

Cerivastatin and atorvastatin inhibit IL-3-dependent differentiation and IgE-mediated histamine release in human basophils and downmodulate expression of the basophil-activation antigen CD203c/E-NPP3

Yasamin Majlesi,* Puchit Samorapoompichit,[†] Alexander W. Hauswirth,* Gerit-Holger Scherthaner,*[‡] Minoo Ghannadan,* Mehrdad Baghestanian,[‡] Abdolreza Rezaie-Majd,[‡] Rudolf Valenta,[§] Wolfgang R. Sperr,* Hans-Jörg Bühring,[¶] and Peter Valent*

Departments of *Internal Medicine I, Division of Hematology & Hemostaseology, [‡]Internal Medicine II, Division of Angiology, and [§]Pathophysiology, and [†]Institute of Histology & Embryology, University of Vienna, Austria; and [¶]Department of Internal Medicine II, University of Tübingen, Germany

Abstract: Recent data suggest that the statins, apart from their lipid-lowering activity, exhibit profound anti-inflammatory effects. Basophils are major proinflammatory effector cells in diverse pathologic reactions. We have examined the in vitro effects of five different statins on primary human basophils, their progenitors, and the basophil cell line KU-812. Preincubation of blood basophils with cerivastatin or atorvastatin (0.1–100 μ M) for 24 h reduced their capacity to release histamine on immunoglobulin E (IgE)-dependent stimulation in a dose-dependent manner. These statins also inhibited IgE-dependent up-regulation of the basophil-activation antigen CD203c. Moreover, both statins suppressed interleukin-3-induced differentiation of basophils from their progenitors as well as ³H-thymidine uptake in KU-812 cells. All inhibitory effects of cerivastatin and atorvastatin were reversed by mevalonic acid (200 μ M). The other statins tested (lovastatin, simvastatin, pravastatin) did not show significant inhibitory effects on basophils. Together, these data identify cerivastatin and atorvastatin as novel inhibitors of growth and activation of human basophils. *J. Leukoc. Biol.* 73: 107–117; 2003.

Key Words: IgE receptor · allergy · CD203c · progenitor cells

INTRODUCTION

Basophils are hemopoietic effector cells involved in allergic and other inflammatory reactions [1–4]. These cells produce a number of biologically active mediator substances and express high-affinity immunoglobulin E (IgE) binding sites [1–6]. In response to IgE-receptor cross-linking or other stimuli, basophils release their mediators into the extracellular space [4–8]. Thus, exposure of basophils to a specific allergen results in mediator secretion in allergic patients. In

common with other hemopoietic cells, basophils originate from uncommitted hemopoietic progenitors [9–12]. The most potent differentiation factor for human basophils appears to be interleukin-3 (IL-3) [10, 11]. In fact, when normal bone marrow (BM)-derived progenitor cells are cultured in the presence of IL-3 for 14 days, a substantial number of cultured cells are basophils [11]. Other cytokines, such as granulocyte macrophage-colony stimulating factor (GM-CSF) or IL-5, can also influence basophil differentiation [10–12]. In addition, these cytokines (IL-3, IL-5, GM-CSF) are known to up-regulate the releasability in mature basophils [13–18]. The effects of IL-3, GM-CSF, and IL-5 on basophils are exerted through high-affinity cell-surface receptors (R). Particularly, human basophils express significant amounts of IL-3R α (CD123), the common β chain of IL-3R/GM-CSFR/IL-5R, and also the α chains of the GM-CSFR and IL-5R [18–21].

A number of inflammatory and neoplastic disorders are associated with an increased production or activation of basophils. In chronic myeloid leukemia (CML), basophilia is a common finding [22, 23]. In CML patients, basophils express the *bcr-abl* fusion gene, indicating that they belong to the neoplastic clone [24]. A significant overproduction of basophils is typically found during disease progression (accelerated phase) of CML [23]. Apart from CML, basophilia is also commonly seen in other myeloproliferative disorders and sometimes also in myelodysplastic syndromes [25, 26]. In addition, increased numbers of (polyclonal) basophils can be found in allergic reactions, late-phase reactions, and chronic inflammation [27–30]. In allergic reactions, basophil activation may represent a serious clinical problem.

Correspondence: Peter Valent, M.D., Department of Internal Medicine I, Division of Hematology & Hemostaseology, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria. E-mail: peter.valent@akh-wien.ac.at

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Statins are potent inhibitors of hydroxymethylglutaryl CoA (HMG CoA) reductase [31–35], 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 [31]. A well-established concept is that the MVA pathway is involved in the post-translational isoprenylation of important cellular proteins, including growth regulators and oncogene products such as RAS [36–38]. Clinically, statins were first described and introduced as cholesterol-lowering agents [32, 39]. However, statins exert multiple effects on different cells and cell lines of various origin [40–50]. Recently, the statins were found to modulate growth and the functional properties of hemopoietic (effector) cells, including monocytic cells and murine mast cells [46–50]. In the present study, the effects of five different statins on growth and mediator release in human basophils were examined.

MATERIALS AND METHODS

Monoclonal antibodies (mAb) and other reagents

The mAb E124.2.8 (anti-IgE), B1.49.9 (CD25), CLB-gran12 (CD63), and fluorescein isothiocyanate (FITC)-labeled CLB-gran12 were purchased from Immunotech (Marseille, France); YB5.B8 (CD117) was from Becton Dickinson (San Jose, CA); and K20 (CD29) was from Dako (Glostrup, Denmark). The mAb PL1 (CD162) was obtained from the VIth International Workshop on Human Leukocyte Differentiation Antigens (Kobe, Japan). The mAb 97A6 (CD203c) [51] was produced at the University of Tübingen (Germany). PE-labeled 97A6 mAb was kindly provided by Dr. André van Agthoven (Immunotech). A specification of mAb is shown in **Table 1**. Recombinant human (rh)IL-3 was purchased from Strathmann Biotech (Hannover, Germany); RPMI-1640 medium, gentamycin, penicillin, streptomycin, amphotericin B, and fetal calf serum (FCS) were from PAA Laboratories (Linz, Austria); L-glutamine was from Gibco-Life Technologies (Gaithersburg, MD), and ethanol was from Merck (Darmstadt, Germany). Calcium ionophore A23187, MVA, geranylgeraniol, and squalene were purchased from Sigma Chemical Co. (St. Louis, MO), and trans,trans-Farnesol was from Fluka (Buchs, Switzerland).

Preparation of statins

Cerivastatin was provided by Bayer (Wuppertal, Germany), atorvastatin by Pfizer (Karlsruhe, Germany), simvastatin and lovastatin by Merck and Co. (Linden, NJ), and pravastatin by Bristol-Myers Squibb (Munich, Germany). Atorvastatin was dissolved in 100% ethanol at 55°C and cerivastatin in 7% ethanol at room temperature. Simvastatin, lovastatin, and MVA were provided in lactone form. These reagents were prepared by dissolving in ethanol and 0.1 N NaOH. The resulting solutions were heated (50°C, 2 h) and then adjusted to

pH 7.2 by adding 0.1 M HCl. Stock solutions of statins were prepared by dissolving in distilled water and stored at –70°C until used. Pravastatin was dissolved in distilled water. In control experiments, water-soluble pravastatin was also dissolved in ethanol. Final work concentrations of statins were prepared by dissolving in RPMI-1640 medium supplemented with 10% FCS.

