Abstract. – BACKGROUND AND AIM: Statins are HMG-CoA reductase inhibitors within the framework of cholesterol biosynthesis and used to lower the low-density lipoprotein (LDL). There are other aspects of statins can deploy a protective effect, even without the LDL’s lowering. The aim of this study is to investigate the effects of different type of statins on proliferative and migrative behaviors of Hepatocyte Growth Factor (HGF) induced human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS: Human umbilical vein endothelial cells were isolated and cultured. Groups were designed in order to observe the effects of every individual substance. HUVECs were stimulated with HGF, statins and farnesylyphosphat ammonium salt (FPP) or geranylgeranyl-pyrophosphate (GGPP), respectively. Cell proliferations were counted 48 hours after initial stimuli and distances between migration fronts were used in migration analyses.

RESULTS: All types of statins showed significant anti-migrative and anti-proliferative characters. Simvastatin and fluvastatin but not cerivastatin, were able to inhibit the HGF depending migration and showed a significant effect on the inhibition of the isoprenylation (GGPP). Only simvastatin influenced the HGF depending migration via inhibiting the isoprenylation process through GGPP. Cerivastatin significantly decreased the proliferation and Fluvastatin significantly enhanced the migration behaviors of HUVECs when they were co-incubated with methyl-β-cyclodextrin (MCD).

CONCLUSIONS: Statins countermand the pro-proliferative and as well as the promigrative effect of HGF on HUVECs. The mechanisms which provoke this effect are dependent on the type of statin. Direct interactions of statins with lipid rafts play a significant role in the endothelial cell mechanisms.

Key Words: Statins, Proliferation, Migration, Endothelial cells, HGF, Lipid rafts.
tion and healing of the tissues. It has an effect on hepatocytes and endothelial cells as a mitogen factor\textsuperscript{9,10}. HGF has also an anti-apoptotic effect on endothelial cells\textsuperscript{11,12}. It was shown that the damaged smooth muscle cells express HGF receptors and, therefore, they are induced for proliferation and migration\textsuperscript{13}. Owing to its mitogenic and motogenic qualities, HGF has a function both in endothelial regeneration after an injury and atherosclerotic process as well. Hence existence of HGF can be proven in arteriosclerotic lesions, while it doesn’t exist in healthy vessels\textsuperscript{12}.

Statins are a class of drugs used to lower cholesterol levels through MHG-CoA-reductase inhibition, which plays central role in the production of cholesterol in the liver. It was observed for a long time that among patients with the same LDL-values those who carried clearly less risk for cardiovascular incidents were the ones treated with statins\textsuperscript{14}. Hence, it can be concluded that further mechanisms must play a role for the prevention of cardiovascular incidents other than pure reduction of serum LDL levels. These pleiotropic effects are against the arteriosclerotic risk factors such as inflammation, thrombosis and oxidation\textsuperscript{15}. The most important possible mechanism is the inhibition of mevalonate synthesis, a preliminary stage of farnesyldiphosphate and geranylgeranyl pyrophosphate. Prenylation is a posttranslational modification of proteins which are responsible for the correct positioning of proper proteins in the cell membrane. Proper positioning is again a prerequisite for their biologic function\textsuperscript{16,17}. It was demonstrated that the nitric oxide (NO) availability and the relaxation ability of endothelial cells can be drastically improved by statins\textsuperscript{18}. Decreased NO effect on endothelial cells is an important component of endothelial dysfunction.

Typical cell membrane of eukaryotes is comprised of lipids and proteins. The lipids, mainly the phospholipids, are oriented in a way that the hydrophilic carbohydrates are butt-jointed and double layered. In the heterogeneous, asymmetric membrane there are relatively organized special microdomains, so called lipid rafts, which consist of glycosphingolipids, cholesterol and glycosylphosphatidylinositol (GPI) binding proteins, which involve in signal transduction\textsuperscript{19,20}. Smaller rafts can merge with bigger platforms through protein-protein or protein-lipid interactions\textsuperscript{21}. Proteins positioned on the GPI belongs a variety of receptors, i.e. insulin receptor, which explains the importance of lipid rafts on many signal paths\textsuperscript{22}. Receptors functioning as tyrosine kinase are often encountered especially in lipid rafts. HGF receptors also belong to the class of tyrosine kinase receptors.

