Diverse effects of inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase on the expression of VCAM-1 and E-selectin in endothelial cells

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The expression of monocyte adhesion molecules, such as VCAM-1 (vascular cell adhesion molecule-1) and E-selectin, on the surface of the endothelium is an important step in the initiation and progression of atherosclerotic lesions. We hypothesized that the inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in endothelial cells could influence the expression of VCAM-1 and E-selectin. Using cultured human umbilical vein endothelial cells, we found that mevastatin (0.1–1 μ M) significantly reduced the expression of VCAM-1 protein in cells activated by tumour necrosis factor- α (TNF- α) for 7 h. In contrast, TNF- α -induced E-selectin protein expression was augmented after mevastatin treatment. Mevastatin inhibited the mRNA expression of both VCAM-1 and E-selectin in TNF- α -stimulated endothelial cells. The activity of the transcription factor nuclear factor- κ B, which is known to regulate the transcription of VCAM-1 and E-selectin, was significantly reduced after incubation with mevastatin. Analysis of the time-dependent

variation in the TNF- α -induced expression of E-selectin, and estimation of the rate of surface disappearance of E-selectin together with measurement of the amounts of E-selectin molecules secreted, indicated that mevastatin inhibited the surface removal of E-selectin. This is compatible with the observed increase in E-selectin expression after statin treatment. All observed effects of mevastatin were reversed by mevalonate, the product of the HMG-CoA reductase reaction. In conclusion, inhibition of HMG-CoA reductase in endothelial cells attenuates VCAM-1 expression, but increases E-selectin expression, after cytokine induction. These diverse effects are associated with changes in the transcriptional regulation of the two adhesion molecule genes and modulation of the surface removal of E-selectin.

Key words: endocytosis, endothelium, mevastatin, NF- κ B, shedding.

INTRODUCTION

The clinical efficacy of the inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase by statins in reducing cardiovascular morbidity and mortality has been convincingly demonstrated in primary and secondary prevention trials [1,2]. Evidence has indicated that the effects of these agents are not completely explained by the decrease in plasma low-density lipoprotein cholesterol levels, and it has been proposed that they may also exert important actions in addition to their effects on plasma lipids [3,4]. This suggestion fits well with experimental observations *in vitro* indicating that statins inhibit vascular smooth muscle cell proliferation [5,6], decrease the production of reactive oxygen species from monocytes/macrophages [7], enhance the levels of endothelial cell nitric oxide synthase [8,9] and decrease the production of endothelin [9] in endothelial cells. Thus both clinical and experimental data support the hypothesis that inhibition of HMG-CoA reductase by statins may directly influence important vessel wall functions related to the development of atherosclerosis.

A key event in the initiation and progression of atherosclerotic lesions is the accumulation of monocytes/macrophages in the arterial wall. The recruitment of monocytes is initiated by the expression of adhesion molecules on the luminal surface of vascular endothelial cells. These molecules bind to ligands on

circulating monocytes, thereby paving the way for cellular attachment and migration into the vascular wall [10]. VCAM-1 (vascular cell adhesion molecule-1) and E-selectin are two inducible endothelial cell adhesion molecules which bind surface molecules on monocytes: VCAM-1 belongs to the Ig superfamily of adhesion molecules [11], whereas E-selectin is a C-type lectin [12]. A number of cytokines, including tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), can induce the expression of VCAM-1 and E-selectin in endothelial cells *in vitro* [13,14], and these cytokines may act as inducers of adhesion molecules during the atherogenic process *in vivo* [15,16].

E-selectin and VCAM-1 are not stored in the endothelial cells, and their surface expression depends on activation of gene transcription and *de novo* protein synthesis. The intracellular transduction pathway leading to mRNA synthesis from the E-selectin and VCAM-1 genes after cytokine induction involves activation of the transcription factor nuclear factor- κ B (NF- κ B), a central molecule in the induction of inflammatory responses in several cell types [17]. However, it has been shown that additional factors are important in the regulation of the level of adhesion molecules on the endothelial surface. Specifically, endothelial expression of E-selectin is also dependent on the membrane disappearance rate of the adhesion molecule, since a majority of the molecules are rapidly internalized [18–20] or proteolytically cleaved [21] and shed to the extracellular space. In the present

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, hydroxymethylglutaryl-CoA; HRP, horseradish peroxidase; IL-1 β , interleukin-1 β ; NF- κ B, nuclear factor- κ B; TMB, tetramethylbenzidine; TNF- α , tumour necrosis factor- α ; VCAM-1, vascular cell adhesion molecule-1.

