CCR5 in multiple sclerosis : expression, regulation and modulation by statins

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Statins & microglia cell function

Simvastatin affects cell motility and actin cytoskeleton distribution of microglia

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Glia, 2006, 53: 115-123

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Abstract

Statin treatment is proposed to be a new potential therapy for MS, an inflammatory demyelinating disease of the central nervous system. The effects of statin treatment on brain cells, however, are hardly understood. We therefore evaluated the effects of simvastatin treatment on the migratory capacity of brain microglial cells, key elements in the pathogenesis of MS.

It is shown here that exposure of human and murine microglial cells to simvastatin reduced cell surface expression of the chemokine receptors CCR5 and CXCR3. In addition, simvastatin treatment specifically abolished chemokine-induced microglial cell motility, altered actin cytoskeleton distribution and led to changes in intracellular vesicles. These data clearly show that simvastatin inhibits several immunological properties of microglia, which may provide a rationale for statin treatment in MS.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS), hallmarked by multiple demyelinated lesions throughout the brain and spinal cord. Activation of microglia, the resident macrophages of the CNS, is thought to be a key element in the development of neurological disorders such as MS [1-3]. Microglia are the first cell type to respond to a variety of CNS injuries and can even display an activated phenotype without the occurrence of obvious neuropathological changes. In the normal (adult) CNS ramified microglia, in contrast to peripheral macrophages, display a dormant phenotype, characterized by a lack of phagocytic activity and low expression of membrane bound molecules essential for the induction of macrophage functions [4,5]. Upon activation, microglia develop a range of innate immune functions, including proliferation, upregulation of cell surface receptors such as major histocompatibility complex class II (MHC-II), secretion of immunoregulatory factors and recruitment to the site of damage [4,5].

Various studies have provided evidence for enhanced motility of microglia to sites of CNS injury or inflammation [6-9]. Under normal circumstances, microglia constitutively express low levels of various chemokine receptors [10-14]. This expression is upregulated during *in vitro* activation and in various neurodegenerative diseases [10-12,14]. In particular, the chemokine receptors CCR2, CCR3, CCR5 and CXCR3 are implicated in the pathogenesis of MS [11,12]. For functional cell chemotaxis, clustering of chemokine receptors in restricted membrane microdomains, named lipid rafts, and redistribution of these rafts leading to polarization of the cell is needed [15-17]. In addition, throughout this process, rearrangement of the actin cytoskeleton is essential for the generation of spatial asymmetry of the cytoskeleton and formation of membrane extensions [17].

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key enzyme in cholesterol biosynthesis [18]. Due to their ability to inhibit the synthesis of cholesterol, statins are widely used in medical practice as the main therapy for hypercholesterolemia and have been shown to lower cardiovascular-related morbidity and mortality [19,20]. In addition to the effect of statins on atherosclerosis, there is evidence to suggest that statins have potential immunomodulatory capacities. For example, statins are able to inhibit the production of pro-inflammatory cytokines by several cell types [21-23]. In addition, recent studies have shown that statins are able to inhibit the constitutive and IFN-γ-induced expression of MHC-II molecules on various cell types [23-26]. Furthermore, we have recently shown that simvastatin treatment affects the integrity of lipid rafts, resulting in loss of cell surface expression of several other immuno-regulatory molecules, including the chemokine receptor CCR5 [26]. Finally, statins have been shown to inhibit lymphocyte functions, such as proliferation and natural killer-cell cytotoxicity [25,27].

The putative immunomodulatory properties of statins would potentially be beneficial for the treatment of patients with autoimmune disorders. Therefore, statins

are currently considered as possible treatment agents for Multiple Sclerosis (MS) and other neurodegenerative diseases [25,28-30]. A number of studies in mouse and rat experimental autoimmune encephalomyelitis (EAE), a Th1-mediated central nervous system (CNS) demyelinating disease with symptoms similar to MS, have shown that oral treatment of EAE susceptible mice with statins not only protects these mice from developing this disease, but also reverses already established disease. Statin treatment clearly results in a delayed disease onset, milder clinical signs, normalization of the symptoms and protection against loss of myelin and perivascular inflammatory infiltrates [23,31,32].

With regard to microglia, data about the effects of statins on this cell type are limited. It has been shown that statins inhibit the production of nitric oxide and cytokines by cultured murine and rat microglia [33,34]. In addition, statins reduce IFN-γ-induced MHC-II and CD40 expression on murine microglial cells *in vitro* [23,34] and microglial MHC-II expression in mice affected by EAE *in vivo* [23]. However, little is known about the effect of statins on human microglia and on microglial cell functions.

In the present study we have investigated the impact of simvastatin treatment on expression of chemokine receptors, cell motility and cytoskeleton integrity of cultured human and murine microglial cells. We show that exposure to simvastatin resulted in down regulation of chemokine receptor expression and inhibition of chemotactic behavior of cultured microglia. Furthermore, disruption of cholesterol-containing intracellular microdomains and cytoskeleton integrity in cultured microglia was noted following simvastatin exposure. Together, our results demonstrate that exposure to simvastatin not only affects cell surface expression of the chemokine receptors CCR5 and CXCR3 but also results in intracellular alterations, with apparent bearing on the migratory capacity of microglia.