Histamine release experiments

Blood cells were obtained from 16 normal donors, three patients allergic to Phl p 5, and one patient suffering from CML in accelerated phase (with marked basophilia). Basophils were enriched by Ficoll density centrifugation. In normal/allergic donors, enriched cells contained 1–6% basophils (remaining cells were lymphocytes and monocytes as well as some neutrophils). Enriched CML basophils showed a purity of 65% after short-term culture (remaining cells being blasts and basophilic progenitors as well as some promyelocytes and lymphocytes). Histamine release was performed essentially as described [18]. In typical experiments, cells (3×10^6 /ml) were incubated with various concentrations (0.1–100 μ M) of statins in RPMI-1640 medium containing 10% FCS and 1 U/ml rhIL-3 at 37°C (5% CO₂) for 24 h. In selected experiments, basophils were incubated with statins in the presence or absence of MVA (200 μ M), farnesol (10 μ M), geranylgeraniol (10 μ M), or squalene (10 μ M) for 24 h before exposed to agonists. In control experiments, ethanol (same concentrations as in statin solutions) was applied as solvent control before anti-IgE incubation. In all experiments, the viability of basophils after exposure to statins or other compounds was 90%. After statin pretreatment, cells were centrifuged, resuspended in histamine release buffer (HRB; Immunotech), and exposed to various concentrations (0.03–3 μ g/ml) of anti-IgE (E124.2.8 diluted in HRB) in 96-well microtiter plates (TPP, Trasadingen, Switzerland) for 30 min (37°C). In allergic donors, cells were also exposed to rPhl p 5 (0.1 and 0.01 μ g/ml) for 30 min [52]. In time-course experiments, normal basophils were preincubated with statins for various times (0.5, 1, 4, 8, 16, 24 h) before exposed to anti-IgE. In selected experiments, statin-pretreated basophils (24 h) were exposed to Ca-ionophore A23187 (1 μ g/ml) for 30 min (37°C). After incubation with agonists, cells were centrifuged at 4°C for 10 min. Then, cell-free supernatants were recovered, and histamine was measured by a commercial radioimmunoassay (RIA; Immunotech). Histamine release was expressed as percentage of total (extracellular+cellular) histamine measured in lysates of cells that were prepared by freeze-thawing.

Basophil differentiation assay

To study the effects of statins on cytokine-dependent basophil differentiation from normal progenitor cells, BM culture experiments were performed as described using rhIL-3 as a growth factor [11]. BM cells were obtained from four patients with normal BM (staging of lymphoma). Informed consent was obtained in each case. BM mononuclear cells (MNC) were isolated using Ficoll. The MNC (0.5×10^6 per ml) were cultured in 24-well culture plates (TPP; 1 ml per well) in RPMI-1640 medium containing 10% FCS, rhIL-3 (100 U/ml), and various concentrations of statins (0.01–10 μ M) or control medium at 37°C/5% CO₂. After 14 days, cell cultures were harvested and examined for the total number of cells, presence of basophils (Wright-Giemsa staining), and cell viability (trypan blue exclusion test). The absolute numbers of basophils and other cell types were calculated from total cell numbers and differential counts. All culture experiments were performed in triplicates.

Proliferation assay

To determine the effects of the statins on proliferation of leukemic (CML-derived, factor-independent) basophils, we performed ³H-thymidine incorporation experiments using the bcr-abl-positive, CML-derived basophil cell line KU-812 [53]. KU-812 cells were kindly provided by Kenji Kishi (Niigata University, Japan) and kept in RPMI-1640 medium with 10% FCS. In proliferation experiments, KU-812 cells (4×10^4 cells per well) were placed in 96-well microtiter plates (Costar, Cambridge, MA) and incubated with various concentrations of statins (0.1–100 μ M) at 37°C/5% CO₂ for 24 h. Then, 1 μ Ci ³H-thymidine (Amersham, Buckinghamshire, UK) was added to each well and kept for 6 h (37°C). Cells were then harvested on filter membranes (Packard Bioscience, Meriden, CT) in a Filtermate 196 harvester (Packard Bioscience). Filters were air-dried, and the bound radioactivity was counted in a β -counter (Top-Count NXT, Packard Bioscience). All experiments were performed in triplicates.

TABLE 1. Specification of mAb

CD	Reactive antigen	Clone	Ig class	Source
25	IL-2R α	B1.49.9	G2a	m
29	Integrin β 1	K20	G2a	m
63	LIMP-1	CLB-gran12	G1	m
117	SCFR	YB5.B8	G1	m
162	PSGL-1	PL1	G1	m
203c	E-NPP3	97A6	G1	m

CD, Cluster of differentiation; LIMP-1, lysosomal integral membrane protein-1; SCFR, stem-cell factor receptor; PSGL-1, P-selectin glycoprotein ligand-1; E-NPP3, ectonucleotide pyrophosphatase/phosphodiesterase 3; m, mouse.

Evaluation of apoptosis by DNA fragmentation assay

To study the mechanism of statin-induced inhibition of cell growth, DNA analysis was performed on KU-812 cells essentially as described [54]. In brief, KU-812 cells (2×10^6) were cultured in 100 mm petri-culture dishes (Costar) and exposed to statins (each 1–100 μM) for 24 h (37°C , 5% CO_2). As a positive control, cells were exposed to camptothecin (3 μM), known to induce apoptosis in KU-812 cells. After 24 h, cells were harvested and lysed in “lysing solution” containing 5 mM Tris, 1% sodium dodecyl sulfate, and 5 mM EDTA (pH 7.4). Lysates were incubated in RNase A (50 $\mu\text{g}/\text{mL}$; Boehringer Mannheim, Germany) for 2 h and then in proteinase K for 16 h (37°C). Thereafter, DNA was extracted by incubation in phenol/chloroform/isoamylalcohol and then, in chloroform alone. DNA was precipitated in ethanol with sodium acetate (-20°C , overnight). Precipitates were centrifuged and then dissolved in Tris-EDTA buffer (=10 mM Tris-HCl plus 1 mM EDTA, pH 7.4). Gel electrophoresis was performed on 2% agarose dissolved in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide. Samples were prepared as 20- μL solutions containing 10 μg DNA and 10% loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose). Gels were examined by an UV transilluminator. Apoptosis was defined as typical ladder-type fragmentation of DNA.

Electron microscopy

To further evaluate the mechanism of statin effects on KU-812 cells (increased apoptosis, necrosis, or reduced mitosis), electron microscopy was performed as reported [54]. In brief, KU-812 (10^7) was exposed to statins (50 mM), camptothecin (3 μM), or control medium for 16 or 24 h. After incubation, cells were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl_2 in 0.1 M cacodylate buffer (pH 7.4, 60 min). Then, cells were washed in cacodylate buffer, resuspended in 2% agar, and centrifuged. Agar pellets were postfixed in 1.3% OsO_4 , buffered in 0.66 M collidine, and stained en bloc with 2% uranyl acetate in sodium maleate buffer (pH 4.4) for 2 h. Pellets were then rinsed, dehydrated in alcohol, and embedded in Eponate 812 (Serva, Heidelberg, Germany). Ultrathin sections were prepared and contrasted in 1% uranyl acetate and lead citrate. Sections were viewed under a JEOL 1200 EX II transmission electron microscope (Tokyo, Japan). In each sample, a total number of 100 cells were examined for typical ultrastructural signs of mitosis, apoptosis, and necrosis.

