Activated platelets in patients with severe hypertriglyceridemia: effects of triglyceride-lowering therapy

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Abstract

Hypertriglyceridemia, a risk factor for cardiovascular disease, has been associated with hypercoagulability, but whether platelet activation is implicated is unknown. This study was designed to compare the in vivo platelet activation status between patients with severe hypertriglyceridemia and age- and sex-matched control subjects, and to evaluate the effects of triglyceride-lowering therapy. Sixteen patients with primary hypertriglyceridemia were included in a double-blind, placebo-controlled cross-over trial with 400 mg bezafibrate once daily. Platelet activation was analysed by double label flow cytometry, using monoclonal antibodies against GP53, P-selectin, and platelet-bound fibrinogen. Surface expression of the lysosomal membrane protein GP53 was significantly higher in the hypertriglyceridemic patients at baseline as compared to the group of age- and sex-matched controls (16.3 ± 4.8% vs. 8.9 ± 3.4%, respectively, P < 0.001). No differences in the expression of P-selectin and fibrinogen binding were observed. In response to bezafibrate therapy, the expression of GP53 in the patient group decreased from 16.3 ± 4.8% to 13.1 ± 4.1% (P = 0.018). The expression of P-selectin and fibrinogen binding was not affected by bezafibrate therapy. In conclusion, patients with hypertriglyceridemia have an increased in vivo platelet activation status, which can be improved by bezafibrate therapy. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Triglycerides; Flow cytometry; Bezafibrate; Platelet activation

1. Introduction

Platelets play an important role in the pathogenesis of atherosclerosis and acute coronary syndromes [1,2]. The beneficial effects of anti-platelet agents in secondary prevention trials support this hypothesis [3]. Activation of platelets has been demonstrated in patients with unstable angina pectoris and acute myocardial infarction [4,5]. Also the classical cardiovascular risk factors such as diabetes, smoking and hyperlipoproteinemia have been associated with increased platelet reactivity or platelet activation [6–8].

Hypertriglyceridemia (HTG) is a risk factor for cardiovascular disease in both men and women [9]. In addition to the characteristic lipoprotein profile, which includes low levels of high density lipoprotein (HDL) cholesterol and the presence of atherogenic small, dense low density lipoprotein (LDL) particles, hypertriglyceridemia has been associated with various derangements of the hemostatic system. Plasma fibrinogen levels are often elevated in hypertriglyceridemic patients [10,11]. Also, activated factor VII and plasminogen activator inhibitor-1 levels are elevated in these patients and correlate with total plasma triglyceride levels [12,13]. These results indicate that hypertriglyceridemia is associated with a hypercoagulable state and impaired fibrinolysis. The effects of triglycerides on platelet function are less uniformly described [7,14]. Previous studies used platelet aggregometry to indirectly assess platelet reactivity ex vivo. This technique is difficult to perform...
in turbid plasma samples. The development of whole blood flow cytometry [15] provides a tool to address the platelet activation status directly and specifically, by measuring the presence of activation antigens on the platelet surface.

Activation of platelets is initiated by the binding of agonists to specific receptors [16]. As a result, the glycoprotein (GP) IIb–IIIa complex changes its conformation, thereby revealing the binding site for fibrinogen. In addition, the membranes of the α-granules, dense granules and lysosomes fuse with the platelet cell-membrane to release the granular contents. As a consequence, novel activation-dependent membrane glycoproteins become exposed, which in the resting platelet were present only in the granule membranes. In the present study, three of these activation-dependent antigens were determined: P-selectin (CD62P), an α-granule-derived transmembrane protein; GP53 (CD63), a lysosomal membrane protein; and platelet-bound fibrinogen.

The present study was designed to directly assess the platelet activation status in patients with hypertriglyceridemia as compared to healthy age- and sex-matched control subjects. In addition, the effects of triglyceride-lowering therapy were studied in a double-blind, placebo-controlled trial with 400 mg bezafibrate once daily.

2. Materials and methods

2.1. Patients and control subjects

The study group consisted of 16 unrelated patients (14 males and two females) with primary hypertriglyceridemia who were recruited from the outpatient lipid clinic of the Leiden University Medical Centre. The diagnosis primary hypertriglyceridemia was based on the means of two fasting blood samples obtained after a dietary period of at least 8 weeks. The diagnostic criteria for endogenous hypertriglyceridemia were: total serum triglyceride (TG) > 4.0 mmol/l; very low density lipoprotein (VLDL) cholesterol > 1.0 mmol/l; and low density lipoprotein cholesterol (LDL-C) < 4.5 mmol/l. Exclusion criteria were the apoE2E2 phenotype, secondary hyperlipidemia (renal, liver or thyroid disease, fasting glucose > 7.0 mmol/l, and alcohol consumption of more than 40 g/day) and the use of anti-platelet or anti-coagulant drugs. Patients with a medical history of cardiovascular disease were not included in the study. Lipid lowering drugs, if used, were stopped 6 weeks before entering the study. Patients were urged to adhere to the dietary advice during the study. Seventeen normolipemic, age- and sex-matched control subjects (16 males and one female) were recruited in response to a newspaper advertisement.