Materials and Methods

Statin activation

Prior to use, simvastatin (Calbiochem, Darmstadt, Germany) and L-mevalonate (Sigma-Aldrich, Steinheim, Germany) were converted to their active forms as described earlier [35].

Cell culture and stimulation

Murine microglial cells were prepared and purified from primary cultures of newborn mice as described previously [36]. In brief, brains were removed under sterile conditions from the skull and meninges, and blood vessels were carefully removed. The total brain was trypsinized for 5 min. After centrifugation, the pellet was resuspended in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 IU/ml streptomycin and 50 IU/ml penicillin (all Gibco BRL, Life technologies,

The Netherlands), and washed twice. Finally, the cell suspension was plated in poly-L-lysine-coated tissue culture flasks (Greiner, Alphen a/d Rijn, The Netherlands; cells from two brains per flask).

After 7–10 days in culture, microglia were detached from the astrocytic monolayer by manually shaking the cultures for 2–3 min. For immunocytochemical staining, isolated microglia were seeded on glass coverslips at a nonconfluent density of 3 x $10^4/cm^2$.

Human brain tissue was obtained by rapid autopsy according to standardized procedures under the management of the Netherlands Brain Bank, Amsterdam, The Netherlands. Brain samples were obtained from patients without neurological complications. Isolation of human adult microglia was performed as described previously [37]. Brain tissue samples were dissected from various regions of the brain and collected in DMEM/HAMF10 (Gibco). Independent cell cultures were established from each single sample derived from a specific brain region. Tissue samples were minced into small fragments (<2 mm^3) and digested with 0.25% trypsin solution (Sigma, St. Louis, USA) containing 0.05% DNase (Boehringer Mannheim, Germany). Next, cells were passed through a nylon mesh filter (Becton & Dickinson Falcon, Belgium) and centrifuged. The cell pellet was taken up in a percoll/myelin-gradient buffer and centrifuged to remove cell debris and myelin. After lysis of erythrocytes, cells were washed in culture medium and plated in uncoated 6 wells plates (Greiner).

One day after seeding microglial cells (human and murine) into 6 wells, the medium was changed and simvastatin alone or in combination with L-mevalonate was added to the cultured microglial cells at a final concentration of 10 μM and 100 μM, respectively. The stimulation was repeated the following day.

Cell viability assay

Cytotoxic effects of the inhibitors were determined by measuring the cell viability using Trypan blue (Gibco) staining. Dead cells stain blue with this agent, whereas living cell exclude the dye.

Flow cytrometric analysis

Cells were trypsinized and scraped, and washes with PBS containing 2% bovine serum albumine (BSA; Roche). After washing, cells were stained with a R-phycoerythrin (PE)-conjugated rat monoclonal antibodies (mAb) against murine I-A/I-E and CCR5 or mouse mAb against human HLA-DR or CCR5 or a mouse mAb against human CXCR3 and a PE-conjugated goat-anti-mouse IgG as second Ab (all Becton Dickinson, San Jose, California). As a control, cells were stained with PE-conjugated isotype-matched IgG controls or anti-mouse IgG as second Ab. Fluorescence-activated cell sorting (FACS) analysis was performed on a FACScan flow cytometer (Becton Dickinson) using Cell Quest programming.

Chemotaxis Assay:

For chemotaxis assays, microglial cells were trypsinized, washed once with serum-free DMEM and directly used. Cell migration assays in response to CCL5, CCL3, CXCL10 (R&D Systems) and C5a (Sigma) were performed in a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD) as previously described [38]. Briefly, lower wells were loaded with chemokines diluted in serum-free DMEM. DMEM was used as a negative control. Upper and lower wells were separated by a polycarbonate filter (8-μm pore size; Ge Osmonics, Inc., Herentals, Belgium). Microglial cells (2–3 x 10^4) in 50 μl serum-free DMEM medium were seeded in the upper compartment of the chamber. After incubation for 150 min at 37°C and 5% CO_2, cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were stained with Diff-Quick (Merz-Dade AG, Switzerland). Experiments were performed in triplicates. Rate of microglial migration was calculated by counting cells in four random fields of each well using a 20X bright field objective.

Migration was calculated as percentage of unstimulated migration of untreated cells (control migration). Chemokine specific migration was calculated by normalizing for basal levels of migration. Significance levels were calculated using a two-tailed Student's *t* test for unpaired data.

Actin staining

Microglia (1.5 x 10^4) were allowed to adhere to glass cover slips for 24 h followed by simvastatin stimulation for the indicated time points. The cells were fixed in 2% Formaldehyde in PBS for 5 minutes and permeabilized with Triton X-100 in PBS for 5 minutes at room temperature. For actin skeleton staining FITC-labeled Phalloidin (Molecular Probes, The Netherlands) was used. Staining was analyzed by fluorescence microscopy using a 20X inverted fluorescence optic. Photographic images were captured using a conventional microscope (Axioskop, Zeiss, Oberkochen, Germany) equipped with epifluorescence illumination.

Electron microscopy

Human microglial cells were cultured in small petri dishes and were stimulated with IFN-γ (500 IU/ml) to ensure full activation of the cells and treated with simvastatin (10 μM) for 48 hr. For ultrastructural morphology, cells were fixed in 1.5% glutaraldehyde, postfixed in 1% OsO_4 and embedded in Epon (LX-112). Ultrathin sections were stained with uranyl acetate and lead citrate. All sections were viewed with a Philips CM 10 electron microscope.

