HMG-CoA-reductase inhibition-dependent and -independent effects of statins on leukocyte adhesion

Research Article

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Summary

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, a key enzyme for cholesterol biosynthesis and isoprenoid intermediates. Increasing evidence suggests that statins might affect inflammatory processes including leukocyte recruitment, yet, the underlying mechanisms are not defined. In this study two different pathways for inhibition of leukocyte adhesion by statins are described. (i) Coincubation with lovastatin inhibited adhesion of LFA-1 (CD11a/CD18, L 2)-transfected K562 cells to ICAM-1 and of p150.95 (CD11c/CD18, X 2)-transfected K562 cells to both ICAM-1 and fibrinogen (FBG), whereas adhesion of Mac-1 (CD11b/CD18, M 2)-transfected K562 cells was not affected. Moreover, only LFA-1-mediated adhesion to ICAM-1 but not Mac-1-mediated adhesion to FBG or urokinase-receptor (uPAR)-mediated adhesion to vitronectin (VN) of myelo-monocytic U937 cells was blocked by coincubation with lovastatin. The antiadhesive effect of lovastatin was independent of HMG-CoA-reductase inhibition, as it was not reversible in the presence of mevalonate, farnesyl-pyrophosphate or geranyl-pyrophosphate. In purified systems, lovastatin only blocked the ICAM-1/LFA-1 interaction but not the ICAM-1/Mac-1, FBG/Mac-1 or the VN/uPAR interactions. (ii) In contrast, preincubation of U937 cells for up to 18 h with lovastatin completely abrogated LFA-1-, Mac-1- and uPAR-dependent cell adhesion to the respective ligands. This anti-adhesive function of lovastatin was dependent on HMG-CoA reductase inhibition, since mevalonate or the isoprenoid intermediates restored adhesion, while no downregulation of integrin- or uPAR-expression was observed. Thus, two distinct pathways, involving a direct interaction with LFA-1 and p150.95 and an indirect inhibition of cell adhesion through disruption of cholesterol and/or isoprenoid metabolite biosynthesis are induced by statins. These functions can explain at least in part the inhibition of leukocyte adhesion and the associated antiinflammatory role of statins

I. Introduction

When leukocytes emigrate from the blood-stream into sites of inflammation or injury, they undergo a complex sequence of adhesion and locomotion steps requiring the expression and upregulation of various adhesion receptors on the surface of leukocytes and vascular cells. During their transmigration phase leukocytes adhere to provisional matrix substrates such as fibrinogen (FBG), fibronectin or vitronectin (VN) at sites of increased vascular permeability or damage. The prominent adhesion receptors on leukocytes are integrins, such as VLA-4 (4 1), that can bind to fibronectin, whereas adhesion to FBG is mediated by the 2 integrins Mac-1 (CD11b/CD18, M 2, CR3) and p150.95 (CD11c/CD18). Mac-1 together with LFA-1 (CD11a/CD18, L 2) also provide firm adhesion to and transmigration through the endothelium by recognition of their counter-receptor ICAM-1 on endothelial cells; evidence exists that p150.95 binds ICAM-1 as well (Springer, 1994; Carlos and Harlan, 1994; Stewart et al, 1995; Blackford et al, 1996; Gahmberg, 1997). The functional properties of integrins in general can be modulated by lateral (cis) interaction with integrin

associated protein (CD47), members of the tetraspanin family, syndecans, caveolin-1 or urokinase type plasminogen activator receptor (uPAR) (CD87), leading to the formation of transient multireceptor complexes that facilitate the dynamic recruitment of signaling molecules to sites of cellular contacts or focal adhesions (Ossowski and Aguirre-Ghiso, 2000; Preissner et al, 2000). Besides its ability to regulate integrin-dependent adhesion phenomena, uPAR can also directly mediate leukocyte adhesion to matrix-associated VN (Wei et al, 1994; Sitrin et al, 1996; May et al, 1998).

Recently, attention has been drawn to the role of microdomain structures of the plasma membrane, denoted lipid rafts, in cell adhesion. Lipid rafts are enriched in glycosphingolipids, cholesterol, transmembrane proteins and signaling molecules. GPI-anchored proteins may become sequestered into the microdomains as well, which have a lower fluidity than the surrounding membrane allowing the formation of multireceptor adhesion complexes. On epithelial cells, caveolin is a unique raft component, that has the intrinsic propensity to oligomerize and, thereby, contribute to formation of membrane invaginations termed caveolae (Horejsi et al, 1999; Kurzchalia and Parton, 1999; Smart et al, 1999; Simons and Toomre, 2000). Although leukocytes lack caveolin expression, they still contain lipid rafts that may facilitate the formation of adhesion complexes. The possibility that lipid rafts might regulate leukocyte adhesion by modulating integrin avidity has already been suggested (Krauss and Altevogt, 1999).