2.2. Study design and blood sampling

The patients were randomised to receive in a double-blind cross-over fashion a fixed dose of bezafibrate, 400 mg once daily, and placebo for 6 weeks. The two periods in which medication was taken were separated by a wash-out period of 6 weeks. Before and after each treatment period of 6 weeks, fasting venous blood samples were obtained from the participants for lipid measurements. The platelet activation status was assessed at the end of the placebo and treatment periods. From the control subjects, fasting blood samples were obtained at baseline under identical conditions. Informed consent was obtained from each patient and the protocol was approved by the institutional Medical Ethics Committee (protocol number P183/96).

2.3. Lipids and lipoproteins

Venous blood was collected after an overnight fast. Serum was obtained after centrifugation at 1500 × g for 15 min at room temperature. Three millilitres of fresh serum was ultracentrifuged for 15 h at 232 000 × g at 15°C in a TL-100 tabletop Ultracentrifuge, using a TLA-100.3 fixed angle rotor (Beckman, Palo Alto, CA, USA). The ultracentrifuge was carefully divided in a density (d) < 1.006 and d = 1.006–1.25 g/ml fraction, designated as the VLDL and LDL–HDL fraction, respectively. The triglyceride and cholesterol concentrations were measured enzymatically using test kits (Boehringer, Mannheim, Germany). HDL cholesterol was measured in the LDL–HDL fraction after precipitation of apoB-containing particles with phosphotungstic acid and MgCl₂. ApoE phenotyping was performed by isoelectric focusing according to Havekes et al. [17]. ApoB48 was measured in the d < 1.006 g/ml fraction with SDS-PAGE. The mean apoB48 concentration was 4 ± 3% (range 0–10%) of apoB100 concentration.

2.4. Preparation of whole blood samples for flow cytometry

Blood was obtained from an antecubital vein through a butterfly needle (1.0 mm) with a light tourniquet. After discarding the first 2 ml of blood, a 1.8-ml sample of blood was collected in 0.2 ml of 3.2% tri-sodium citrate. Within 5 min, 5 μl aliquots of the citrated blood were diluted in 30 μl of HEPES buffer (137 mmol/l NaCl, 2.7 mmol/l KCl, 1.0 mmol/l MgCl₂, 5.6 mmol/l glucose, 20 mmol/l HEPES, 1 mg/ml bovine serum albumin, 3.3 mmol/l NaH₂PO₄, pH 7.4; buffer A), 5 μl of biotinylated anti-GPⅠb and 5 μl of a second fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (MoAb). Final concentrations were 0.75 μg/ml biotinylated anti-GPⅠb, 5 μg/ml FITC-anti-GP53, 5
μg/ml FITC-anti-P-selectin, 16 μg/ml FITC-anti-fibrinogen (platelet-bound fibrinogen) and 5 μg/ml FITC-labelled control antibody (IgG1 isotype). After careful mixing and 15 min incubation at room temperature in the dark, 5 μl of ten-fold diluted phycoerythrin-GPIb conjugated streptavidin was added. Incubation continued for another 15 min at room temperature. Finally, 2.5 ml of buffer A was added, containing 0.2% formaldehyde (final concentration).

2.5. Antibodies

The following murine MoAbs were used. Anti-GPIb (CLB-MB45), directed against the α-chain of GPIb, was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Anti-GPIb was biotinylated as described by Hnatowitch et al. [18]. FITC-labelled MoAbs against P-selectin (CD62P) and GP53 were obtained from Immunotech (Marseille, France). FITC-labelled chicken anti-human fibrinogen polyclonal antibodies were obtained from Biopool AB (Umeå, Sweden) and FITC-labelled IgG1 control antibodies were purchased from Becton & Dickinson (San Jose, CA, USA). Phycoerythrin-conjugated streptavidin was obtained from Dakopatts (Glostrup, Denmark).

