3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Decrease Fas Ligand Expression and Cytotoxicity in Activated Human T Lymphocytes

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Background—HMG-CoA reductase inhibitors reduce cardiovascular mortality, although the mechanisms of action have not been completely elucidated. The presence of T cells and apoptotic cells in atherosclerotic plaques is well established, the reduction of cellular content being a marker of their vulnerability. One of the main mechanisms of cell death activation is the Fas-Fas ligand (FasL) system.

Methods and Results—We studied whether HMG-CoA reductase inhibitors can regulate FasL expression and cytotoxicity in human T cells (Jurkat cells). Activation of Jurkat cells with phorbol esters and ionomycin increased FasL expression, an effect prevented by atorvastatin or simvastatin. Mevalonate and geranylgeranylpyrophosphate but not farnesylpyrophosphate prevented the effect of atorvastatin, indicating that protein geranylation was involved in FasL expression. The C3 exotoxin, which selectively inactivates Rho proteins, also decreased FasL expression on T cells. Overexpression of constitutively active RhoA increased FasL expression in Jurkat cells, and dominant-negative RhoA decreased FasL expression in activated cells, indicating that RhoA is implicated in FasL expression. Atorvastatin also decreased cytotoxic activity of activated Jurkat cells on FasL-sensitive cells. Finally, atorvastatin treatment reduced FasL expression in peripheral blood mononuclear cells and human carotid atherosclerotic plaques.

Conclusions—Atorvastatin regulates FasL expression in T cells, probably because of the inhibition of RhoA prenylation. These results provide novel information by which atorvastatin may regulate the cytotoxic activity of T cells and the number of cells in the atherosclerotic plaque.

Key Words: lymphocytes • apoptosis • molecular biology

Vascular lesions are caused by inflammatory and fibroproliferative responses to injury of the endothelium and vascular smooth muscle cells (VSMCs).1 Atherosclerotic lesion formation involves macrophage and T-cell infiltration of the vessel wall. Activated T cells can induce apoptosis in resident and infiltrating cells of atherosclerotic plaques by the action of the Fas-Fas ligand (FasL) system. Fas is a type I membrane protein belonging to the tumor necrosis factor receptor family that induces a death signal when bound to FasL.2 FasL expression has been reported in activated T cells, natural killer cells, and several nonlymphoid organs, including the testis, small intestine, and kidney.3 Fas is expressed ubiquitously, and expression of Fas and FasL has been detected in the diseased vessel wall; it has been proposed that Fas-mediated apoptosis is a feature of atherogenesis4 and atherosclerotic plaque instability.5

FasL expression in activated T cells implicates 2 different signals: activation of the protein kinase C pathway and release of intracellular Ca2+ stores, subsequent Ca2+ influx, and activation of the serine phosphatase calcineurin.8 These signals can be mimicked by treatment of cells with the pharmacological agents phorbol 12-myristate 13-acetate (PMA) and ionomycin. These agents induce activation of different transcription factors involved in the regulation of FasL expression in T cells.9 FasL expression in T lymphocytes contributes to their cytotoxic function; it mediates the elimination of autoreactive peripheral T cells10 and participates in the activation-induced cell death.

HMG-CoA reductase inhibitors (statins) have been shown to be efficacious in reducing cardiovascular events.11,12 Although the salutary effects of these agents may be explained by their beneficial actions on lipid profile, increasing evidence suggests that they exhibit effects unrelated to lipid reduction.13 The mevalonate pathway inhibition affects several metabolites implicated in the regulation of different cell functions. GTP-binding proteins are prenylated by...
mevalonate-derived isoprenoid compounds, such as farnesy1pyrophosphate (FPP) and geranylgerany1pyrophosphate (GGPP). The attachment of an isoprenoid residue to the small G proteins is necessary for their anchorage to cell membranes and full functionality. Small G proteins include various proteins, such as Ras, Rac, and Rho. These proteins are involved in different functions of the cells, such as gene expression, organization of the actin cytoskeleton, membrane trafficking, programmed cell death, proliferation, and transformation.

