Inflammation-sensitive proteins and erythrocyte aggregation in atherothrombosis

Einor Ben Assayag, Nathan Bornstein, Itzhak Shapira*, Tamar Mardi, Yelena Goldin, Tatiana Tolshinski, Yafa Vered, Vera Zakuth, Michael Burke, Shlomo Berliner1, Dorit Samocha Bonet2

Department of Internal Medicine “D”, Tel-Aviv Sourasky Medical Center, and Sackler Faculty of Medicine, 6 Weizman Street, Tel Aviv University, Tel Aviv 64239, Israel

Received 3 June 2003; received in revised form 4 December 2003; accepted 24 December 2003

Available online 16 April 2004

Abstract

Objective: To find the relative contribution of various inflammation-sensitive proteins including fibrinogen, immunoglobulins (IgG, IgM and IgA), ceruloplasmin and high sensitivity C-reactive protein (hs-CRP) to the induction and/or maintenance of enhanced erythrocyte adhesiveness/aggregation in the peripheral blood of individuals with atherothrombotic risk factors.

Methods: The degree of erythrocyte adhesiveness/aggregation was determined by a simple slide test and image analysis. In addition, we measured various inflammation-sensitive protein levels including fibrinogen, ceruloplasmin, immunoglobulins and hs-CRP in a group of 234 individuals with atherothrombotic risk factors and healthy ones. Pearson partial correlations and multiple linear regression analysis were performed.

Results: Fibrinogen was found to be the major protein contributing to the enhanced erythrocyte adhesiveness/aggregation, explaining 30% of the model. Fibrinogen and IgG together explained 32.4% of the model. Other inflammation-sensitive proteins did not reach statistical significance and were excluded from the model.

Conclusions: Among inflammation-sensitive proteins measured in our cohort, fibrinogen is the dominant contributor to erythrocyte adhesiveness/aggregation in the peripheral blood of individuals with atherothrombotic risk factors and healthy ones. These findings may pave the way for the development of therapeutic strategies directed at the attenuation of erythrocyte aggregability in individuals with atherothrombosis.

© 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: Inflammation-sensitive proteins; Erythrocyte aggregation

1. Introduction

It has been repeatedly shown that atherothrombosis is associated with the presence of a low grade, subclinical and smoldering internal inflammation [1–3]. The presence of the inflammatory response has pathogenetic [4,5], prognostic [6] as well as therapeutic implications [7]. Thus, the elucidation of the various pathophysiological conditions associated with low-grade inflammation might be relevant when new therapeutic interventions to attenuate the inflammatory response are considered.

The enhanced erythrocyte adhesiveness/aggregation in the peripheral blood of individuals with low-grade inflammation might be of special interest due to its potential association with microcirculatory slow flow [8–11] and tissue deoxygenation [12,13]. In fact, we have recently shown that individuals with atherothrombotic risk factors have enhanced erythrocyte adhesiveness/aggregation in their peripheral blood [14,15]. Moreover, various researchers have implicated enhanced erythrocyte aggregation in the development of ischemic conditions [16–18] and therapeutic interventions to attenuate this phenomenon have been found beneficial [19,20]. Thus, it might be relevant to elucidate which factors are involved in the appearance of enhanced erythrocyte adhesiveness/aggregation in the peripheral blood of individuals with atherothrombosis.

* Corresponding author. Tel.: +972-3-6974254; fax: +972-3-6973635. E-mail address: shapirai@tasmc.health.gov.il (I. Shapira).
1 Shlomo Berliner is a shareholder in the Inflamet Ltd. Company, Tel Aviv, Israel.
2 Part of this study constitutes a part of the PhD thesis of E Ben Assayag, other parts are from the PhD thesis of D Samocha Bonet. These two authors contributed equally to the present work.
atherothrombotic risk factors and healthy ones. The identification of these factors might be relevant for future attempts to develop strategies aimed at attenuation of these cell-to-cell interactions.

