

## Effects of simvastatin on angiogenic growth factors released at the site of microvascular injury

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Dear Sir,

Vascular endothelial growth factor (VEGF), secreted by a number of cells, can induce not only endothelial cell migration and growth, regeneration and angiogenesis, but also activation of monocytes and their recruitment into atherosclerotic plaques (1, 2), thus leading to plaque progression. Previous studies indicate that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, are able to modulate angiogenesis in cell cultures and animal models. It has been shown that statins have proangiogenic effects at low therapeutic levels, while inhibition of angiogenesis is induced by high concentrations of these agents (3). Atorvastatin (20 mg/d for two months) was reported to reduce plasma VEGF levels in 14 hypercholesterolemic patients with coronary artery disease (CAD) (4). There has also been one report showing a decrease in plasma VEGF levels following fibrate therapy (5). However, a major source of VEGF are activated platelets (6), forming thrombi upon vascular injury of arteries, e.g. following atheromatous plaque rupture. Upon activation by thrombin *in vitro*, platelets release large amounts of VEGF (7). Weltermann et al. (8) reported that substantial quantities of VEGF are released from platelets during hemostatic plug formation in healthy men aged 20 to 35 years. It is not known whether statin or fibrate therapy affects the release of VEGF at the site of microvascular injury. Given numerous sources of VEGF detected in circulating blood and controversies around the actual impact of both hypolipemic modes of therapy on VEGF-mediated processes, we sought to investigate the effect of simvastatin versus fenofibrate on levels of angiogenic factors in a model of blood coagulation *in vivo* that directly examines coagulant processes involving activated platelets at the site of hemostatic plug formation. Apart from VEGF,  $\alpha$ -granules of platelets activated during blood coagulation release platelet-derived growth factor (PDGF) BB, a potent mitogen and chemotactic agent, which is able to upregulate the expression of VEGF, and thus acts as an indirect inducer of angiogenesis (9). Therefore, we decided to compare the release profiles of

VEGF and PDGF BB upon vascular injury, together with additional platelet activation marker such as soluble CD40 ligand (sCD40L).

A study was conducted in 34 hypercholesterolemic subjects, aged 35 to 65 years, who denied taking any hypolipemic drugs for at least six weeks. The individuals enrolled in this study represented a part of the study population from our recent article published in *Thrombosis and Haemostasis* (9). Subjects were randomly allocated to receive simvastatin (Zocor<sup>®</sup>, Merck) 40 mg daily or micronized fenofibrate (Lipanthyl Supra<sup>®</sup>, Fournier) 160 mg daily. The duration of the study was four weeks.

The University Ethical Committee approved the study, and patients provided a written, informed consent. All the investigations were performed at baseline and at days 3 and 28 after the start of simvastatin administration. To evaluate the effect of both drugs on proteins released at the site of injury, we used a model of microvascular injury that allows to measure levels of various mediators in blood samples collected from standardized bleeding-time wounds (10). Briefly, blood oozing from two standardized bleeding-time wounds, performed with a Simplate II device (Organon Teknika) was collected into heparinized capillary tubes (Kabe Labortechnik) every 30 seconds until cessation of bleeding. Blood samples were then passed into an anticoagulant cocktail, centrifuged and frozen, as described previously (10). Given interindividual bleeding time values, the final analysis was limited to the first 10 samples.

In supernatant samples and plasma obtained from venous blood, VEGF and PDGF-BB levels were determined using commercially available sandwich enzyme-linked immunosorbent assays (R & D Systems). The detection threshold was 9 pg/ml and 15 pg/ml, respectively. The VEGF assay detects the soluble isoforms of human VEGF (VEGF 165 and 121). In addition, sCD40L, a marker of platelet activation, was also measured in venous blood and the bleeding-time blood samples (R & D Systems). The coefficients of intra- and inter-assay variations were about 7%.

Data are given as mean  $\pm$  SEM. Intergroup comparisons were performed using the Mann-Whitney-U test. Serial data obtained in the three time points were analyzed by Friedman's repeated measures ANOVA. Spearman's rank correlation coefficient was calculated to test the association between two variables with a non-normal distribution. A p-value below 0.05 was considered significant.

