Reduced platelet aggregation after fluvastatin therapy is associated with altered platelet lipid composition and drug binding to the platelets

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Aims High plasma cholesterol concentration and increased platelet activity are two major risk factors for atherosclerosis. Lovastatin, the lipophilic drug was shown to inhibit platelet aggregation whereas pravastatin, the hydrophilic drug had no such effect. Analysis of the effect of fluvastatin which is both a lipophilic and hydrophilic drug, on platelet aggregation was the goal of the present study.

Methods Fluvastatin 40 mg daily was administered to 25 patients with hypercholesterolaemia for up to 24 weeks. Normal subjects acted as controls. The influence of fluvastatin on plasma lipids and on platelet aggregation and fluidity was studied. The direct effect of fluvastatin on platelets was compared with that of other statins.

Results Fluvastatin therapy (40 mg day$^{-1}$ for a period of 4 weeks) in hypercholesterolaemic patients resulted in a 23% and 29% reduction in plasma levels of total cholesterol and LDL-cholesterol respectively. Platelet cholesterol/phospholipids molar ratio was reduced by 26% and platelet aggregation was significantly ($P<0.02$) reduced by 10% after 4 weeks of fluvastatin treatment. On continuing fluvastatin therapy for additional 20 weeks, no further decrement in plasma LDL cholesterol levels or in platelet cholesterol/phospholipid ratio were noted. However, platelet aggregation was further significantly ($P<0.01$) reduced by up to 15%. Incubation of platelets with increasing concentrations of fluvastatin or lovastatin, demonstrated a dose-dependent reduction in platelet aggregation, whereas pravastatin showed no effect. This inhibitory effect of fluvastatin or lovastatin on platelet aggregation (up to 34% or 22% respectively at a concentration of 1 mg statin ml$^{-1}$) was found both in platelet rich plasma and in washed platelet suspensions. Fluvastatin and lovastatin (but not pravastatin), seem to share similar platelet binding sites, as non labelled fluvastatin or lovastatin were able to displace [$H$]-labeled-fluvastatin from its binding sites on platelets.

Conclusions Fluvastatin therapy reduces platelet aggregation via a dual effect which involves its in vivo hypocholesterolaemic action on platelet cholesterol content, and also a direct effect of the drug binding to the platelets. The antiatherogenicity of fluvastatin may be related, in addition to its plasma cholesterol lowering ability, to its inhibitory effect on platelet activation.

Keywords: fluvastatin, pravastatin, lovastatin, hypercholesterolaemia, platelet aggregation

Introduction Atherosclerosis is a complicated process involving the interaction of plasma lipoproteins, blood platelets and arterial wall cells [1–4]. Blood platelets have been shown to be intimately involved in atherosclerosis [5–7]. Activated platelets can affect macrophage cholesterol accumulation, and foam cell formation, by altering the uptake of low density lipoprotein (LDL) by arterial wall macrophages [8–14]. Plasma LDL in turn, can activate platelets in a process which involves its binding to the platelets [1, 19, 16].

Activation of platelets occurs in hypercholesterolaemic patients, as enhanced platelet responsiveness was noted when exposed to aggregatory agonists ex vivo [1, 2, 5, 17, 18].

Several hypolipidaemic drugs as well as plasmapheresis were shown to reduce platelet tendency to aggregation [19–22]. Statins are most potent hypocholesterolaemic agents which inhibit cellular hydroxy-methyl glutaryl coenzyme A (HMG-CoA) reductase, the key enzyme in cholesterol biosynthesis [23, 24]. Although all statins share the inhibitory effect on HMG-CoA reductase, the different formulations of the various statins are associated with differences in their effects on macrophage foam cell formation [25–27]. Administration of statins to hypercholesterolaemic patients also affects platelets function [28]. Recently, lovastatin (which is converted to its active form within the gastrointestinal tract) administration for 20 weeks to hypercholesterolaemic patients was shown to decrease significantly the extent of their platelet aggregation [28]. We thus hypothesized that the relatively new active drug fluvastatin can similarly affect platelet function. The purpose of the present study was to...
analyze the effect of another new statin, fluvastatin, which is an active drug, on platelet aggregation in hypercholesterolemic patients, in relation to its effect on platelet cholesterol content, as well as to its binding characteristics to the platelets.

