The influence of statin therapy on platelet activity markers in hyperlipidemic patients after ischemic stroke

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Abstract

Introduction: Low-density lipoprotein cholesterol (LDL-C) has been reported to increase platelet activation. Reducing the level of LDL-C with statins induces important pleiotropic effects such as platelet inhibition. This association between platelet activity and statin therapy may be clinically important in reducing the risk of ischemic stroke. We investigated the effect of simvastatin therapy on platelet activation markers (platelet CD62P, sP-selectin, and platelet-derived microparticles (PDMPs)) in hyperlipidemic patients after ischemic stroke.
Material and methods: The study group consisted of 21 hyperlipidemic patients after ischemic stroke confirmed by CT, and 20 healthy subjects served as controls. We assessed the CD62P expression on resting and thrombin-activated blood platelets. CD62P and PDMPs were analyzed by the use of monoclonal antibodies anti-CD61 and anti-CD62 on a flow cytometer. The level of sP-selectin in serum was measured by the ELISA (enzyme-linked immunosorbent assay) method. All markers were re-analyzed after 6 months of treatment with simvastatin (20 mg/day).
Results: Hyperlipidemic patients presented a significantly higher percentage of CD62+ platelets and higher reactivity to thrombin compared to control subjects. After simvastatin therapy hyperlipidemic patients showed a reduction of the percentage of resting CD62P(+) platelets ($p = 0.005$) and a reduction of expression and percentage of CD62P(+) platelets after activation by thrombin (median $p < 0.05$; percentage: $p = 0.001$). A decrease of sP-selectin levels ($p = 0.001$) and percentage of PDMPs ($p < 0.05$) in this group was also observed.
Conclusions: HMG-CoA reductase inhibitor therapy in stroke patients with hyperlipidemia may be useful not only due to the lipid-lowering effect but also because of a significant role in reduction of platelet activation and reactivity.

Key words: HMG-CoA reductase inhibitor, hyperlipidemia, ischemic stroke, platelets, CD62P, sP-selectin, platelet-derived microparticles.

Introduction

The increase of platelet activity plays a key role in atherothrombotic events. It has been proven during the acute and chronic phase of ischemic stroke [1–3]. Several glycoproteins are expressed on the platelet surface during platelet activation. One of the most important markers of platelet activation is P-selectin expression [4]. This adhesion molecule is

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a component of the platelet α-granule membrane and of the Weibel-Palade body membrane of endothelial cells. Activation of these cells augments P-selectin expression on the cell surface [5]. It has been shown that the state of P-selectin hyperexpression caused by platelet activation and degranulation is short-lasting and platelets rapidly shed the surface P-selectin to the plasma pool. Thus, an increased soluble P-selectin (sP-selectin) plasma level may indicate thrombotic disorders [6].

It has been demonstrated that low-density lipoproteins enhance platelet activation and reactivity to platelet agonists. Platelet activation caused by native and oxidized low-density lipoprotein (LDL) leads to the release of platelet-derived growth factor (PDGF) [7]. The PDGF stimulates low-density lipoprotein cholesterol (LDL-C) accumulation in macrophages by increasing the number of LDL-C receptors on these cells. Also, LDL-C causes intra-platelet acidification by Na^+/H^+ exchange inhibition, leading to the augmentation of susceptibility to platelet agonists [8]. This association between platelet reactivity and hyperlipidemia may play a significant role in atherothrombosis development.

Platelet activation and aggregation is considered as a crucial step in initiation and aggravation of arterial thrombosis. The inhibition of platelet activation appears to be an attractive strategy for stroke prevention. It has been demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co-A) reductase inhibitors (statins) can regulate the atherothrombotic events not only by cholesterol lowering [9]. Downregulation of platelet activity is one of the non-lipid-related statin mechanisms. Statins are known to reduce platelet aggregation and TxA2 metabolite excretion [10, 11]. Evidence also exists that chosen statins may be protective against intimal hyperplasia. The studies showed that lipophilic (fluvastatin, simvastatin) but not hydrophilic (pravastatin) statins cause reduction of smooth muscle cell proliferation and migration, independent of plasma cholesterol reduction [12, 13]. Thus, simvastatin influences the mechanism related to LDL-C reduction, as a platelet cholesterol content as well as LDL-C independent mechanism.