We studied 17 simvastatin-treated subjects (12 men and 5 women) and 17 fenofibrate-treated subjects (13 men and 4 women). There were no intergroup differences in age, lipid profile, concomitant medication, bleeding time and platelet count. Following a 3-day simvastatin administration, total cholesterol (TC) and LDL cholesterol (LDL-C) were reduced from 249.2  $\pm$  8.4 mg/dl and 164.5  $\pm$  7.6 mg/dl to 220.1  $\pm$  8.2 mg/dl (p<0.001)

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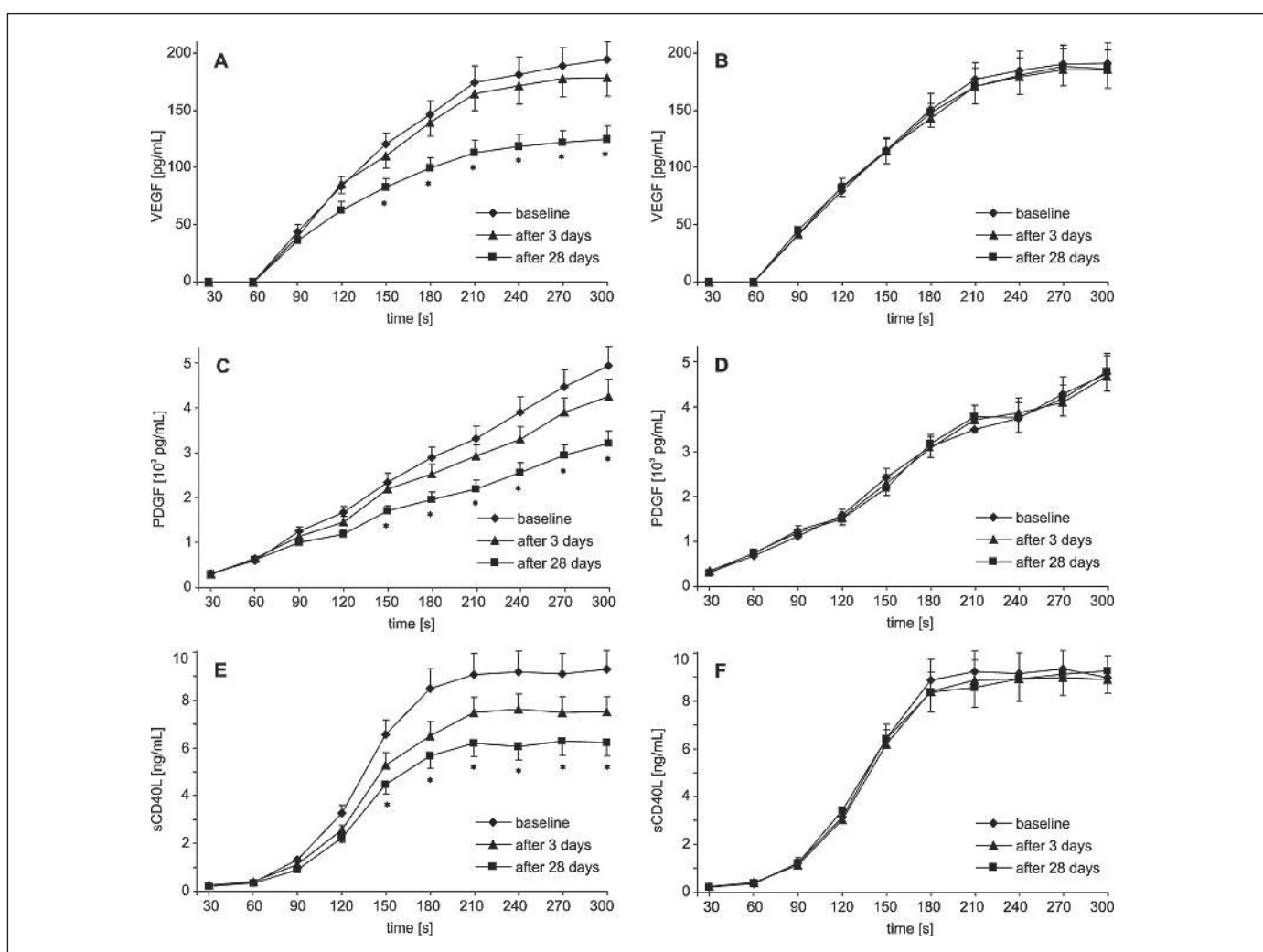
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and  $141.2 \pm 8.8$  mg/dl ( $p < 0.001$ ), respectively, while triglyceride (TG) and HDL cholesterol remained unaltered. In subjects treated with fenofibrate for three days, TC and LDL-C did not change ( $246.1 \pm 9.2$  vs.  $239.1 \pm 8.8$  mg/dl and  $148.2 \pm 7.9$  vs.  $149.3 \pm 8.4$  mg/dl, respectively). Fenofibrate lowered only TG from  $188.1 \pm 24.4$  to  $150.2 \pm 19.7$  mg/dl ( $p = 0.03$ ). After 28 days of simvastatin therapy, TC, LDL-C, and TG declined to  $173.5 \pm 7.3$  mg/dl ( $p < 0.001$ ),  $90.2 \pm 4.8$  mg/dl ( $p < 0.001$ ), and  $139.3 \pm 16.9$  mg/dl (from  $190 \pm 22.2$  mg/dl;  $p = 0.005$ ), respectively, as compared to day 3. In fibrate-treated subjects, TC and LDL-C did not decrease significantly at 28 days ( $220.1 \pm 7.6$  mg/dl and  $135.9 \pm 8.4$  mg/dl, respectively); however, TG fell to  $130.2 \pm 5.5$  mg/dl ( $p = 0.004$  vs. day 3). Simvastatin and fenofibrate did not affect platelet count as compared to the baseline values ( $229,000 \pm 9,000$  vs.  $222,000 \pm 10,000$  on day 28, and  $220,000 \pm 10,000$  vs.  $218,000 \pm 8,000$  on day 28, respectively). Bleeding time in both treatment groups became slightly prolonged ( $348 \pm 42$  vs.

