

Original article

Effects of various statins on cytokine-dependent growth and IgE-dependent release of histamine in human mast cells

Background: Statins are inhibitors of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase, a key enzyme in mevalonic acid (MVA)-dependent signaling. Recent data suggest that statins exhibit profound inhibitory effects on growth and function of various immune cells. In the present study, we examined the *in vitro* effects of five different statins on primary human mast cells (MCs), MC progenitors, and the human MC line HMC-1.

Methods: Histamine release experiments were conducted on isolated MCs using statins and an anti-immunoglobulin E (IgE) antibody. Culture experiments were performed with stem cell factor (SCF) and interleukin (IL)-6, and cord blood-derived progenitors.

Results: Preincubation of primary lung MCs with cerivastatin or atorvastatin (1–50 μ M) for 24 h resulted in inhibition of anti-IgE-induced release of histamine. The effects of both statins were dose-dependent. Moreover, both statins, and to a lesser degree lovastatin, were found to inhibit the SCF-induced differentiation of MCs from their progenitors. The other statins tested (simvastatin, pravastatin) did not affect mediator release or growth of MCs.

Conclusions: Cerivastatin and atorvastatin act as inhibitors of growth and function of human MCs.

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Mast cells (MCs) are multifunctional effector cells of the immune system (1, 2). These cells produce an array of biologic mediators and play a crucial role in allergic and inflammatory reactions (1–3). MCs also express a number of critical cell surface antigens including cytokine receptors and the high affinity receptor for immunoglobulin E (IgE; Fc ϵ RI; 4, 5). In response to IgE receptor cross-linking or other stimuli, MCs release their mediators into the extracellular space (3–5).

In common with all other hemopoietic cells, MCs originate from CD34⁺ progenitors (6, 7). The most potent differentiation factor for human MCs appears to be stem cell factor (SCF; 8–10). In particular, when uncommitted hemopoietic progenitors are cultured in SCF for several weeks, a substantial number of cultured cells are MCs (8–10). In addition, SCF upregulates the

capacity of MCs to release histamine in response to IgE-dependent stimuli (11–13). The effects of SCF on MCs are exerted through a specific tyrosine kinase receptor (c-kit) that is expressed on uncommitted and MC-committed progenitors as well as on mature MCs (14, 15).

Statins are potent inhibitors of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase, a critical enzyme in the mevalonic acid (MVA) pathway that leads to the formation of various isoprenoid compounds and their products including cholesterol, dolichol, and ubiquinone (16–18). The MVA pathway is critically involved in the post-translational isoprenylation of cellular proteins including growth regulators and oncogene products-like Ras (19, 20). Statins were first described and introduced in clinical trials as cholesterol-lowering agents (21). However, statins exert a variety of effects on different

cells including immune cells known to participate in inflammatory and allergic reactions (22–24). Likewise, it has been shown that certain statins can inhibit secretory responses in rat MCs (24). We have recently shown that certain statins inhibit cytokine-dependent growth and IgE-mediated release of histamine in human basophils (25). In the present study, the effects of five different statins on SCF-dependent growth and IgE-dependent mediator release in human MCs were examined.

Materials and methods

Reagents

Recombinant human (rh) SCF and rh interleukin (IL)-6 were purchased from Strathmann Biotech (Hamburg, Germany), collagenase type II from Worthington (Lakewood, NJ, USA), RPMI-1640 medium and fetal calf serum (FCS) from PAA Laboratories (Pasching, Austria), and Iscove's Modified Dulbecco's medium (IMDM) from Gibco Life Technologies (Gaithersburg, MD, USA), myeloma IgE from Chemicon (Temecula, CA, USA), and MVA and the Ca-ionophore A23187 from Sigma (St Louis, MO, USA). Cerivastatin was provided by Bayer (Wuppertal, Germany), atorvastatin by Pfizer (Karlsruhe, Germany), simvastatin and lovastatin by Merck & Co (Linden, NJ, USA), and pravastatin by Bristol-Myers Squibb (Munich, Germany). Stock solutions of statins and MVA were prepared as described previously (25).