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study we examined whether the surface expression of VCAM-1 and E-selectin, and the underlying regulatory mechanisms, were influenced by the inhibition of HMG-CoA reductase.

EXPERIMENTAL

Materials

TNF- α and IL-1 β were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Mevastatin and mevalonate (mevalonic acid lactone) were obtained from Sigma (St. Louis, MO, U.S.A.). Simvastatin, kindly provided by Merck, Sharp and Dohme, was added in the active form in 0.1 M NaOH, after adjustment to pH 7.4 with HCl.

Cell cultures

Human endothelial cells obtained from collagenase-digested umbilical veins [12] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 2 μ g/ml ciprofloxacin, 100 μ g/ml ampicillin, 1% L-glutamine, 40 μ g/ml endothelial cell growth factor, 15 units/ml heparin and 5 mM glutamine in gelatin-coated plates (0.65 μ g/cm²), and maintained at 37 °C in an atmosphere of 5% CO₂/95% atmospheric air. The cells were subcultured after detaching with trypsin solution and replating. Cell counting was performed after trypsin treatment, using a Bürker-Türk chamber. Viability of cells was evaluated by morphology and Trypan Blue exclusion. The endothelial phenotype was confirmed using phase-contrast microscopy (i.e. cuboidal, cobblestone-appearing monolayer of cells) and positive immunofluorescence staining with antibodies against von Willebrand Factor (results not shown). Cells from more than 10 different healthy donors were used.

ELISA procedures

Cellular content of VCAM-1 and E-selectin

A modified ELISA procedure was used to measure the cellular E-selectin and VCAM-1 content. Cells grown in 96-well plates and exposed to cytokines and drugs as indicated were washed once with 150 μ l of PBS, fixed in 150 μ l of 100% (v/v) methanol for 10 min, air-dried and stored at 4 °C. Dried cells were rehydrated and blocked in 150 μ l of PBS containing 0.1% Tween-20 and 0.5% BSA (PBS/Tween/BSA) for 30 min and then washed twice in PBS/Tween. The wells were then incubated for 2 h at room temperature with either a monoclonal antibody against human E-selectin (BBA-16; R&D Systems) diluted 1:500 in PBS/Tween/BSA, or a polyclonal goat antibody against human VCAM-1 (BBA-19; R&D Systems) diluted 1:500 in PBS/Tween/BSA. After two washes in PBS/Tween, wells were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in PBS/Tween/BSA: rabbit anti-(mouse Ig)-HRP (NA9310; Amersham Life Sciences) diluted 1:4000 for E-selectin measurements, and rabbit anti-(goat Ig)-HRP (P0160; Dako A/S) diluted 1:4000 for VCAM-1 analysis. After a 1 h incubation at room temperature, wells were washed five times in PBS/Tween, and they were subsequently coloured using 100 μ l of TMB (tetramethylbenzidine) reagent (Dako S 1600) as substrate for the bound HRP. After 5 min of incubation, the reaction was stopped by adding 100 μ l of 3 M H₂SO₄. Absorbance was read at 450 nm in an ELISA reader.

