperlipidemic patients, whereas IgG, smoking, AGP and gender were significant independent predictors in healthy subjects ($r = 0.79$, $p < 0.0001$). The correlations obtained for the models predicting the adhesive strength between RBCs were 0.69 in patients (Alb, HDL-C, IgG, $p < 0.002$) and 0.71 in healthy individuals (AGP, BMI, $p < 0.0001$).

Conclusion. This study suggests that acute-phase proteins such as IgG, Hp, AGP and Alb influence significantly and in an independent way the level of RBC aggregation. The close association between RBC aggregation and cardiovascular risk factors further strengthens its clinical importance.

Introduction

Numerous clinical studies have consistently reported that hyperlipidemia (1-4), coronary artery disease (5-9), cerebrovascular disease (10-15), and arterial and venous thromboembolic events (16-18) are associated with an elevated level of red blood cell (RBC) aggregation. Pathological RBC aggregation characterized by strong intercellular links and compact aggregates is believed to be linked with microcirculatory disorders, which affect the peripheral vascular resistance, and reduce capillary perfusion and oxygen transfer to tissues (12, 19-23). The presence of flow stasis triggered by high levels of RBC aggregation seems also to play an important role in the mechanism of thrombosis in veins, arteries and heart chambers (24-25). The possibility that RBC hyperaggregation may be an independent risk factor for cardiovascular disease has stimulated the interest in further understanding its determinants.

The influence of acute-phase proteins such as fibrinogen (Fib) and immunoglobulins (IgG, IgA and IgM) on RBC aggregation was widely studied *in vitro* and these proteins are since recognized as RBC aggregation promoters (26-28). On the other hand, albumin (Alb) is known to produce the opposite effect of inhibiting IgG-induced RBC aggregation and accelerating Fib-induced aggregation (29). A recent *in vitro* study by our group (30) showed that haptoglobin (Hp), ceruloplasmin (Cp), and C-reactive protein can also affect RBC aggregation. However, it remains unknown if these acute-phase proteins exert their function on RBC aggregation under *in vivo* conditions, where exists a complex interrelationship and numerous interactions between variables throughout various stages of the cardiovascular disease.

The relationship between hemorheological parameters, lipids and lipoproteins has also been studied although conflicting results were obtained. According to Ruhenstroth-Bauer et al. (5), the serum concentration of high-density lipoprotein (HDL) cholesterol is negatively correlated with RBC aggregation in both patients with coronary artery disease and in the normal population. Razavian et al. (2) found a significant negative correlation between RBC aggregation and HDL_2-cholesterol (HDL_2-C) in hypercholesterolemic patients. However, Neumann et al. (6) reported no significant relationship between HDL-C and hemorheological parameters including plasma viscosity, a photometric RBC aggregation index, and RBC filtrability following multivariate analysis of data from patients with coronary artery disease. In lovastatin-treated patients with familial hypercholesterolemia, a decrease in RBC aggregation was noted following the reduction of plasma lipids (low-density lipoprotein cholesterol [LDL-C], apoB/A-I ratio, and total cholesterol [TC]) (4).

The main objective of the present study was to identify the impact of acute-phase proteins on RBC aggregation in hyperlipidemia. Another objective of the study was to provide insight into the role and interactions of known cardiovascular risk factors on RBC aggregation. From the literature, it was shown that RBC aggregation is enhanced in obesity and is affected by smoking, age, gender and blood pressure (31-34).

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Table 1 Comparison of studied variables between both populations

Variables	Patients (n = 35)	Controls (n = 32)	*p* value
S_{10}	27 ± 4	24 ± 3	***
γS (s^{-1})	142 ± 49	128 ± 17	*
Fib (g/l)	3.0 ± 0.5	2.7 ± 0.3	**
IgG (g/l)	11 ± 2	12 ± 3	NS
Alb (g/l)	45 ± 3	46 ± 2	NS
Hp (g/l)	1.9 ± 0.7	1.5 ± 0.8	NS
Cp (g/l)	0.27 ± 0.05	0.29 ± 0.13	NS
AGP (g/l)	0.85 ± 0.20	0.75 ± 0.20	NS
AT (g/l)	1.9 ± 0.3	2.1 ± 0.3	*
TC (mmol/l)	7.0 ± 0.8	5.1 ± 0.8	***
TG (mmol/l)	2.7 ± 2.3	1.1 ± 0.6	**
HDL-C (mmol/l)	1.1 ± 0.3	1.4 ± 0.4	***
LDL-C (mmol/l)	4.8 ± 0.6	3.2 ± 0.6	***
BMI (kg/m^2)	26 ± 5	23 ± 3	**
$M_{pressure}$ (mmHg)	76 ± 13	89 ± 9	NS