Isolation and culture of human mast cells

Lung tissue was obtained at surgery (lobectomy) from eight patients with bronchiogenic carcinoma after informed consent was given. Juvenile foreskin was obtained from one pediatric patient at circumcision after informed consent was obtained from parents. Tissue MCs were isolated as described (26, 27). Briefly, tissue was chopped into small fragments and washed extensively in Mg^{2+}/Ca^{2+} -free Tyrode's buffer. Then, tissue fragments were incubated in collagenase type II (2 mg/ml) at 37°C for 1–3 h. Dispersed cells were then recovered by filtration through Nynet cloth, washed, and recovered in RPMI-1640 medium plus 10% FCS. The MC leukemia line HMC-1 (28) was kindly provided by Dr J. H. Butterfield (Mayo Clinic, Rochester, MN, USA). The HMC-1 cells were cultured in IMDM supplemented with 10% FCS.

Histamine release experiments

The histamine release assay was performed essentially as described (29). In typical experiments, cells (1×10^6 /ml) were incubated with various concentrations (1–50 μ M) of statins in RPMI-1640 medium containing 10% FCS at 37°C for 24 h. In select experiments, lung MCs were incubated with statins (10 μ M) in the presence or absence of MVA (200 μ M). After statin pretreatment, cells were incubated with IgE for 3 h, washed, resuspended in histamine release buffer (Immunotech, Marseille, France), and then exposed to anti-IgE antibody E124.2.8 (10 μ g/ml) for 30 min (37°C). In separate experiments, cells were exposed to the Ca-ionophore A23187 (0.1–10 μ g/ml) at 37°C for 30 min. Cells were then centrifuged (4°C), and the cell-free supernatants as well as the cell suspension (extracellular plus intracellular phase) examined for histamine content by radioimmunoassay (Immunotech). Histamine release was calculated as percentage (released histamine) of total histamine (=extracellular plus intracellular histamine). Statin-induced inhibition of IgE-

mediated histamine release was expressed as percentage of anti-IgE-induced histamine release (=100%).

Mast cell differentiation assay

To study the effects of the statins on MC differentiation, cord blood (cb) cell cultures were prepared using SCF and IL-6 as growth factors as reported (8–10). The cb was obtained from umbilical veins at full-term deliveries ($n = 4$) after informed consent was given by mothers. The cb mononuclear cells (MNC) were isolated by Ficoll and cultured in 24-well plates (TPP, Trasadingen, Switzerland; 0.5×10^6 cells/ml) in RPMI-1640 medium containing 10% FCS, rhSCF (100 ng/ml) and rhIL-6 (100 ng/ml), and various concentrations of statins (0.1–10 μ M) or control medium, at 37°C and 5% CO₂. Medium and growth factors were changed on day 14. After 28 days, cells were counted and examined for the percentage of MCs and other cell types (mostly macrophages) by Wright-Giemsa staining. The numbers of MCs and of macrophages were calculated from total cell numbers and differential counts.

Proliferation assay

To determine statin effects on proliferation, ³H-thymidine incorporation experiments were performed as described (30, 31). The HMC-1 cells (4×10^4 cells/well) were placed in 96-well microtiter plates and incubated with various statins (0.1–100 μ M) at 37°C for 24 h. In select experiments, MCs were incubated with statins in the presence or absence of MVA (200 μ M). After incubation, 1 μ Ci of ³H-thymidine (Amersham, Buckinghamshire, UK) was added for 6 h at 37°C. Cells were then harvested on filter membranes (Packard Bioscience, Meriden, CT, USA). Bound radioactivity was counted in a β -counter (Top-Count NXT; Packard Bioscience).