We have presently focused on several inflammation-sensitive proteins that might be implicated in the induction and/or maintenance of increased erythrocyte adhesiveness/aggregation including fibrinogen, immunoglobulins (IgG, IgM and IgA), ceruloplasmin and high sensitivity-C reactive protein (hs-CRP). We found fibrinogen to be the dominant inflammation-sensitive protein and the major contributor to the enhanced erythrocyte aggregation.

2. Methods

2.1. Study population and design

The participants were all part of the Tel Aviv Medical Center Inflammation Survey (TAMCIS). The TAMCIS is a cross-sectional study that includes healthy employees of the Tel Aviv Medical Center and the Tel Aviv Municipality (Israel) as well as individuals with atherothrombotic risk factors who visit the outpatient clinics of the Tel Aviv Medical Center along with those who have a history of a clinically overt vascular disease including coronary artery bypass grafting (CABG), myocardial infarction (MI), ischemic cerebrovascular accident (CVA) or peripheral artery occlusive disease (PAOD). All of them gave their written informed consent to participate in the survey, according to the instructions of the Local Ethics Committee. Recruitment for the study was based on local announcements in the monthly salary slips of the medical and municipality personnel, as well as personal appeal to the patients in the various outpatient clinics to participate in the survey. Excluded were individuals with an underlying inflammatory disease (e.g., arthritis, inflammatory bowel disease) as well as individuals with any infection or other inflammatory condition, including myocardial or cerebrovascular infarction, surgery or angiography during the 6 months prior to their recruitment to the present study. Moreover, we excluded individuals treated with steroid or non-steroidal anti-inflammatory medications, except for low-dose aspirin (less than 325 mg/day).

2.2. Laboratory methods

2.2.1. The erythrocyte adhesiveness/aggregation test (EAAT)

The EAAT was measured using a simple slide test as described [14]. Briefly, venous blood from the antecubital vein was obtained between 8 and 11 AM following an overnight fast. Blood was drawn into a sodium citrate (3.8%) containing syringe (one volume of sodium citrate and three volumes of whole blood). One drop of the citrated whole blood was trickled onto a slide inclined at an angle of 30° and allowed to run down by gravity, leaving a fine film. The slides were left to dry at the same angle, at room temperature. A technician who was blinded to the clinical and laboratory results of the patients scanned the slides by using an image analysis system (INFLAMET™, Inflamat, Tel Aviv, Israel) [15].

2.2.2. The inflammation meter (Inflamet™)

This system consists of a Pentium Win 95 equipped with a Matrox Meteor (Matrox, Montreal, Canada) color frame grabber, a color charge-coupled device (CCD) camera and a microscope which was operated at × 200 magnification, resulting in an image resolution of 0.4 µm per pixel. Nine images were taken from each slide. The fields of view were chosen systematically to sample different regions of the slide. Each image is processed separately and the outputs are then averaged to form the final slide outputs. The nine fields of view cover a total area of 0.6 mm². A variable named erythrocyte percentage (EP) was chosen to represent the degree of erythrocyte adhesiveness/aggregation. This is essentially a measurement of the slide area covered by the red blood cells. In the absence of aggregation, the area is 100% whereas with the appearance of enhanced erythrocyte adhesiveness/aggregation the percentage is lower. Thus, the higher the aggregation degree, the smaller is the area covered by the cells, and vice versa. This is due to the fact that during cell aggregation, free spaces are formed between the aggregated cells. These free spaces are subtracted from total slide area covered by red blood cells. A typical example is presented in Fig. 1.

2.2.3. Inflamet variabilities

The coefficient of variation for the EAAT when one person prepared and read the different slides of a patient with inflammation was 0.14. We repeated this evaluation in five different patients with the same results. When nine different individuals prepared slides from the same patient the coefficient was 0.07. It was 0.1 when the same person read the same slide 10 times. The interobserver variability of this test was discussed previously in a study that included 273 individuals with various degrees of infection/inflammation [21]. We found a substantial interobserver agreement. In addition, we have recently reported the day-to-day variation of EAAT in a group of 30 individuals who repeated the EAAT determinations (7–13 examinations per patient) [22]. In this study, we could clearly show that the daily fluctuations of the test are comparable to other commonly used variables of the acute phase response, including hs-CRP, white blood cell count and fibrinogen concentrations [22]. In addition, a highly significant correlation (r=0.94, p<0.001, n=50) was obtained when the same slide was analyzed by two independent individuals (unpublished data).