Rate of disappearance of VCAM-1 and E-selectin from the cell surface

Estimation of disappearance rates for E-selectin from the surface of endothelial cells was carried out by a modified ELISA

procedure. Briefly, cells were grown in 96-well plates and, after treatment with mevastatin and TNF- α for various time periods, a monoclonal antibody against E-selectin (BBA-16; R&D Systems) or a polyclonal goat antibody against human VCAM-1 (BBA-19; R&D Systems) was added directly to the growth medium at a final dilution of 1:100 and allowed to bind to surface molecules for 1 h at 37 °C. Unbound antibodies were removed by three washes with 1% (w/v) BSA in DMEM, preheated to 37 °C. The cells were left for various time periods (0–80 min) at 37 °C, after which the biological functions of the cells in individual tissue culture plates were stopped by incubating on ice. The amount of surface-bound antibodies was measured after incubation with a secondary antibody: rabbit anti-(mouse Ig)-HRP (NA9310; Amersham Life Sciences) for E-selectin detection, and rabbit anti-(goat Ig)-HRP (P0160; Dako A/S) for VCAM-1, diluted 1:2000 with 1% BSA/DMEM for 1 h, followed by four washes with 1% BSA/DMEM. Incubation with the secondary antibody and the washing procedure were done on ice. Finally, wells were coloured using 100 μ l of TMB reagent (Dako S 1600) as substrate for the bound HRP. After 5 min of incubation, the reaction was stopped by adding 100 μ l of 3 M H₂SO₄. Absorbance was read at 450 nm in an ELISA reader. The results are presented as absorbance values after different incubation periods, expressed as percentage of the absorbance at the time at which excess antibodies were washed away.

Soluble E-selectin

For the determination of soluble E-selectin shed from the cells into the conditioned medium, a commercially available sandwich ELISA was used, as specified by the manufacturer (Parameter-BBE2B; R&D Systems). Samples were diluted 1:3.

RNA isolation and reverse transcriptase-PCR analysis

Total RNA was extracted from cells grown in six-well culture dishes, using 500 μ l of Trizol reagent (Gibco BRL), according to the instructions from the manufacturer. The amount and quality of isolated RNA was assessed by measurement of absorbance at 260 and 280 nm. From each sample, 1 μ g of RNA was used for reverse transcription in 30 μ l of reaction mixture containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM dTTP, 30 pmol of random hexamers and 200 units of MMLV (Moloney murine leukaemia virus) reverse transcriptase (Gibco BRL). The samples were incubated for 75 min at 37 °C, followed by incubation for 15 min at 95 °C.

PCR was subsequently performed using 2 μ l of reverse transcriptase solution in a reaction mixture of 20 μ l containing 20 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM dTTP, 2.5 units of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) and 500 pmol of each of the specific primers. The following primers were used. E-selectin (EMBL accession number M24736): E-sel-1100, 5'-ATCATCCTGCAACTTCACC-3' (exon 5); E-sel-1445, 5'-ACACCTCACCAAACCCTTC-3' (exon 7). VCAM-1 (EMBL accession number M73255): VCAM-1750, 5'-GGGAAAACAGAAAAGAGGG-3' (exon 4); VCAM-2425, 5'-CAATGGTAGGGATGAAGG-3' (exon 6). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EMBL accession number M17851): GAPDH-379, 5'-GCCAAAAGGGTCA-TCATCTC-3'; GAPDH-665, 5'-GTAGAGGCAGGGATG-ATGTT-3'. Amplification of cDNA was performed in a Hybaid Thermocycler with two initial cycles of 95 °C for 60 s, 54 °C for 60 s and 73 °C for 60 s. These cycles were followed by a number

of rounds at 94 °C for 45 s, 54 °C for 45 s and 73 °C for 45 s. For the final cycle, the samples were incubated for 2 min at 73 °C. The PCR products were separated by gel electrophoresis in a 2 % (w/v) agarose gel containing ethidium bromide. All PCR reactions gave rise to products of the expected molecular size. The intensity of visualized bands was measured in arbitrary absorbance units with a Bio-Rad UV-gel camera, and used as an estimate of mRNA amount.

To ensure a dose-dependent PCR for the quantitative PCR protocol, pilot experiments were performed to determine the optimal number of cycles for each primer set. Pools of cDNA made from RNA isolated from both TNF- α -treated and control endothelial cells were subjected to GAPDH, E-selectin and VCAM-1 PCR with different cycle numbers. The number of cycles which gave rise to a weak band was selected for further evaluation of the samples, i.e. 27 cycles for GAPDH, 27 cycles for E-selectin and 30 cycles for VCAM-1. Negative controls, including RNA samples treated without reverse transcriptase and samples without RNA, did not give rise to DNA amplification (results not shown).