*** $p < 0.001$; **$p < 0.01$; *$p < 0.05$

Methods

Study Population

For all individuals participating to the study, the body mass index (BMI) was assessed as the weight/height^2 (kg/m^2) and the mean blood pressure $M_{pressure}$ was calculated using: $M_{pressure}$ = (systolic blood pressure + 2 × diastolic blood pressure)/3. All participants completed a questionnaire inquiring about age, gender, medical history, current medication, smoking habits, and conditions known to influence RBC aggregation. These included cardiovascular disease, hypertension, diabetes mellitus, arterial and venous thromboembolic disease, plasmocytic dyscrasias, neoplasia, and anemia. There were no pregnant women among the candidates.

Thirty-five patients with primary hyperlipidemia (22 males) were selected from the Lipid Clinic of the Clinical Research Institute of Montreal. The mean age of patients was 48 ± 13 years (range of 18 to 70 years). The patient selection criteria were a total cholesterol concentration above 6.0 mmol/l and a LDL-C concentration greater than 4.1 mmol/l. Eighty-eight percent of all patients presented the IIa and IIb phenotypes according to the WHO/Fredrickson classification of hyperlipidemias. Patients with familial hypercholesterolemia were excluded from this study. Among patients, 3 were taking medication for lowering lipids and 3 were active smoker. Therapeutic agents were also given to two patients for conditions such as menopause and benign prostate hypertrophy.

A total of 32 healthy normolipidemic individuals (20 males) were also studied and recruited from our institutional staff. Their mean age was 40 ± 13 years (range of 22 to 69 years). These controls had no history of dyslipidemia, hypertension, cardiovascular disease, diabetes mellitus or carcinoma. No abnormalities were found on the electrocardiogram and blood chemistry results. Four of them smoked on a regular basis. Normal subjects and patients included in the study gave informed consent. The protocol was approved by the human ethical committee of our Institution.

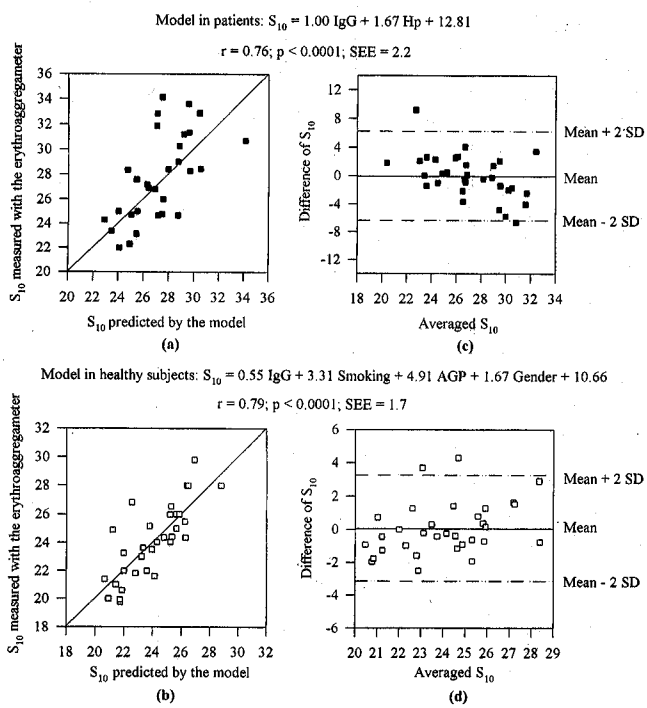

Fig. 1 The left panels show the relationship between the RBC aggregation kinetics (S_{10}), measured with the erythroaggregometer (y axis), and that predicted by the multivariate linear regression models (x axis) for both patients with hyperlipidemia (a) and controls (b). The right panels present the Bland-Altman plot of the differences of S_{10} as a function of their averaged values for patients with hyperlipidemia (c) and controls (d). The parameter "r" represents the correlation coefficient and SEE is the standard error of the estimate

Erythrocyte Aggregation

After a 12 h overnight fast, blood was drawn by venipuncture in standard Vacutainer tubes containing EDTA as the anticoagulant (1.5 g/l). All measurements were performed within 4 h after blood collection. The blood samples were centrifuged at 1,300 g for 10 min and the hematocrit was adjusted at 0.40 by addition or subtraction of autologous plasma. The hematocrit was measured using a microcentrifuge (Haemofuge, Heraeus Instruments) operating at 14,980 g for 10 min. The blood samples were incubated in a warm bath (37° C) for 5 min before measuring the erythrocyte aggregability.