The aim of our study was to investigate the effect of simvastatin therapy (20 mg/day) on platelet activation markers (platelet CD62P and sP-selectin, and platelet-derived microparticles (PDMPs)) in hyperlipidemic patients after ischemic stroke.

Material and methods

We investigated 21 patients (12 males, 9 females, mean age: 62 ±10.17 years) with hyperlipidemia (LDL-C: 5.27 ±0.66 mmol/l; total cholesterol (TC): 7.48 ±0.84 mmol/l). All patients had suffered from an ischemic stroke at least 3 months before they were recruited to the study. Control subjects (CS) consisted of 20 age- and sex-matched patients hospitalized for discopathy ($n = 14$) or tension-type headache ($n = 6$); blood analyses of these patients were within the standard values. In these subjects past or present symptomatic cerebrovascular disease was excluded. The ischemic stroke diagnosis was established using medical history of the patients, neurological examination and cranial computed tomography (CT). All patients received aspirin (75 mg/day) and antihypertensive drugs (ACE inhibitors, β-blockers, calcium channel blockers). Patients after ischemic stroke received neuroprotective drug therapy (piracetam and vinpocetine). Patients did not receive lipid-lowering therapy before the study. Exclusion criteria for all subjects included: diabetes mellitus, cancer, systemic and chronic inflammatory diseases, hemorrhagic diathesis, severe liver disease, renal failure and anticoagulant treatment. Lacunar and cardiogenic ischemic stroke also excluded patients from the study to avoid the influence of stroke subtype on the results of our research.

All participants authorized data use for investigational purposes by signed informed consent.

Simvastatin was administered in hyperlipidemic patients at a dose of 20 mg/day for an average of 6 months. Routine laboratory parameters, complete blood cell counts, total cholesterol (TC), LDL-C, high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), glucose levels and markers of platelet activation (platelet CD62P, sP-selectin and PDMPs) were evaluated at baseline and 6 months after the initiation of simvastatin treatment. Total cholesterol, HDL-C and TG levels were measured enzymatically with an Olympus AU400 analyzer. The LDL-C levels were measured by the Friedewald formula.

P-selectin expression (CD62P) on resting and thrombin-activated platelets was measured by means of flow cytometry (FACScan, Becton Dickinson, San Jose, USA). Venipuncture of the forearm vein was performed in a fasting state. We did not use stasis to avoid platelet activation. Two blood samples were taken. The first sample reflected the state of platelet activity *ex vivo* (0.1 ml of blood into a tube containing 1 ml of 0.5% solution of paraformaldehyde in PBS). The second sample was used to assess platelet ability to be activated by 0.08 U of bovine thrombin (0.5 ml of blood and 0.5 ml of EDTA). For the flow cytometry analysis, the following monoclonal antibodies (mAb) were used: anti-CD61-FITC (DAKO) and anti-CD62P-PE (Becton Dickinson). Platelets were identified by flow cytometry based on size and CD61 surface expression. The data were presented as the percentage of platelets with P-selectin expression and median of fluorescence reflecting density of

glycoprotein on the platelet surface. Platelet-derived microparticles were recognized as CD61+ microparticles. They were identified based on their characteristic flow cytometric profile of forward scatter channel (FSC) (size < 0.2 μm). Data collected with flow cytometry were analyzed using WinMDI 2.8.

Soluble P-selectin level was assessed by ELISA (Quantikine human sP-selectin/CD62P ELISA Kit, R&S Systems, Abingdon, UK). After 10 min centrifugation, the blood plasma obtained from EDTA anticoagulated samples was stored at –80°C until measurements. Measurement of soluble P-selectin was performed according to the manufacturer's instructions. All markers were re-analyzed after 6 months of treatment with simvastatin.