Nuclear isolation, extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cultures of confluent endothelial monolayers by a mini-extraction protocol, as described by Johnson et al. [22] with slight modifications. Cells were placed on ice and harvested into PBS by scraping. After one wash in ice-cold PBS, cells were lysed in 50 μ l of 10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.25 % Nonidet P40, 0.5 mM PMSF, 0.5 mM *N*-ethylmaleimide and 0.02 mM benzamide (lysis buffer) for 15 min on ice. The released nuclei were collected by centrifugation (500 *g*, 4 min, 4 °C) and washed once in lysis buffer. The nuclear pellet was resuspended in 50 μ l of 20 mM Hepes, pH 7.9, 0.45 M NaCl, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM *N*-ethylmaleimide and 0.02 mM benzamide (extraction buffer) and incubated for 15 min on ice. Nuclei were pelleted by centrifugation (14000 *g*, 4 min, 4 °C) and the resulting extract was diluted 1:1 (v/v) in 20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA and 20 % (v/v) glycerol. The protein concentration was determined using a Pierce Micro BCA Protein Assay (Pierce, Rockford, IL, U.S.A.) with BSA as standard.

Consensus double-stranded κ B DNA oligonucleotides (sc-2505; Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.) (5'-AGTTGAGGGGACTTTCCAGGC-3') were labelled with [γ -³²P]ATP (Amersham Life Sciences) using polynucleotide kinase (Boehringer Mannheim) in 50 mM Tris/HCl, pH 8.2, 10 mM MgCl₂, 0.1 mM EDTA, 5 mM dithiothreitol and 0.1 mM spermidine. Unincorporated nucleotides were removed after passage over a Sephadex G-25 spin column (Amersham Pharmacia Biotech). The binding mixture for EMSA was prepared in a total volume of 20 μ l and contained 1 μ g of nuclear extract, 1 μ g of double-stranded non-specific DNA [poly(dI)·poly(dC); Pharmacia] and 5 μ l of ³²P-labelled probe in a final buffer solution of 10 mM Hepes, pH 8.2, 56 mM NaCl, 12.5 mM KCl, 0.15 mM EDTA and 2.5 % glycerol. The reaction mixture was incubated at room temperature for 20 min, before electrophoretic separation in a 6 % (w/v) polyacrylamide gel (Novex, San Diego, CA, U.S.A.) in 0.25 $\times$ TBE buffer (1 $\times$ TBE = 45 mM Tris/borate and 1 mM EDTA). Gels were dried under vacuum and exposed to film (Hyperfilm; Amersham Life Sciences) for 24–48 h. Competition experiments were performed with the addition of an approx. 40-fold excess of non-labelled double-stranded consensus κ B DNA or mutated DNA (5'-AGTTGAGGCGACTTTCC

AGGC-3; sc-2511; Santa Cruz Biotechnologies) prior to the addition of labelled DNA. Supershift EMSA was performed as above, but with the addition of 1 μ g of rabbit antibodies against the p65 subunit of NF- κ B (sc-109X; Santa Cruz Biotechnologies), 1 μ g of rabbit anti-(NF- κ B p50 subunit) antibodies (sc-114X; Santa Cruz Biotechnologies) or 1 μ g of rabbit anti-(von Willebrand Factor) antibodies (Dako A0082) to the EMSA mixtures before incubation for 20 min. Visualized bands from autoradiograms were quantified by densitometry, using a Bio-Rad 700 densitometer.