RBC aggregation measurements were performed at 37° C with a validated erythroaggregameter based on a Couette flow arrangement (Regulest, Florange, France) (35). Each blood sample of 1.5 ml was placed into the instrument and sheared for 10 s at 550 s^{-1} to provide rouleau disruption. After abrupt cessation of the rotation of the co-axial outer cylinder, the variation of the scattered light intensity was recorded during the rouleau formation process. An aggregation kinetics index (S_{10}, no unit) was calculated as the ratio of the area above the light intensity curve during the first 10 s after flow stoppage to the total area within the same period of time. Immediately following the measurement of the kinetics of rouleau formation, the blood sample was sheared at 96 different levels from 6 to 720 s^{-1} to measure the shear rate necessary to break the adhesion between RBCs. A curve describing the scattered light intensity as a function of the shear rate was obtained for each blood sample. The total dissociation threshold of rouleaux (γS) was evaluated as the shear rate corresponding to the maximum scattered light intensity. A total of 5 min was needed to measure both S_{10} and γS, approximately. Reported intra-essay reproducibility values ranged from 2 to 2.3% for S_{10}, and 8 to 12.9% for γS (36).

Serum Acute-phase Protein Concentrations

The clotting assay method (Clauss method) was used for the measurement of the concentration of fibrinogen (Fib) in citrated plasma and the bromocresol green dye binding method was used to assess the concentration of serum albumin (Alb). The concentration of the other serum acute-phase proteins [immunoglobulin G (IgG), haptoglobin (Hp), ceruloplasmin (Cp), α_1-acid glycoprotein (AGP), and α1-antitrypsin (AT)] was determined using the immunonephelometric method. All measurements were performed by the Biochemistry Service of the Hôtel-Dieu Pavilion of the University of Montreal Hospital.

Lipid and Lipoprotein Cholesterol Concentrations

Enzymatic methods were used to measure the total cholesterol (TC) and triglyceride (TG) concentrations. The high-density lipoprotein cholesterol (HDL-C) concentration was determined after precipitation of apo B – con-

taining lipoproteins by dextran sulphate/$MgCl_2$. The low-density lipoprotein cholesterol (LDL-C) concentration was calculated using the Friedewald formula. This formula was used only for triglyceride concentrations below 4.5 mmol/l.

Statistical Analyses

All data were expressed as means ± one standard deviation (SD). The general linear model (Minitab statistical software, version 10.2), using age, gender and smoking habit as covariates was used to assess differences between groups for all variables measured (Table 1). Multivariate forward stepwise linear regression analyses (SigmaStat statistical software, Jandel Scientific, version 1.0) were performed to assess the correlation (r) between the RBC aggregation indices (S_{10} and γS), and biochemical (Fib, IgG, Alb, Hp, Cp, AGP, AT, TC, TG, HDL-C and LDL-C) and clinical (BMI, $M_{pressure}$, age, gender and smoking habit) variables (Figs. 1 and 2). Because the variables studied covered wide ranges of values and units, they were also transformed into the standardized residual form Z using the equation: $Z = (X-\mu)/\sigma$, where X represents the value of each variable, μ is the mean and σ is the SD computed over the whole population. To compare the relative contribution of each variable Z in the multivariate models, they were expressed in percent. The agreement between the RBC aggregation parameters and the multivariate models was assessed using the Bland Altman method (37). For statistical inference, a value of $p < 0.05$ was considered significant.

Results

Comparison of Studied Variables between both Populations

As seen in Table 1, statistically significantly higher levels of RBC aggregation kinetics (S_{10}) and adhesive strength between RBCs (γS) were found in patients. The concentrations of Fib, AT, TC, TG, LDL-C and the BMI were significantly increased, and the concentration of HDL-C was lower in patients.