Statins inhibit the key enzyme of cholesterol biosynthesis 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase). In addition to lowering plasma cholesterol, increasing evidence suggests that statins play a pleiotropic role in the vascular system by effects on nitric oxide synthesis, smooth muscle cell proliferation, fibrinolysis or the immune system (Soma et al, 1993; Aikawa et al, 1998; Essig et al, 1998; Guisarro et al, 1998; Laufs and Liao, 1998; Laufs et al, 1998, 1999; Kwak et al, 2000; Diomede et al, 2001; Kwak and Mach, 2001). In particular, statins could inhibit leukocyte recruitment by regulating the expression of monocyte chemoattractant protein-1 (Romano et al, 2000) and of adhesion receptors (Weber et al, 1997; Ganne et al, 2000; Yoschida et al, 2001) or they might modulate integrin affinity by preventing geranyl-geranylation of RhoA (Liu et al, 1999). Cholesterol depletion by statins might also disrupt lipid rafts and, thereby, affect cell adhesion (Kraus and Altevogt, 1999; Simons and Toomre, 2000). Finally, a recent report suggested that different statins selectively bind to LFA-1, thereby blocking LFA-1 mediated leukocyte adhesion (Kallen et al, 1999; Weitz-Schmidt et al, 2001).

These observations prompted us to investigate in more detail the role of lovastatin in β_2-integrin- and uPAR-mediated leukocyte interactions. Two distinct mechanisms, a HMG-CoA reductase-dependent and an –independent, for inhibition of leukocyte adhesion are described, which further help to understand the antiinflammatory role of statins.

II. Materials and methods

A. Reagents

Two-chain high molecular weight urokinase type plasminogen activator (uPA) was from American Diagnostica (Bergstrasse, Germany). VN was purified from human plasma and converted to the multimeric form as previously described (Chavakis et al, 1998). FBG and fibronectin were purchased from Sigma (Munich, Germany). Vitamin D3 was from Biomol (Hamburg, Germany), transforming growth factor- was from R & D Systems (Boston, MA), and interleukin-3 was from PBH (Hannover, Germany). Phorbol 12-myristate 13-acetate (PMA) was from Gibco (Paisley, Scotland,UK). The blocking monoclonal antibody against human CD18, 60.3, was kindly provided by Dr. J. Harlan (University of Washington, Seattle, WA), the blocking monoclonal antibody against human CD11a, L15, was a generous gift from Dr. C. Figdor (University of Nijmegen, The Netherlands) and anti-uPAR monoclonal antibodies R3 and R4 (Chavakis et al, 1999) were given by Dr. G. Hoyer-Hansen (The Finsen Laboratory, Copenhagen, Denmark). Monoclonal antibodies K20 against β_1-integrins (CD29), 6.5B5 against ICAM-1, 2LPM19c against CD11b, KB90 against CD11c, MHM24 against CD11a and polyclonal rabbit-anti-FBG were from Dako (Hamburg, Germany). Isolated Mac-1, LFA-1 and ICAM-1 were kindly obtained from Dr. S. Bodary (Genentech, San Francisco, CA). Recombinant soluble uPAR was kindly provided by Dr. D. Cines (University of Pennsylvania, Philadelphia, PA). Lovastatin, mevalonate, farnesyl-pyrophosphate and geranyl-pyrophosphate were from Sigma (Munich, Germany). Peroxidase-conjugated secondary anti-mouse and anti-rabbit immunoglobulins were from DAKO (Hamburg, Germany).

B. Cell culture

Myelomonocytic cells (U937) obtained from American Type Culture Collection (ATCC) (Rockville, MD) were cultured in RPMI-1640 medium containing 10% (vol/vol) fetal calf serum. K562 cells transfected with Mac-1 were kindly provided by Dr. M. Robinson (Celltech Ltd, Slough, England) and K562 cells transfected with LFA-1 or p150.95 were a generous gift from Dr. Y. van Kooyk (University of Nijmegen, The Netherlands) and were cultivated in a mixture of 75% RPMI containing 10% fetal calf serum and 25% ISCOVE´s medium containing 5% fetal calf serum. Expression of the respective β_2-integrins was tested by FACS analysis (see below). All culture media were from Gibco (Eggenstein, Germany), and the cell culture plastic was from Nunc (Rocksilde, Denmark).

C. Cell adhesion assays

Cell adhesion to VN, ICAM-1 and FBG coated plates (and to BSA-coated wells as control) was tested according to previously described protocols (Chavakis et al, 1999, 2000, 2001, 2002). Briefly, multiwell plates were coated with 5 µg/ml ICAM-1, FBG or 2 µg/ml VN (dissolved in bicarbonate buffer, pH 9.6), respectively, and blocked with 3% (wt/vol) BSA. U937 cells, which had been differentiated for 24 h with vitamin D3 (100 nM) and transforming growth factor- (2 ng/ml), or K562 cells were washed in serum-free RPMI and plated onto the precoated wells for 60-90 min at 37°C in the absence or presence of competitors in serum-free RPMI as indicated in the figure legends. Where indicated, U937 cells were preincubated for various time periods without or together with lovastatin in the absence or presence of mevalonate, farnesyl-pyrophosphate or geranyl-pyrophosphate. Following the incubation period for the adhesion assay, the wells were washed and the number of adherent cells was quantified by

crystal violet staining at 590 nm.