2.6. Flow cytometric analysis

Double label flow cytometry was performed as described previously [19,20]. After collection, blood samples were analysed in a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA). Forward light scatter and sideward light scatter markers was determined in a population of 5000 platelets (R2) by analysing the fluorescence intensity of the FITC-labelled antibodies at 515 nm. The threshold for platelet activation was set at 2% fluorescence-positive platelet activation with an FITC-labelled IgG1 control antibody. Platelet count and volume were measured on a Sysmex™ SE-9000 analyser (TOA Medical Electronics, Kobe, Japan).

To verify our method of platelet activation analysis by expression of the activation markers, whole blood obtained from eight healthy volunteers, and coagulated with 0.10 mmol/l sodium citrate (final concentration) was stimulated with 10 μmol/l adenosine diphosphate in vitro. The mean fluorescence intensity (MFI) for FITC-labelled anti-P-selectin (CD62P) increased from 3.6 ± 0.5 to 10.8 ± 4.3 and MFI for FITC-labelled anti-GP53 (CD63) increased from 5.6 ± 0.8 to 9.7 ± 1.5 (mean ± S.D.). These data indicate that the procedure used to label and fix the platelets can detect platelet activation by increased antigen expression of activation markers.

2.7. Statistical analyses

Results are presented as the mean ± S.D. Mean differences between the control and patient groups were calculated with the Mann–Whitney U-test. Only differences in sex distribution between the patient group and control group were calculated with the Fisher’s exact test. The results at the end of the placebo and bezafibrate period were analysed pairwise using the Wilcoxon paired signed-ranks test. Findings were regarded to be statistically significant when the probability of these data under the null hypothesis was less than 0.05. Statistical analyses were performed with SPSS/PC+™ software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Patient characteristics

As shown in Table 1, the patient and control groups were comparable with regard to age and sex. Serum triglyceride levels were significantly higher in the HTG patients as compared to the control subjects. Although both LDL-C and high density lipoprotein cholesterol (HDL-C) levels were lower in the patient group, very low density lipoprotein cholesterol (VLDL-C) concentrations were markedly elevated and accounted for the elevation in total serum cholesterol concentrations.
Table 2
Effects of treatment with placebo and bezafibrate on serum lipids and lipoproteins*

<table>
<thead>
<tr>
<th></th>
<th>Before placebo</th>
<th>During placebo</th>
<th>Before bezafibrate</th>
<th>During bezafibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>TTG (mmol/l)</td>
<td>10.48 ± 5.31</td>
<td>12.20 ± 8.58</td>
<td>11.41 ± 7.77</td>
<td>3.83 ± 1.51*</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>8.01 ± 2.01</td>
<td>7.65 ± 2.46</td>
<td>8.08 ± 2.75</td>
<td>5.90 ± 1.32**</td>
</tr>
<tr>
<td>VLDL-C (mmol/l)</td>
<td>4.35 ± 2.25</td>
<td>4.29 ± 2.13</td>
<td>4.52 ± 3.06</td>
<td>1.45 ± 0.66*</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>2.86 ± 1.04</td>
<td>2.65 ± 0.61</td>
<td>2.76 ± 0.52</td>
<td>3.55 ± 0.89**</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.80 ± 0.20</td>
<td>0.72 ± 0.13</td>
<td>0.81 ± 0.13</td>
<td>0.91 ± 0.13***</td>
</tr>
</tbody>
</table>

* Values are presented as the mean ± S.D. TC, total cholesterol; TTG, total triglycerides; All values are mean ± S.D. based on triplicate measurements.

** P < 0.01 as compared to the corresponding value before bezafibrate therapy using the Wilcoxon paired signed-ranks test.

*** P < 0.05 as compared to the corresponding value before bezafibrate therapy using the Wilcoxon paired signed-ranks test.

3.2. Effect of therapy on serum lipid and lipoprotein levels

All subjects concluded the study without any side-effects. The effects of bezafibrate and placebo are shown in Table 2. Placebo therapy did not influence serum lipid and lipoprotein levels. Bezafibrate therapy resulted in a significant reduction in serum TG (−66%, P < 0.001), serum cholesterol levels (−27%, P < 0.001) and VLDL-C levels (−68%, P < 0.001). LDL-C and HDL-C levels increased by 29% (P = 0.003) and 12% (P = 0.011), respectively.

3.3. Platelet analyses

The first objective was to compare the expression of GP53, P-selectin and platelet fibrinogen binding between control subjects and HTG patients. Since placebo therapy did not influence serum lipid levels in the patient group, the platelet activation data obtained during placebo therapy were used as ‘baseline’ values and compared with the platelet activation data of the control group. As shown in Table 3 and Fig. 1, no differences in the expression of P-selectin and fibrinogen binding were found between the control subjects and HTG patients (during placebo therapy). However, the expression of GP53 was significantly higher in the HTG patients (16.3 ± 4.8%) as compared to the group of control subjects (8.9 ± 3.4%, P < 0.001; range 3.1–16.6% and median 9.1%). Platelet count and mean platelet volume did not differ between the HTG patients and control subjects (Table 3).