2.2.4. Variables of the acute phase response

Complete blood count with white cell differential were performed by using the Coulter STKS (Beckman Coulter,
Nyon, Swiss) automatic cell analyzer, fibrinogen concentration was determined by the method of Clauss [23] and a Sysmex 600 (Sysmex, Hyaga, Japan) autoanalyser and the hs-CRP was measured using the Boering BN II nephelometer (DADE Boering, Marburg, Germany) according to Rifai et al. [24]. Ceruloplasmin was determined by an adaptation of the Roche Diagnostics (Indianapolis, IN, USA), Tina-quant method (immunoturbidimetric test) to the Bayer Advia 1650 Analyzer (Bayer, Tarrytown, NY, USA). The immunoglobulins IgG, IgM and IgA were measured by ELISA using a Beckman Coulter Array 360 system (Beckman Coulter, Galway, Ireland).

2.3. Statistical analysis

The results are presented as mean ± S.D. Pearson and Spearman correlations were performed to evaluate the associations between the various inflammation-sensitive proteins and the erythrocyte adhesiveness/aggregation (measured in EP), and the age-adjusted Pearson correlations are presented. A Multiple Linear Regression was constructed with EP as the dependent variable and the various inflammation-sensitive proteins as independent variables. To improve symmetry, hs-CRP and IgM were transformed to the natural log scale. The Multiple Linear Regression was performed in a stepwise algorithm with an entry probability value criterion of 0.05 and a removal probability of 0.1. In addition, we performed forward and backward regressions to validate the results and to avoid co-linearity. The SPSS statistical package was used to perform statistical evaluation (SPSS, Chicago, IL, USA).

3. Results

The study included 234 individuals, 140 women and 94 men with a mean age of 58.7 ± 15.3 years. There were 42 individuals (17.9%) with diabetes mellitus, 92 (39.3%) with hypertension and 121 (51.7%) with hyperlipidemia. Twenty-eight individuals (11.9%) had a past history of MI or CABG, five (2.1%) had a history of CVA and six individuals (2.6%) had a history of PAOD.

Table 1 presents the mean ± S.D. and ranges of the various inflammation-sensitive proteins, serum lipid concentrations and the EP measured in our cohort. Table 2 presents the age-adjusted Pearson correlations between the various inflammation-sensitive proteins and the EP. It can be seen that fibrinogen, Ln(hs-CRP), IgG and Ln(IgM) correlated with the EP. Moreover, it can be seen that the various inflammation-sensitive proteins correlated with each other. Of special significance is the strong and positive correlation between fibrinogen and Ln(hs-CRP). Table 3 presents the Multiple Linear Regression results with EP as the dependent variable and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mean ± S.D. and range of the inflammation-sensitive protein concentrations, serum lipid concentrations and the erythrocyte adhesiveness/aggregation (measured in erythrocyte percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation-sensitive proteins</strong></td>
<td>Mean ± S.D., Range</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>317.6 ± 66, 176–586</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>4.9 ± 5.9, 0.25–38.8</td>
</tr>
<tr>
<td>IgG (mg/l)</td>
<td>11.6 ± 2.5, 2.34–19.7</td>
</tr>
<tr>
<td>IgM (mg/l)</td>
<td>1.3 ± 1.0, 0.22–7.7</td>
</tr>
<tr>
<td>IgA (mg/l)</td>
<td>2.5 ± 1.1, 0.11–7.1</td>
</tr>
<tr>
<td>Cp (mg/dl)</td>
<td>25.5 ± 7.6, 10.6–70.9</td>
</tr>
<tr>
<td><strong>Serum lipids concentrations</strong></td>
<td>Mean ± S.D., Range</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>213 ± 41, 132–359</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>132 ± 35, 58–245</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>55 ± 16, 26–131</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>134 ± 62, 40–539</td>
</tr>
<tr>
<td><strong>Erythrocyte adhesiveness/aggregation</strong></td>
<td>Mean ± S.D., Range</td>
</tr>
<tr>
<td>EP (%)</td>
<td>79.8 ± 15.0, 34.3–100</td>
</tr>
</tbody>
</table>