The purpose of this experiment was to analyze the effects of different statins on proliferative and promigrative effect of HGF induced HUVECs. The role of prenylation and lipid rafts on HGF and statin stimulated endothelial cells was analyzed as well.

**Materials and Methods**

**Isolation and Culture of HUVECs**

Isolation of human umbilical vein endothelial cells (HUVECs) was performed according to methods described by Jaffe et al\textsuperscript{23}.

HUVECs were resuspended in 15 ml Endothelial Basal Medium (EBM; Promo Cell, Heidelberg, Germany) after they had been obtained from centrifuge. Thereafter, the EBM was filled with additional substances: 0.4% ECGS/H, Epidermal Growth Factor, 0.1 ng/ml, hydrocortisone, 1 ug/ml, basic Fibroblast Factor, 1 ng/ml, amphotericin, 50 ng/ml, gentamycin 50 µg/ml (Promo Cell, Heidelberg, Germany) and 20% fetal call serum (FCS). The cultures were kept in an incubator at 37°C in a fully humidified atmosphere with 5% CO\textsubscript{2} concentration until they grow into confluent stage (2-3 days). Cell identification was accomplished with immunofluorescence technique using antibodies against Von Willebrand factor (Dakopatts, Hamburg, Germany).

The cultivation of HUVECs was performed in an incubator at 37°C with 5% CO\textsubscript{2} concentration. Cells were flushed with Henk’s balanced salt solution (HBSS; PAA H15-00) in order to bathe the calcium, which blocks the effect of trypsin. Afterwards, cells were incubated for a minute with trypsin (Sigma T 4299, Saint Louis, MO, USA). Finally, the effect of trypsin was controlled with a light microscope and stopped by adding EBM. Thereafter, the HUVECs were resuspended and laid on gelatin covered D12 shells (Falcon 353003). The cells were later incubated with EBM by adding 10% FCS (Biowest, Hannover, Germany). The medium was changed every 2-3 days. HUVECs were observed daily with a light microscope.

**Proliferation Analysis**

HUVECs were seeded on gelatin added 12-Well-Plates. The cells were cleaned with HBSS
Migration Analysis

The cells were seeded in the middle of each 12-Well plate. After silicone parts were placed, cells were separated from the shells and seeded on the plates with a growth medium of 100,000 cells/ml enriched with 2% FCS. The cells were incubated for 48 hours.

Before the stimulation, silicone parts were removed with a sterilized forceps and the free space was measured. This value was pointed as a reference value for the upcoming migration value. Subsequently, the cells were stimulated as described above. After the stimulation, the cells were incubated for another 48 hours.

Analyses of migration experiments were performed via low magnified microscopic captures (2x). The initial values were measured before the stimulation. The distance between migration fronts was measured multiple times and an average value was calculated, which served as a reference for the future measurements. Calculations were performed after the incubation in a same way. In order to determine the actual migration, the average value after incubation was subtracted from initial average value and the net result was divided into two. Division by two let us to measure the migration from one front.

Statistical Analysis

All results were obtained from average values of multiple measurements which were obtained from various cell preparations. Standard error of the mean is shown on each graph. The Kruskal-Wallis test was used to analyze our data. Results are expressed as mean ± SD. A p-value less than 0.05 was considered statistically significant.

Results

Effects of Statins on HGF-Induced HUVEC Proliferation

The proliferative effect of HGF on HUVECs was calculated with the help of Kuhlmann et al’s research, where the maximum value of proliferation was set as 15 ng/ml\textsuperscript{24}. A significant proliferative effect of HGF was verified compared with non-HGF-induced control group (p < 0.05). A complete nullifying effect of statins (cerivastatin, simvastatin and fluvastatin) on HGF was shown (Figure 1). Even after the statin treatment, no significant differences were observed between HGF stimulated and non-HGF stimulated HUVECs in terms of cell proliferation (p > 0.05).
The effects of different types of statins on proliferation and migration of HGF-induced HUVECs

**Effects of Statins on HGF-Induced HUVEC Migration**

All three types of statins showed highly significant anti-migrative character ($p < 0.05$) Cerivastatin failed to inhibit the promigrative effect of HGF significantly ($p > 0.05$). Although simvastatin and fluvastatin significantly inhibited the promigrative effect of HGF ($p < 0.05$), no significant difference was found between them ($p > 0.05$) (Figure 2).