Methods

Patients

Thirty male patients were recruited for this study. Inclusion criteria were 40–70 years old, plasma total cholesterol levels above 6 mmol l\(^{-1}\) but less than 8 mmol l\(^{-1}\) despite dietary therapy, plasma triglycerides level below 3 mmol l\(^{-1}\) for up to 24 weeks. Blood samples were taken before therapy, and 4, 8, 12 and 24 weeks after fluvastatin administration. Blood samples were taken for analyses of plasma lipid concentrations, as well as for determinations of platelet aggregation in response to collagen. The study was approved by the Helsinki Ethical Committee, Rambam Medical Center, Haifa, Israel.

Platelet separation

For platelet studies, venous blood (40 ml) was collected through siliconized syringes into sodium citrate, 3.8% at a ratio of 9:1 (v:v) for platelet-rich plasma (PRP) preparation or into acid citrate dextrose solution (1.4% citric acid, 2.5% sodium citrate, and 2% dextrose) at a ratio of 9:1 (v:v) for washed platelets (WP) preparations. PRP was prepared by low-speed centrifugation (100 \( \times \) g for 10 min) at 25 °C, and the remaining sample was recentrifuged at 1000 \( \times \) g for 10 min to obtain platelet-poor plasma (PPP). Platelets in PRP were counted and diluted with PPP to achieve a uniform concentration of \( 3 \times 10^{8} \) cells/ml. Washed platelets (WP) were prepared from PRP by centrifugation at 240 \( \times \) g for 20 min. The platelet pellet was washed twice in 5 mmol l\(^{-1}\) Hepes buffer, pH 7.4. Platelet- associated radioactive statin was then measured in scintillation fluid, using a \( \beta \)-counter and expressed as a percentage of the added labelled statin.

Platelet cholesterol and phospholipids content

Platelets from hypercholesterolaemic patients were washed three times with Hepes buffer, and then sonicated twice for 20 s at 80 watt. Platelet lipids were extracted with hexane: isopropanol (3:2, v:v). The cholesterol content was measured in the dried hexane phase by the method of Chianuon et al. [30]. Total platelet phospholipid content was also determined in the dried hexane phase by the method of Rouser et al. [31]. Platelet protein was determined using the method of Lowry et al. [32].

Statistical analysis

Data were analyzed for significance of the results by Student’s t-test. Values are given as means \( \pm \) s.d. Differences at a level of less than 0.05 were considered significant.

Results

Effect of fluvastatin therapy on plasma lipids

Fluvastatin therapy in 25 patients (40 mg day\(^{-1}\) for a period of 24 weeks) resulted in 23% and 29% decrement in the plasma levels of total cholesterol and LDL cholesterol, respectively (Figure 1). This hypercholesterolaemic effect was achieved after 4 weeks of therapy (Figure 1). Maximal decrement in plasma triglyceride levels (26%) was obtained after 8 weeks of treatment (Figure 1). Fluvastatin therapy however did not affect plasma high density lipoprotein content, as well as to its binding characteristics to the platelets.
Fluvastatin and platelet aggregation

Time on fluvastatin therapy (weeks)

0 1 0 2 0 3

Plasma lipid concentration (mmol l$^{-1}$)

300
200
100

Figure 1 Effect of fluvastatin therapy on plasma lipid concentration. Fluvastatin (40 mg day$^{-1}$) was administered to 25 hypercholesterolaemic patients for a period of 24 weeks. Blood samples were taken at 0 time and after 4, 8, 12 and 24 weeks of drug therapy. Total cholesterol ($\|$), triglycerides ($\|$) and HDL-cholesterol ($\|$) concentrations were determined in the plasma. Results represent mean ± s.d. ($n$ = 25).

Effect of fluvastatin therapy on platelet aggregation and fluidity

Platelet aggregation was significantly ($P<0.02$) reduced after 4 weeks of fluvastatin therapy by 6% and 10% as analyzed by determination of the maximal aggregation amplitude and the aggregation curve slope respectively (Figure 2a,b). After 24 weeks of therapy a further significant reduction, by 11% and 15% respectively, was obtained (Figure 2a,b).