hs-CRP = high sensitivity-C reactive protein, Cp = ceruloplasmin, EP = erythrocyte percentage.
all inflammation-sensitive proteins as independent variables. It can be seen that fibrinogen and IgG were the only inflammation-sensitive proteins significant, explaining together approximately 32% of the model. Furthermore, it can be seen that fibrinogen alone was a major and dominant contributor, explaining 30% of the model. All other inflammation-sensitive proteins measured were excluded from the model, as they did not reach statistical significance. Since fibrinogen and Ln(hs-CRP) strongly correlated (Table 2), we assessed a different model with EP as the dependent variable and all inflammation-sensitive proteins but fibrinogen as the independent variables. In that model, Ln(hs-CRP) alone explained approximately 15% of the model and together with Ln(IgM) explained approximately 18% of the model. All other inflammation-sensitive proteins were excluded, as they did not reach statistical significance. Another regression model was performed to evaluate the contribution of serum lipoproteins to the erythrocyte adhesiveness/aggregation. We found that none of them contributed to the model (results not shown).

4. Discussion

Previously, it became evident that the smoldering inflammatory response present in individuals with atherothrombotic risk factors and healthy individuals is not necessarily harmless [25–28]. Several acute phase response markers including fibrinogen [29,30], hs-CRP [4,5,25–28] and additional inflammation-sensitive proteins [31,32] have been identified as having a potential pathogenetic role in the disease. Furthermore, several of these proteins have been associated with enhanced erythrocyte aggregation [33,34], suggesting that they are associated with an unfavorable hemorheological profile.

The notion that the increased tendency of erythrocyte to adhere to each other and aggregate might have a pathogenetic role in ischemic conditions is not new. In fact, increased plasma concentrations of fibrinogen as well as erythrocyte aggregation are associated with hyperviscosity and an unfavorable outcome in atherothrombosis conditions [35–37]. We have recently conducted several studies in which we have shown that an enhanced erythrocyte adhesiveness/aggregation can be detected in the peripheral venous blood of individuals with atherothrombotic risk factors and in healthy ones [14,15,38–42]. A significant finding in these studies was the clear association between the multiplicity of atherothrombotic risk factors and the degree of erythrocyte adhesiveness/aggregation [14].

Following the above-mentioned observations, it became clear that the enhanced erythrocyte adhesiveness/aggregation could be induced by plasmatic factors [43,44] and be abolished following plasmapheresis [45]. Consequently, we wanted to identify which of the multiple reported macromolecules present in the plasma contribute to the induction and/or maintenance of the enhanced erythrocyte adhesiveness/aggregation. One of these studies was conducted in a group of patients before and following LDL apheresis and showed the predominant role of fibrinogen [46]. We have presently focused on a group of healthy individuals as well

---

Table 2

Age-adjusted correlations between inflammation-sensitive proteins and erythrocyte adhesiveness/aggregation (in terms of Erythrocyte Percentage)