**Effects of Mevalonate on Proliferation Behaviors of HGF and Statin Incubated HUVECs**

Mevalonate alone and combination of mevalonate with HGF showed no significant difference in proliferation behaviors compared to the control groups ($p > 0.05$). Mevalonate addition to the statin groups significantly reversed the antiproliferative effect of statins ($p < 0.05$). HGF effect was no longer re-

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**Figure 1.** Figure showing the effects of statins on HGF induced endothelial cell proliferation. All statins inhibited the HGF induced proliferation of HUVECs ($p < 0.05$).

**Figure 2.** Although simvastatin and fluvastatin significantly inhibited the effect of HGF on endothelial cell migration ($p < 0.05$), cerivastatin didn’t show an anti-migrative effect ($p > 0.05$).
produced through mevalonate. However, co-incubation of mevalonate, fluvastatin and HGF showed a significant antiproliferative effect on HUVECs compared with non-HGF stimulated group ($p < 0.05$ mevalonate + fluvastatin vs. HGF + fluvastatin + mevalonate).

**Effects of Mevalonate on the Migration Behaviors of HGF and Statin Incubated HUVECs**

Mevalonate alone or with HGF showed no significant changes in the migration behavior of HUVECs. Anti-migratory effect of cerivastatin and fluvastatin was significantly reversed by addition of mevalonate, even at the control level ($p < 0.05$). However, in the experiments with simvastatin, mevalonate couldn’t reverse the anti-migrative effect ($p > 0.05$). The further addition of HGF showed a statistically meaningful change only in simvastatin group.

**Effects of Farnesylpyrophosphate (FPP) on Proliferation Behaviors of HGF and Statin Incubated HUVECs**

FPP alone or in combination with HGF had an antiproliferative effect on endothelial cells ($p < 0.05$). FPP significantly inhibited the anti-proliferative effects of cerivastatin ($p < 0.05$). In simvastatin group, the anti-proliferative effect of statin was removed by additional incubation with FPP ($p < 0.05$). In contrast, FPP didn’t reverse the effect of fluvastatin ($p > 0.05$). Additional stimulation of cells with HGF led no further increase in cerivastatin and simvastatin groups. Further addition of HGF showed an antiproliferative tendency against fluvastatin + FPP group.

**Effects of Farnesylpyrophosphate (FPP) on the Migration Behaviors of HGF and Statin Incubated HUVECs**

FPP itself or combined with HGF had no effect on the migration behavior of the cells ($p > 0.05$). The anti-migratory effects of cerivastatin and fluvastatin were reversed significantly by FPP ($p < 0.05$). Yet, simvastatin didn’t show any reversing effect. Not any statistically meaningful changes observed in migration behavior with the further addition of HGF to statin groups ($p > 0.05$).

**Effects of Geranylgeranyl-Pyrophosphate (GGPP) on the Migration Behaviors of HGF and Statin Incubated HUVECs**

In comparison to the control group, GGPP alone or with HGF did not show any significant differences on the growth behaviors of the cells. GGPP significantly reversed the antiproliferative effect of cerivastatin and simvastatin and fluvastatin ($p < 0.05$). Additional stimulation with HGF did not cause any further increase in proliferation in cerivastatin group ($p > 0.05$). In contrast, a significantly increased proliferation was observed in simvastatin and fluvastatin group with additional stimulation with HGF ($p < 0.05$).

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**Importance of Lipid Rafts for the Proliferation Behaviors of HGF and Statin Incubated HUVECs**

Cells which were pre-incubated with MCD and cerivastatin showed a significant antiproliferative behavior compared to purely cerivastatin incubated cells ($p < 0.05$) (Figure 3). This significant difference was reversed by the addition of HGF again. In the experiments with simvastatin or fluvastatin, pre-incubation with MCD didn’t show statistically significant results ($p > 0.05$). Although the co-incubation with HGF in simvastatin group showed a significant decrease in proliferation ($p < 0.05$), but not any significant changes were observed in HGF induced fluvastatin group ($p > 0.05$).