Maximal decrement in platelet cholesterol/phospholipid molar ratio (26%) was achieved after four weeks of fluvastatin therapy and this effect was not further changed on continuing hypercholesterolaemic patients in response to 4 mg day$^{-1}$ of fluvastatin therapy up to 24 weeks (Figure 2a). Platelet cholesterol/phospholipid molar ratio was decreased as a result of the decrement in platelet cholesterol content from 38±2 μg of cholesterol per mg cell protein to 28±2 μg of cholesterol per mg cell protein after 24 weeks of fluvastatin therapy, with no significant change in platelet phospholipid content (92±3 or 86±3 μg of phospholipids mg$^{-1}$ cell protein before and after 24 weeks of fluvastatin therapy respectively).

The in vitro effect of fluvastatin on platelet aggregation

All three studied statins, at a concentration of 0.01 μg ml$^{-1}$, had no effect on platelet aggregation (data not shown). At a concentration of 0.1 μg ml$^{-1}$, fluvastatin inhibited platelet aggregation (in PRP) by 26% as determined by analysis of the maximal aggregation amplitude (Figure 3a). Lovastatin and pravastatin had no effect on aggregation at this low concentration. At 1.0 μg ml$^{-1}$, both fluvastatin and lovastatin had their maximal inhibitory effect on platelet aggregation (Figure 3a). At this concentration, fluvastatin and lovastatin inhibited maximal aggregation amplitude by 34% and 22% respectively ($P<0.01$). Pravastatin had no effect on platelet aggregation at all studied concentrations (Figure 3a). Upon using washed platelets (WP), free of plasma constituents, the maximal inhibitory effects of fluvastatin and lovastatin on platelet aggregation was also obtained at 1 μg ml$^{-1}$ (Figure 3b).

Maximal aggregation amplitude was inhibited by 16% or 11% for fluvastatin or lovastatin respectively ($P<0.01$) (Figure 3b). Platelet aggregation slope also decreased, by 24% and 14%, for fluvastatin and lovastatin respectively, whereas pravastatin, had no inhibitory effect on the platelet aggregation slope (data not shown).

PRP from hypercholesterolaemic patients exhibited a significant ($P<0.01$) increment of 14% in the aggregation curve slope in comparison to normal volunteers (Figure 4).
patients or from healthy control subjects, with fluvastatin reduction in plasma cholesterol concentration. Increased

The present study demonstrates that fluvastatin therapy in addition to its hypercholesterolaemic effect on plasma LDL, significantly reduce platelet aggregation in hypercholesterolaemic patients. These results were shown in 25 male hypercholesterolaemic patients. The relevance of these results to women or to hypertriglyceridaemic patients, needs to be evaluated.

The inhibitory effect of fluvastatin on platelet aggregation could be associated with the decrement in platelet cholesterol/phospholipid molar ratio, which paralleled the decrement in plasma cholesterol concentration. Increased platelet cholesterol content contributes to platelet activation in hypercholesterolaemic patients [33], and this is related to

**Table 1** Fluvastatin binding to platelets derived from hypercholesterolaemic patients and from normolipidaemic subjects.

<table>
<thead>
<tr>
<th>Statin concentration (µg ml(^{-1}))</th>
<th>Fluvastatin binding to platelets (% of added statin)</th>
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<tbody>
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<td></td>
<td>Normals</td>
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<tr>
<td></td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>1</td>
<td>0.10±0.02</td>
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<tr>
<td>10</td>
<td>0.21±0.03</td>
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[\(^{3}H\)-Fluvastatin, at increasing concentrations (0–10 µg ml\(^{-1}\)) was added to PRP (3×10\(^8\) platelets ml\(^{-1}\)) that was obtained separately from five healthy control subjects or from five hypercholesterolaemic patients. After an incubation period of 30 min at 37°C, washed platelets were prepared as described under Methods. The platelet-associated radioactivity was determined and the drug binding to platelets was expressed as percentage of the added statin. Results represent mean±S.D. (n=3).]