Results

Simvastatin affects cell surface expression of MHC-II and chemokine receptors on microglia.

First, we investigated the effect of simvastatin on cell surface expression of MHC-II molecules and chemokine receptors on primary microglia of murine and human origin. It should be noted that due to the isolation procedure used, both human and murine microglial cells already displayed an activated phenotype reflected by activation-induced expression of MHC-II molecules.

In line with previous observations with different cell types, simvastatin treatment (10μM, 48hr) of murine microglia resulted in a marked decrease of cell surface expression of MHC-II (Figure 1A). In addition, microglial cell surface expression of the chemokine receptor CCR5 was almost completely suppressed by simvastatin (Figure 1A). In human microglia simvastatin (10μM, 48hr) similarly reduced cell surface expression of MHC-II (data not shown) and also inhibited the expression of CCR5 (Figure 1B), though this effect was less pronounced than in murine microglia. In addition, we evaluated the

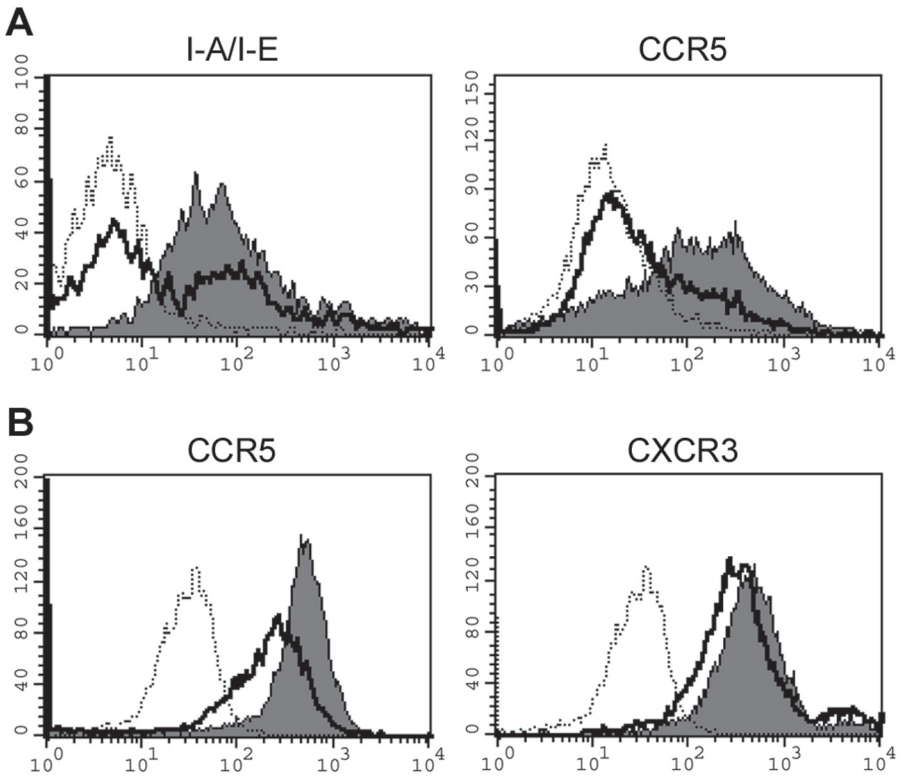

Figure 1. Simvastatin reduces expression of MHC-II and chemokine receptors on microglial cells. FACS analysis of MHC-II and chemokine receptor expression on the cell surface of primary murine (A) and human (B) microglia. Microglial cells were left untreated (filled histograms), or treated with simvastatin (48 hr, 10 μM, dark line) and subsequently assayed for cell surface expression of MHC-II and the indicated chemokine receptors. Shown are representatives of 6 (A) and 3 (B) independent experiments. Dotted lines represent isotype control staining.