Because activated T lymphocytes are present in atherosclerotic lesions and statins are used for the treatment of atherosclerosis, we explored the effect of atorvastatin on FasL expression in activated T cells. Moreover, we analyzed the cytotoxicity of T cells on target cells and the regulation of this function by atorvastatin.

**Methods**

**Reagents**

RPMI-1640, penicillin, streptomycin, and trypsin-EDTA were obtained from BioWhittaker. FBS was from Gibco. Atorvastatin (sodium salt, dissolved in Tris-borate, pH 8) was from Pfizer. C3 exoenzyme was from Calbiochem, and FuGENE 6 transfection reagent was from Roche. The remaining reagents were obtained from Sigma unless specified otherwise.

**Cell Cultures**

Fas-sensitive A20 and Fas-resistant A20R murine B lymphoma cells were a gift from Dr Jürg Tschopp (Lausanne University, Switzerland). A20R cells were generated by continuous culture in the presence of FasL and have downregulated Fas receptor expression. Jurkat cells (ATCC) and A20 and A20R cells were cultured in RPMI-1640 supplemented with 10% decomplemented FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO₂.

**Northern Blot Analysis**

Total RNA (20 μg) was separated in 1% agarose gels containing 2.3% formaldehyde and transferred to nylon membranes (GeneScreen Plus; NEN) as described previously. The human probes were prepared by reverse transcription–polymerase chain reaction of total RNA isolated from Jurkat cells. The following primers were used for human FasL: antisense, 5'-TCAGCAGCTGTTAAGATTG-3' and sense, 5'-CTGGAATGGGAAGACAC-3', and GAPDH: antisense, 5'-AATTGACATCTGACCAACACAA-3' and sense, 5'-ATCTGTGTTTCTTACGAGTGC-3'. Quantification was performed with Image-Quant Software (Molecular Dynamics).

**Cell Transfections**

pcDNA3B (empty vector), wild-type RhoA (pcDNA3-wtRhoA), constitutively active Q63L Rho A, and dominant-negative N19 RhoA were a gift from Dr Piero Crespo (Instituto de Investigaciones Biomédicas, Madrid, Spain). Reporter fusion plasmid that contained 1.2 kb FasL promoter and the firefly luciferase gene (pFasL-LUC) was from Dr Douglas Green (La Jolla Institute for Allergy and Immunology, San Diego, Calif). Transfection was carried out by the FuGENE 6 method according to the manufacturer’s instructions. Jurkat cells were maintained in RPMI-1640 supplemented with 10% FBS. Cells were seeded at a density of 5 × 10⁵ cells per well. The next day, transfections were performed using 3 μg of the human FasL promoter and 0.5 μg of Renilla luciferase reporter vector pRL-TK. Twenty-four hours after transfection, cells were activated with PMA/ionomycin for another 18 hours. For experiments of RhoA mutant overexpression, cells were cotransfected with FasL promoter by pRL-TK vectors along with the indicated RhoA expression vectors. Cells were harvested and lysed in 100 μL of lysis buffer. Finally, Renilla and firefly luminescences were measured.

**Western Blot**

Cells from different experimental conditions were collected and pelleted. Western blots were performed as previously described. The blots were incubated with polyclonal anti-FasL antibody (sc-834; Santa Cruz) or polyclonal anti-RhoA antibody (sc-179; Santa Cruz).

**FasL Bioactivity**

FasL killing activity was assessed by incubating 1 × 10⁵ to 1 × 10⁶ effector Jurkat cells with 1 × 10¹⁰ bromodeoxyuridine-labeled target A20 or A20R cells. Higher effector-to-target ratios were abandoned because Jurkat cells became overconfluent at effector-to-target ratios > 1. The release of bromodeoxyuridine was determined after 20 hours of coincubation by use of a cellular DNA fragmentation ELISA (Boehringer Mannheim). As a positive control, Jurkat cells were replaced by 10 ng/mL recombinant FasL. For the calculation of the percentage of dead cells, cell death induced in Fas-sensitive cells by recombinant FasL was considered to be 100%. Results are expressed as mean ± SEM of independent experiments each consisting of quadruplicate wells.