Statistical analysis

Data are presented as means $\pm$ S.E.M. Differences between groups were compared with one-way ANOVA and Student's *t* test. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

Cytokine-induced expression of VCAM-1 and E-selectin after inhibition of HMG-CoA reductase

TNF- α and IL-1 β strongly induced the expression of VCAM-1 and E-selectin in endothelial cells (Figure 1). However, pretreatment with mevastatin for 18 h, before the addition of TNF- α for 7 h, had a pronounced effect on the expression pattern. First, incubation with mevastatin (1 μ M) reduced the expression of VCAM-1 after stimulation with the cytokine (Figure 1, upper panel). This effect was completely reversed by addition of the product of the HMG-CoA reductase enzyme, mevalonate (100 μ M). In contrast, the cytokine-inducible amount of E-selectin was significantly increased after mevastatin treatment (Figure 1, lower panel). Again, this effect was reversed by co-incubation with mevalonate. Similar results were obtained when

Figure 1 Effects of the inhibition of HMG-CoA reductase on the TNF- α -induced expression of adhesion molecules

The cellular content of VCAM-1 (upper panel) and E-selectin (lower panel) is presented as absorbance values (see the Experimental section) from experiments with endothelial cell cultures after treatment with 1 μ M mevastatin (MSTAT) with or without 200 μ M mevalonate (MEV) for 28 h, and/or TNF- α (1 ng/ml) for the last 7 h as indicated. Values are means $\pm$ S.E.M. for *n* = 12 cultures. Significance: **P* < 0.05 compared with cultures treated with TNF- α but without mevastatin.

Figure 2 Dose-dependent effects of mevastatin on the TNF- α -induced expression of adhesion molecules

The cellular content of VCAM-1 (upper panel) and E-selectin (lower panel) is shown in endothelial cell cultures after treatment with the indicated concentrations of mevastatin and mevalonate for 28 h, and/or TNF- α (1 ng/ml) for the last 7 h as indicated. Values are means $\pm$ S.E.M., $n = 4$ cultures. Significance: * $P < 0.05$ compared with cultures treated with 1 ng/ml TNF- α , without the addition of mevastatin and mevalonate.

IL-1 β was used as inducer of VCAM-1 and E-selectin surface expression (results not shown). As can be seen in Figure 2, it was possible to obtain dose-dependent effects of mevastatin when concentrations greater than 0.1 μ M were used. Concentrations greater than 1 μ M, however, had no increased effects on the amounts of VCAM-1 or E-selectin. In addition, treatment with simvastatin (10 μ M), another HMG-CoA reductase inhibitor, under similar experimental conditions produced the same pattern of results [VCAM-1 expression (arbitrary density units): control, 322 ± 43 ; + TNF/-simvastatin, 831 ± 48 ; + TNF/+simvastatin, 738 ± 35 ($2P < 0.05$ compared with cultures without simvastatin); E-selectin expression: control, 66 ± 8 ; + TNF/-simvastatin, 286 ± 15 ; + TNF/+simvastatin, 366 ± 14 ($2P < 0.05$ compared with cultures without simvastatin); means $\pm$ S.D., $n = 4$].

In a series of control experiments, we found that, within a time frame of 48 h, there were no observable adverse effects of mevastatin (below 3 μ M) on endothelial cell viability and morphology (results not shown).

Effects of mevastatin on VCAM-1 and E-selectin mRNA expression

As shown in Figure 3, TNF- α strongly induced the expression of both VCAM-1 and E-selectin mRNAs, as measured 2 h after cytokine treatment. The induction of mRNAs for these adhesion molecules was significantly inhibited by treatment with mevastatin (1 μ M for 20 h), and this effect was reversed by mevalonate.

Figure 3 Effects of the inhibition of HMG-CoA reductase on TNF- α -induced E-selectin and VCAM-1 mRNA expression

(A) Representative gel showing ethidium bromide-stained, electrophoretically separated, reverse transcriptase-PCR-amplified DNA (with primers for GAPDH, E-selectin and VCAM-1 mRNA) from endothelial cells treated with 1 μ M mevastatin (MSTAT) and/or 100 μ M mevalonate (MEV) for 22 h, with or without 1 ng/ml TNF- α for the last 2 h as indicated. (B) Quantitative data from densitometric gel analysis, presenting results from TNF- α -induced cell cultures treated or not with 1 μ M mevastatin (MSTAT) and 100 μ M mevalonate (MEV) as indicated. Data are presented as means $\pm$ S.E.M., $n = 7$ cultures. Significance: * $P < 0.05$ compared with control (no additions).