Bezafibrate therapy resulted in an improvement of the lipoprotein profile in the patient group (Table 2). The mean effects of this therapy on the expression of platelet activation markers are shown in Table 3. The expression of P-selectin and binding of fibrinogen did not change in response to bezafibrate therapy. During bezafibrate therapy, the high expression of GP53 in HTG patients decreased from 16.3 ± 4.8% to 13.1 ± 4.1% (P = 0.018), but remained higher than the levels in the control group (P < 0.05). Although mean platelet volume was not affected by both therapy modalities, the platelet number increased in response to bezafibrate therapy (+8%, P = 0.011; Table 3). The individual changes in GP53, P-selectin and platelet-bound fibrinogen expression upon bezafibrate therapy are presented in Fig. 1. The P-selectin expression did not change substantially within the patients and was similar to the values obtained in the healthy controls. The changes in platelet-bound fibrinogen were not significant.

4. Discussion

In this study we investigated the in vivo platelet activation status in hypertriglyceridemic patients directly by flow cytometric analysis of activation-depend...
Fig. 1. Expression of platelet activation markers. Expression of GP53 (left panel), P-selectin (middle panel) and platelet-bound fibrinogen (right panel) on platelets obtained from 16 hypertriglyceridemic patients (solid circles) during placebo and bezafibrate therapy compared with 17 age- and sex-matched control subjects (open circles). Analysis of the platelet activation markers was performed by double label flow cytometry, using antibodies against GP53, P-selectin and platelet-bound fibrinogen. The surface expression of the lysosomal membrane protein GP53 was significantly higher in the hypertriglyceridemic patients as compared to the group of controls. No differences in the expression of P-selectin and fibrinogen binding were observed. In response to bezafibrate therapy, the expression of GP53 in the patient group decreased, whereas the expression of P-selectin and fibrinogen binding was not affected.

dent platelet surface antigens. In previous studies, platelet reactivity was assessed in vitro by platelet aggregometry [7,14]. A major problem of this technique is that light transmission measurements are influenced by turbidity of plasma, a common phenomenon in this patient group. Other investigators have tried to avoid this problem by defining an upper limit of the serum triglyceride level [21] or using an impedance aggregometer technique [14]. A second problem of platelet aggregometry is the fact that the in vivo platelet activation status is not assessed directly but is merely implied from platelet reactivity in vitro. In contrast, the flow cytometric analysis of activation-dependent platelet surface antigens is not influenced by turbidity of blood samples and directly reflects the platelet activation status in vivo. To our knowledge, this is the first report describing the in vivo platelet activation status of hypertriglyceridemic patients.

Our first objective was to compare the platelet activation status of HTG patients with age- and sex-matched control subjects. The surface expression of GP53 was higher in the patient group as compared to the group of healthy volunteers. The platelet-activation markers P-selectin and platelet-bound fibrinogen were comparable in both groups. Thus, only one out of three platelet activation markers showed a higher expression in the HTG group. GP53 is a member of the lysosome-associated membrane proteins (LAMP) which become expressed on the platelet surface upon activation as the membrane of the lysosomal granules fuse with the plasma membrane [22,23]. Several studies have shown that LAMPs become exposed at the platelet surface when platelets are activated in vitro [24]. In addition, an increased platelet expression of LAMPs has been demonstrated in metastatic cancer and preeclampsia, which are associated with thrombo-embolic complications [20,24]. These studies indicate that GP53 and other LAMPs are good markers for platelet activation. Accordingly, the increased expression of GP53 in the HTG group indicates that hypertriglyceridemia is associated with an increased platelet activation status. The results of the triglyceride-lowering therapy with bezafibrate on both the improvement of lipid parameters and the reduction of GP53 expression further support this hypothesis. It should be emphasised, however, that despite a considerable reduction in plasma TG levels, normotriglyceridemia was not achieved. In accordance, the extent of increased GP53 expression was reduced but remained significantly higher than in controls. In contrast to GP53, no differences were found in expression of P-selectin between platelets obtained from HTG patients and healthy volunteers. P-selectin is a transmembrane protein of the α-granules and becomes expressed only on the platelet surface upon activation. Michelson et al. [25] showed that retransfusion of activated platelets that expressed P-selectin resulted in a
rapid loss of P-selectin in vivo. This decrease was paralleled by an increase in soluble P-selectin. Thus, P-selectin is exposed transiently at the platelet surface after activation and dissociates from the platelet, implying that the expression of P-selectin may not be a good marker of platelet activation in vivo. Also in contrast to GP53, no differences were found in the amount of fibrinogen bound to the surface of patient versus control platelets. Upon platelet activation, fibrinogen binds to the activated GPIIb–IIIa complex. Resting platelets do not bind fibrinogen, but when platelets are activated the GPIIb–IIIa complex changes its conformation and thus becomes eligible to bind fibrinogen. This change in the fibrinogen receptor conformation is highly reversible [26]. Therefore, the extent of surface binding of fibrinogen may be a transient phenomenon, which makes it less suitable as a marker for the platelet activation status. The finding that different platelet activation markers provide seemingly discrepant results is not uncommon in clinical studies. A number of studies have reported increased expression of GP53 only, or of one of the other markers, in a variety of diseases [20,27–29]. We conclude that an increased platelet activation status, as measured by surface GP53 exposure, is present in hypertriglyceridemic patients.