Surface marker studies

To evaluate the effects of cerivastatin and atorvastatin on expression of cell-surface antigens, KU-812 cells and primary basophils obtained from patients with CML ($n=3$) were examined. KU-812 cells were preincubated with statins (50 μM) for 24 h at 37°C and then stained with mAb against CD25, CD29, CD63, CD117, CD162, and CD203c. Cells were also incubated with statins in the presence or absence of MVA (200 μM). Expression of CD antigens on KU-812 cells was quantified by single color-flow cytometry on a FACScan (Becton Dickinson). In case of CD203c, the dose- and time-dependent effects of cerivastatin and atorvastatin were measured: In time-course experiments, KU-812 cells were incubated with statins (50 μM) for various time periods (1, 4, 8, 16, and 24 h). Dose-dependencies were determined by applying statins at 0.1–50 μM . To study the effects of the statins on IgE-dependent up-regulation of CD203c and CD63, primary CML-derived basophils were examined. CML basophils were incubated with atorvastatin or cerivastatin (each 50 μM) for 24 h (37°C , 5% CO_2). Then, cells were exposed to anti-IgE mAb E.124.2.8 (1 $\mu\text{g}/\text{mL}$) or control buffer for 15 min at 37°C . After incubation, cells were washed and analyzed for expression of CD63 and CD203c by two-color flow cytometry using labeled mAb (CD63-FITC; CD203c-PE) and a FACScan. Staining reactions were controlled using isotype-matched antibodies.

Statistical evaluation

To determine the level of significance in our results, appropriate statistical tests, including ANOVA and the paired Student's *t*-test, were applied. For multiple comparisons, *P* values were adjusted according to Bonferroni. Results were considered significantly different when *P* was <0.05 .

RESULTS

Cerivastatin and atorvastatin inhibit IgE-mediated histamine release in human basophils

Preincubation of normal blood basophils with cerivastatin or atorvastatin (each 5 μM) for 24 h inhibited histamine release provoked by anti-IgE [anti-IgE (1 $\mu\text{g}/\text{mL}$), $31.8 \pm 13.9\%$ histamine release; vs. anti-IgE (1 $\mu\text{g}/\text{mL}$)+cerivastatin (5 μM),

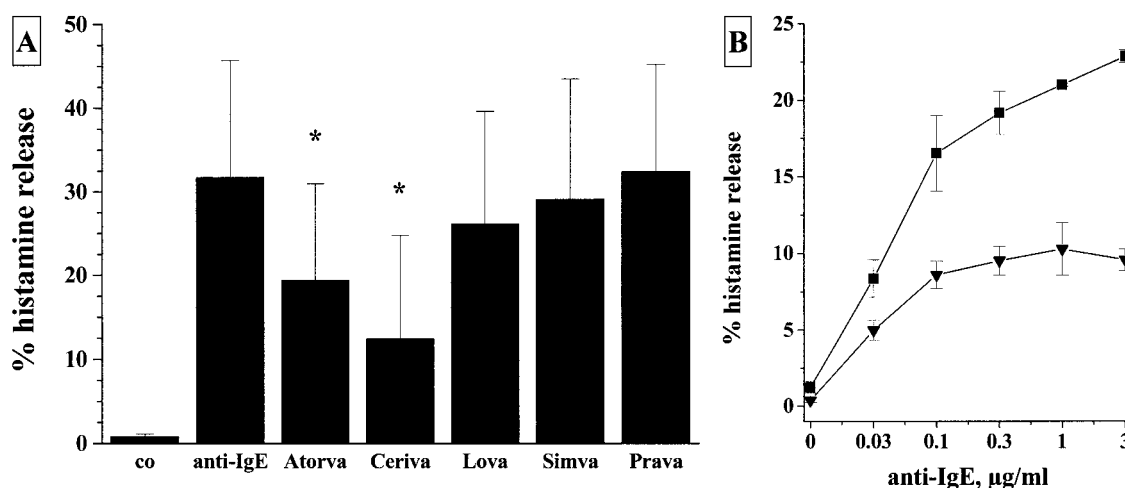


Fig. 1. Effects of various statins on anti-IgE-induced histamine release. (A) Basophils of 11 healthy donors were incubated with statins [atorvastatin (Atorva), cerivastatin (Ceriva), lovastatin (Lova), simvastatin (Simva), pravastatin (Prava); each 5 μM] or control medium together with rIL-3 (1 U/ml) at 37°C for 24 h. Then, cells were exposed to anti-IgE (1 $\mu\text{g}/\text{mL}$) for 30 min. Cells were then centrifuged, and cell-free supernatants were analyzed for histamine content. Histamine release is expressed as percentage of total histamine. Values represent the mean $\pm$ SD ($n=11$). co, Spontaneous histamine release; *, significant inhibition. (B) The effects of atorvastatin on basophil histamine release induced by various concentrations of anti-IgE. Anti-IgE (■) induced a dose-dependent histamine release from basophils preincubated in control medium. Preincubation of basophils with atorvastatin (50 μM) for 24 h (▼) resulted in a substantial inhibition of IgE-dependent histamine secretion. Results from one donor are shown. Values represent the mean $\pm$ SD of triplicates.

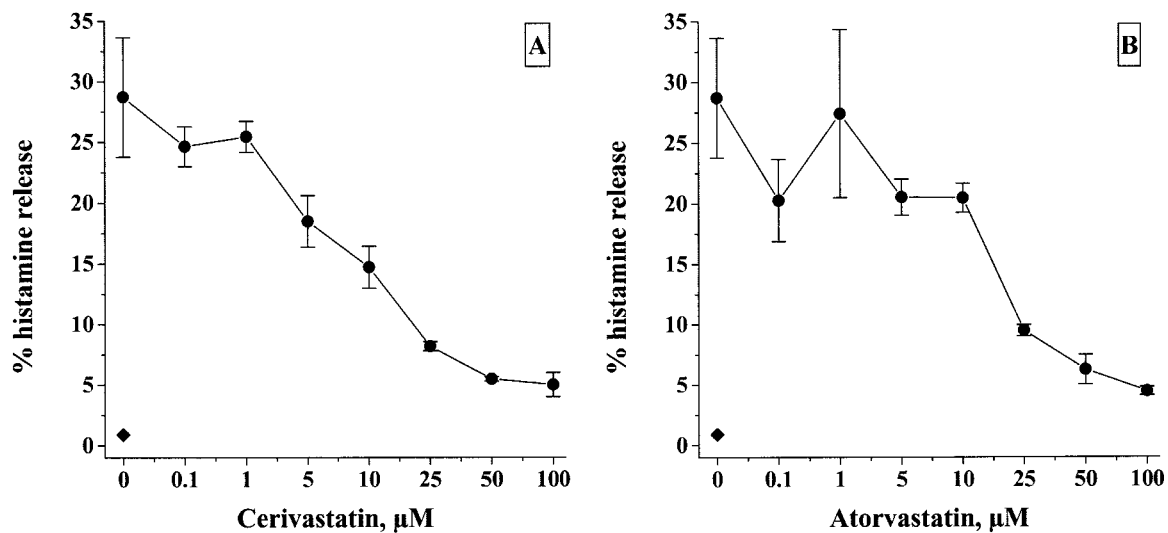


Fig. 2. Dose-dependent effects of cerivastatin and atorvastatin on IgE-dependent histamine release in human basophils. Normal blood basophils were incubated with various concentrations (as indicated) of cerivastatin (A) or atorvastatin (B) at 37°C for 24 h. Thereafter, cells were exposed to anti-IgE antibody (1 $\mu\text{g}/\text{ml}$) for 30 min and then centrifuged. Histamine was measured in cell-free supernatants by RIA. Histamine release is expressed as percentage of total histamine. Results represent the means $\pm$ SD of triplicates.