Evaluation of apoptosis by DNA analysis

To study the mechanism of statin-induced inhibition of cell growth, DNA analysis was performed on HMC-1 cells essentially as described (30, 31). In brief, HMC-1 cells (2×10^6) were exposed to statins (each 1–100 μ M) or camptothecin, 3 μ M (positive control) for 24 h (37°C, 5% CO₂). After incubation, cells were lysed, incubated in RNase A (50 μ g/ml) and proteinase K at 37°C for 16 h. Thereafter, DNA was extracted and precipitated in ethanol with sodium acetate (–20°C, overnight). Gel electrophoresis was performed on 2% agarose as described (30, 31). Apoptosis was defined as typical ladder-type fragmentation of DNA.

Surface marker analysis

HMC-1 cells were incubated with the statins (each 50 μ M) for 24 h (37°C) and then were examined by flow cytometry using the following monoclonal antibodies (mAb): T11 (CD2), 84H10 (CD54), and Clb-gran12 (CD63) from Immunotech, E11 (CD35), 23C6 (CD51/CD61), YB5.B8 (CD117), and 9F5 (CD123) from Becton Dickinson (San Jose, CA, USA), and W17/1 (CD88) from Serotec (Düsseldorf, Germany). The mAb K20 (CD29), 14A2.H1 (CD151), and PL1 (CD162) were obtained from the Vth and VIth Workshop on Human Leukocyte Differentiation Antigens. The mAb SE5A5 (CD172a) and 97A6 (CD203c) were a kind gift of Dr H. J. Bühring (University of Tübingen, Germany). Primary MCs were preincubated with atorvastatin (10 or 50 μ M) or cerivastatin (10 or 50 μ M) for 24 h (37°C), then exposed to IgE (3 h), and then were incubated with control medium or anti-IgE (1 μ g/ml) for up to 180 min. The MCs were examined by multicolor flow cytometry using

CD117-APC, CD45-PerCP (Becton Dickinson), CD63-FITC (Immunotech), and CD203c-PE (Immunotech).

Statistical analysis

To determine the level of significance in statin inhibition experiments, standard statistical tests including the Student's *t*-test, were applied. A *P*-value of <0.05 was considered to indicate statistical significance.

Results

Effects of statins on histamine release in primary mast cells

Preincubation of lung MCs with cerivastatin or atorvastatin (10 and 50 μM) for 24 h resulted in a significant inhibition of histamine release provoked by anti-IgE, 10 $\mu\text{g/ml}$ (anti-IgE = 100% vs anti-IgE + cerivastatin, 50 μM : $40.2 \pm 14.8\%$ vs anti-IgE + atorvastatin, 50 μM : $26.3 \pm 13.5\%$, $P < 0.05$; Fig. 1). Almost identical results were obtained with skin MCs (not shown). The effects of cerivastatin and atorvastatin were dose-dependent and were counteracted by addition of MVA (200 μM ; Fig. 2). The other statins applied (simvastatin, lovastatin, pravastatin) showed no significant effects on histamine release in MCs ($P > 0.05$; Fig. 1). Neither cerivastatin nor atorvastatin showed a significant effect on Ca-ionophore A23187-induced release of histamine in