$398 \pm 13$  s, and  $376 \pm 14$  vs.  $420 \pm 10$  s, at baseline vs. day 28, respectively;  $p = 0.04$  for both comparisons).

In venous blood, neither simvastatin ( $45.4 \pm 5.8$  vs.  $47.8 \pm 5.9$  pg/ml) nor fenofibrate ( $46.1 \pm 3.9$  vs.  $44.9 \pm 5.2$  pg/ml) reduced VEGF levels significantly. Similarly, plasma PDGF levels were not affected by either hypolipemic agent ( $561 \pm 43$  vs.  $549 \pm 50$  pg/ml for simvastatin and  $552 \pm 57$  vs.  $538 \pm 48$  pg/ml for fenofibrate). In contrast, plasma sCD40L levels decreased from  $1.32 \pm 0.21$  to  $0.63 \pm 0.14$  ng/ml after 28 days of simvastatin therapy ( $p = 0.019$ ) and  $1.44 \pm 0.2$  to  $0.61 \pm 0.09$  ng/ml after 28 days of fenofibrate therapy ( $p = 0.012$ ), with no significant changes on day 3 of the drug administration.

Levels of VEGF, PDGF and sCD40L in shed blood rose gradually at each time point in all individuals (Fig. 1). Prior to therapy, a maximum rate of the VEGF level increase was similar in both treatment groups ( $1.26 \pm 0.11$  pg/ml/s for simvastatin and  $1.23 \pm 0.11$  pg/ml/s for fenofibrate). Similarly, maximum VEGF



**Figure 1: The time-course of the release of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and soluble CD40 ligand (sCD40L) at the site of microvascular injury and its modulation by simvastatin (40 mg/day) or fenofibrate (160 mg/day) assessed after 3 and 28 days of the therapy.**

Results are shown for the simvastatin group (A, C, E) and the fenofibrate group (B, D, F) for the three variables measured, respectively. \*  $P < 0.05$  vs. baseline. E) Differences between baseline and day 3 with respect to sCD40L levels were significant in the last four time points.

levels did not differ between treatment groups ( $194.2 \pm 18.3$  vs.  $188.5 \pm 14.6$  pg/ml;  $p > 0.05$ ). Maximum PDGF levels ( $4936 \pm 432$  vs.  $4723 \pm 458$  pg/ml;  $p > 0.05$ ) and the rate of their increase ( $18.2 \pm 2.1$  pg/ml/s vs.  $21.6 \pm 2.4$  pg/ml/s;  $p > 0.05$ ) were also similar in simvastatin- or fenofibrate-treated subjects, respectively.

After the first three days of hypolipemic therapy, VEGF and PDGF levels did not change significantly (Fig. 1). A 28-day simvastatin therapy, but not fenofibrate administration, resulted in significantly lower VEGF and PDGF levels at the site of microvascular injury starting from the 150-second blood samples (Fig. 1). Posttreatment maximum rates of the VEGF level increase and maximum VEGF levels were markedly lower in simvastatin-treated patients than at baseline ( $0.78 \pm 0.06$  pg/ml/s and  $125.4 \pm 10.9$  pg/ml, respectively;  $p < 0.001$  for both comparisons). In the fenofibrate group, no significant reductions were observed, and the corresponding values were  $1.14 \pm 0.13$  pg/ml/s and  $184.5 \pm 16.6$  pg/ml (Fig. 1). Maximum levels of PDGF decreased significantly after 28 days of simvastatin administration to  $3201 \pm 283$  pg/ml ( $p < 0.001$ ) but did not change in fenofibrate-treated individuals (Fig. 1). Simvastatin, but not fenofibrate, reduced the maximum rates of the PDGF level increase (to  $11.6 \pm 1.3$  vs.  $16.7 \pm 1.7$  pg/ml/s,  $p = 0.04$ , respectively). Comparing the time-course for sCD40L in the bleeding-time blood with those of VEGF and PDGF, we observed a similar reduction (by about 30% as compared to the baseline value,  $p = 0.011$ ) in sCD40L levels after a 28-day simvastatin administration and no change after a 28-day fenofibrate treatment (Fig. 1). Interestingly, significant decreases in the maximum rate of sCD40L release ( $1.09 \pm 0.08$  vs.  $0.87 \pm 0.06$  ng/ml/s,  $p = 0.03$ ) and its maximum levels ( $9.31 \pm 0.9$  vs.  $7.31 \pm 0.7$  ng/ml,  $p = 0.023$ ) at the site of injury were found as early as following three days of simvastatin administration as compared to the baseline values. Fenofibrate did not affect any of these parameters (Fig. 1).