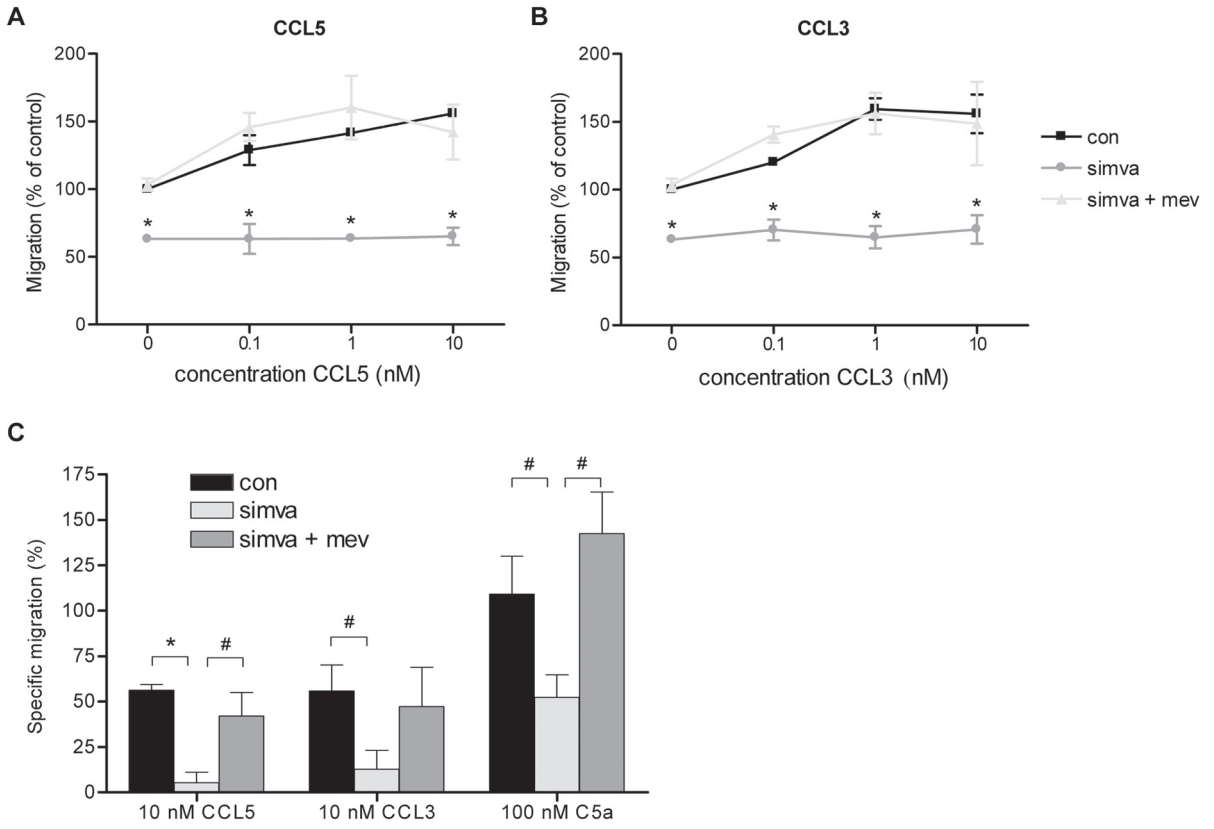

Figure 2. Simvastatin inhibits chemotaxis in primary murine microglia. **A**,**B** Migratory behavior towards CCL5 and CCL3. Depicted are percentages of migrating cells relative to basal levels of migration (basal level = 100%). Microglial cells were left untreated (con), treated with simvastatin (48 hr, 10 μM, simva) alone or in combination with L-mevalonate (100 μM, mev) and subsequently assayed for their migratory capacity. **C** Chemokine specific migration (as percentage of basal migration) induced by CCL5, CCL3 and C5a in control cells and cells treated with simvastatin alone or in combination with L-mevalonate. A-C Depicted are mean + SEM of at least three independent experiments, measured in triplo, and statistically significant differences between control cells (con) and simvastatin treated cells (simva), and simvastatin treated cells and simvastatin and L-mevalonate-treated cells (simva + mev), according to two-tailed student's T test: * $P < 0.05$, # $P < 0.1$.

expression of the chemokine receptor CXCR3, which was also lowered by simvastatin on human microglia (Figure 1B).

This effect of simvastatin on the cell surface expression of the chemokine receptors CCR5 and CXCR3 indicates that simvastatin could affect microglia chemotaxis.

Simvastatin impairs microglia chemotaxis

To evaluate the effect of simvastatin on murine microglial cell chemotaxis, we investigated the effect of simvastatin in an *in vitro* chemotaxis assay in response to two ligands for CCR5, i.e.CCL5 (RANTES) and CCL3 (MIP-1α), and the chemotactic anaphylatoxin C5a, using concentrations of simvastatin that are in the range of therapeutical concentrations. Under control conditions, microglial cells displayed a considerable rate of spontaneous migration. The chemokines CCL5 and CCL3 both induced a significant increase in chemotaxis of cultured microglia compared with

unstimulated control cells at a concentration range of 0.1 nM –10 nM (Figure 2A and B). Applying the chemokines CCL5 and CCL3 (10 nM) induced an increase in migrating murine microglial cells by > 150%, as compared with the basal level of migration. The chemotactic anaphylatoxin C5a was used as a control to discriminate between chemotaxis induction triggered by lipid raft-dependent and raft-independent receptors (C5aR, CD88) and induced pronounced chemotaxis in cultured murine microglial cells (> 200 %) compared to unstimulated microglia (Figure 2C).

Simvastatin (10μM, 48hr) induced a significant inhibition of baseline migration of cultured murine microglia. In addition, both chemokines did not significantly increase the number of migrating cells, indicating that simvastatin treatment inhibits chemokine-induced migration of murine microglial cells (Figure 2A-C). However, C5a still induced a significant increase in murine microglial cell migration after simvastatin treatment (60% increase, $p < 0.5$, Figure 2C). Nevertheless, in comparison to the untreated C5a response, maximum levels of migration were never observed.

Restoring the mevalonate pathway by co-incubation with L-mevalonate (100 μM) completely reversed the inhibitory effect of simvastatin on both basal and chemokine-induced murine microglial cell migration (Figure 2A-C). This indicates that the effect of simvastatin on the migratory capacity of microglial cells is due to its property to inhibit the mevalonate pathway.