Inhibition of TNF- α -induced NF- κ B activation by mevastatin

Next we determined the nuclear activity of the transcription factor NF- κ B in the endothelial cells, employing EMSAs on extracts of isolated nuclei. Treatment with mevastatin (1 μ M for 20 h) inhibited TNF- α -induced nuclear NF- κ B activation, and this inhibition was abolished when cells were co-incubated with mevalonate (Figures 4A and 4B). The specificity of the EMSA procedure was ascertained in control experiments, where non-labelled κ B consensus oligonucleotides, added at 40-fold excess compared with the labelled probe, could compete for binding, whereas mutated oligonucleotides could not (Figure 4C). This

Figure 4 Effects of mevastatin on TNF- α -induced nuclear NF- κ B activity

(A) Representative autoradiogram from an EMSA experiment. The binding activity of 32 P-labelled κ B oligonucleotides in extracts from endothelial cell nuclei is presented after treatment of the endothelial cells with 1 μ M mevastatin (MSTAT) and/or 100 μ M mevalonate (MEV) for 20 h, with TNF- α induction for the last 45 min. (B) Quantitative data from a series of similar NF- κ B EMSAs. Data are presented in densitometric intensity units derived from autoradiograms of 32 P-labelled κ B activity. Values are means $\pm$ S.E.M., $n = 8$ cultures. Significance: * $P < 0.05$ compared with control. (C) Specificity of the NF- κ B EMSA. Nuclear extracts from TNF- α -treated or control endothelial cells, as indicated, were subjected to EMSA with the addition of a 40-fold molar excess of unlabelled κ B consensus (CON) or mutated κ B (MUT) oligonucleotide as shown. (D) Supershift EMSA, with the use of different antibodies added to the EMSA mixtures before electrophoretic separation. Antibodies were against the p65 subunit of NF- κ B (A-p65), the p50 subunit of NF- κ B (A-p50) and von Willebrand Factor (A-vWf).

finding strongly supports the specificity of the observed activity. The content of the NF- κ B complex in the endothelial cells was also assessed by supershift EMSA. These experiments showed that antibodies against the p65 subunit of NF- κ B, when added to the EMSA reaction mixture, were able to bind to the complex formed, creating a large slower-migrating polymer in the polyacrylamide gels (Figure 4D).

Time-dependent expression of VCAM-1 and E-selectin proteins after induction by TNF- α

As shown in Figure 5, the time-dependent expression of VCAM-1 and E-selectin after TNF- α treatment showed different patterns.

Figure 5 Time-dependent variation in the cellular content of VCAM-1 (upper panel) and E-selectin (lower panel) in endothelial cells after induction with TNF- α with (◆) or without (●) pretreatment with mevastatin for 18 h before addition of the cytokine

TNF- α was added at 0 h. Values are means $\pm$ S.E.M., $n = 9$ cultures per data point. Significance: * $P < 0.05$ compared with cultures in the absence of mevastatin.

The cellular amount of VCAM-1 increased after cytokine induction, reaching a maximum after approx. 14 h, after which levels remained stable for at least 24 h post-induction (Figure 5, upper panel). Mevastatin treatment significantly reduced the expression of VCAM-1 at 5 h post-induction and at every time point examined thereafter. In contrast, the time-dependent expression of E-selectin reached a maximum at approx. 15 h after induction with TNF- α , after which it declined (Figure 5, lower panel). When cells were treated with mevastatin, the levels of TNF- α -inducible E-selectin were comparable with the levels in non-treated cells at 2 and 5 h after cytokine activation. However, after 10 h and at subsequent time points, levels of E-selectin were significantly increased (Figure 5, lower panel).

Surface disappearance of VCAM-1 and E-selectin

In a series of experiments we determined the rate of removal of VCAM-1 and E-selectin from the surface of TNF- α -treated cells by measuring the time-dependent disappearance of antibodies bound to VCAM-1 or E-selectin on the cell surface. As seen in Figure 6, VCAM-1 was removed only slowly from the surface, whereas E-selectin molecules were removed rapidly from the cellular surface, with a half-life of approx. 30 min. In contrast with VCAM-1, for which no statistically significant differences were seen, the rate of disappearance of E-selectin was strongly repressed when cells were treated with mevastatin at both 1 μ M and 0.3 μ M (results not shown).