D. Analysis of uPAR and integrin expression by flow cytometry

After incubation for 18 h in the absence or presence of lovastatin differentiated U937 cells were washed twice with HEPES-buffered saline and were incubated with saturating concentrations of primary antibody (10 µg/ml) for 60 min at 4°C. Cells were washed again, resuspended in HEPES buffer and phycoerythrin-conjugated F(ab)2 fragment of goat anti-rabbit (or mouse) IgG (Dianova, Hamburg, Germany) was added in saturating concentrations for 60 min at 4°C. After washing and resuspension, mean fluorescence of 10,000 cells was measured in a flow cytometer (Beckton Dickinson, Heidelberg, Germany). Nonspecific fluorescence was determined using control species- and isotype-matched primary antibody.

E. ELISA for ligand-receptor interactions

Maxisorp plates (high binding capacity; Nunc) were coated with Mac-1 or LFA-1 (5 µg/ml) dissolved in 20 mM HEPES, 150 mM NaCl, 1 mM Mn^{2+}, pH 7.2 and then blocked with 3% (wt/vol) bovine serum albumin (BSA) in the same buffer. Binding of FBG (10 µg/ml) or ICAM-1 (10 µg/ml) to the immobilized integrin was performed in a final volume of 50 µl of the same buffer as above together with 0.05% (wt/vol) Tween-20 and 0.1 % (wt/vol) BSA in the absence or presence of different competitors as indicated in the figure legends. After incubation for 2 h at 22°C and a washing step, bound ligands were detected by the addition of polyclonal rabbit anti-FBG or monoclonal mouse anti-ICAM-1 followed by the addition of 1:1000 diluted peroxidase-conjugated antibody against rabbit or mouse immunoglobulins, respectively. The conversion of the substrate 2,2-azino-di-(3-ethly)benzthiazoline sulphate (Boehringer, Mannheim, Germany) was monitored at 405 nm in a Thermomax microtitre plate reader (Molecular Devices, Menlo Park, CA). Nonspecific binding to BSA-coated wells was used as blank and was subtracted to calculate the specific binding. The same protocol was used when binding of multimeric VN (2 µg/ml) to immobilized uPAR (5 µg/ml, dissolved in bicarbonate buffer, pH 9.6) was tested, except that the binding buffer was TBS containing 0.05 % (wt/vol) Tween-20 0.1 % (wt/vol) BSA. Bound VN was detected with the anti-VN monoclonal antibody VN7 and additional steps of quantitation were the same as mentioned above.

III. Results

A. HMG-CoA reductase independent regulation of leukocyte adhesion by lovastatin

As previously established, the adhesion of myelo-monocytic U937 cells [differentiated with TGF (2 ng/ml) and vitamin D3 (100 nM) for 24 h] to immobilized FBG is predominantly mediated by Mac-1, whereas both Mac-1 and LFA-1 mediate adhesion to immobilized ICAM-1. U937 cell adhesion to FBG and ICAM-1 is enhanced by Mn^{2+} or phorbol ester (PMA). Moreover, U937 cell adhesion to VN is uPAR-dependent; uPA can stimulate adhesion, as it increases the affinity of the uPAR/VN-interaction (Chavakis et al, 2000, 2001 Preissner et al, 2000). In the presence of lovastatin, adhesion of U937 cells to ICAM-1was markedly reduced, whereas adhesion to FBG or VN was not affected at all (**Figure 1A**). The
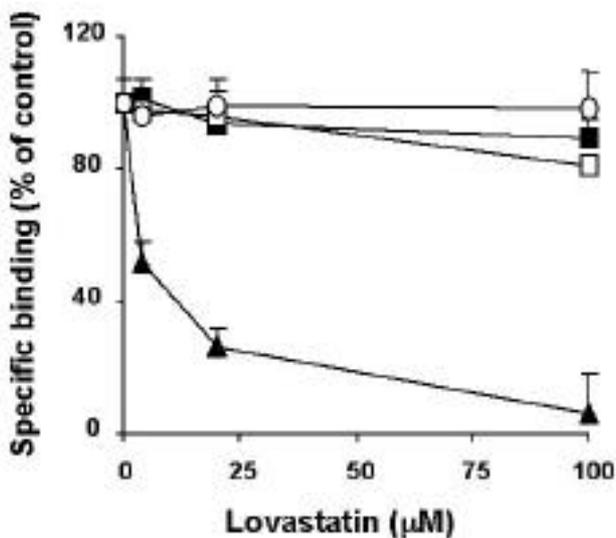
inhibitory effect of lovastatin on ICAM-1 adhesion was unchanged in the presence of the isoprenoid metabolites mevalonate, farnesyl-pyrophosphate, or geranyl-pyrophosphate (**Figure 1B**). None of these three metabolites alone could affect U937 cell adhesion to ICAM-1 (not shown).