In human microglial cells, simvastatin treatment (10μM, 48hr) induced the same inhibition of migration. Similar to the effect in murine microglia, simvastatin treatment resulted in a reduction of both basal levels of migration (not shown) and specific migration towards the chemokines CCL5, CCL3 and CXCL10 (IP-10, a ligand for CXCR3) (Figure 3). In contrast, the migratory response elicited by C5a in human microglia, was not affected by simvastatin treatment (Figure 3).

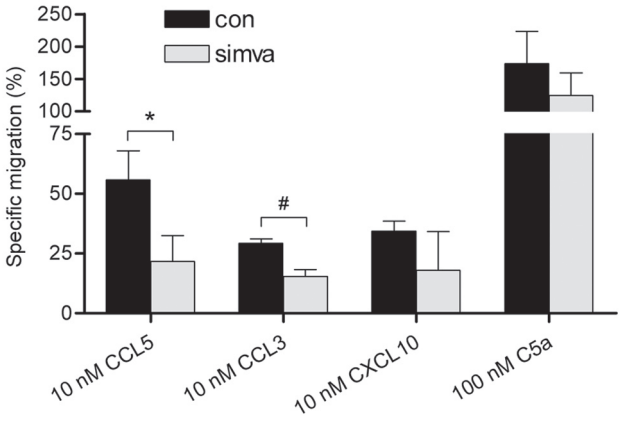

Figure 3. Simvastatin impairs chemotaxis in primary human microglia. Migration towards CCL5, CCL3, CXCL10 and C5a by primary human microglia left untreated (con) or treated with simvastatin (48 h, 10 μM , simva). Depicted are mean + SEM of at least three independent experiments, measured in triplo, and statistically significant differences between control cells and simvastatin treated cells according to two-tailed student's T test: * $P < 0.05$, # $P < 0.1$.

Taken together, these data indicate that simvastatin reduces basal migratory capacity as well as chemokine-induced migration of both murine and human microglia. It should be noted that under these experimental concentrations, simvastatin treatment did not influence cell viability of either murine or human microglial cells as determined by trypan blue staining (data not shown).

The inhibition of chemotaxis by simvastatin is time dependent and reversible

In additional experiments, we investigated the time-course and reversibility of simvastatin treament in murine microglia. After 24 hr basal migratory capacity seemed unaltered, whereas chemokine induced migration was already significantly reduced by simvastatin (10μM; Figure 4). After 48 hr simvastatin significantly reduced basal motility and completely blocked chemokine-specific migration. However, 24 hr after simvastatin removal, normal migratory behavior was restored (Figure 4), indicating that the effect of simvastatin on basal and chemokine-induced chemotaxis is completely reversible.

Cytoskeleton alterations following simvastatin exposure

Simvastatin does not only affect chemokine specific migration, but also basal migration of microglia. We therefore hypothesized that this effect was not only due to lowered expression of chemokine receptors, but could also be due to disturbance of other cellular processes needed for chemotaxis, such as actin rearrangement. This was supported by our observations that simvastatin treatment of microglia altered their shape and adhesive capacity. Therefore, we visualized actin distribution of microglia treated with a concentration range of simvastatin up to 48 hr to evaluate at

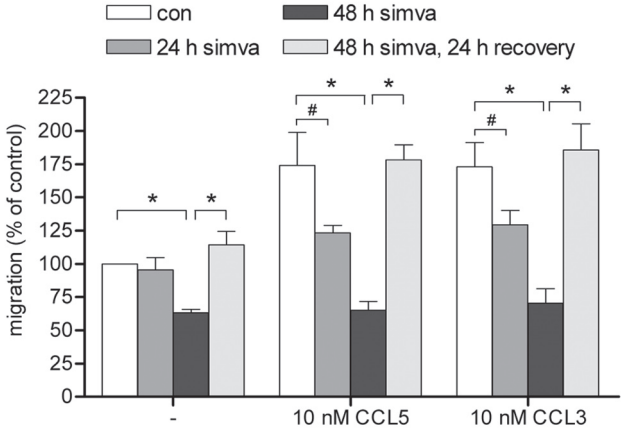

Figure 4. The effect of simvastatin on microglial migration is time-dependent and reversible. Migratory capacity of primary murine microglia left untreated (con), treated with simvastatin (10μM, simva) for 24 hr and 48 hr and treated with simvastatin for 48 hr followed by washing and 24 hr recovery. Basal migration (–) and migration towards CCL5 and CCL3 was determined. Depicted are mean + SEM of three independent experiments, measured in triplo, and statistically significant differences according to two-tailed student's T test: * $P < 0.01$, # $P < 0.1$.

which concentrations and time points an effect of simvastatin on the cytoskeleton was visible.