This study was approved by the Ethics Committee of the Medical University of Lodz. (no. RNN/205/04/KB).

Statistical analysis

All variables were logarithmically transformed to approximate the normal distribution before analysis. Results were analyzed by standard statistical analysis, Student's *t* test, the Shapiro-Wilk test and Levene's test. Data were expressed as the mean value with standard deviation for normally distributed data. Statistical analysis was performed using SPSS PC 11.5 and Statistica 6.0. For all the statistical analyses, the results were considered significant when $p < 0.05$.

This study was approved by the Ethics Committee of the Medical University of Lodz. (no. RNN/205/04/KB).

Results

The clinical characteristics of patients before and after treatment with simvastatin are presented in Table I.

After 6 months of treatment with 20 mg/day of simvastatin we observed a significant reduction of total and LDL-C and TG levels in hyperlipidemic stroke patients (TC: 7.48 ±0.84 mmol/l to 5.35 ±0.82 mmol/l, $p = 0.001$; LDL-C: 5.27 ±0.66 mmol/l to 3.20 ±0.75, $p = 0.001$ and TG: 1.95 ±0.83 mmol/l to 1.56 ±0.52 mmol/l, $p < 0.05$).

Our data revealed a significant influence of statin therapy on platelet activation in hyperlipidemic patients after ischemic stroke. The percentage of resting CD62P(+) platelets in hyperlipidemic patients was significantly lower after 6 months of treatment with simvastatin (HL at baseline: 2.72 ±1.13 vs. HL after treatment: 1.09 ±0.78, $p = 0.005$) (Figure 1 A), and there was no significant difference in the percentage of resting CD62P(+) platelets between this group after treatment with simvastatin and control subjects (HL after treatment: 1.09 ±0.78 vs. CS: 1.01 ±0.2, $p > 0.05$). However, we did not observe a decrease of CD62P expression after statin therapy (HL at baseline: 10.57 ±4.67 vs. HL after treatment: 11.12 ±3.42, $p > 0.05$) (Figure 1 B). Our data also indicated that treatment with simvastatin caused a reduction of platelet reactivity to thrombin. Hyperlipidemic patients treated with simvastatin showed a reduction of expression and percentage of thrombin-activated platelets CD62(+) (median: HL at baseline: 126.8 ±24.3 vs. HL after treatment: 78.2 ±54.7, $p = 0.02$; percentage: HL at baseline: 80.3 ±13.2 vs. HL after treatment: 39.3 ±27.0, $p = 0.001$) (Figures 1 C, D). There were no significant differences in CD62P expression or percentage of thrombin-activated platelets CD62(+) between hyperlipidemic patients after statin therapy and control subjects (median: HL after treatment: 78.2 ±54.7 vs. CS: 79.6 ±30.5, $p > 0.05$; percentage: HL after treatment: 39.3 ±27.0 vs. CS: 48.2 ±16.6, $p > 0.05$) (Figures 1 C, D).

We also observed the influence of statin therapy on sP-selectin serum concentrations. In hyperlipidemic patients treated with simvastatin the levels of sP-selectin were significantly lower in comparison with baseline values (baseline: 124.0 ±60.1 vs. after treatment: 77.6 ±38.1; $p < 0.001$), indicating the reduction of the platelet release reaction. In addition, after sP-selectin level downregulation, we did not observe any difference in

Table I. Clinical characteristics in study subjects (mean ± SD)

Parameters	HL at baseline	HL after 6 months of treatment	Value of *p*
TC [mmol/l]	7.48 ±0.84	5.35 ±0.82	0.001
HDL-C [mmol/l]	1.39 ±0.44	1.39 ±0.25	0.925
LDL-C [mmol/l]	5.27 ±0.66	3.20 ±0.75	0.001
TG [mmol/l]	1.95 ±0.83	1.56 ±0.52	< 0.05
MPV [fl]	8.52 ±0.90	8.69 ±0.86	0.052
PLT [× 10³ μl]	285.3 ±83.8	271.3 ±130.7	0.156