U937 cells engage both Mac-1 and LFA-1 for ICAM-1-dependent adhesion; however, the lack of inhibitory activity of lovastatin on Mac-1-related adhesion to FBG indicated that lovastatin interacts only with LFA-1 directly.
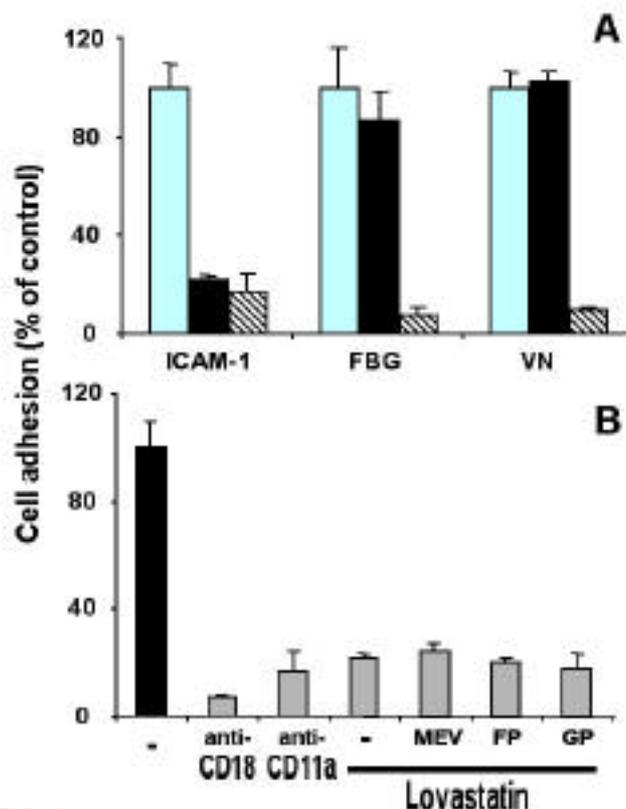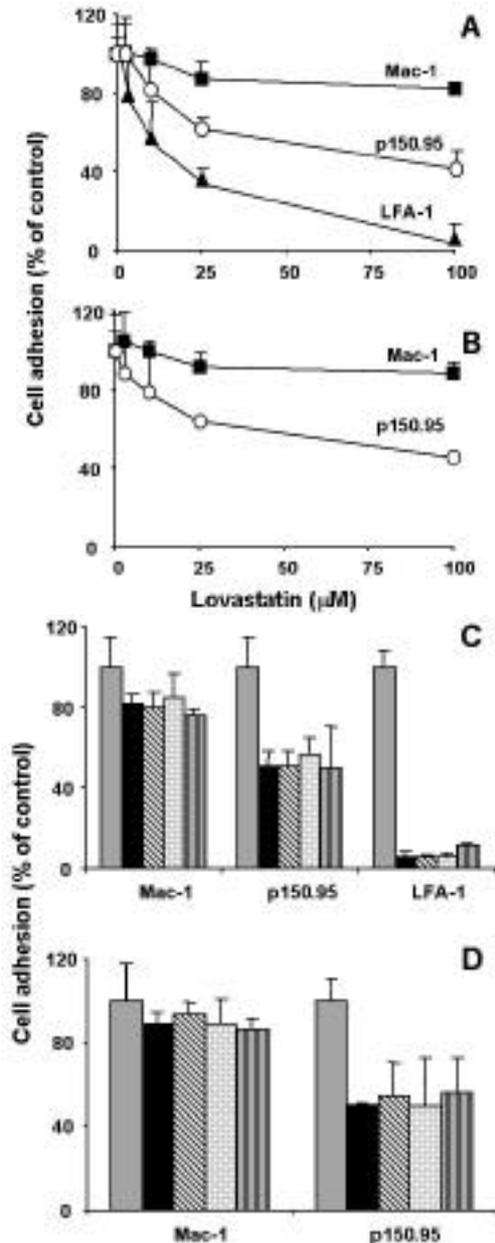