**Patients**

Ten patients with carotid atherosclerosis were randomized to receive atorvastatin 80 mg/d (n = 5) or usual care (n = 5) during 6 weeks before they underwent carotid endarterectomy at Hospital Clínico San Carlos, Madrid, Spain (Prof J. Serrano). Informed consent was obtained before enrollment in all cases. The study was approved by the Hospital’s ethics committee in accordance with the institutional guidelines.

**Isolation of Peripheral Blood Mononuclear Cells**

Blood samples were diluted 1:1 in PBS, and cells were separated in 5 mL Ficoll gradient (lymphocyte isolation solution, Rafer) by centrifugation at 2000g for 30 minutes. Peripheral blood mononuclear cells were collected, and RNA was extracted. Approximately 95% of the cells are mononuclear cells (flow cytometry, not shown).

**Immunohistochemistry**

Specimens were collected and stored in parformaldehyde for 24 hours and later in ethanol until they were embedded in paraffin. Paraffin-embedded carotid plaques were cross-sectioned into 4-μm-thick pieces, dewaxed, and rehydrated. Anti-FasL antibody was applied overnight. Secondary antibody was applied for 1 hour at 1:200 dilution. Then, ABComplex/horseradish peroxidase (Dako) was applied overnight. Secondary antibody was applied for 1 hour at 1:200 dilution. Then, ABCComplex/horseradish peroxidase (Dako) was added for 30 minutes. The sections were stained for 10 minutes with 3,3'-diaminobenzidine (Dako), counterstained with hematoxylin, and mounted in Pertex (Medite). In each experiment, negative controls using the corresponding IgG were included to check for nonspecific staining.

**Quantification**

Computer-assisted morphometric analysis was performed with the Olympus semiautomatic image analysis system with Micro Image software (version 1.0 for Windows). Preparations were digitized via an Olympus microscope (BH-2) at 400 magnification connected to a CCD video camera. Results are expressed as percentage of positive staining per square millimeter.

**Statistical Analysis**

All experiments were performed at least 3 times. Statistical analysis was performed with GraphPAD InStat (GraphPAD Software). Results are expressed as mean ± SEM and were analyzed by ANOVA and Student’s t test (differences were considered significant at a value of P < 0.05).

**Results**

**Atorvastatin Downregulates FasL Expression in Activated T Cells**

As in previous studies, treatment with PMA/ionomycin induced FasL mRNA in Jurkat cells in a time-dependent
manner, peaking at 4 hours (data not shown). To study whether treatment with atorvastatin could regulate FasL mRNA expression induced by PMA plus ionomycin, Jurkat cells were preincubated for 2 hours with different concentrations of atorvastatin and then incubated in the presence of PMA/ionomycin for 4 hours. We showed that atorvastatin treatment decreases FasL mRNA expression in a dose-dependent manner (Figure 1A). To assess the effect of atorvastatin on the promoter activity of FasL gene, Jurkat cells were transiently transfected with a luciferase reporter construct containing the 1.2-kb FasL promoter. When Jurkat cells were activated with PMA/ionomycin, FasL reporter activity was increased and treatment with atorvastatin significantly reduced FasL reporter activity (4.2 ± 0.5 control; 19.8 ± 1.7 PMA/ionomycin; 12.3 ± 2 atorvastatin 10 μmol/L; \( P < 0.05 \) control versus PMA/ionomycin and PMA/ionomycin versus atorvastatin) (Figure 1B). Similarly, treatment with 2 different statins, atorvastatin (Fig 1C) and simvastatin (not shown), also decreased FasL protein expression induced by PMA/ionomycin in a dose-dependent manner.