Several mechanisms may explain the increased platelet activation status in patients with hypertriglyceridemia. First, platelet activity in hyperlipidemias may be related to changes in the lipid composition of platelet membranes. Increased plasma cholesterol levels have been shown to decrease the platelet membrane fluidity [30]. These cholesterol-enriched rigid platelet membranes show an enhanced platelet responsiveness by increasing the number and affinity of platelet thrombin receptors [31]. Malle et al. [32] studied platelet membrane fluidity in other types of dyslipidemia. Interestingly, platelets from HTG patients demonstrated increased membrane fluidity as compared to healthy control subjects. So, platelet membrane rigidity does not seem to be a plausible explanation for the enhanced platelet activation status in the HTG group.

A second mechanism that may be involved is oxidative stress. Several studies have demonstrated that oxidative stimuli may activate platelets [33,34]. A characteristic lipoprotein pattern that can be observed in hypertriglyceridemia includes relatively low concentrations of LDL and HDL cholesterol, which are caused by exchange of lipids between the VLDL pool on one hand and the LDL–HDL pool on the other. In this process, the LDL particles become triglyceride-enriched and relatively cholesterol ester-depleted. As the LDL particles are progressively lipolysed in the circulation, the triglycerides are degraded and a small, dense particle remains. These small, dense LDL particles are regarded as potentially atherogenic since they show a

low resistance to oxidative modification [35]. However, earlier we reported that small dense LDL of HTG patients is not associated with low resistance to oxidation [36]. At present it is not clear whether small, dense LDL particles in these patients are associated with an increased tendency to oxidative modification [35,37] and, consequently, with an increased platelet activation [38].

A third explanation may be the platelet activating potential of VLDL, the lipoprotein fraction that primarily accumulates in hypertriglyceridemia. Several studies have demonstrated that VLDL stimulates platelet aggregation [39,40]. Van Willigen et al. [41] studied the effect of LDL on fibrinogen binding to the GPIIb–IIIa complex. Interestingly, incubation of platelets with LDL induced a rapid and dose-dependent increase in fibrinogen binding to platelets. No increase was observed after modification of the lysine residues of LDL, and therefore the effect was suggested to be receptor-mediated. It was speculated that LDL changes the exposure of the GPIIb–IIIa complex into a more active configuration. The principal candidate ligand of LDL is apolipoprotein (apo) B-100, which mediates the clearance of LDL from the circulation, as ligand for the LDL-receptor. Interestingly, apoB-100 contains a substantial number of lysine residues that play an important role in the receptor-mediated clearance [42]. In hypertriglyceridemia, LDL concentrations are low and the principal lipoprotein fraction that accumulates is VLDL. Since VLDL, like LDL, contain apoB-100, VLDL may influence the platelet activation status as well by changing the conformation of the GPIIb–IIIa complex via apoB-100. Another study, however, found that LDL augments platelet reactivity via a receptor-independent mechanism, by inhibiting the Na$^+$/H$^+$ antiport in the platelet membrane [43]. Whether this ion exchanger is influenced by VLDL as well remains to be determined. Further studies are needed to elucidate the mechanisms that could be involved in the interaction between VLDL and platelets.

In conclusion, our data indicate that hypertriglyceridemia is associated with an increased expression of the lysosomal membrane protein GP53, which is indicative of an increased in vivo platelet activation status. Triglyceride-lowering therapy by bezafibrate results in a significant reduction of the serum lipid levels and a modest reduction in GP53 expression.

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