Figure 1. U937 cell adhesion to ICAM-1, FBG and VN. (**A**) PMA (50 ng/ml)-stimulated U937 cell adhesion to immobilized ICAM-1 (5 µg/ml) and FBG (5 µg/ml) or uPA (50 nM)-stimulated U937 cell adhesion to immobilized VN (2 µg/ml) was studied in the absence (open bars) or the presence of lovastatin (100 µM, filled bars) or the following blocking antibodies (hatched bars): anti-CD18 (15 µg/ml) for ICAM-1- and FBG-mediated adhesion, anti-uPAR (10 µg/ml) for VN-dependent adhesion. (**B**) PMA (50 ng/ml)-stimulated U937 cell adhesion to immobilized ICAM-1 (5 µg/ml) was studied in the absence (-) or presence of a blocking anti-CD18 antibody (15 µg/ml), a blocking anti-LFA-1 (CD11a) antibody (15 µg/ml), lovastatin alone (100 µM), or in combination with mevalonate (100 µM, MEV), farnesyl-pyrophosphate (100 µM, FP), or geranyl-pyrophosphate (100 µM, GP). Cell adhesion is expressed as percent of control, which is represented by the adhesion in the presence of PMA (or uPA, where adhesion to VN is shown) and in the absence of any competitor. Data are mean ± SEM (n=3) of a typical experiment; similar results were obtained in at least three separate experiments.

In order to test this hypothesis in detail, the inhibitory capacity of lovastatin was tested in two further systems: (**i**) In a purified system, lovastatin inhibited only binding of ICAM-1 to LFA-1, whereas the binding of ICAM-1 to immobilized Mac-1, the binding of FBG to Mac-1 or the binding of VN to immobilized uPAR were not affected at all (**Figure 2**). (**ii**) The effect of lovastatin on adhesion of differently transfected erythroleukemic K562 cells was studied: While non-transfected K562 cells did not adhere to FBG or ICAM-1, respectively, cells became adherent to both substrates upon transfection with Mac-1 or p150.95, whereas LFA-1 transfected cells only adhered to ICAM-1 (not shown). As expected, adhesion of Mac-1 transfected cells to ICAM-1 and FBG was not changed in the presence of lovastatin, whereas adhesion of LFA-1 transfected cells was completely inhibited by lovastatin with an IC50 of approximately 20 µM. Interestingly, adhesion of p150.95 transfected cells to both FBG and ICAM-1 was partially blocked by lovastatin with an IC50 of about 70 µM (**Figure 3A** and **Figure 3B**). The antiadhesive effect of lovastatin on adhesion of both LFA-1- and p150.95- transfected cells was not abolished in the presence of mevalonate, farnesyl-pyrophosphate or geranyl-pyrophosphate (**Figure 3C** and **Figure 3D**). Taken together, these data indicate that lovastatin selectively interacts with LFA-1 and with a lower potency with p150.95 but not with Mac-1. Lovastatin thereby can block LFA-1-mediated cell adhesion to ICAM-1 and to a lower extent p150.95-mediated adhesion to FBG and ICAM-1 in a manner independent of inhibition of HMG-CoA reductase.
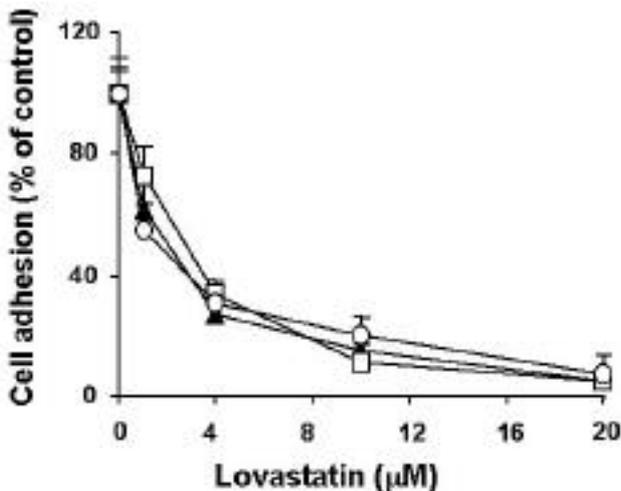

Figure 3: Influence of lovastatin coincubation on the adhesion of K562 cells. PMA (50 ng/ml)-stimulated adhesion of Mac-1-transfected K562 cells (filled squares), p150.95-transfected K562 cells (open circles) and LFA-1-transfected K562 cells (filled triangles) to immobilized ICAM-1 (5 µg/ml) (**A**) and PMA (50 ng/ml)-stimulated adhesion of Mac-1-transfected K562 cells (filled squares) and p150.95-transfected K562 cells (open circles) to immobilized FBG (5 µg/ml) (**B**) was studied in the presence of increasing concentrations of lovastatin. PMA (50 ng/ml)-stimulated adhesion of Mac-1-transfected K562 cells, p150.95-transfected K562 cells and LFA-1-transfected K562 cells to immobilized ICAM-1 (5 µg/ml) (**C**) and PMA (50 ng/ml)-stimulated adhesion of Mac-1-transfected K562 cells and p150.95-transfected K562 cells to immobilized FBG (5 µg/ml) (**D**) was studied in the absence (open bars) or presence of lovastatin alone (100 µM, filled bars), or in combination with mevalonate (100 µM, hatched bars), farnesyl-pyrophosphate (100 µM, dotted bars), or geranyl-pyrophosphate (100 µM, vertical lines). Cell adhesion is shown as percent of control, which is represented by the adhesion of cells in the absence of any competitor. Data are mean ± SEM (n=3) of a typical experiment; similar results were obtained in at least three separate experiments.