Mevalonate and GGPP Prevent the Effect of Atorvastatin on FasL Expression
Mevalonate is the metabolite that is directly synthesized by the HMG-CoA reductase. We analyzed the effect of mevalonate on FasL expression. Jurkat cells were preincubated for 2 hours with atorvastatin (10 μmol/L) in the presence of mevalonate (100 μmol/L) and then incubated with PMA/ionomycin for 4 hours. Mevalonate reversed the inhibitory effect of atorvastatin on FasL mRNA expression (Figure 2A). Moreover, Jurkat cells were transiently transfected with FasL promoter in the presence of PMA/ionomycin and exposed to atorvastatin plus mevalonate. As shown in Figure 2B, mevalonate reversed the inhibitory effect of atorvastatin on FasL promoter activity (22.3 ± 2.7 mevalonate; 10.2 ± 1.1 atorvastatin; \( P < 0.05 \)). Mevalonate also reversed the effect of atorvastatin or simvastatin in FasL protein expression (Figure 1C).

Furthermore, we analyzed the importance of isoprenoids related to the mevalonate pathway in the FasL expression and observed that GGPP (5 μmol/L) but not FPP (5 μmol/L) reversed the inhibitory effect of atorvastatin on FasL mRNA expression (Figure 2A). Only GGPP was able to reverse the FasL promoter activity reduced by atorvastatin (17.4 ± 3.1 GGPP; \( P < 0.05 \)) (Figure 2B). These results indicate that GGPP, probably through geranylgeranylation of proteins, can be involved in the regulation of FasL expression.

C3 Exoenzyme Downregulates FasL Expression in Activated T Cells
GGPP is involved in posttranscriptional modification of small G proteins. In this sense, Rho proteins are known to be geranylgeranylated. These proteins are inactivated by the action of the ADP-ribosyltransferase C3 toxin from Clostridium botulinum. As shown in Figure 3A, treatment of Jurkat cells with C3 exoenzyme decreases FasL mRNA expression and promoter activity elicited by PMA/ionomycin in a dose-dependent manner (26.4 ± 1.2 PMA/ionomycin; 12.8 ± 2.4 C3 exoenzyme 5 μg/mL; \( P < 0.05 \)). These results suggest that Rho proteins can regulate FasL expression in Jurkat cells.

Figure 1.

**Figure 1.** Atorvastatin downregulates FasL mRNA expression and reduces human FasL (hFasL) promoter activity. A, Northern blot showing effect of atorvastatin (Atv) on FasL mRNA expression. B, Cells were transfected with pFasL-LUC plus pRL-TK and treated with PMA/ionomycin. C, Immunoblot showing effect of atorvastatin or D, simvastatin alone or in combination with mevalonate (MVA) on FasL expression.
RhoA Can Regulate FasL Expression
To evaluate the involvement of Rho proteins in FasL expression, we analyzed the effect of different expression vectors that coded for wild-type RhoA, constitutively active RhoA (Q63L-RhoA), and dominant-negative RhoA (N19 RhoA) on FasL mRNA expression and promoter activity. As shown in Figure 3B, constitutively active RhoA significantly increased FasL mRNA expression and promoter activity (16.4 ± 5.8 Q63LRhoA versus 6.9 ± 3.2 empty vector; \(P < 0.05\)). In addition, dominant-negative RhoA significantly prevented FasL mRNA expression and promoter activity elicited by PMA/ionomycin (18.6 ± 1.2 N19 RhoA versus 25.7 ± 4.5 empty vector plus PMA/ionomycin; \(P < 0.05\)). These data support the proposition that RhoA is specifically involved in the control of FasL expression. Furthermore, atorvastatin decreased the presence of RhoA in the membrane with reciprocal increases in the cytosol (Figure 4).

Atorvastatin Decreases Cytotoxic Capacity of Activated T Cells
To study whether diminution of FasL expression induced by atorvastatin can regulate the cytolytic activity of activated T cells, Fas-sensitive cells (A20) were cocultured with Jurkat cells in the presence of atorvastatin. As shown in Figure 5A, apoptosis induced by activated Jurkat cells on A20 cells was prevented by atorvastatin. This effect was also reversed by mevalonate, indicating the direct effect of the mevalonate pathway inhibition on the cytotoxicity of activated T cells. Furthermore, constitutively active RhoA significantly increased cell death of A20 cells, and dominant-negative RhoA prevented, at least in part, apoptosis induced by activated Jurkat cells on target cells (Figure 5C). In contrast, apoptosis induced by activated Jurkat cells was not present in Fas-resistant A20R cells under the same culture conditions, and apoptosis in A20 cells was reduced by 68% when neutralizing anti-FasL antibodies were added to the coculture medium (Figure 5B). These data indicate that FasL participates in the apoptosis induced by Jurkat cells on target cells.