12.7 $\pm$ 12.3%; vs. anti-IgE (1 $\mu\text{g}/\text{ml}$)+atorvastatin (5 μM), 19.5 $\pm$ 11.5%; $P<0.05$; $n=11$; **Fig. 1A**]. Figure 1B shows the inhibitory effects of atorvastatin on histamine release induced by different concentrations of anti-IgE. The effects of cerivastatin and atorvastatin on anti-IgE-induced histamine release were dose-dependent with IC_{50} values ranging between 5 and 25 μM for cerivastatin and between 25 and 50 μM for atorvastatin (**Fig. 2**). The effects of these statins were also time-dependent with significant inhibition occurring after 8 h (**Fig. 3**). The other statins applied (simvastatin, lovastatin, pravastatin) showed no significant effects on anti-IgE-induced histamine secretion (Fig. 1A). The inhibitory effects of cerivastatin and atorvastatin on anti-IgE-induced histamine release in basophils could be reversed by

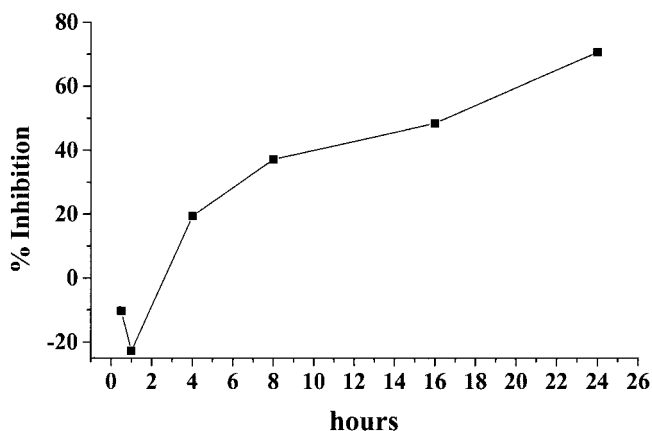


Fig. 3. Time-dependent effect of cerivastatin on anti-IgE-induced histamine release from blood basophils. Basophils from a healthy donor were preincubated with cerivastatin (10 μM) for various time periods as indicated and then exposed to anti-IgE antibody (1 $\mu\text{g}/\text{ml}$) for 30 min (37°C). Cells were then centrifuged, and the cell-free supernatants were recovered and examined for histamine content by RIA. Results represent the mean $\pm$ SD of triplicate determinations. As visible, exposure to cerivastatin was followed by a time-dependent decrease in basophil releasability (increase in inhibition).

addition of MVA. These statin effects were also reversed by addition of farnesol and geranyl-geraniol but not by squalene. **Figure 4** shows the effects of MVA (200 μM), farnesol (10 μM), geranylgeraniol (10 μM), and squalene (10 μM) on the inhibitory action of cerivastatin on anti-IgE-induced histamine release in basophils. Neither MVA (Fig. 4) nor the other compounds tested (farnesol, geranylgeraniol, squalene) were found to modulate anti-IgE-induced histamine release per se. Also, ethanol per se did not inhibit anti-IgE-induced histamine release at the concentrations applied (the same as used in experiments with ethanol-dissolved statins). In contrast to anti-IgE-induced histamine release, the statins did not suppress Ca-ionophore-induced release of histamine from basophils ($P>0.05$; $n=3$). Basophils from three Phl p 5-allergic donors were also examined. As in normal basophils, atorvastatin and cerivastatin inhibited histamine release provoked by anti-IgE. In addition, these statins inhibited the Phl p 5-induced release of histamine in sensitized basophils (not shown). To show that atorvastatin and cerivastatin inhibited histamine release through direct interaction with basophils but not through modulation of accessory cells (apart from basophils, the MNC in normal donors contained lymphocytes, monocytes, and some neutrophilic and eosinophilic granulocytes), CML basophils were enriched and examined. These CML basophils were 65% pure, the remaining cells being mostly blast cells and basophilic progenitors. Again, both statins showed substantial inhibitory effects on these CML basophils (**Fig. 5**).

Cerivastatin and atorvastatin inhibit IL-3-dependent differentiation of basophils

IL-3 induced the differentiation of basophils in day 14 BM suspension culture and also promoted the formation of eosinophils as described previously [11]. The addition of cerivastatin (0.01–10 μM) or atorvastatin (0.01–10 μM) to these cultures resulted in a dose-dependent inhibition of basophil formation (**Fig. 6A**). In addition, these statins inhibited the

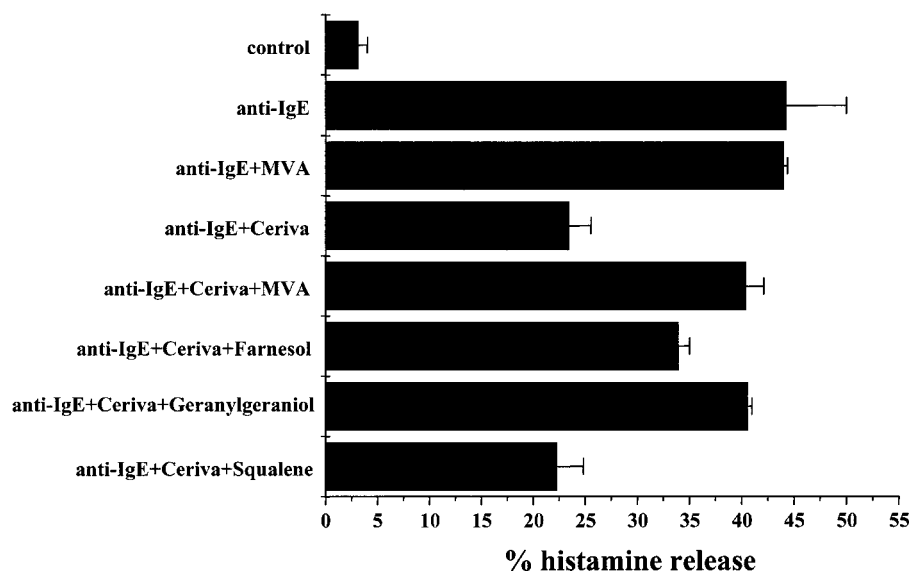


Fig. 4. Effect of MVA and other compounds on inhibitory effects of statins on IgE-dependent histamine release from basophils. Basophils were preincubated with cerivastatin (10 μ M) in the presence or absence of MVA (200 μ M), farnesol (10 μ M), geranylgeraniol (10 μ M), squalene (10 μ M), or control buffer for 24 h. After preincubation, basophils were exposed to anti-IgE (1 μ g/ml) for 30 min at 37°C. Then, cells were centrifuged, and the supernatants were recovered and analyzed for the presence of histamine by RIA. Histamine release is expressed as percentage of total histamine. As visible, preincubation of basophils with cerivastatin (anti-IgE+Ceriva) resulted in an inhibition of anti-IgE-induced histamine release, whereas no effect was seen with MVA (anti-IgE+MVA). However, MVA, farnesol, and geranylgeraniol were found to be capable of counteracting the effect of cerivastatin on anti-IgE-induced histamine release. By contrast, no such counteracting effect was seen with squalene. Results represent the means $\pm$ SD of triplicate determinations.