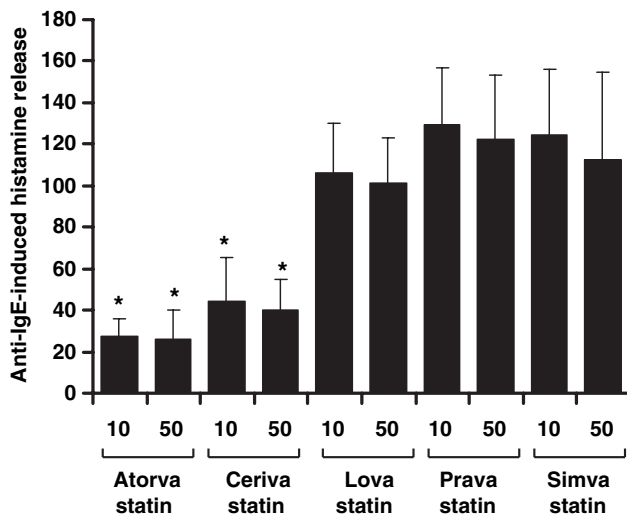


Figure 1. Effects of various statins on releasability of lung mast cells. Isolated lung mast cells were preincubated with various statins (10 and 50 μM) for 24 h at 37°C, and then exposed to anti-immunoglobulin E (IgE; 10 $\mu\text{g/ml}$) for 30 min. Statin-induced changes in mediator release are expressed as percentage of histamine release provoked by anti-IgE (=100%). Results represent the mean $\pm$ SD of three independent experiments (mast cells from three different donors). Significant changes in anti-IgE-induced histamine release are indicated by an asterisk ($P < 0.05$). Cell viability did not change by addition of statins for 24 h.

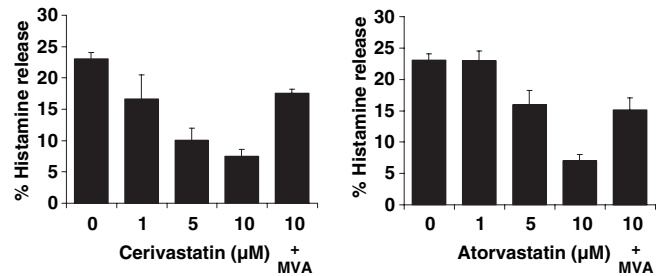


Figure 2. Dose-dependent effects of cerivastatin and atorvastatin on immunoglobulin E (IgE)-dependent histamine release in human lung mast cells. Isolated lung mast cells were preincubated with various concentrations of cerivastatin (A) or atorvastatin (B) or control medium (0) for 24 h (37°C). Mevalonic acid (MVA) was added (at 200 μM) to statins (each 10 μM) as indicated. After statin exposure, cells were incubated with anti-IgE (10 $\mu\text{g/ml}$) for 30 min. Results show the percentage of histamine release (percentage of total histamine) and are expressed as mean $\pm$ SD of triplicates performed in one representative experiment.

human MCs (not shown), confirming previous data obtained with RBL cells (24). As assessed by trypan blue exclusion, exposure to statins did not affect the viability of MCs (or other cell types) during the 24-h incubation period.

Effects of statins on cytokine-dependent differentiation of human MCs

According to previous observations (8–10), SCF plus IL-6 induced the formation of MCs in cb-derived progenitors within 28 days. Addition of cerivastatin (0.1–10 μM) or atorvastatin (0.1–10 μM) resulted in a dose-dependent inhibition of cytokine-induced development of MCs in these cultures (Fig. 3A). A less pronounced effect on MC growth was also found with lovastatin (Fig. 3A). The other statins tested (pravastatin, simvastatin) did not suppress cytokine-dependent differentiation of MCs in these cultures (Fig. 3A). An unexpected observation was that none of the statins tested was found to affect total cell numbers in our long-term cultures (control: $0.35 \pm 0.12 \times 10^6/\text{ml}$; atorvastatin, 1 μM : 0.31 ± 0.08 ; cerivastatin, 1 μM : 0.26 ± 0.11 ; lovastatin, 1 μM : 0.34 ± 0.12 ; pravastatin, 0.36 ± 0.22 ; simvastatin, 1 μM : 0.36 ± 0.2 ; $n = 4$; $P > 0.05$), contrasting the dramatic effect of atorvastatin and cerivastatin on MC growth. To explore the nature of this phenomenon, we also examined the numbers of macrophages (the second type of cells growing from cb progenitors) in our long-term cultures. In these experiments, it was found that none of the statins decreased the numbers of macrophages. Rather, in case of atorvastatin and cerivastatin (and to a lesser degree lovastatin), an increase in total macrophage numbers was observed (Fig. 3B). These data suggest a statin-induced shift from