Figure 2: Influence of lovastatin on different ligand receptor interactions. The binding of ICAM-1 (10 µg/ml) to immobilized Mac-1 (open squares) or to immobilized LFA-1 (filled triangles), the binding of FBG (10 µg/ml) to immobilized Mac-1 (filled squares) or the binding of VN to immobilized uPAR (open circles) is analyzed in the absence or presence of increasing concentrations of lovastatin. Specific binding is expressed as percent of control, which is represented by the binding of the ligand to the respective immobilized receptor in the absence of lovastatin. Data are mean ± SEM (n=3) of a typical experiment; similar results were obtained in at least three separate experiments.

B. HMG-CoA reductase-dependent regulation of leukocyte adhesion by lovastatin

In contrast to the described direct antiadhesive effect of lovastatin on cells during coincubation, a completely different pattern of inhibition was observed when lovastatin was preincubated with leukocytes for up to 18 h followed by removal of excess reagent prior to the cell adhesion experiment. In particular, lovastatin preincubated for 18 h with U937 cells dose-dependently inhibited their adhesion to ICAM-1, FBG or VN. The inhibitory capacity was almost identical in all three systems (IC50 of about 1-2 µM) (**Figure 4**). Furthermore, the following differences were observed between U937 cell adhesion to ICAM-1 and adhesion to FBG and VN: In the time course, after 5 h of incubation with lovastatin about 30 % inhibition of U937 cell adhesion to FBG and VN was observed and inhibition was almost complete after 12 h. At all time points the effect of lovastatin was restored by mevalonate. Farnesyl-pyrophosphate or geranyl-pyrophosphate as well could completely reverse the antiadhesive effect of lovastatin on cell adhesion to FBG and VN (**Figure 5**). In contrast, already after 2 h of lovastatin preincubation adhesion to ICAM-1 was inhibited by 50% but could not be restored by mevalonate. Again, after 12 h lovastatin preincubation U937 cell adhesion to ICAM-1 was completely abolished However, this effect was only partially (50% of initial adhesion) reversed in the presence of mevalonate reaching a cell adhesion level that was comparable to cell adhesion after 2 h lovastatin preincubation (**Figure 5**). Thus, the action of lovastatin preincubation on U937 cell adhesion to ICAM-1 consists of two components, a HMG-CoA reductase-independent

direct blocking effect on LFA-1 and a HMG-CoA reductase-dependent effect.

The HMG-CoA reductase-dependent antiadhesive effect of lovastatin preincubation might result from a downregulation of the expression of respective adhesion receptors, namely 2-integrins or uPAR. However, lovastatin preincubation for 18 h did not affect the expression level of uPAR, 2-integrins (no change in CD11a, CD11b and CD18 expression) or 1 integrins (CD29) (**Table 1**). The CD11c chain was not detected on U937 cells, explaining the lack of inhibition of U937 cell adhesion to FBG by coincubation with lovastatin (**Figure 1**).

In conclusion, these findings indicate that lovastatin preincubation can regulate both 2-integrin and uPAR-mediated leukocyte adhesion in a cholesterol biosynthesis-dependent manner without changing the expression level of 2-integrins or uPAR.
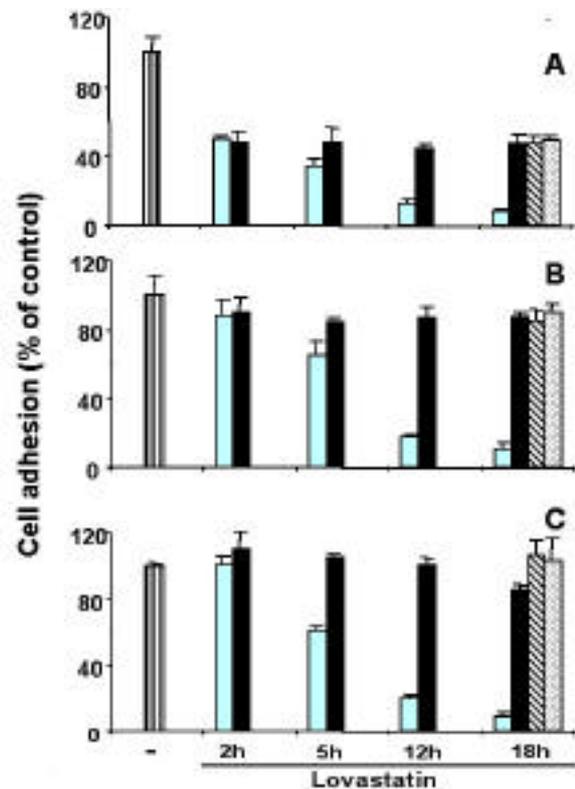
Figure 5: Influence of preincubation of lovastatin and isoprenoid metabolites on U937 cell adhesion. Following preincubation for various time periods as indicated, PMA (50 ng/ml)-stimulated U937 cell adhesion to (**A**) immobilized ICAM-1 (5 µg/ml), to (**B**) immobilized FBG (5 µg/ml) or (**C**) uPA (50 nM)-stimulated U937 cell adhesion to immobilized VN (2 µg/ml) was studied in the absence (vertical lines) or presence of lovastatin (20 µM) alone (open bars) or in combination with mevalonate (100 µM, filled bars). In the 18 h preincubation setting lovastatin was also reacted together with farnesyl-pyrophosphate (100 µM, hatched bars) or geranyl-pyrophosphate (100 µM, dotted bars). Cell adhesion is expressed as percent of control, which is represented by the adhesion in the presence of PMA (or uPA, where adhesion to VN is shown) and in the absence of any competitor. Data are mean ± SEM (n=3) of a typical experiment; similar results were obtained in three separate experiments.