Figure 6 Effects of mevastatin treatment on the rate of disappearance of VCAM-1 (upper panel) and E-selectin (lower panel) from the surface of endothelial cells

Confluent cells were treated with 1 ng/ml TNF- α for 4 h and then incubated with anti-VCAM-1 or anti-E-selectin antibodies. Unbound antibodies were removed by washing after 1 h, and the amount of immunoglobulins left on the surface of the cells was measured in individual cell culture plates, incubated at 37 °C, at various time points thereafter. The amounts of antibodies bound are presented as percentages of the level at the time when excess antibodies were removed. ●, Untreated control cells; ○, cells treated with 1 μ M mevastatin for 20 h before the addition of TNF- α . Values are means $\pm$ S.E.M., $n = 6$ cultures per data point.

Figure 7 Shed extracellular E-selectin, secreted into the conditioned medium of endothelial cells

The cells were treated with 1 μ M mevastatin (MSTAT) for 40 h and/or with 1 ng/ml TNF- α for the last 24 h, as indicated. Values are expressed as means $\pm$ S.E.M., $n = 10$. Significance: * $P < 0.05$ compared with cultures treated with TNF- α , but without mevastatin.

E-selectin shedding

E-selectin can be removed from the surface of cells either by internalization [19] or by proteolytic cleavage (shedding) [21]. As shown in Figure 7, we measured the amount of soluble E-selectin

in conditioned media from cells treated with mevastatin for 42 h and TNF- α for 24 h, and found that TNF- α -induced E-selectin levels increased markedly after mevastatin treatment.

DISCUSSION

In the present paper, we provide evidence for a significant effect of the inhibition of HMG-CoA reductase on the cytokine-inducible expression of the endothelial adhesion molecules VCAM-1 and E-selectin. First, we found that mevastatin treatment reduced the expression of VCAM-1, but augmented the expression of E-selectin, after stimulation with either TNF- α or IL-1 β . This effect was due to specific inhibition of HMG-CoA reductase, since it was reversed by the addition of mevalonate. Moreover, the effect of mevastatin was dose-dependent and could be mimicked by another HMG-CoA reductase inhibitor, simvastatin, at higher concentrations, in accordance with previous studies [9,24]. These results expand previous data indicating that inhibition of HMG-CoA reductase in endothelial cells leads to alterations in the cellular expression of endothelial cell nitric oxide synthase [8,9,23,24], endothelin-1 [9] and fibrinolytic activity [25]. Secondly, we observed that the cytokine-induced increases in the levels of mRNAs for both VCAM-1 and E-selectin were attenuated after mevastatin treatment. This observation was in agreement with the finding of reduced amounts of VCAM-1 protein after statin treatment, but was clearly in conflict with the results indicating increased E-selectin protein expression. The parallel decrease in mRNAs for both adhesion molecules indicated that a common determinant of the expression level of these genes was affected by statin treatment.

One major determinant of the rate of transcription of both VCAM-1 and E-selectin is the activity of NF- κ B [17,26,27]. This transcription factor is present in the cytoplasm of endothelial cells, where it can be activated by a variety of different stimuli, including TNF- α and IL-1 β [28]. The present results suggest that statin treatment attenuates the activation of NF- κ B. Although only a moderate decrease was seen, this observation suggests that the reduced VCAM-1 and E-selectin mRNA levels after mevastatin treatment can be explained, at least partly, by a decrease in the activity of this transcription factor. This observation is in accordance with previous studies of statin-mediated effects in other cell types, which indicated that activation of NF- κ B with either TNF- α in vascular smooth muscle cells [29] or lipopolysaccharide in mesangial cells [30] was reduced after the inhibition of HMG-CoA reductase. Taken together, these findings imply that a part of the cellular signalling machinery which determines the activity of NF- κ B may depend on the presence of mevalonate or mevalonate-derived metabolites. Also, this notion agrees with results showing that statin inhibits Rho activity in endothelial cells [25], since the activity of Rho seems to be necessary for the functioning of NF- κ B [30,31].