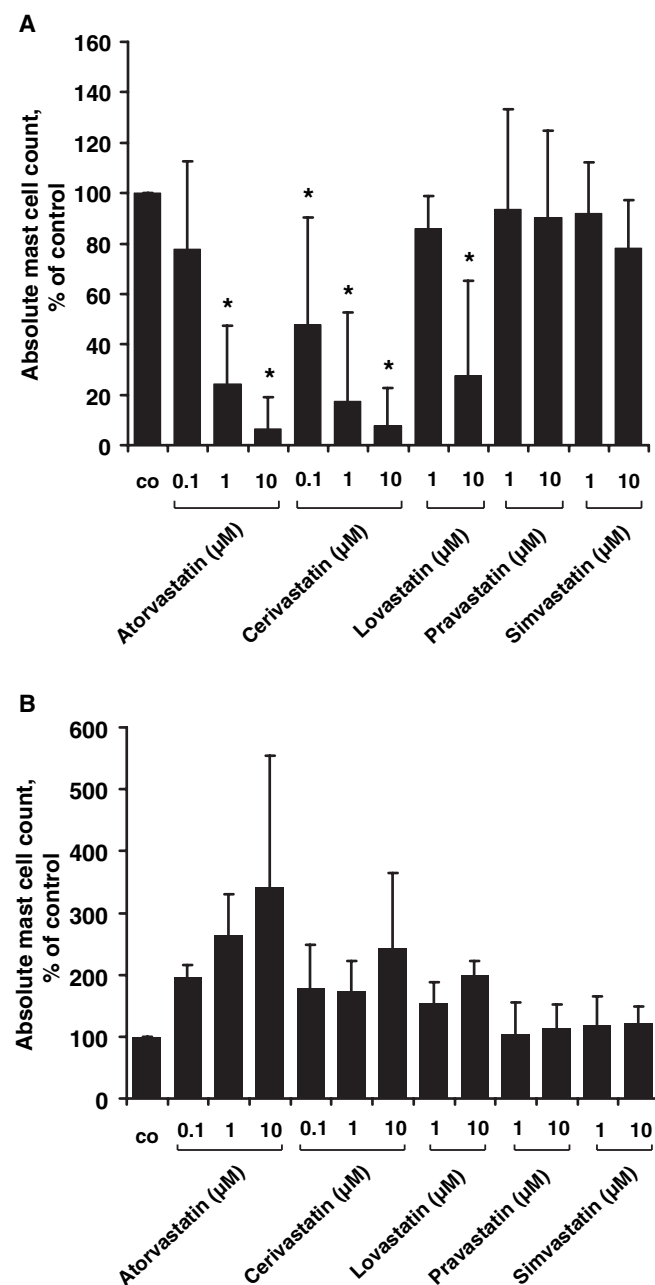


Figure 3. Effects of statins on stem cell factor (SCF)-induced differentiation of human mast cells. Cord blood cells were grown in the presence of SCF and interleukin (IL)-6. Statins were added at various concentrations as indicated. After 28 days, most cultured cells were found to be mast cells or macrophages. The absolute numbers of mast cells (A) and macrophages (B) per well were determined from percentage counts and absolute cell numbers. Results are expressed as percentage of mast cells (A) or macrophages (B) relative to control (co = cell count in SCF and IL-6 without statins) and represent the mean $\pm$ SD of four independent experiments. Significant changes in cell numbers are indicated by an asterisk ($P < 0.05$). Remarkably, the absolute cell numbers in culture (mast cells plus macrophages) did not change in statin-supplemented cultures compared with control.

the MC to the macrophage differentiation compartment in cultured cells.

Effects of statins on cytokine-independent growth of human mast cells

To study statin effects on factor-independent growth of MCs, we performed experiments on HMC-1, a human MC leukemia cell line. In these experiments, cerivastatin, atorvastatin, and lovastatin were found to inhibit spontaneous uptake of ^3H -thymidine into HMC-1 cells at relatively high concentrations (10–100 μM). The effects of these statins were dose-dependent (Fig. 4A–C). The other statins tested did not inhibit proliferation of HMC-1 cells (not shown). The inhibitory effects of the statins on HMC-1 growth could be abolished by addition of MVA, 200 μM (Fig. 4D).