Influence of Acute-phase Proteins and Cardiovascular Risk Factors on RBC Aggregation

Due to the fact that the biochemical and clinical variables may be interrelated under *in vivo* conditions, they were combined to investigate their impact on RBC aggregation. As seen in Figure 1a, the linear combination of IgG ($p < 0.0001$) and Hp ($p < 0.05$) was significantly correlated with S_{10} ($r = 0.76$) in patients. The standardized residual form of this model was:

$$S_{10} = 0.57\ IgG + 0.33\ Hp - 0.10, \qquad \text{(Eq. 1a)}$$

which emphasizes the strong influence of IgG on S_{10} in patients with hyperlipidemia (57% of the variation of the model)[1]. For each patient, the difference between S_{10} calculated with Eq. 1a and that measured with the erythroaggregameter was expressed as a function of their means (Bland-Altman plot). According to Figure 1c, a good agreement was obtained because only two samples lay outside the mean ± 2 SD. This suggests that the aggregation index S_{10} can be accurately predicted by the multiple linear regression model.

[1]It is important to note that IgG does not explain 57% of the variation of S_{10} because the model is not perfectly correlated with S_{10}.

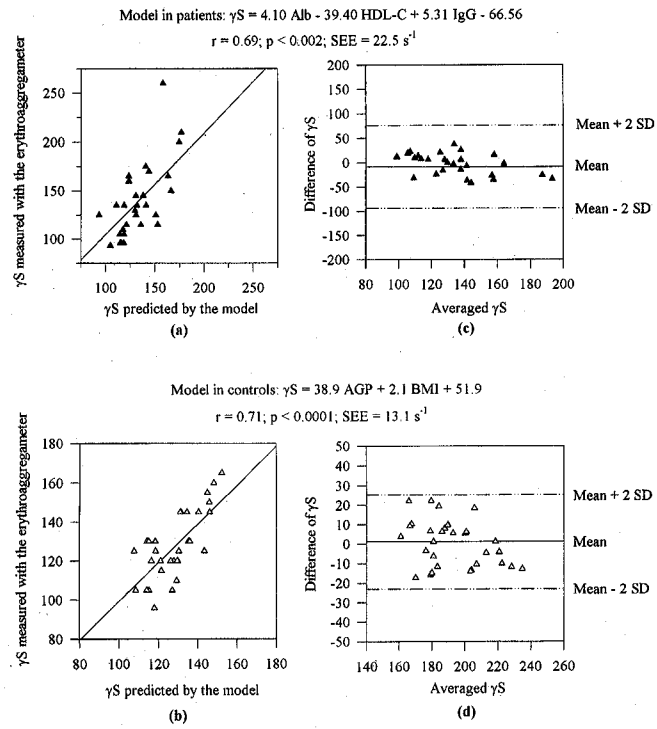

Fig. 2 The left panels show the relationship between the total dissociation threshold of rouleaux (γS), measured with the erythroaggregameter (y axis), and that predicted by the multivariate linear regression models (x axis) for both patients with hyperlipidemia (a) and controls (b). The right panels present the Bland-Altman plot of the differences of γS as a function of their averaged values for patients with hyperlipidemia (c) and controls (d). The parameter "r" represents the correlation coefficient and SEE is the standard error of the estimate

In controls (Fig. 1b), IgG ($p < 0.01$), cigarette smoking ($p < 0.01$), AGP ($p < 0.01$) and gender ($p < 0.05$) predicted S_{10}. The standardized residual form of this model, that resulted in a correlation coefficient of 0.79, was:

$$S_{10} = 0.32\ IgG + 0.26\ Smoking + 0.22\ AGP + 0.19\ Gender - 0.002. \qquad \text{(Eq. 1b)}$$

In this last equation, the IgG concentration and smoking were the most important determinants of S_{10}, contributing to 58% of the variation of the model. The Bland-Altman plot presented in Figure 1d showed a satisfactorily agreement between the linear model and S_{10}.

According to Figure 2a, γS could be predicted by the positive contribution of Alb ($p < 0.05$) and IgG ($p < 0.05$), and the negative contribution of HDL-C ($p < 0.05$) in patients ($r = 0.69$). In healthy individuals, it was determined by the positive combination of AGP ($p < 0.01$) and BMI ($p < 0.01$), as seen in Figure 2b ($r = 0.71$). The standardized residual form of these models were:

$$\gamma S = 0.28\ Alb - 0.27\ HDL\text{-}C + 0.24\ IgG - 0.21 \text{ in patients, and} \qquad \text{(Eq. 2a)}$$
$$\gamma S = 0.51\ AGP + 0.48\ BMI - 0.007 \text{ in healthy subjects.} \qquad \text{(Eq. 2b)}$$

All variables of the model (Alb, HDL-C, IgG and the constant) contributed almost equally to the increased adhesive strength between RBCs in patients. In healthy subjects, γS was determined almost equally by AGP and BMI. The agreement between the measured erythro-

aggregameter value and the calculated value obtained with these models used to assess γS is shown in Figures 2c and d: the Bland-Altman plots showed a perfect agreement.