Figure 4: Influence of lovastatin preincubation on U937 cell adhesion. Following preincubation for 18 h in the absence or presence of increasing concentrations of lovastatin, adhesion of PMA (50 ng/ml)-stimulated U937 cells to immobilized ICAM-1 (5 µg/ml) (filled triangles), to immobilized FBG (5 µg/ml) (open squares) or uPA (50 nM)-stimulated U937 cell adhesion to immobilized VN (2 µg/ml) (open circles) was studied. Cell adhesion is expressed as percent of control, which is represented by the adhesion in the presence of PMA (or uPA, where adhesion to VN is shown) and in the absence of lovastatin. Data are mean ± SEM (n=3) of a typical experiment; similar results were obtained in three separate experiments.

Table 1: Influence of lovastatin on integrin and uPAR expression.

Receptors	Control	Lovastatin
CD11a	100±8.2	92.6±3.1
CD11b	100±6.4	97.3±5.3
CD18	100±12.9	110.7±9.1
CD29	100±8.4	106.7±4.5
uPAR	100±8.9	97.9±1.7

The expression of CD11a, CD11b, CD18, CD29 and uPAR on U937 cells that were preincubated for 18 h in the absence or presence of lovastatin (40 μM) as measured by FACS-analysis is shown. The expression of the various integrins or uPAR is presented as percent of control, which relates to the expression of the respective adhesion molecule in the absence of lovastatin. Data are mean ± SEM (n=3) of a typical experiment; similar results were obtained in three separate experiments.

IV. Discussion

Leukocyte activation and adhesion to the endothelium and the subsequent transendothelial migration are pivotal steps in the recruitment of cells to the inflammatory /injured tissue. This highly coordinated multistep process requires tight regulation of adhesive events (Carlos and Harlan, 1994; Springer, 1994) including the induction of genes coding for participating adhesion receptors including integrins, their change in avidity as well as the modification of ligand-binding properties (Porter and Hogg, 1998; Woods and Couchman, 2000). Conversely, in pathological situations associated with organ transplantation, atherosclerosis and ischemia/reperfusion injury, arthritis and psoriasis the antagonism of these adhesive leukocytic interactions may become a promising therapeutic apppproach (Nahakura et al, 1996; Issekutz, 1998; Kruegeret al, 2000; Martin et al, 2000; Poston et al, 2000). In this respect, recent evidence points to an immunomodulatory role of statins (Katznelson and Kobashigawa, 1995; Maron et al, 2000; Kwak and Mach, 2001) which are commonly used to reduce plasma cholesterol levels in order to decrease the risk of cardiovascular disease (Corsini et al, 1995). In this study we define the direct and indirect role of statins in leukocyte adhesion and the possible underlying mechanisms. Two distinct pathways, a HMG-CoA reductase-dependent and an –independent were distinguished and appear to be relevant for the antiadhesive effects of statins.

In particular, coincubation of monocytes with lovastatin resulted in a dramatic reduction of LFA-1-dependent cell adhesion to ICAM-1, but not of Mac-1-dependent adhesion to FBG or uPAR-dependent adhesion to VN. This direct antiadhesive effect of lovastatin was unrelated to HMG-CoA reductase inhibition, as it was not reversed by mevalonate or other isoprenoid metabolites. Rather, it was attributed to the direct inhibition of the LFA-1/ICAM-1 interaction by lovastatin as corroborated in a purified system. Whereas Mac-1 binding to its ligands ICAM-1 and FBG as well as uPAR interaction with VN were not directly affected by lovastatin, binding of another 2-integrin, p150.95, to FBG and ICAM-1 was partially blocked directly by lovastatin. Our data are in accordance with and extend a recent report showing that statins inhibit LFA-1 by binding to an allosteric L-site located within the I-domain of the chain (Weitz-Schmidt et al, 2001). Thus, lovastatin binds to LFA-1 as well as with lower affinity to p150.95, but not to Mac-1, thereby directly affecting leukocyte adhesion.