There were no associations between TC or LDL-C and the rate of increase in the VEGF levels or their maximum values on days 3 or 28 ( $r < 0.2$ ;  $p > 0.05$ ). This held also true for PDGF and sCD40L levels in the bleeding-time blood. VEGF levels positively correlated with PDGF levels in the simvastatin and fenofibrate groups ( $r = 0.73$ ;  $p < 0.001$  and  $p = 0.8$ ;  $p < 0.001$ , respectively). Associations between sCD40L and VEGF or PDGF were also significant, though weaker ( $r$  between 0.3 and 0.5;  $p < 0.05$ ).

The present study shows that in hypercholesterolemic subjects, simvastatin 40 mg daily significantly decreases levels of angiogenic factors released from platelets, i.e. VEGF and PDGF, as well as sCD40L at the site of vascular injury. Similar to other additional actions of statins (12), this effect did not depend on cholesterol-lowering properties of simvastatin. In addition, this study is the first to demonstrate an association between statin administration and decrease in PDGF levels in the microvasculature.

Blann et al. (5) found a decrease in VEGF levels in circulating blood following fenofibrate therapy. There has been only one report regarding fibrate-mediated changes in PDGF levels that demonstrated an unexpected significant increase in concentrations of PDGF after a 2-month ciprofibrate therapy (13). However, in our model fenofibrate given for one month did not

affect these growth factors when determined at the site of microvascular injury.

Importantly, analyses of sCD40L levels in the bleeding-time blood indicate that a release of this protein from platelets differs from the patterns observed for VEGF and PDGF and can be detected as early as after a 3-day statin treatment. At day 28, however, simvastatin lowered sCD40L at the site microvascular injury like in case of VEGF and PDGF. Although simvastatin and fenofibrate decreased plasma sCD40L levels, in the model of microvascular injury only simvastatin is able to reduce sCD40L, which is in line with previous findings (14). At present, reasons for this discrepancy are elusive and may be related to the fact that sCD40L is released not only from platelets, but also, e.g. from endothelial cells, monocytes and activated T cells (14). Moreover, our results show that some drug-induced changes, especially related to thrombus formation, can be detected only at the site of vascular injury, or they are quite different from those seen in plasma of venous blood.

A mechanism underlying statin-induced decrease in amounts of platelet-derived VEGF may be related to impaired platelet activation at least in part due to reduced thrombin generation. Weltermann et al. (8) showed that treatment with hirudin, a selective thrombin inhibitor, reduced VEGF levels determined at the site of microvascular injury. Thrombin-lowering effect of simvastatin reported in our model (10) may also explain the current findings. Nevertheless, fibrate has been found to attenuate thrombin generation (10); however, this effect has not been associated with inhibition of the VEGF release in the present study, suggesting that HMG-CoA reductase inhibitors might exert some additional properties likely related to decreased isoprenoid synthesis (12). An alternative explanation is the thrombin-independent inhibition of platelet activity by statins as suggested by some investigators (15, 16) and us (10).

Our findings have potentially important implications. Since increased VEGF levels have been reported to correlate with worse clinical outcome in patients with acute coronary syndromes (17), statin-induced reduction in VEGF released *in loco* might contribute to beneficial effects of HMG-CoA reductase inhibition. Moreover, a release of VEGF and PDGF during the early stages of coagulation activation is also likely important in wound healing. It is unclear whether statins might impair this process.

In view of data suggesting a differential effect of low versus high doses of statins on angiogenesis (3), the impact of lower doses of simvastatin on the release of VEGF and PDGF in the Simplate model deserves investigation. However, in 12 subjects treated with 20 mg simvastatin daily for three months, we did not observe any significant changes in the levels of VEGF or PDGF measured in the bleeding-time blood (A. Undas, unpublished data).

The current study suggests that simvastatin (40 mg daily) may inhibit atherosclerosis in part by decreased release of angiogenic and mitogenic agents from platelets at the site of vascular injury.

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