To clarify whether the inhibitory effects of the statins on HMC-1 cell growth are associated with increased apoptosis, DNA analysis was performed. In these experiments, neither cerivastatin nor atorvastatin induced necrosis excluding a toxic effect of these statins on MCs even at a concentration of 100 μM . Both statins also failed to produce apoptosis in HMC-1 cells, confirming results obtained with the basophil cell line KU812 (25). By contrast, exposure of HMC-1 to camptothecin resulted in typical ladder-type fragmentation of DNA suggesting apoptotic cell death (not shown).

Effects of statins on expression of surface antigens on human mast cells

As assessed by flow cytometry, HMC-1 cells were found to express CD2, CD29, CD35, CD51, CD54, CD63, CD87, CD117, CD151, CD162, CD172a, and CD203c. However, neither atorvastatin nor cerivastatin (each 0.1–50 μM) were found to upregulate or downregulate expression of lineage-related or activation-linked CD antigens (CD2, CD29, CD35, CD51, CD54, CD61, CD63, CD87, CD88, CD117, CD123, CD151, CD162, CD172a, CD203c) on HMC-1 cells (not shown). Also, no effects of cerivastatin or atorvastatin on expression of CD63 or CD203c on lung MCs (anti-IgE-induced or resting cells) were seen.

Discussion

Recent data suggest that apart from their lipid-lowering activity, statins exhibit inhibitory effects on growth and function of various hemopoietic cells including those considered to play an important role in inflammatory and allergic reactions (22–25). In the present study, we have examined the effects of five different statins on growth and function of human MCs and their progenitors. The results of our study show that cerivastatin and atorvastatin inhibit anti-IgE-induced release of histamine in