IL-3-induced formation of eosinophils (**Table 2**). The other statins tested did not suppress IL-3-dependent differentiation of human basophils or eosinophils in BM cultures ($P>0.05$). To further document the effects of cerivastatin and atorvastatin on basophil differentiation, we also measured cellular histamine levels as a nonsubjective basophil parameter in our BM cultures. As expected, it was found that the IL-3-induced production of histamine in these cultures was inhibited by both statins (**Fig. 6B**). The inhibitory effects of cerivastatin and atorvastatin on basophil growth could be neutralized by addition of MVA (200 μ M; **Table 2** and **Fig. 6**). All in all, these data show that cerivastatin and atorvastatin inhibit factor-dependent in vitro differentiation of human basophils.

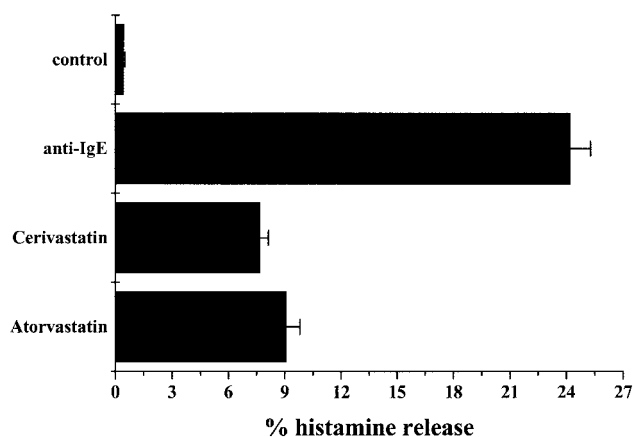


Fig. 5. Inhibitory effects of cerivastatin and atorvastatin on histamine release in enriched basophils. Basophils were enriched from a patient with CML in accelerated phase and marked basophilia. Enriched basophils (65% pure) were cultured in the presence or absence of cerivastatin (10 μ M) or atorvastatin (10 μ M) at 37°C for 24 h. Thereafter, cells were exposed to anti-IgE mAb E124.2.8 (1 μ g/ml) for 30 min. Then, cells were centrifuged, and the cell-free supernatants were recovered and analyzed for histamine content. Histamine release is expressed as percentage of total histamine. Results represent the means $\pm$ SD of triplicate determinations. As visible, anti-IgE produced a substantial release of histamine from CML basophils compared with control buffer (control). Preincubation with cerivastatin or atorvastatin inhibited this anti-IgE-induced histamine release.

Effects of statins on factor-independent growth of leukemic basophils (KU-812)

To study the effects of the statins on factor-independent growth of leukemic basophils, we performed experiments using the CML-derived leukemic basophil cell line KU-812. Cerivastatin and atorvastatin were found to inhibit the spontaneous uptake of ^{3} H-thymidine in KU-812 cells. The effects of both statins were dose-dependent. IC₅₀ values ranged between 1 and 10 μ M for atorvastatin and between 0.1 and 1 μ M for cerivastatin (**Fig. 7**). The other statins tested did not inhibit proliferation of KU-812 cells ($P>0.05$). The inhibitory effects of cerivastatin and atorvastatin could be reversed by addition of MVA (200 μ M; **Fig. 7**). To clarify whether the inhibitory effects of the statins on basophil growth are associated with reduced mitosis, increased apoptosis, or cell necrosis, DNA and electron microscopy analyses were performed. As assessed by electron microscopy, cerivastatin and atorvastatin reduced the rate of mitosis in KU-812 cells [percentage of mitotic cells in control medium, $12.4 \pm 3.0\%$; vs. atorvastatin (50 μ M, 24 h), $2.3 \pm 0.6\%$; vs. cerivastatin (50 μ M, 24 h), $2.9 \pm 0.2\%$; $P<0.05$], whereas the numbers (percentage) of apoptotic and necrotic cells remained nearly unchanged compared with untreated cells (**Fig. 8**). In contrast, exposure of KU-812 cells to camptothecin resulted in an increased percentage of apoptotic cells. Camptothecin also induced typical ladder-type fragmentation of DNA in KU-812 cells, suggesting apoptosis, whereas no ladder-type fragmentation was seen when KU-812 cells were incubated with cerivastatin or atorvastatin (not shown). These results suggest that cerivastatin and atorvastatin inhibited cell growth of KU-812 by inhibiting mitosis but not via induction of apoptosis or a direct toxic effect.

Cerivastatin and atorvastatin downmodulate expression of the basophil activation antigen CD203c/E-NPP3

Basophils express a number of activation-linked surface antigens including CD63 and E-NPP3 (=CD203c) [20, 21, 51]. During IgE-dependent cell activation, the levels of CD63 and

Fig. 6. Effects of statins on IL-3-induced differentiation of human basophils. (A) BM MNC of four donors was incubated with IL-3 (100 U/ml) in the presence or absence of various concentrations of cerivastatin or atorvastatin as indicated. After 14 days, cultures were harvested and analyzed for absolute cell numbers and the percentages of basophils. Basophil numbers were calculated from total cell numbers and percentage counts. Results are given as percent of control (IL-3 alone=100%) and represent the mean $\pm$ SD from four donors. *Significant inhibition ($P<0.05$). As visible, both statins induced significant and dose-dependent inhibition of IL-3-dependent differentiation of basophils in culture. (B) Cellular histamine levels in a BM culture grown in the presence of IL-3 (100 U/ml) with or without various concentrations of atorvastatin for 14 days as indicated. IL-3 induced a significant formation of histamine in these cultures compared with control medium (control). This IL-3-induced production of histamine (which parallels IL-3-dependent basophil differentiation) was inhibited by atorvastatin in a dose-dependent manner. Addition of MVA (+MVA, 200 μ M) to atorvastatin (1 μ M) neutralized the inhibitory effect of this statin.