**Importance of Lipid Rafts for the Migration Behaviors of HGF and Statin Incubated HUVECs**

MCD significantly decreased the migration profile of HUVECs. ($p < 0.05$) Co-incubation with
The effects of different types of statins on proliferation and migration of HGF-induced HUVECs

HGF didn’t show a significant change in the migration compared to purely MCD-stimulated cells. Cerivastatin or simvastatin combined with MCD showed no significant changes in the migration compared to purely statin-stimulated cells \( (p > 0.05) \). Although the additional stimulation with HGF didn’t lead any significant change in cerivastatin group, a significant increase in the migration was shown in simvastatin group \( (p < 0.05) \) (Figure 4).

On the contrary, fluvastatin stimulated cells pre-incubated with MCD showed a very significant pro-migrative effect compared to cells that were treated only with fluvastatin \( (p < 0.05) \). The additional incubation with HGF showed no further increase in migration behavior \( (p > 0.05) \).

Discussion

Angiogenesis involves both proliferation and migration processes of vascular cells. Both factors are critically involved in the formation of new blood vessels. In our experiment, the proliferation and migration behaviors of human umbilical endothelial cells were examined separately from each other to eliminate the responses of individual factors of angiogenesis. Furthermore, it was investigated whether the inhibition of HMG-CoA reductase and/or the role of prenylation of proteins are responsible for the effect of statins. The role of lipid rafts for HGF-induced proliferation was examined as well.

Statin concentrations which had proved to be antiproliferative were: Cerivastatin 0.1 \( \mu \text{mol/l} \), simvastatin 2.5 \( \mu \text{mol/l} \) and fluvastatin 1 \( \mu \text{mol/l} \).25. Weis et al26 showed that cerivastatin and atorvastatin in low concentrations (0.005-0.01 \( \mu \text{mol/l} \)) has a capability to induce proliferation of human adult dermal microvascular endothelial cells (HMVECs) and HMEC-1 of an immortalized human dermal endothelial cell line, however in high concentration (0.05-1 \( \mu \text{mol/l} \)) they showed an inhibitory effect. Also Frick et al27 showed that the effects of statins are concentration and cell type dependent.

The proliferative effect of HGF and the antiproliferative effect of statins were analyzed in our study. All three types of statins inhibited the HGF effect on HUVECs. In the migration experiments, although simvastatin and fluvastatin were able to remove the promigrative potential of HGF, HGF effect was still detectable in experiments with cerivastatin. These results correlate with results of other research groups that examined the reaction of HGF-stimulated HUVECs for statins. Uruno et al28 investigated the behavior of angiogenesis of HGF and fluvastatin stimulated HUVECs. It was concluded that fluvastatin in low doses favors the
HGF-induced angiogenesis and had an inhibitory effect in high doses. Recently, simvastatin was shown to reduce the VEGF-induced proliferation dose-dependent in retinal endothelial cells. A correlation between VEGF concentration in the blood and statin treatment has also been found in clinical studies. The serum level of VEGF was significantly reduced, after 4 months of treatment with pravastatin.

Co-incubation with mevalonate partially removed the statin effect on migration and proliferation. This means that the inhibition of HMG-CoA reductase plays a crucial role in the effect of statins on HUVECs. Weis et al. demonstrated similarly the antiproliferative effect of cerivastatin on HMEC-1 which can be removed by the addition of mevalonate. Veillard et al. described the reversing effects of mevalonate on the simvastatin in human vascular endothelial cells.

No group effects of the statins were shown at the additional stimulation of the cells with HGF. LDL reduction of statins indeed represents a group effect, however, vary the non-lipid-reducing, so-called pleiotropic effects of statins structure dependent strongly and, thus, represent substance effects. In the proliferation and in the migration analysis, only in fluvastatin, an additional proliferative or promigrative effect against cells that were stimulated only with statin+mevalonate could be detected. The inhibition of the HGF-effect by cerivastatin and simvastatin does not seem to be caused decisively due to the blocking of the HMG-CoA reductase. It is believed that statins, for example, intervene directly in the cell signaling, which regulates apoptosis, proliferation and metabolism. Therefore, it is necessary to find out whether the prenylation of proteins for the HGF-induced angiogenesis plays a role. In order to find out which of the two different prenylation pathways are relevant for the HGF-effect, both substances were tested sequentially. FPP was able to remove significantly the inhibitory effect of statins in the proliferation experiments, except for fluvastatin. A group-effect of the statins showed itself in the migration analysis. FPP caused a significant increase in migration behavior in all cases. In the literature there are many reports stating that FPP is insufficient to remove significantly the effect of statins. However, there are also cases stating that FPP can remove the statin-effect significantly. With a further addition of HGF, restoration of the HGF effect was shown neither in the proliferation nor in the migration studies. A rather opposite effect was evident in the prolifera-