Discussion

It is recognized that RBC aggregation is determined by the properties of plasma proteins (concentration, molecular mass and structure), the characteristics of RBCs (deformability, surface electrical charges, shape and hematocrit), the properties of the suspending medium (pH, temperature, viscosity, osmolarity and ionic strength), and the shear forces of the flow (38-41). Therefore, pathological changes affecting directly or indirectly any of these factors should contribute to the variations of the aggregation kinetics and adhesive strength between RBCs. RBC aggregation is the major determinant of the viscosity of blood at low shear rates (38). As reviewed by Lowe (42), low shear rate conditions can occur in the human vasculature at arterial bends and bifurcations (due to flow separation), which may be relevant to atherogenesis; distal to arterial stenoses (due to flow recirculation), which may be relevant to arterial thrombogenesis following plaque rupture; in the ischemic microcirculation (distal to an atherothrombotic stenosis or occlusion), which may be relevant to the pathogenesis of both acute ischemia and chronic intermittent ischemia; and in the deep leg veins of immobile persons, which may be relevant to venous thrombogenesis. In addition to that, there are strong evidences to support RBC aggregation as a cause of the flow stagnation in the left atrium of patients with mitral valve disease and atrial fibrillation, which may be relevant to the thrombogenesis of prosthetic heart valves (43).

The possible physiopathological consequences of high levels of RBC aggregation include not only the effect of the viscosity on the flow resistance and the activation of the coagulation system under flow stasis condition, but also the increased platelet adhesion to the vessel wall (due to mechanical displacement by RBC aggregates) and the increased platelet activation by mechanical stresses imposed by RBCs (42, 44). All this may promote vascular wall lesions as well as the interaction of atherogenic components such as lipoproteins and fibrinogen with the endothelium and smooth muscle cells. Hyperlipidemia and the development of atherosclerosis may cause changes in the physicochemical properties of plasma proteins, RBC membrane structure, and plasma constituents that lead to an increase of the aggregation. RBC hyperaggregation may also aggravate the disease by reducing the oxygen supply to tissues, and by slowing down the blood flow due to its effect on blood viscosity.

In the present study, acute-phase proteins, HDL-C and cardiovascular risk factors were related to RBC aggregation in hyperlipidemic patients and normolipidemic controls. The originality of the study is of several orders. The different models obtained to explain RBC aggregation emphasized the complex interrelationship between the tested variables. These models also showed that different parameters can affect the kinetics of rouleau formation (S_{10}) and the adhesive strength between RBCs (γS) for both populations. Using the standardized residual transformations, the relative contribution of each variable in the models could be clearly assessed. To our knowledge, no study reported similar results for patients with the IIa and IIb phenotypes of hyperlipidemia.

Impact of Acute-phase Proteins on Erythrocyte Aggregation

It is widely accepted that Fib is a major promoter of RBC aggregation (27-28, 45) and a significant risk factor of cardiovascular disease (46-48). In our study on normal subjects and patients with hyperlipidemia, although Fib was not a significant predictor in multivariate analyses, it was correlated with RBC aggregation when univariate regression analyses were used (results not presented). For instance, Fib was statistically correlated with S_{10} ($r = 0.56$, $p < 0.001$) and γS ($r = 0.39$, $p < 0.05$) in patients with hyperlipidemia. In normal subjects, correlations of 0.34 ($p = 0.06$) and 0.42 ($p < 0.05$) were found with S_{10} and γS, respectively.

It is accepted that other plasma proteins can have a determinant role on RBC aggregation. In the study by Neumann et al. (8), the high levels of RBC aggregation in patients with coronary artery disease could not be explained by a high concentration of Fib. In that study, RBC aggregation was found more predictive of acute myocardial infarction than the fibrinogen concentration. In young stroke patients (15), enhanced levels of RBC aggregation compared to young controls were found, both in the early and in the late phases, whereas Fib was normal. In the study by Poggi et al. (31), a prolonged very low calorie diet in obese subjects reduced RBC aggregation and globulins, increased albumin and had no effect on fibrinogen. The present study emphasized the fact that besides Fib, other factors can produce variability in RBC aggregation levels. The absence of Fib in Figures 1 and 2 may be related to its correlation with other parameters (47). Thus, Fib cannot be considered as an independent predictor of S_{10} and γS in our population of normal subjects and patients with hyperlipidemia.