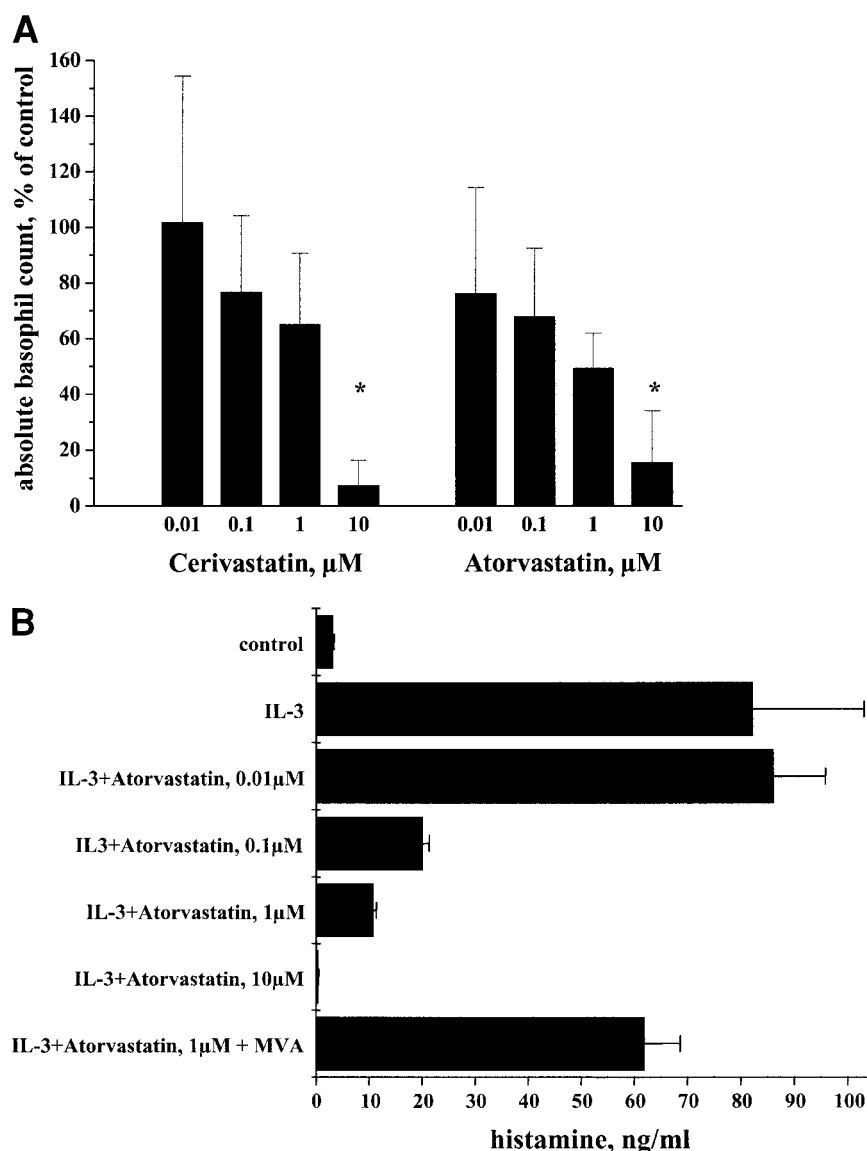


TABLE 2. Formation of Basophils and Other Cell Types in BM Culture

Stimulating agent(s)	Absolute numbers ($\times 10^5/\text{ml}$) of cells recored on day 14								Histamine content (ng/ml suspension)
	Blast	PMC	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes	Total cell counts	
None (control medium)	0.02	0.02	0.31	0.11	0.09	0.24	1.41	2.20	3.14
IL-3, 100 ng/ml	0.28	0.78	0.17	0.78	1.11	1.11	1.33	5.56	82.26
IL-3 + cerivastatin, 0.01	0.00	0.37	0.29	0.29	1.29	0.21	1.71	4.16	66.51
IL-3 + cerivastatin, 0.1	0.04	0.57	0.45	0.45	0.78	0.04	1.76	4.09	84.65
IL-3 + cerivastatin, 1.0	0.00	0.27	0.24	0.20	1.01	0.17	1.48	3.37	90.63
IL-3 + cerivastatin, 10	0.02	0.13	0.11	0.06	0.17	0.09	1.58	2.16	09.41
IL-3 + cerivastatin, 1.0 + MVA	0.05	0.59	0.16	0.59	1.23	0.11	2.29	5.02	74.64
IL-3 + atorvastatin, 0.01	0.10	0.25	0.15	0.25	1.34	0.40	2.48	4.97	86.9
IL-3 + atorvastatin, 0.1	0.09	0.59	0.41	0.59	0.73	0.23	1.92	4.56	20.07
IL-3 + atorvastatin, 1.0	0.10	0.28	0.28	0.08	0.66	0.05	1.09	2.54	11.08
IL-3 + atorvastatin, 10	0.06	0.18	0.12	0.04	0.39	0.12	1.12	2.03	00.42
IL-3 + atorvastatin, 1.0 + MVA	0.11	0.59	0.37	0.64	1.55	0.11	1.97	5.34	61.92

The BM culture was established as described in the text using IL-3 (100 ng/ml) with or without statins as indicated. MVA was applied at a concentration of 200 μ M. On day 14, the culture was harvested and analyzed for differential cell counts. Absolute numbers of cells were calculated from total cell numbers and differential counts. The histamine content was determined by RIA. Results obtained in one donor are shown. Similar data were obtained in three other donors. PMC, Promyelocytes.

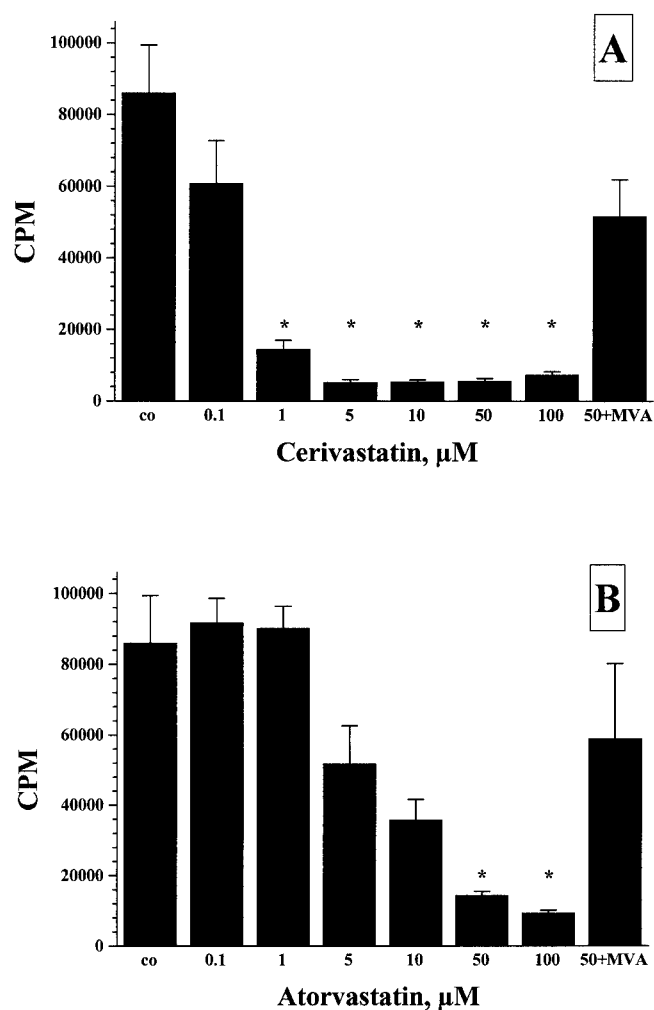


Fig. 7. Effects of cerivastatin and atorvastatin on thymidine uptake in KU-812 cells. KU-812 cells were incubated with various concentrations (0.1–100 μM) of cerivastatin (A) and atorvastatin (B) or control medium (co) for 24 h (37°C, 5% CO_2) in triplicate cultures. Then, cells were exposed to ^3H -thymidine, and the uptake was measured as described in the text. Results represent the mean $\pm$ SD of triplicate determinations. As visible, both statins inhibited the proliferation of KU-812 cells in a dose-dependent way. Addition of MVA (+MVA), 200 μM , reversed the inhibitory effects of cerivastatin and atorvastatin (50 μM). *Significant inhibition ($P < 0.05$). CPM, Counts per minute.