SD – standard deviation, TC – total cholesterol, LDL-C – LDL-cholesterol, HDL-C – HDL-cholesterol, TG – triglycerides, MPV – mean platelet volume, PLT – platelets, HL – hyperlipidemic group.
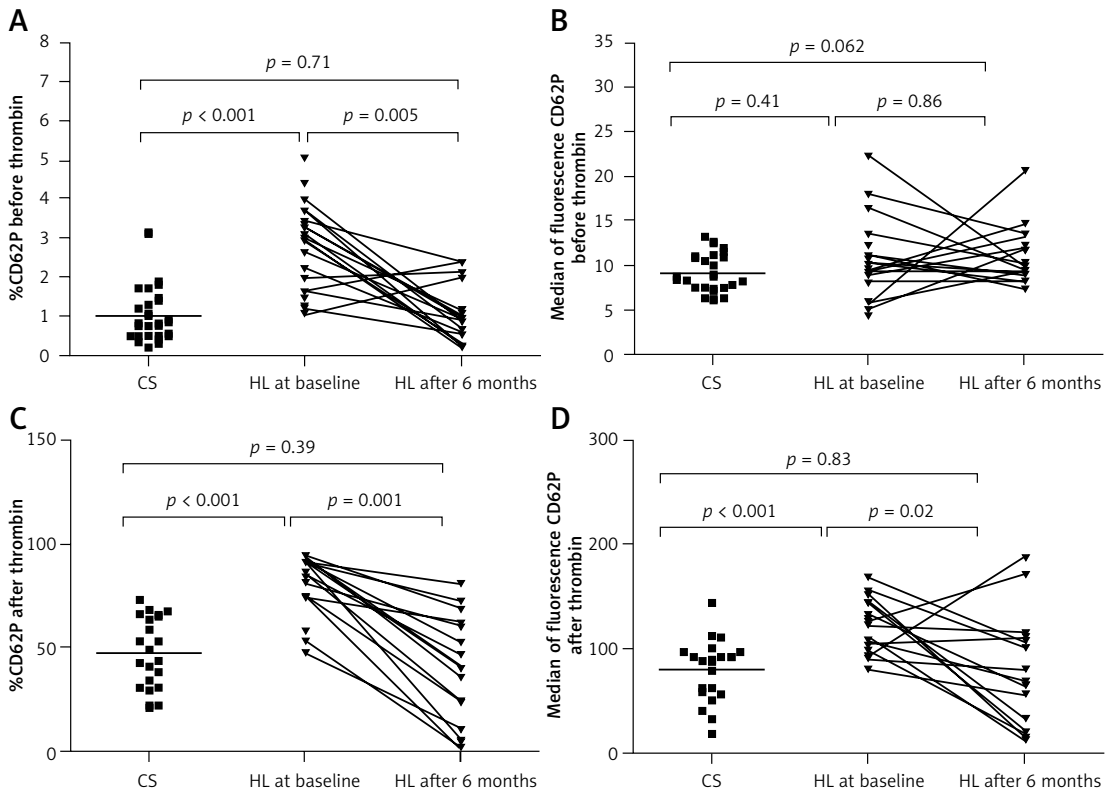
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Figure 1. Percentage and expression of resting (**A**, **B**) and thrombin-activated (**C**, **D**) CD62P(+) platelets in hyperlipidemic stroke patients at baseline and after simvastatin therapy

HL – hyperlipidemic group, CS – control subjects.

sP-selectin concentration between HL and CS (HL after treatment: 77.6 ±38.1 vs. CS: 81.6 ±34.3, *p* > 0.05) (Figure 2).

We also observed a significant reduction of percentage of PDMPs after treatment with simvastatin (baseline: 4.39 ±2.5 vs. after treatment: 2.64 ±1.43, *p* < 0.05). There was no significant difference in the PDMP percentage between the hyperlipidemic group after statin therapy and control subjects (HL after treatment: 2.64 ±1.43 vs. CS: 2.53 ±1.69, *p* > 0.05) (Figure 3).