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**Figure 4.** Effect of MCD on the migration behavior of fluvastatin and HGF-stimulated endothelial cells. Cells stimulated with fluvastatin + MCD showed a statistically significant promigrative changes ($p < 0.05$).
tion experiments with cerivastatin and simvastatin, the additional incubation with HGF caused a significant reduction in cell proliferation. It must, therefore, be assumed that the way of prenylation over FPP has no relevance with the proliferative or promigrative behavior of the HGF.

GGPP significantly reversed the inhibitory effects of statins in terms of proliferation and migration. At an additional stimulation of the cells with HGF, a further significant increase of the proliferation in simvastatin and fluvastatin group was observed. Co-incubation of cerivastatin with HGF showed no increase in proliferation. However, in terms of migration behavior, additional HGF significantly increased the migration potential only in simvastatin group. In the literature, many observations have been described that GGPP is capable of reversing the effect of statins on endothelial cells. Park et al.35 showed that GGPP is capable to remove the angiostatic effect of simvastatin on HMEC. Villard et al.31 described their observations that simvastatin in endothelial cells reduces chemokines and their receptor expression by inhibiting GGPP pathway. Additionally, GGPP has also been shown to remove the inhibitory effects of cerivastatin and atorvastatin on HMVECs.26 However, there are only few studies on how GGPP behaves towards cells which were treated with statins and growth factors. Our study demonstrated GGPP is an important agent for the proliferative and promigrative functions of HGF. The inhibitory effect of cerivastatin on HGF-stimulated HUVECs does not seem to be dependent on the prenylation mechanism. The effect of simvastatin on the growth factor works with the prenylation. The effect of fluvastatin on HGF-stimulated HUVECs in the proliferation is in connection with the prenylation, yet this could not be proved for the migration. In total, therefore, it shows that the inhibition of the prenylation is not alone responsible at all statins to inhibit the effect of the growth factor HGF, which means it is a substance effect and not a group effect of statins. Other research groups as well could describe observations that different statins show different behaviors.37 The isoprenylation, however, is also only a hypothesis to explain the pleiotropic effects of statins.

Another approach is an assumption, that the modification of lipid rafts caused by statins plays an important role for the pleiotropic effects of HMG-CoA reductase inhibitors.38 Hillyard et al.39 described their observations, that simvastatin and fluvastatin in NK-cells dose-dependently reduced the number of lipid rafts. To observe the role of lipid rafts, the cells were incubated with MCD, which promotes in low concentrations the absorption of cholesterol in the cell and in higher concentrations supports the degradation of lipid rafts.35 MCD significantly reduced the growth and the migration of HUVECs. Furthermore, similar to statins, it was able to remove the preproliferative, or the promigrative effect of HGF. This indicates that the presence of lipid rafts plays a crucial role in the effectiveness of the hepatocyte growth factor. Wu et al.41 was shown a reduction in the activation of astrocytes, as well as the production of IL-1, after simvastatin reduced the expression and the phosphorylation of the epidermal growth factor receptor (EGFR) within the lipid rafts. According to our results, it is quite conceivable that a similar effect takes place at the HGF receptor, which would explain an effect of statins on the HGF-effect, beyond the prenylation. Li et al.40 showed that simvastatin causes a decreased raft/caveolae formation and inactivation of the Akt-pathway and triggers apoptosis in cancer cells.

### Conclusions

Statins were shown to significantly inhibit the proliferation and migration behavior of HGF-stimulated HUVECs. This effect can be attributed, especially for simvastatin, to the inhibition of GGPP-dependent isoprenylation. However, this mechanism appears to be only partially relevant for fluvastatin and have no role in cerivastatin. Inhibitory effects of statins on HGF-stimulated HUVECs are substance-depending over various mechanisms, which act partly via the inhibition of isoprenylation, partly depending on lipid rafts, but remain to a large extent still unclear.

### Conflict of Interest

The Authors declare that there are no conflicts of interest.

### References


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