Upon in vitro incubation of PRP from hypercholesterolaemic patients or from healthy control subjects, with fluvastatin (0.1 µg ml\(^{-1}\)), the aggregation curve slope was decreased by 20% or by 30%, respectively (Figure 4).
the effect of platelet cholesterol on the interaction between platelets and the aggregating agents. Hochgraf et al. [26] found that in hypercholesterolaemic patients, lovastatin therapy attenuates the increased platelet cholesterol/phospholipid molar ratio and reduced the increased platelet aggregatory response in these patients to normal values. It seems reasonable to postulate that fluvastatin increased platelet fluidity, which also affects platelet aggregation, by reducing platelet cholesterol/phospholipid molar ratio. Plasma LDL, and more so oxidatively modified LDL, triggers platelet activation and enhances platelet aggregation and secretion via specific binding sites for the lipoprotein on the platelet surface which differ from the classical apolipoprotein B/E receptors on fibroblasts [15, 34–36].

As plasma LDL has been shown to increase platelet aggregation [15], reducing plasma LDL levels by statins therapy can be expected to reduce the platelets’ tendency to aggregate. Indeed lipid lowering treatment in hypercholesterolaemic patients resulted in a substantial reduction in platelet aggregation [37]. Recently, it was shown that cholesterol lowering therapy using pravastatin reduced platelet thrombus formation and hence the risk of acute thrombosis and coronary events in hypercholesterolaemic patients [38]. In the present study the inhibitory effect of fluvastatin on platelet aggregation increased with time beyond the first 4 weeks of therapy even though there was no further decrement in platelet cholesterol/phospholipid molar ratio. This may be the result of the reduction (by 26%) of plasma triglycerides levels after 8 weeks of drug therapy, as very low density lipoprotein (VLDL) has been found to enhance platelet aggregation [15]. Fluvastatin can also reduce platelet aggregation by other mechanisms such as a direct drug interaction with the platelets which can affect platelet response to the aggregating agents. Fluvastatin was shown to be more potent than lovastatin or pravastatin as an inhibitor of platelet aggregation. Indeed, fluvastatin showed a pronounced in vitro inhibitory effect on platelet aggregation at concentrations of 0.1–1 μg mL⁻¹, which are comparable with the in vivo drug concentration in the plasma.

The peak concentration (Cₚₑak) of fluvastatin in the plasma after multiple doses is about 0.4 μg mL⁻¹ [39]. Unlike fluvastatin, lovastatin was less potent at this concentration; the Cₚₑak of lovastatin in plasma is as low as 0.07 μg mL⁻¹ [40].

Fluvastatin and pravastatin are used in their active hydroxy acid forms, whereas lovastatin is used as an inactive lactone. This inactive lactone form of lovastatin is converted to its active form (β-hydroxy acid) in the liver by the enzyme lactonase [41]. In the present study we compared fluvastatin to the inactive form of lovastatin and demonstrated a direct inhibitory effect in vitro of both drugs on platelet aggregation.

The inhibitory in vitro effects of fluvastatin and lovastatin on platelet aggregation was more prominent in PRP than in WP. This may be due to an effect of plasma constituents which can influence the interaction between the statin and...
the platelets. Both fluvastatin and lovastatin inhibit platelet aggregation in vitro and this phenomenon may be explained by the binding capacities of these lipophilic statins to specific binding sites on the platelet surface. Although lovastatin is converted in vivo to an active metabolite, structural domains of this metabolite similar to that of its precursor and also to fluvastatin, may be responsible for their in vitro inhibitory effects on platelet aggregation. In contrast, pravastatin, a hydrophilic statin, neither binds to platelets nor inhibits platelet aggregation in vitro. In line with this observation is the finding that pravastatin did not affect platelet activation in patients with mild hypercholesterolemia [42]. The maximal binding of fluvastatin and lovastatin to platelets was achieved at a concentration of 4 μg/ml [4]. This dissociation between the statin binding capacity to the platelets and its platelet inhibitory effect, may result from factors in addition to the number of platelet specific binding sites, such as a steric effect of the statin on platelet interaction with the aggregating agent (collagen). It is also possible that a maximal inhibitory effect of the statins on platelet aggregation can be reached by occupying only part of the binding sites on the platelet surface.

In conclusion, we have demonstrated that fluvastatin therapy significantly reduced platelet activation in hypercholesterolaemic patients. This effect may be secondary to its hypocholesterolaemic effect on platelet cholesterol content, as well as a direct effect of the drug binding to platelets. Thus, fluvastatin may be considered antatherogenic [43] not only because of its plasma hypocholesterolaemic characteristics, but also as a result of its inhibitory effect on platelet aggregation.

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