Discussion

Hyperlipidemia is a well-established risk factor for atherothrombotic events, in which platelet activation plays a significant role. The study by Labios *et al.* indicated a significant correlation between platelet function normalization and the decrease in plasma total and LDL cholesterol levels after statin therapy [14]. Huhle *et al.* [15] assessed the effect of fluvastatin therapy on platelet activity. They noticed that the cholester-
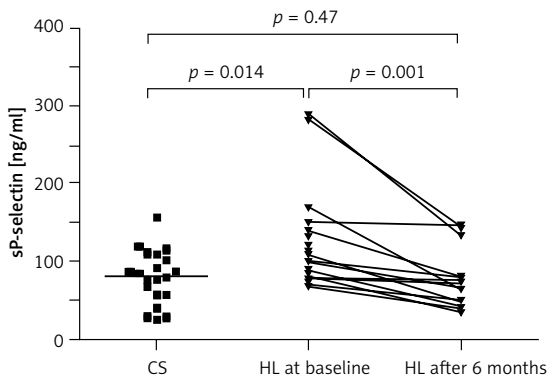

Figure 2. Soluble P-selectin (sP-selectin) concentration at baseline and after treatment with simvastatin in study subjects
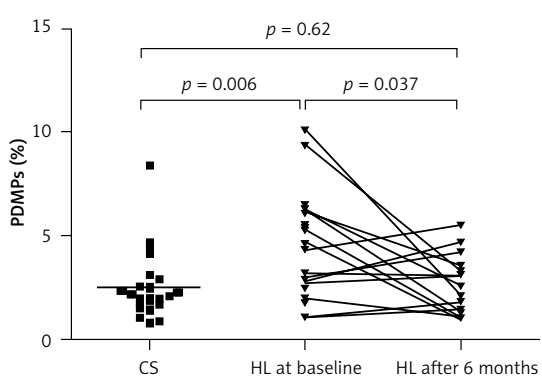
HL – hyperlipidemic group, CS – control subjects.

Figure 3. Percentage of platelet-derived microparticles (PDMPs) at baseline and after treatment with simvastatin in study subjects

HL – hyperlipidemic group, CS – control subjects.

ol-lowering effect is accompanied by a significant downregulation of the platelet membrane activation markers CD62 and CD63, reflecting reduced platelet activity. Our findings are concordant with these observations. We observed a significant influence of statin therapy on platelet function, and that fact was associated with a reduction of LDL and total cholesterol levels. We noted a significant decrease of the percentage of resting and thrombin-activated CD62P(+) platelets in hyperlipidemic stroke patients after 6 months of treatment with simvastatin in comparison with baseline values. Furthermore, after this downregulation we did not observe a significant difference in the percentage of CD62P(+) platelets between HL and CS.

The study of Hwang *et al.* investigated the effects of short-term atorvastatin treatment (8 weeks) on platelet P-selectin expression in patients with hypercholesterolemia. The major finding was that statin therapy in this group of patients resulted in significant downregulation of P-selectin expression [16]. Cha *et al.* [17] investigated the beneficial effect of statin to reduce platelet P-selectin expression in atherosclerotic ischemic stroke. The expression was significantly reduced after treatment with simvastatin 20 mg for 12 weeks; however, the effect of statin disappeared after 12 weeks of treatment cessation. The P-selectin changes induced by statin were independent of the LDL cholesterol level changes. The study by Puccetti *et al.* [18] showed a significant reduction of P-selectin expression and platelet aggregation after 6 weeks of treatment with statin as well as a platelet hyperactivation state in the second week after treatment discontinuation.