<table>
<thead>
<tr>
<th>n=234</th>
<th>Fib</th>
<th>Ln(hs-CRP)</th>
<th>IgG</th>
<th>IgA</th>
<th>Ln(IgM)</th>
<th>Cp</th>
<th>EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fib</td>
<td>1</td>
<td>$r=0.59$</td>
<td>$r=0.23$</td>
<td>$r=0.25$</td>
<td>$r=0.4$</td>
<td>$r=0.53$</td>
<td>$r=0.4$</td>
</tr>
<tr>
<td>Ln(hs-CRP)</td>
<td>1</td>
<td>$r=-0.015$</td>
<td>$r=-0.015$</td>
<td>$r=0.25$</td>
<td>$r=0.015$</td>
<td>$r=0.014$</td>
<td>$r=0.002$</td>
</tr>
<tr>
<td>IgG</td>
<td>1</td>
<td>$r=0.29$</td>
<td>$r=0.02$</td>
<td>$r=0.015$</td>
<td>$r=0.02$</td>
<td>$r=0.03$</td>
<td>$r=10^{-4}$</td>
</tr>
<tr>
<td>IgA</td>
<td>1</td>
<td>$r=0.007$</td>
<td>$r=-0.08$</td>
<td>$r=0.02$</td>
<td>$r=0.02$</td>
<td>$r=0.02$</td>
<td>$r=0.02$</td>
</tr>
<tr>
<td>Ln(IgM)</td>
<td>1</td>
<td>$r=0.9$</td>
<td>$r=0.25$</td>
<td>$r=0.82$</td>
<td>$r=0.035$</td>
<td>$r=0.002$</td>
<td>$r=0.036$</td>
</tr>
<tr>
<td>Cp</td>
<td>1</td>
<td>$r=0.15$</td>
<td>$r=0.22$</td>
<td>$r=0.07$</td>
<td>$r=0.07$</td>
<td>$r=0.07$</td>
<td>$r=0.36$</td>
</tr>
</tbody>
</table>

Table 3

Multiple linear regression analysis with erythrocyte percentage as the dependent variable

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>$\beta$</th>
<th>S.E. ($\beta$)</th>
<th>$P$ value</th>
<th>Model $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>$-0.126$</td>
<td>0.014</td>
<td>$&lt;0.001$</td>
<td>0.3</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>$-0.127$</td>
<td>0.014</td>
<td>$&lt;0.001$</td>
<td>0.324</td>
</tr>
<tr>
<td>IgG</td>
<td>$-0.927$</td>
<td>0.365</td>
<td>0.012</td>
<td></td>
</tr>
</tbody>
</table>

A predictive model was derived by stepwise linear regression analysis. The following independent variables were assessed: fibrinogen, IgG, hs-CRP, IgM, ceruloplasmin and IgA. hs-CRP and IgM were transformed to the natural log scale in order to improve symmetry and comparability of effect estimates. Only those variables with a $P$ value of $<0.05$ were included in the final fitted model. $\beta$ indicates linear regression coefficient; S.E.($\beta$) = standard error of $\beta$ (for detailed information, see Results).
as those with atherothrombotic risk factors. Several inflammation-sensitive proteins were analyzed, including fibrinogen, immunoglobulins (IgG, IgM and IgA), ceruloplasmin and hs-CRP, due to previous studies implicating these proteins as involved in erythrocyte aggregation [31–34]. The results of the present multiple linear regression analysis are significant in that they show the dominant and major role of fibrinogen in the phenomenon, conversely to the other proteins herewith evaluated.

Our cohort included individuals treated with medications that have potential anti-inflammatory activities, including statins, fibrates as well as aspirin and angiotensin II modulators. However, it should be emphasized that the correlation was performed between the given protein concentration and the degree of erythrocyte aggregation. Moreover, our measurements are based on the fact that the erythrocytes react to the concentrations of the adhesive proteins in their milieu, unrelated to the question whether these concentrations are elevated or reduced as a result of drug-related effects.

The results of the present study might have practical implications because they pave the way for therapeutic interventions directed at attenuating peripheral erythrocyte adhesiveness/aggregation. These interventions could be life style modifications that attenuate the intensity of the inflammatory response [47–49], anti-inflammatory medications [50] or therapeutic apheresis [20]. In case of apheresis, specific reduction of fibrinogen concentrations might be of special importance.

We conclude that out of several inflammation-sensitive proteins, including fibrinogen, immunoglobulins, ceruloplasmin and hs-CRP, fibrinogen dominated regarding its contribution to the enhanced erythrocyte adhesiveness/aggregation in the peripheral blood. These findings are relevant for future studies focusing on new directions and modifications to attenuate the unfavorable hemorheological effect that this phenomenon might have in individuals with atherothrombosis.

References