Untreated murine microglial cells showed a normal morphology with normally distributed actin, spread out throughout the cell and concentrated mainly in the cell cortex (Figure 5A). Upon treatment with simvastatin, murine microglial cells

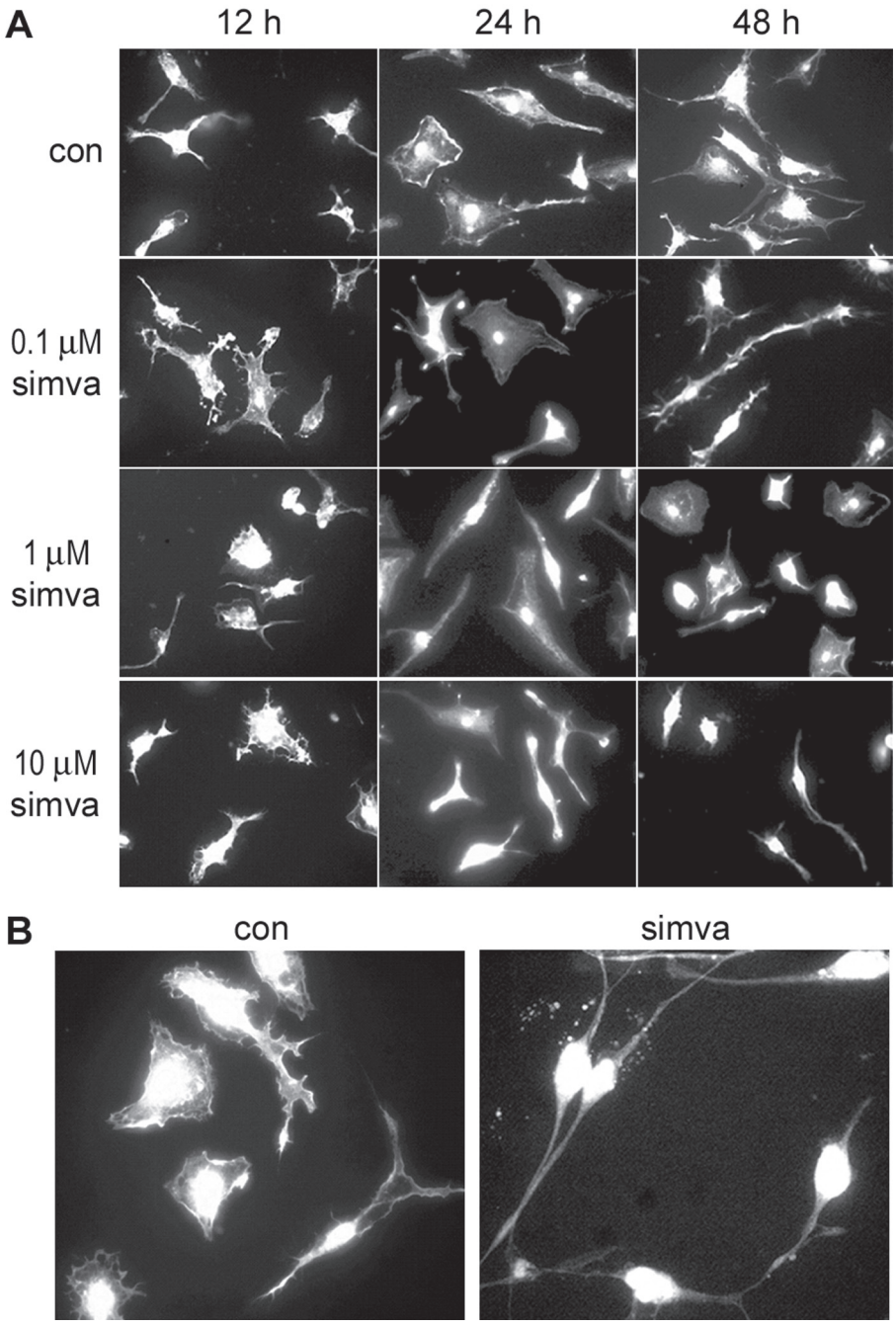

Figure 5. Simvastatin induces actin reorganization in microglial cells. Localization of F-actin in murine (A) and human (B) microglia cells. **A** Time and concentration dependence of simvastatin-induced actin reorganization in primary murine microglia visualized by F-actin staining. **B** F-actin staining of untreated primary human microglia (con) or microglia treated with simvastatin (24 hr, 10 µM, simva).

rounded up and the dense net of actin fibers, observed in control cells, disappeared. At low concentration of simvastatin (0.1 μM and 1 μM) the cell spanning fibers began to disassemble. A higher concentration of simvastatin (10 μM) lead to a complete disintegration of cytosolic fibers. At these concentrations an effect on actin distribution could already be observed after 12 hr. Furthermore, after 48 hr microglia were mostly rounded up and displayed a spindle-like appearance with actin condensed around the nucleus. The same effects were observed in human microglia (Figure 5B). However, these cells seemed to be more resistant to simvastatin treatment than murine microglial cells, with less rounding up occurring after simvastatin treatment (10μM, 48hr). These results indicate that simvastatin not only affects cell surface expression of membrane bound molecules, including chemokine receptors, but also the actin arrangement of cultured murine and human microglial cells.

Simvastatin promotes intracellular vesicle degradation in cultured human microglial cells

Because simvastatin clearly affects cell motility and actin distribution in microglia we questioned to which degree simvastatin treatment leads to changes in basal cellular structures in microglia. We therefore investigated the effect of simvastatin on the morphological structure of cultured human microglial cells by ultra-structural morphology electron microscopy (EM) on simvastatin treated microglia. Figure 6 shows that the cytoplasm of untreated human microglial cells was filled with mitochondria, ribosomal endoplasmatic reticulum (rER), fat deposits and vesicles for the transport of proteins. In simvastatin treated microglial cells the appearance of mainly the latter structures was altered. In these cells, normal appearing vesicles were hardly detectable, whereas clusters of larger vacuolar structures were frequently observed (arrowheads). These newly formed structures might represent remnants of the smaller vesicles found in untreated cells. Interestingly, the mitochondria and ER structure were unaltered by simvastatin treatment (arrows), indicating that energy-management and protein assembly and modification is probably unaffected by simvastatin in cultured human microglial cells under the applied concentrations.