The immunoglobulin G was shown to be the strongest determinant of S_{10} in patients with hyperlipidemia and healthy subjects (Figs. 1a and 1b). IgG had also a strong influence on γS in patients with hyperlipidemia (Fig. 2a). This finding is in agreement with previous studies reporting a strong effect of IgG on RBC aggregation in *in vitro* experiments (26, 29). In Figure 1a, Hp was a significant independent predictor of S_{10} in patients. This finding agrees well with a recent *in vitro* study performed by our group (30) that showed a significant impact of Hp on S_{10}, but no impact on γS. In that study, the plasma concentrations of Hp (2.22-4.99 g/l), obtained by adding this protein to the blood of normal subjects, were above those reported in the present study (see Table 1). This may suggest variations in the functional capacity of this protein to promote RBC aggregation and/or changes in the affinity of RBCs with this protein in patients with hyperlipidemia.

It was surprising to observe a positive correlation between Alb and γS in patients with hyperlipidemia (Fig. 2a), because Alb was shown *in vitro* to inhibit IgG-induced RBC aggregation (29). Because albumin may compete for adsorption sites with other plasma proteins, its role may differ between *in vitro* and *in vivo* conditions. Alb alone has no cellular aggregating effect (49). When combined to more than one protein, it may both promote or inhibit RBC aggregation, as shown by Maeda and Shiga (29). In our *in vitro* study previously reported (30), the effect of AGP on RBC aggregation was not significant when a high plasma concentration (2.22 g/l) of this protein was present in the blood of normal subjects. In the present study, AGP affected both S_{10} and γS in normal subjects, at a mean plasma concentration of 0.75 g/l (see Table 1). Like Alb that has a molecular weight of 66.5 kDa, AGP is a relatively small plasma glycoprotein formed by a single polypeptide chain of 181 amino acids having a molecular weight of 40 kDa (50). Consequently, the macromolecular bridging theory (38) cannot be appropriate to explain the effect of AGP on RBC aggregation. Thus, a direct effect of AGP on RBC aggregation is not probable. As demonstrated for Alb (29), AGP may also compete with other plasma proteins for adsorption sites on the RBC membrane. Its presence in the multivariate models may thus be explained by an interaction with other plasma proteins, such as Fib and IgG.

Contribution of HDL-C in Inhibiting Erythrocyte Aggregation in Hyperlipidemic Patients

Our study refocused attention on the role of HDL-C as a significant independent predictor of RBC aggregation. In patients with hyperlipidemia, HDL-C was inversely correlated with the adhesive strength (γS) between RBCs. These results are in agreement with Ruhenstroth-Bauer et al. (5) and Razavian et al. (2) that found a negative correlation between HDL-C and RBC aggregation. In the latter study, the negative correlation with HDL-C was shown to be associated with HDL_2-C but not with HDL_3-C. This is particularly interesting because of the evidence suggesting that a reduction in the concentration of the HDL_2 lipoprotein subfraction promotes atherosclerosis, whereas changes in the concentration of the HDL_3 component have no effect on cardiovascular disorders (51). As summarized by Simon et al. (52), HDL_2-C does not inhibit fibrinogen-induced RBC aggregation *in vitro*. Hence, its effect on RBC aggregation should be attributed to another unknown mechanism.

Conclusion

The importance of IgG as an independent promoter of the kinetics of rouleau formation and adhesive strength between RBCs was shown in patients with hyperlipidemia. In normal subjects, this protein also affected the kinetics of rouleau formation. The present study confirmed our recent *in vitro* investigation (30) that suggested a role for Hp in promoting RBC aggregation. AGP turned out to be an important factor affecting RBC aggregation in healthy subjects. Alb was also positively correlated with the adhesive strength between RBCs in patients. No such results had previously been reported and it is believed that AGP and Alb affect RBC aggregation through mutual interactions with other proteins. HDL-C was shown to be negatively correlated with RBC aggregation (γS) in hyperlipidemic patients, as shown by others. Furthermore, RBC aggregation was related to other established cardiovascular risk factors (smoking, gender, BMI). Our results thus support the hypothesis of a relationship between RBC hyperaggregation and atherosclerosis.

Acknowledgments

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