CD203c increase [20, 51]. In the present study, we asked whether the statins can interfere with constitutive and/or anti-IgE-induced expression of basophil activation antigens. In a first step, KU-812 cells were analyzed. Exposure of KU-812 cells to cerivastatin (0.1–50 μM) or atorvastatin (0.1–50 μM) for 24 h resulted in a dose-dependent inhibition of expression of CD203c (**Fig. 9**). The effects of cerivastatin and atorvastatin on CD203c expression on KU-812 cells were also time-dependent with maximum inhibition occurring after 8 h. The inhibitory effects of the statins were neutralized by addition of MVA (not shown). Apart from CD203c, cerivastatin and atorvastatin were also found to modulate expression of CD63 and CD117 in KU-812 cells; however, in contrast to CD203c, CD63 and CD117 were up-regulated by the statins (**Table 3**). No effects of the statins on expression of CD25, CD29, and CD162 on KU-812 were found (**Table 3**). We also examined the effects of

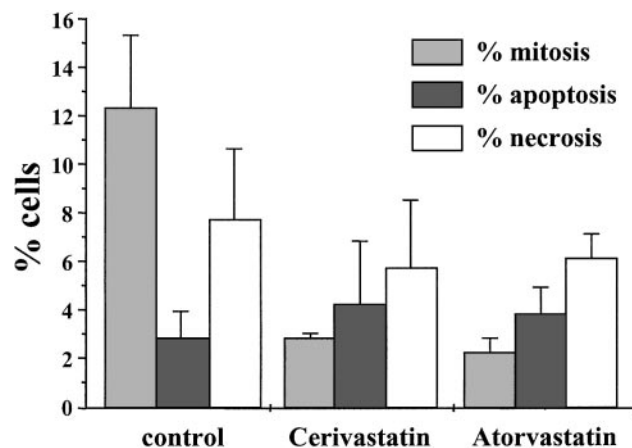


Fig. 8. Electron microscopic examination of KU-812 cells. KU-812 cells were exposed to control medium (control), cerivastatin (50 μM), or atorvastatin (50 μM) for 24 h at 37°C. Then, cells were subjected to electron microscopy. The number and percentages (%) of mitotic, apoptotic, and necrotic cells were determined. As visible, exposure to cerivastatin or atorvastatin was followed by a significant decrease in the number (%) of mitotic cells, whereas no significant changes in the numbers of apoptotic or necrotic cells were found ($P > 0.05$). Data represent the mean $\pm$ SD of three independent experiments.

the statins on IgE-dependent up-regulation of CD203c and CD63 on basophils. For this purpose, primary CML basophils were examined. In line with previous data [51], incubation of basophils with anti-IgE (1 $\mu\text{g}/\text{ml}$) resulted in an increase of expression of CD203c and CD63. Preincubation of basophils with cerivastatin or atorvastatin (each 50 μM) for 24 h decreased their capacity to up-regulate CD203c in response to IgE receptor cross-linking (**Fig. 10**), whereas no significant

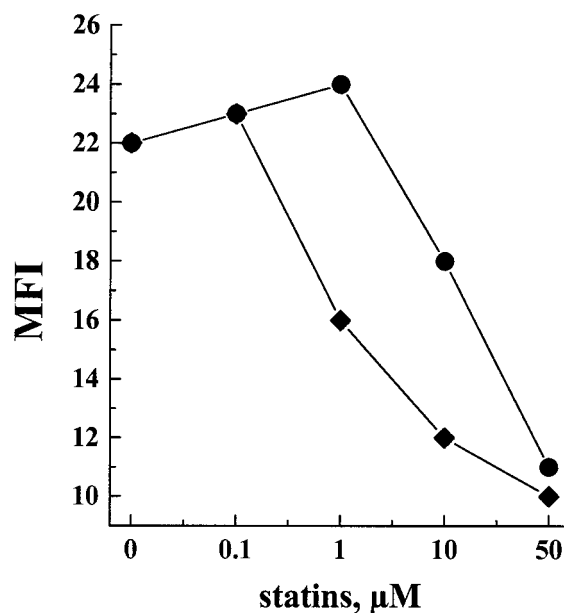


Fig. 9. Dose-dependent effects of statins on expression of CD203c on KU-812 cells. KU-812 cells were incubated with various concentrations of cerivastatin (◆) or atorvastatin (●). After incubation (24 h, 37°C), cells were stained with 97A6 antibody (CD203c) and analyzed by flow cytometry. As visible, both statins induced dose-dependent inhibition of expression of CD203c. Data represent mean fluorescence intensities (MFI).

TABLE 3. Expression of Surface Markers on KU-812 Cells—Effects of Statins

	MFI expressed in % of control		
	Control medium	Cerivastatin	Atorvastatin
PBS control	100	88.68 ± 9.84	88.67 ± 9.27
FITC control	100	90.74 ± 18.27	88.05 ± 15.19
CD25	100	93.59 ± 20.50	88.78 ± 13.90
CD29	100	95.19 ± 15.71	95.10 ± 17.58
CD63	100	157.22 ± 53.95	186.37 ± 93.10
CD117	100	182.72 ± 34.71*	231.41 ± 31.55*
CD162	100	93.76 ± 20.01	87.73 ± 28.37
CD203c	100	65.36 ± 17.97*	53.77 ± 14.97*

KU-812 cells were incubated with cerivastatin (50 μ M), atorvastatin (50 μ M), or control medium for 24 h. Then, cells were analyzed for expression of surface antigens by the indirect immunofluorescence-staining technique. Results are expressed as percentage of MFI obtained in comparison with medium control and represent the mean $\pm$ SD of nine independent experiments.

* Significant changes in surface marker expression ($P < 0.05$). PBS, Phosphate-buffered saline.

effect on CD63 up-regulation was seen (not shown). Exposure to statins also resulted in a slight decrease of (constitutive) expression of CD203c in untreated CML basophils.

DISCUSSION

Recent data suggest that statins exhibit inhibitory effects on growth and function of various myeloid cells and their progenitors [44–50]. It has also become evident that the statins are potent immunomodulating agents [44, 45]. In the present study, we have examined the effects of five different statins on growth and function of human basophils and their BM-derived precursor cells. The results of our study show that cerivastatin and atorvastatin inhibit cytokine-dependent and leukemic (factor-independent) growth of basophil progenitor cells as well as anti-IgE-induced release of histamine in blood basophils. Furthermore, these statins were found to suppress expression of

the basophil-activation antigen CD203c, a novel surface enzyme that is up-regulated in response to IgE-receptor cross-linking [51]. All in all, our data show that cerivastatin and atorvastatin are potent regulators of growth and function (activation) of human basophils.

Statins are inhibitors of HMG CoA reductase, the rate-limiting enzyme in the MVA metabolic pathway, which is required for the formation of cholesterol (squalene pathway) as well as isoprenylation of various regulator molecules involved in cell growth and function, such as G-proteins or members of the RAS family [31–35]. Such regulator molecules become farnesylated or geranylgeranylated to acquire their functional activity. To demonstrate that the statin effects on basophils were mediated through inhibition of the isoprenoid pathway, we performed experiments using MVA, farnesol, geranylgeraniol, and squalene. In these experiments, it was found that the statin-induced inhibition of basophil function can be reversed by MVA, farnesol, and geranylgeraniol but not by squalene. These data strongly suggest that indeed the isoprenoid pathway (and respective regulator/signaling molecules) was targeted by atorvastatin and cerivastatin in human basophils. These data are also in line with previous studies performed on murine mast cells [48, 55–57].