The observed attenuation of mRNA expression for VCAM-1 and E-selectin, associated with reduced NF- κ B activation, was in accordance with the observed decrease in VCAM-1-protein expression, but clearly contrasted with the elevated E-selectin expression in statin-treated cells. However, the results from our studies on the time-dependent expression and rate of disappearance of the E-selectin molecules demonstrate that E-selectin is removed within hours of its appearance on the endothelial cell surface. Furthermore, our data show that the rate of removal of E-selectin was depressed after statin treatment. It therefore appears likely that the observed paradoxical increase in cytokine-inducible E-selectin expression, despite decreased mRNA levels after statin treatment, could be due to statin-

induced inhibition of the removal of E-selectin. The observed lack of a difference in E-selectin protein expression at 5 h (Figure 5) was probably due to reduced E-selectin production because of lowered amounts of mRNA (analogous to VCAM-1). Two different pathways determine the disposal of E-selectin from the endothelial cell surface: a large proportion of the molecules undergo endocytosis and subsequent rapid degradation in the lysosomal compartment [18–20], whereas smaller quantities are proteolytically cleaved and secreted into the extracellular space (shedding) [21,32]. We are not able, from the results in the present study, to determine which pathway is influenced by statins. However, our results show that the amount of shed E-selectin after TNF- α induction was increased after statin treatment, indicating that the inhibition of E-selectin removal was not due to attenuation of proteolytic cleavage of the molecule. Consequently it is likely that the decreased rate of disappearance of E-selectin after inhibition of HMG-CoA reductase is due to inhibition of internalization of this adhesion molecule. The mechanisms behind the endocytosis of E-selectin seem to be guided by a novel signalling pathway, dependent on a non-tyrosine-containing motif in the cytoplasmic tail of E-selectin [33] and driven by a tubulin-dependent process [18]. Whether inhibition of HMG-CoA reductase may influence these intracellular mechanisms awaits further study.

Data from a rabbit model of atherosclerosis have shown that statin treatment diminishes neointimal inflammation and attenuates the expression of vascular monocyte chemoattractive protein-1 [34]. However, in addition to modulation of chemotactic stimuli, our results suggest that statin-induced alterations in the binding of monocytes to endothelial cells, due to changes in the expression of adhesion molecules, could also play an important role. Statin-mediated alterations in the cytokine-inducible endothelial expression of VCAM-1 and E-selectin in endothelial cells are indeed likely to have functional consequences. For example, the integrin $\alpha 4 \beta 1$ on monocytes is a ligand for VCAM-1 [14], and a statin-induced decrease in VCAM-1 expression on the endothelial cell surface may reduce monocyte adhesion. On the other hand, the consequences of statin-induced effects on endothelial E-selectin expression are more difficult to predict. First, our knowledge about ligands for E-selectin is incomplete, although sialylated Lewis X-related carbohydrate moieties, present on several candidate proteins, are of importance [12,34,35]. The putative glycoprotein ligands are present not only on monocytes, but also on lymphocyte subsets [36,37], and alterations in the amounts of E-selectin on the surface of endothelial cells could therefore alter the binding capacity for several inflammatory cell types. Secondly, it has been suggested that E-selectin-mediated binding of leucocytes to the endothelium leads to propagation of various intracellular signals, which may alter several endothelial cell functions [38,39]. Statin-induced alterations in the surface expression of both VCAM-1 and E-selectin and in the handling of surface E-selectin could thus lead to altered inflammatory responses.

In conclusion, we have shown that inhibition of HMG-CoA reductase in endothelial cells leads to a decrease in the cytokine-inducible expression of VCAM-1, but increased expression of E-selectin. Moreover, statin treatment results in reduced endothelial levels of both VCAM-1 and E-selectin mRNAs after cytokine activation, at least partly because of reduced activation of NF- κ B. Our data also indicate that increased endothelial E-selectin expression after statin treatment is due to inhibition of the internalization of this adhesion molecule. These observations show, for the first time, that mevalonate-derived metabolites play important regulatory roles in the expression of endothelial cell adhesion molecules. These results could have important im-

plications for the understanding of endothelial function in patients treated with statins.

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