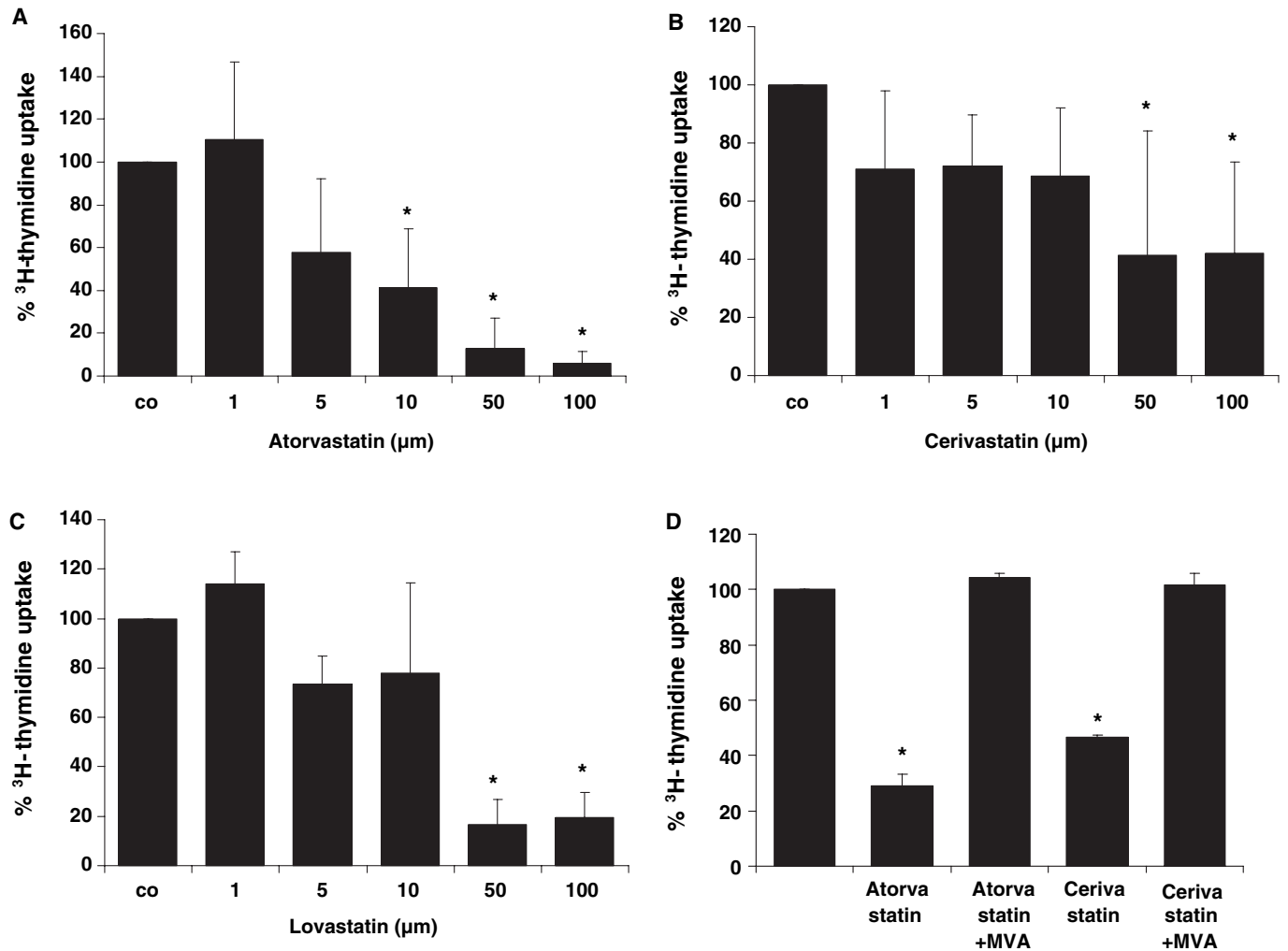


Figure 4. Effects of statins on proliferation of HMC-1 cells. HMC-1 cells were incubated with various concentrations of atorvastatin (A), cerivastatin (B), and lovastatin (C), or control medium (co = 100%) at 37°C for 24 h. Then, ³H-thymidine uptake was determined. Results represent the mean $\pm$ SD of four independent experiments. (D) Effects of mevalonic acid (MVA; 200 μ M) on atorvastatin (50 μ M)-induced and cerivastatin (50 μ M)-induced inhibition of proliferation (³H-thymidine uptake) of HMC-1 cells is shown. Significant changes ($P < 0.05$) are indicated by an asterisk.

mature tissue MCs. Furthermore, these statins were found to suppress cytokine-dependent growth of normal MC progenitors as well as factor-independent growth of leukemic progenitors, i.e. HMC-1 cells. Together, our data identify cerivastatin and atorvastatin as inhibitors of growth and activation of human MCs.

A number of different cell types have been described as targets of statin-induced inhibition of growth and/or cell activation. Likewise, it has been described already that lovastatin can inhibit mediator release in rat MCs (24, 32, 33). It has also been described that cerivastatin and atorvastatin can suppress the growth and IgE-dependent release of histamine in human basophils (25). In the present study, we provide evidence that both statins can also downregulate growth and function of human MCs. However, significant effects of these statins on growth of HMC-1 cells were only observed at high but not low drug

concentrations, confirming earlier studies (34), which contrasts statin-induced inhibition of growth of normal MC progenitors that can be achieved with lower statin concentrations. This discrepancy is best explained by the neoplastic nature of HMC-1 cells, which may be associated with a decreased potential to respond to statins compared with normal MCs (progenitors). From these observations one can also predict that the growth of neoplastic human MCs cannot be controlled by statins at pharmacologic concentrations.

With regard to the growth inhibitory effects of atorvastatin and cerivastatin on (normal) MC progenitors, an important question is whether these effects are restricted to MCs, or can also be demonstrated for other myeloid cells. To address this question, we also examined total cell numbers and macrophage numbers in our long-term culture system. Unexpectedly, none of the statins

tested was found to modulate total cell numbers or to suppress growth of macrophages in these cultures. Rather, in the presence of cerivastatin, atorvastatin, or lovastatin, the numbers of macrophages even increased compared with control. These data are best explained by a statin-induced shift of immature myeloid cells from the MC into the macrophage compartment of differentiation. All in all, the growth inhibitory effects of atorvastatin and cerivastatin on MCs are not associated with a general myelosuppressive effect. On the contrary, growth-suppressive effects of atorvastatin and cerivastatin are not restricted to MCs, but have also been described for human basophils (25).