Statins represent a group of HMG CoA reductase inhibitors with similar biochemical properties and comparable effects on MVA-dependent cell functions [34, 35]. However, the statins differ in their structure and exhibit different relative potencies and tissue-specific activities [34, 35, 58, 59]. In the present study, the statins applied differed significantly in their inhibitory activity on basophil growth and activation. Thus, only cerivastatin and atorvastatin were found to inhibit basophil differentiation and histamine release in a significant manner. A slight but insignificant effect was also observed with lovastatin, whereas no effects were seen with simvastatin or pravastatin. The reason for the differential effects of the statins on human basophils is not known. One explanation could be that the statins exhibit a different uptake into basophils and basophil progenitor cells. An interesting aspect in this regard is that certain statins appear to bind to distinct cell-surface membrane

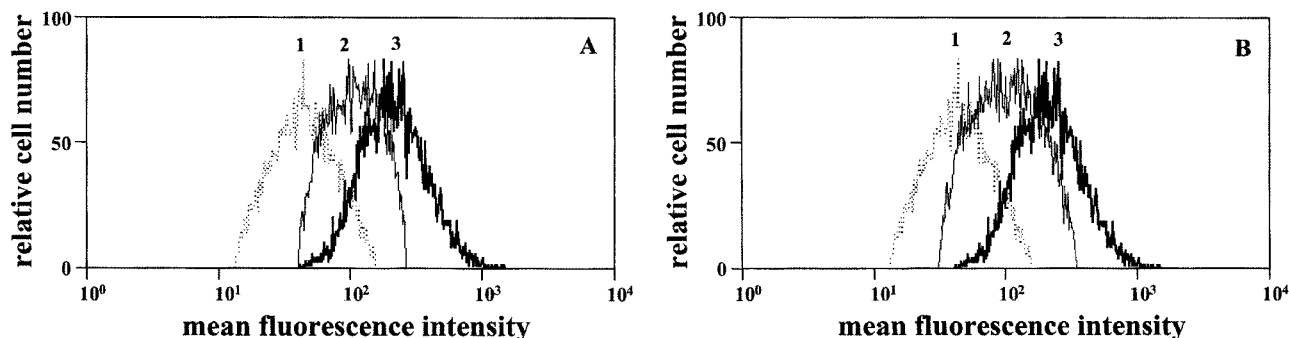


Fig. 10. Effects of cerivastatin and atorvastatin on IgE-dependent expression of CD203c on primary blood basophils. Primary blood basophils were enriched from a patient with chronic myeloid leukemia by Ficoll density centrifugation. Enriched cells were preincubated with cerivastatin, 50 μ M (A; 2), or atorvastatin, 50 μ M (B; 2), or control medium (A and B; 1 and 3) at 37°C for 24 h. Then, cells were incubated with control medium (A and B; 1) or anti-IgE antibody, 1 μ g/ml (A and B; 2 and 3), for 15 min. After washing, basophils were labeled with the PE-conjugated mAb 97A6 (CD203c). Expression of CD203c was analyzed by flow cytometry. Baseline levels of CD203c on basophils (1) increased significantly in response to anti-IgE (A and B; 3). Preincubation of basophils with cerivastatin (A; 2) or atorvastatin (B; 2) inhibited the anti-IgE-induced up-regulation of CD203c.

antigens. Likewise, lovastatin was found to bind to CD11a (lymphocyte function-associated antigen-2 α chain) [60], a molecule that is also expressed on basophils [20, 21]. Whether cerivastatin and atorvastatin bind to basophil surface antigens in a specific (receptor-dependent) manner remains unknown. In this regard, it was also of interest to know whether the statins examined exerted their inhibitory effects on basophils directly or through modulation of accessory cells. In particular, a number of different leukocyte types, including neutrophils and monocytes, are known to be targets of statins [61]. To address this question, we enriched basophils from a patient with accelerated-phase CML and marked basophilia (remaining cells being mostly blast cells and basophilic progenitors in MNC preparations) and incubated these enriched basophils with cerivastatin and atorvastatin. In these experiments, both statins inhibited anti-IgE-induced histamine liberation in the same way as seen with normal (impure) basophils. These data strongly suggest that atorvastatin and cerivastatin inhibited histamine release through a direct action on basophils.

An alternative hypothesis for the different relative potencies of statins would be that statins exhibit a different capability of cellular distribution. Likewise, it has been shown that cerivastatin and atorvastatin have a higher capacity to diffuse through the lipid membranes compared with the other statins [34, 35]. A differential effect of statins on signal transduction cascades following IgE receptor cross-linking seems unlikely. Thus, all the statins tested are known to cause delipidation and inhibition of signaling proteins without major differences observed among statin species.

Recent data have shown that statins can inhibit IgE-dependent cell activation in rat mast cells [48, 55–57]. Our data would be in line with these observations. However, in contrast to rat mast cells showing a good response to lovastatin, no major responses of human basophils to this statin were found. The reason for this discrepancy remains unknown. One explanation could be that secretory cells in different species exhibit different sensitivities against statins. Alternatively, mast cells differ from basophils in their response to statins. Whether the statins applied are capable of inhibiting growth and/or functions of human mast cells remains to be determined.

The surface-enzyme E-NPP3 (CD203c) is a novel, useful marker of human basophils [51, 62]. In fact, CD203c is specifically expressed on basophils but not on any other blood cell [51]. Furthermore, CD203c is a useful new marker of basophil activation. In fact, IgE receptor cross-linking on basophils is associated with a significant increase in expression of CD203c [51]. A similar effect of IgE receptor cross-linking on CD63 expression has also been described [63]. Therefore, we were interested to know whether statins can modulate expression of these CD antigens. The results of our study show that cerivastatin and atorvastatin down-regulate constitutive expression of CD203c on the basophil cell line KU-812, whereas no effects on CD63 expression were found. In addition, we were able to show that these statins inhibit the IgE-dependent increase in CD203c expression on peripheral blood basophils. Whether the statin-induced down-regulation of CD203c on basophils is associated with their decreased capacity to release histamine after IgE receptor cross-linking remains unknown.

Recent data suggest that the statins exert inhibitory effects on growth and proliferation of myeloid progenitor cells [49, 50]. The exact mechanism(s) underlying the inhibitory effects of statins on myeloid cell growth are not completely understood. Some studies have suggested an effect of statins on cell viability and induction of apoptosis [49, 50, 61]. By contrast, in our experiments, exposure of the human basophil cell line KU-812 to cerivastatin or atorvastatin was followed by a significant decrease in the rate of mitosis without causing significant changes in the rate of apoptosis or rate of necrosis. These data suggest that the inhibitory effects of cerivastatin and atorvastatin on basophil growth were a result of direct inhibition of proliferation (mitosis).

A number of disease states are associated with increased production and/or activation of blood basophils [22–26]. Likewise, in CML, especially during disease acceleration, an increase in blood basophils is a common finding [22, 23]. In diverse allergic reactions, basophil activation is often seen and may represent a serious clinical problem [27–30]. A number of attempts have been made in the past to identify drugs that interfere with basophil activation or/and growth. However, most of these drugs, such as cyclosporin A or corticosteroids, have profound effects on the immune system, which has to be taken into account if long-term treatment is considered. The statins may be regarded as a novel class of less immunosuppressive drugs with potential antibasophil activities. An interesting aspect in this regard is that the statins also inhibited allergen-induced degranulation of basophils in sensitized individuals. Conversely, it remains open whether the statin concentrations that can be reached in vivo would be sufficient to inhibit growth and function of basophils. In fact, in most studies, the maximum pharmacologic concentration (in plasma) reached with atorvastatin (given orally) averaged 0.2–0.3 $\mu\text{g/ml}$ [64, 65]. These concentrations are slightly lower than those inducing a significant inhibition of basophil functions in vitro. On the other hand, it has been described that in vivo application of pharmacological doses of statins in rats results in reduced histamine release from mast cells [48]. Therefore, to clarify whether the statins indeed can show inhibitory effects on basophil functions in vivo remains to be determined in clinical studies.

In summary, our data show that atorvastatin and cerivastatin are potent inhibitors of in vitro growth and activation of human basophils. Whether these statins also show beneficial antibasophil effects in vivo remains to be determined.

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