Discussion

Microglial activation and migration has been proposed to be a key element in the development of MS [7,8]. Enhanced expression of chemokines and their receptors most likely underlies this enhanced motility of microglia during MS pathology. Most neurodegenerative diseases, including MS, are associated with expression of chemokines and chemokine receptors in the CNS [11]. It has been shown that expression of the chemokine receptors CCR2, CCR3 and CCR5 is enhanced in activated microglia in chronic active MS lesions [12]. In addition, microglia are able to functionally express

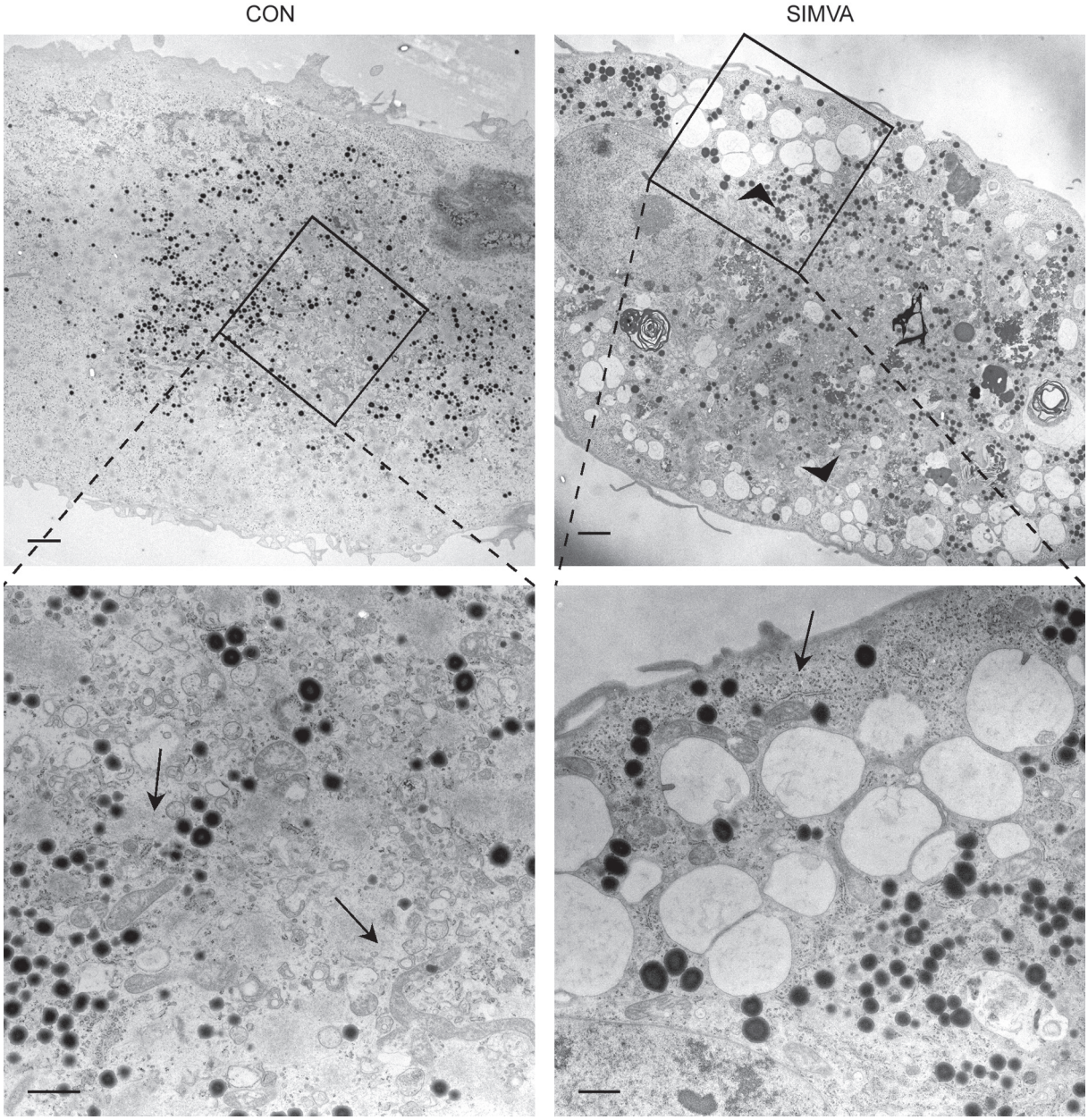

Figure 6. Simvastatin alters intracellular structures in human microglial cells. Ultra structural morphology electron microscopy (EM) of human microglial cells. Microglial cells were left untreated (left panels), or treated for 24 hours with simvastatin (right panels) and then subjected to EM. Bars: upper panels 2.5 µm, lower panels 1 µm. Arrowheads indicate vacuolar structures frequently observed in simvastatin-treated microglia, whereas arrows highlight mitochondria and rER.