An interesting aspect of the current study was that lovastatin did not inhibit anti-IgE-induced mediator release in human MCs as has been described for rat MCs (24). This discrepancy may have several explanations. First, this difference may be due to species-specific responses of MCs to certain statins. A second explanation would be the different route of application. Thus, whereas in rats, lovastatin was applied *in vivo*, the current data were obtained from (*in vitro*) experiments on isolated lung and skin MCs. The possibility that the lovastatin-preparation was inactive could be excluded: thus, lovastatin produced an inhibitory effect on ³H-thymidine uptake in HMC-1 cells and decreased the SCF-induced development of MCs from their cb-derived progenitors.

Statins represent a group of HMG CoA reductase inhibitors with similar biochemical properties and comparable effects on MVA-dependent cell functions (35). However, the statins differ in their structure and exhibit different relative potencies and tissue-specific activities (17, 18, 35). In the present study, the applied statins differed significantly in their inhibitory activity on MC growth and activation. Thus, only cerivastatin and atorvastatin were found to inhibit MC differentiation and histamine release. An additional effect was seen with lovastatin when the growth of MC progenitor cells was tested. The reason for the differential effects of the statins on MC function and growth, are not known. One explanation could be that the statins exhibit a different rate of uptake into MCs and their progenitors. Another hypothesis would be that the various statins exhibit a different distribution in the (sub)cellular compartments. Likewise, it has been shown that cerivastatin and atorvastatin have a higher capacity to spread through lipid membranes compared with the other statins (17, 18, 35). A differential effect of the statins applied on signal transduction cascades relevant to growth, survival, or cell activation, seems unlikely, however. Thus, all the statins tested are known to cause membrane lipid depletion and inhibition of various signaling molecules without major differences observed among statin species (17, 18).

Statins are inhibitors of HMG CoA reductase, the rate-limiting enzyme in the MVA pathway (16–20). In the present study, the effects of cerivastatin and atorvastatin on MCs could be reversed by MVA confirming the specificity of the drug effects observed and the role of the MVA pathway. The notion that MVA counteracted the observed statin effects on HMC-1 cells suggest that factor-independent growth of MCs also involves an isoprenoid-dependent pathway(s) of signaling. However, the isoprenoid-dependent pathways include a number of different cascades and effector molecules such as G proteins or members of the Ras family (18–20). Studies are under way to define MVA-dependent signaling pathways and substrates involved in growth of neoplastic MCs.

A number of recent studies have shown that IgE receptors colocalize with lipid rafts and that 'the lipid raft-environment' facilitates IgE-dependent activation of MCs (36–38). It has also been described that statins can interfere with receptor-dependent signaling by disrupting lipid rafts (39). Whether atorvastatin and cerivastatin affect anti-IgE induced release of histamine in human MCs through targeting of lipid rafts (their components) remains at present unknown. In this regard it is also noteworthy that statins are involved in the regulation of expression of cell membrane molecules in various cells and that the cholesterol content of lipid rafts may be associated with MC activation (40).

Recent data suggest that cerivastatin and atorvastatin inhibit expression of CD203c on human basophils (25). As MCs also express CD203c, we were interested to know whether these statins modulate expression of CD203c or other differentiation- or activation-linked cell surface antigens on human MCs. However, no effects of the statins tested on surface antigen expression on (resting or activated) primary lung MCs or HMC-1 cells were seen. Thus, the mechanisms underlying expression of CD203c on human basophils may differ from those contributing to CD203c expression on MCs.

In conclusion, our data show that atorvastatin and cerivastatin are potent inhibitors of *in vitro* growth and activation of human MCs. Whether these observation have biologic or clinical implications remains at present unknown.

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