Hypercholesterolemia is accompanied by platelet hypersensitivity to various aggregating agents and an increased platelet cell membrane cholesterol content [19]. The plasma membrane and cytosolic calcium levels of platelets appear to be altered in hypercholesterolemic states; thus platelet reactivity could be increased [8]. Piorkowski *et al.* observed the effect of statins on platelet reactivity and noted that P-selectin after ADP stimulation was significantly reduced by 40 mg/day of atorvastatin after 4 weeks of treatment [20]. The study of Ma *et al.* showed significant decreases in ADP-induced platelet P-selectin expression in hypercholesterolemic patients after 8 and 12 weeks of pravastatin therapy. The therapeutic effects of this drug did not vary significantly with length of therapy [21]. In accordance with the previous studies, we found that in our patients both the expression and percentage of thrombin-activated CD62P(+) platelets were lower after a 6-month period of statin therapy than at baseline.

In our study we also investigated the relationship between statin therapy and soluble P-selectin concentration. We found that sP-selectin concentration was significantly decreased after 6 months of treatment with simvastatin. Our data are supported by the study of Romano *et al.* [22], who concluded that fluvastatin administration reduced sP-selectin levels in hypercholesterolemic patients. In other studies the influence of statin therapy on sP-selectin concentrations was also observed [23, 24]. P-selectins are shed from activated platelets and endothelial cells [7]. A question arises whether the raised plasma levels of sP-selectin reflect endothelial dysfunction, platelet activation, or both. Thus, it could be possible that in hypercholesterolemic patients LDL-C increases the release of endothelial P-selectin. On the other hand, the significant increase of sP-selectin concentration in serum may be evidence of the influence of LDL-C on platelet activity [25]. Semenov *et al.* [26] (reporting strong correlations between soluble P-selectin and platelet count) and Fijnheer *et al.* [27] suggest that under normal conditions the majority of the soluble P-selectin derives from platelets. This position is also supported by other evidence, such as a lack of correlation between soluble P-selectin and von Willebrand factor but a better correlation with β-thromboglobulin [28]. In our study we observed a concomitant reduction of both activated platelet P-selectin expression and soluble P-selectin levels after treatment with simvastatin, which may confirm a platelet origin of sP-selectin. Andre *et al.* found that sP-selectin constitutes an endogenous activator of the coagulation process through the generation of circulating microparticles in plasma [29]. They propose that sP-selectin should no longer be considered only as a marker of inflammation or platelet activation but also as a direct inducer of pro-coagulant activity associated with vascular and thrombotic diseases.

Our data demonstrated that platelet-derived microparticles (PDMPs) decreased significantly after treatment with simvastatin. The PDMPs play a role in the normal hemostatic response to vascular injury, but on the other hand, it is also possible that the local generation of PDMPs in atherosclerotic arteries may promote acute arterial occlusion. Relevant studies have also reported a significantly higher percentage of PDMPs in patients with cerebrovascular events [30, 31]. The decrease of PDMPs in hypertensive and hyperlipidemic patients given therapy with simvastatin was also correlated with a significant decrease of LDL [32]. Persistent high blood concentrations of PDMPs may be a marker of increased risk of ischemic stroke. Thus, the decrease of sP-selectin and PDMP concentrations after statin therapy may reduce atherothrombotic events.

Increased platelet glycoprotein expression in our hyperlipidemic patients receiving aspirin

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(75 mg/day) after ischemic stroke suggests that this anti-platelet therapy may not sufficiently prevent platelet activation in post-stroke patients with hyperlipidemia.

In our study, the influence of both statin mechanisms on platelet activation cannot be excluded. We cannot distinguish whether the reduction of platelet activity is due to the direct effect of statin therapy or just an indirect effect of cholesterol lowering, which is the main limitation of the research.

In conclusion, this study demonstrated that the use of statin might be a helpful add-on therapy to regulate platelet-related thrombogenesis in atherosclerotic ischemic stroke. Statin therapy in stroke patients with hyperlipidemia may be useful not only due to the lipid-lowering effect but also because of a reduction of platelet activation. These results may provide a novel basis for the beneficial clinical effects of HMG-CoA reductase inhibitors in hypercholesterolemia.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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