CXCR3 [13]. Our results show that simvastatin treatment of cultured microglia lowered the cell surface expression of the chemokine receptors CCR5 and CXCR3. Accordingly, we show that the chemotactic migration towards the chemokines CCL5, CCL3 and CXCL10 was severely suppressed by simvastatin.

The observed reduction of chemokine receptor expression by simvastatin could be due to impaired transport to the membrane by lipid rafts. These structures, which

are highly enriched in cholesterol and glycosphingolipids, are essential for the membrane expression of various membrane bound molecules on the cell membrane, like chemokine receptors such as CCR5 [15,16,39]. Lipid rafts enable the clustering of these receptors in specific domains, leading to polarization of the cell upon triggering by chemoattractants. By disturbing these structures due to reducing cholesterol synthesis, simvastatin treatment could affect the transport of chemokine receptors to the cell membrane of microglia and their expression on the cell surface. Indeed, we have recently shown that simvastatin treatment affects the integrity of cholesterol-containing microdomains, or lipid rafts, explaining the observed loss of cell surface expression of MHC-II molecules in addition to a variety of other immuno-regulatory molecules, including CCR5 [26]. In contrast, simvastatin does not affect cell surface expression of CD45, a protein that is not associated with lipid rafts, on various primary cultured cells, including microglia (data not shown) [26]. The fact that C5a-induced chemotaxis was less affected by simvastatin than chemokine-specific chemotaxis, is in line with the notion that the presence of the corresponding receptor, C5aR, in lipid rafts has not been established [40].

In addition to cell surface expression of chemokine receptors, lipid rafts are also important for other stages in the process of chemotaxis. For example, several components of intracellular signal transduction pathways activated by receptor binding are incorporated in lipid rafts and it has been shown that early chemokine receptor signaling takes place in lipid rafts [39]. Therefore, disruption of lipid rafts by simvastatin most likely also affects intracellular signaling mediated by chemokine receptors.

However, statins can also affect cell functions by other mechanisms than lipid raft disruption. Indeed, we observed that in comparison to the untreated C5a response, maximum levels of migration induced by triggering the non-raft associated C5aR were impaired by simvastatin treatment, indicating that the effect of simvastatin on microglial cell motility is also caused by disturbance of other cellular processes essential for chemotaxis. The considerable effect of simvastatin on the basal migratory activity of microglia corroborates this assumption.

By inhibiting the mevalonate pathway, statins affect, in addition to cholesterol synthesis, also isoprenylation of a variety of proteins, including Rho GTPases [41,42]. These proteins play a central role in various cellular events, such as cytoskeletal organization, membrane trafficking and intracellular signaling [43,44]. It has been shown that the redistribution of lipid rafts and subsequent cell polarization depends on chemoattractant-induced actin reorganization [15]. This induction of actin reorganization depends on the activation of Rho GTPases [17,43,44]. It has been shown that statins inhibit Rho protein activation and signaling and in turn disrupts the actin cytoskeleton of vascular smooth muscle cells [45]. Using actin staining, we show that the cytoskeleton of microglia was severely altered after simvastatin treatment, rendering it very likely that actin reorganization is impaired in these cells, which could result from lack of Rho GTPase activation and signaling.

Furthermore, EM analysis of microglia reveals a change in the organization of intracellular structures in simvastatin treated cells. In untreated microglia, small intracellular vesicles can be recognized, whereas in simvastatin treated microglial cells larger, vacuolar-like structures are found. We hypothesize that these structures might be remnants of the vesicles found in untreated cells that are fused, due to cholesterol depletion by simvastatin [26]. This suggests that intracellular signaling mechanisms are probably also inhibited by simvastatin treatment. Appearance of mitochondria and ribosomal ER is not altered by simvastatin, indicating that cellular functions necessary for microglial cell survival are still intact. Indeed, under the experimental conditions applied in this study, simvastatin treatment does not influence the viability of human microglial cells nor does it induce any toxic effect as determined by the absence of trypan blue incorporation into the cells.

In addition, we, like others [23], show that statin treatment of microglia affects cells surface expression of MHC-II. When activated, microglia express high levels of MHC-II [8,46,47]. Moreover, microglia are believed to be the most efficient antigen presenting cells within the brain parenchyma [47,48]. In preliminary experiments, we have found that simvastatin, in addition to lowering MHC-II expression, also reduces the expression of the co-stimulatory molecules CD86 and CD40 (not shown). Therefore, in addition to impairing microglia motility, simvastatin treatment of microglia could also affect their ability to present antigens to and activate immune cells. However, more studies with respect to the effect of statins on antigen presentation capacity of microglia are needed to elucidate this matter.

Taken together, we show that simvastatin treatment impairs functional chemokine responses in microglia at several levels as eluted from our *in vitro* assays. Whether statins exhibit similar effects on the migratory capacity of microglia *in vivo* remains to be investigated. Because of their immunomodulatory properties, statins are currently considered as a potential alternative treatment for MS [25,28-30]. This is based on the observations that statins ameliorate or even prevent disease onset in several autoimmune encephalomyelitis animal models [23,31,32]. Considering the involvement of microglial activation and migration, and chemokines and chemokine receptors in the pathogenesis of MS, the results of our study therefore could have a bearing on the potential of statins in the treatment of this disease.

Acknowledgements

This research was supported by a grant from the Dutch MS Research Foundation (MS 00-407 to PvdE).

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