Atorvastatin Decreased FasL Expression in Human Carotid Atherosclerotic Plaques
Finally, in a preliminary study, 10 patients with carotid atherosclerosis were randomized to receive atorvastatin 80 mg/d (n=5) or usual care (n=5) for 6 weeks before they underwent carotid endarterectomy (Table). We observed that treatment with atorvastatin decreased FasL expression in atherosclerotic plaques (Figure 6A). FasL expression did colocalize with macrophages, T lymphocytes, and, to a lesser extent, VSMCs in atherosclerotic plaques (data not shown). In addition, FasL mRNA expression was reduced in peripheral blood mononuclear cells from the same patients treated with atorvastatin (Figure 6B), indicating that atorvastatin can regulate the expression of this protein in vivo.

Discussion
FasL is a proapoptotic protein expressed by inflammatory cells (macrophages and T cells). It has been localized in...
atherosclerotic plaques and can contribute to the apoptosis of resident and infiltrating cells of lesions. In this work, we observed that treatment with the HMG-CoA reductase inhibitor atorvastatin downregulated FasL expression in activated T cells. Atorvastatin treatment did not change the half-life of FasL mRNA (data not shown), indicating that regulation of FasL mRNA expression by atorvastatin was at the transcriptional level. Downregulation of FasL expression induced by atorvastatin was reversed by coincubation with mevalonate, suggesting that isoprenoids from the cholesterol pathway downstream of mevalonate could be important for the FasL expression. GGPP but not FPP prevented the downregulation induced by atorvastatin, suggesting the requirement of an intermediate product modified by geranylgeranylation in the FasL expression. The reduction of FasL expression was also observed in the presence of Clostridium botulinum C3 exotoxin, which selectively inhibits the activation of Rho proteins, but not other small G proteins, such as Cdc42 or Rac. These results indicate that the reduction of FasL expression by atorvastatin is closely related to Rho inhibition. Transfection experiments with expression vectors coding for different forms of RhoA further indicated the participation of this small G protein in the control of FasL expression. Constitutively active RhoA increased FasL expression in T cells, and dominant-negative RhoA decreased the upregulation induced by PMA and ionomycin. These data strongly implicate RhoA in the regulation of FasL expression in T cells. However, we cannot completely exclude the possibility that other GTP-binding proteins can also regulate FasL expression in T lymphocytes.

The precise mechanism by which RhoA regulates FasL expression in T cells remains undefined. Different transcription factors, including nuclear factor (NF)-κB, are implicated in the regulation of FasL expression. Multiple pathways coexist for the activation of NF-κB, and in some of them, small G proteins, such as RhoA, Cdc42, and Rac-1, are critical elements involved in its transcriptional activity. In this sense, treatment with atorvastatin decreased NF-κB in an experimental model of atherosclerosis in rabbits and in cultured VSMCs and macrophages. Furthermore, we have observed that atorvastatin decreases NF-κB activation induced by PMA/ionomycin in Jurkat cells and that parthenolide, an inhibitor of NF-κB activation, also decreases FasL expression and cytotoxicity in activated T cells (data not shown). In addition, different effects of statins mediated by Rho inhibition have been reported. For example, HMG-CoA reductase inhibitors increase fibrinolytic activity and inhibit preproendothelin-1 gene transcription in endothelial cells. Statins also attenuate proliferation of VSMCs.
Activated Jurkat cells cause the death of Fas-bearing cells, such as nonactivated Jurkat cells, and also in the mouse leukemia L1210 cell line. The physiological importance of FasL downregulation by atorvastatin in activated T lymphocytes is because treatment with this HMG-CoA reductase inhibitor decreased cytotoxic activity of T cells in target cells. The implication of small G proteins in the cytotoxicity caused by lymphocytes has been reported inhibiting Rho activation by use of the C3 exoenzyme. These data correlated with the inhibition of FasL expression in activated T cells induced by C3 exoenzyme observed in our experimental conditions. Furthermore, we noted that Jurkat cells transfected with constitutively active RhoA increased its cytotoxic activity, whereas dominant-negative RhoA partially prevented the apoptosis induced by activated T cells. Moreover, in A20 cells, apoptosis was inhibited by neutralizing antibodies to FasL, indicating the requirement of membrane-bound FasL in apoptosis induced by activated T cells. The effect of atorvastatin on FasL expression is related to the inhibition of isoprenylation, indicating that other statins may have a similar effect on the expression of this protein. In this sense, we have observed that simvastatin also decreased FasL expression in activated T cells.