When lovastatin was preincubated with monocytes for up to 18 h, a different inhibition profile was observed: Lovastatin completely blocked all three adhesive events, namely LFA-1/Mac-1-dependent adhesion to ICAM-1, Mac-1-dependent adhesion to FBG and uPAR-dependent adhesion to VN. Inhibition of ICAM-1-related adhesion could be partially attributed to the direct LFA-1 binding property of lovastatin, as (**i**) a significant inhibition by 50% occured already after 2 h, and was not reversed by mevalonate and (**ii**) complete inhibition was observed after longer preincubation times (12-18 h) and could be partially reversed by mevalonate up to the adhesion level obtained after 2 h preincubation with lovastatin. In contrast, both Mac-1- and uPAR-dependent cell adhesion were partially inhibited after 6 h preincubation with lovastatin and were completely blocked after 12-18 h. This effect of lovastatin was dependent on HMG-CoA reductase inhibition, as it was completely reversible in the presence of mevalonate. Interestingly, the IC50 of the HMG-CoA reductase-dependent effect of lovastatin was approximately 1 μM, which is about 20 times (LFA-1) or 70 times (p150.95) lower than the IC50 of the HMG-CoA reductase-independent direct abrogation of both integrin-mediated adhesion reactions. Thus, the antiinflammatory action of statins implied in clinical studies are very likely attributable to the HMG-CoA reductase-dependent pathway, as the higher concentrations of statins required for the direct inhibition of the LFA-1/ICAM-1-, the p150.95/FBG- and the p150.95/ICAM-1-interactions may not be reached with the standard doses (nanomolar range) of approved statin drugs (Frenette, 2001). Indeed, a recent report demonstrated that mevalonate-derived isoprenoid metabolites mediate the antiinflammatory activity of statins in the in vivo air-pouch model of local inflammation (Diomede et al, 2001). Finally, the anti-inflammatory capacity of statins may vary dependent on their individual structure (Weitz-Schmidt et al, 2001).

While direct binding to LFA-1 and p150.95 sufficiently explains the HMG-CoA reductase-independent antiadhesive effect of lovastatin, different mechanisms might be involved in the HMG-CoA reductase-dependent anti-adhesive property of lovastatin: (**i**) Lowering the plasma membrane cholesterol content can affect cell adhesion by disrupting lipid raft integrity (Krauss and Altevogt, 1999; Simons and Toomre, 2000). Recently, the assembly of adhesion complexes containing

adhesion receptors as well as signaling molecules such as focal adhesion kinase or src kinases has been proposed to be confined to glycosphingolipid- and cholesterol-rich, detergent insoluble microdomains of the cell membrane. The antiadhesive effect of lovastatin preincubation presented here could very well be due to raft disruption by cholesterol depletion, as other approaches to disrupt these membrane microdomains result in a very similar downregulation of 2-integrin and uPAR mediated leukocyte adhesion (Chavakis et al., unpublished observations). Moreover, as lipid rafts have been implicated in T-cell receptor-, EGF-receptor-, insulin receptor-, H-Ras-, eNOS- and integrin-dependent signalling phenomena (Simons and Toomre, 2000), the potential modulatory role of HMG-CoA-reductase inhibitors on raft integrity and associated vital cellular functions renders these drugs very attractive for several therapeutic interventions in vascular medicine. (**ii**) Although conflicting results have been reported as to the influence of statins on the cell type specific integrin and uPAR expression (Weber et al, 1997; Liu et al, 1999; Wojeiak-Stothard, 1999; Yoschida et al, 2001), our data are in accordance with these reports showing no change in integrin expression in e.g. myelo-monocytic U937 cells by lovastatin (Weber et al, 1995; Liu et al, 1999). (**iii**) It has been demonstrated that protein geranyl-geranylation is required for 1-integrin-dependent adhesion of leukocytes. It is thus conceivable that statin treatment may affect integrin-dependent leukocyte adhesion via inhibition of the geranyl-geranylation of RhoA, which is thought to be one of the most important effectors involved in regulation of the cytoskeleton network, including the clustering of adhesion molecules during monocyte adherence (Liu et al, 1999; Wojciak-Stothard et al, 1999; Kwak and Mach, 2001; Yoshida et al, 2001). The possibility that statin treatment could thereby directly inhibit RhoA activation and disrupt actin polymerization leading to failure of integrin clustering is a likely interpretation of the presented data, since isoprenoid metabolites could reverse the antiadhesive effect of lovastatin pretreatment. Together, our findings help to decipher the mechanisms underlying the postulated antiinflammatory effects of statins, which, besides atherothrombosis, may prove to be beneficial in arthritis, organ transplantation or psoriasis.

Acknowledgments

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