Figure 5. Atorvastatin decreases cytotoxic activity of Jurkat cells. A, A20 or A20R cells labeled with bromodeoxyuridine (BrdU) were cocultured with Jurkat cells preincubated in different experimental conditions. BrdU release by A20 or A20R apoptotic cells to culture was measured by cellular DNA fragmentation ELISA. *P<0.05 vs control; †P<0.05 vs PMA/ionomycin; ‡P<0.05 vs atorvastatin plus PMA/ionomycin. B, Neutralizing anti-FasL antibodies decrease apoptosis induced by activated Jurkat cells on A20 cells. *P<0.05 vs IgG. C, Cells were transfected with vectors coding for different forms of RhoA for 24 hours. In addition, cells were treated with PMA+ionomycin for 18 hours in presence of empty vector or N19RhoA. Then, A20 or A20R cells labeled with BrdU were cocultured with Jurkat cells in different experimental conditions.
FasL expression may have deleterious consequences in atherosclerotic lesions. On one hand, FasL participates in activation of apoptosis in Fas-bearing cells. Geng et al. reported that Fas is expressed in atherosclerotic lesions. Interaction of Fas-expressing cells with its ligand can induce apoptosis in these cells, contributing to the reduction of the cell number of the lesions. The diminution of FasL expression in T cells can decrease apoptosis in atherosclerotic lesions. In this sense, we have observed that treatment with atorvastatin decreases FasL expression in carotid atherosclerotic plaques and in peripheral blood mononuclear cells and that treatment with pravastatin, another HMG-CoA reductase inhibitor, decreased apoptotic cells in human carotid plaques. On the other hand, recent reports suggest a possible implication of FasL in the recruitment of inflammatory cells to the lesions. Overexpression of FasL in arteries of hypercholesterolemic rabbits can accelerate atherosclerosis lesion formation. In addition, induction of apoptosis by overexpression of Fas-associated death domain protein upregulates the expression of chemoattractant proteins, such as MCP-1 and IL-8, in carotid arteries of rats. In this sense, the treatment with statins has also been related to a reduction of the systemic inflammation.

Finally, the concentration of statins used in our in vitro studies is in excess of the therapeutic concentration ranges (0.5 to 5 μmol/L). However, atorvastatin also reduces FasL expression in atherosclerotic plaques, indicating that our in vitro results can be related to the in vivo situations.

In conclusion, atorvastatin decreases FasL expression in activated T cells and prevents T-cell cytotoxicity. The reduction of the cytolytic effect of T lymphocytes on target cells may contribute to the maintenance of cellularity in the lesions, favoring the stability of the plaque. In addition, the effect of atorvastatin on T-cell activation suggests a potential immunomodulator effect of statins.

Acknowledgments
This study was supported by grants from Fundación Ramón Areces, Ministerio de Ciencia y Tecnología (SAF2001-0717), Instituto Reina Sofia, Mapfre Medicina, and Pfizer-Spain.

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_Circulation_. 2003;108:1506-1513; originally published online September 2, 2003;
doi: 10.1161/01.CIR.0000089086.48617.2B
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/108/12/1506

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