Statins Stimulate *In Vitro* Membrane FasL Expression and Lymphocyte Apoptosis through RhoA/ROCK Pathway in Murine Melanoma Cells[1,2]

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Abstract

The capacity of FasL molecules expressed on melanoma cells to induce lymphocyte apoptosis contributes to either antitumor immune response or escape depending on their expression level. Little is known, however, about the mechanisms regulating FasL protein expression. Using the murine B16F10 melanoma model weakly positive for FasL, we demonstrated that *in vitro* treatment with statins, inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, enhances membrane FasL expression. C3 exotoxin and the geranylgeranyl transferase I inhibitor GGTI-298, but not the farnesyl transferase inhibitor FTI-277, mimic this effect. The capacity of GGTI-298 and C3 exotoxin to inhibit RhoA activity prompted us to investigate the implication of RhoA in FasL expression. Inhibition of RhoA expression by small interfering RNA (siRNA) increased membrane FasL expression, whereas overexpression of constitutively active RhoA following transfection of RhoAV14 plasmid decreased it. Moreover, the inhibition of a RhoA downstream effector p160ROCK also induced this FasL overexpression. We conclude that the RhoA/ROCK pathway negatively regulates membrane FasL expression in these melanoma cells. Furthermore, we have shown that B16F10 cells, through the RhoA/ROCK pathway, promote *in vitro* apoptosis of Fas-sensitive A20 lymphoma cells. Our results suggest that RhoA/ROCK inhibition could be an interesting target to control FasL expression and lymphocyte apoptosis induced by melanoma cells.

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Introduction

APO-1/Fas, now called CD95, triggers apoptosis on binding of its ligand (CD95L/FasL) or specific agonist antibodies [1,2]. Fas have three cysteine-rich extracellular domains and an intracellular death domain essential for signaling. Two different CD95 receptor–induced apoptotic pathways are known to exist, both leading to subsequent caspase-3 activation [3]. Widely expressed in both normal and neoplastic cells [2,4–6], the expression of Fas does not necessarily predict susceptibility to FasL-induced apoptosis. Fas–FasL interaction is involved in normal immune development and homeostasis [7,8] and also in the maintenance of immune privilege in certain organs such as the eye, the central nervous system, and the testis [9]. Although expressing Fas, cells in these organs show resistance to Fas-mediated apoptosis. This Fas-resistance mechanism has also been described in several tumor models contributing to the formation and growth of *neoplasia* [10].

In cancer patients, clinical morbidity and mortality is often associated with the acquired insensitivity of tumor cells to immunologic detection or elimination [11]. FasL expression by tumor cells represents one possible mechanism responsible for this immunologic escape, allowing cells to *counterattack* and induce apoptosis in Fas-expressing cytotoxic T lymphocytes and natural killer cells, infiltrating the tumor or the tumor microenvironment. The expression of FasL on many human tumors, generally associated with poor prognosis supports this hypothesis. However, the apoptosis-inducing capacity of the FasL molecules expressed on melanoma cells and, more generally, the biologic significance of the Fas–FasL implication in human tumors remains a complex matter of debate [12,13]. Indeed, conflicting findings have suggested that tumors use FasL either to counterattack tumor-infiltrating cytotoxic cells or to trigger a neutrophil-mediated inflammatory response and tumor rejection [14,15]. Recently, it has been shown that the effect of FasL may depend on its expression level [14]. At high levels, FasL triggers tumor rejection by both a potent neutrophil-mediated local inflammation response and the start of a T-cell–dependent tumor-specific memory. In contrast, at low levels, FasL enhances tumor growth by counterattacking

Abbreviations: FTI, farnesyl transferase inhibitor; GGTI, geranylgeranyl transferase inhibitor; GST, glutathione *S*-transferase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; RT-PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering RNA

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antitumor effector lymphocytes. Altogether, these observations suggest that the increase of FasL expression on tumor cells could be an interesting goal in cancer immune therapy. However, in all these tumor models, little is known about the mechanisms regulating FasL protein expression.

In the present study, we have investigated the capacity of statins and other Rho protein inhibitors to modulate *in vitro* membrane FasL expression. Statins seemed suitable pharmacological agents with their common use in cardiovascular disease prevention and recent potential as new anticancer agents. Based on preclinical studies on several animal tumor models, such as melanoma, mammary carcinoma, pancreatic adenocarcinoma, fibrosarcoma, glioma, neuroblastoma, and lymphoma, statins have demonstrated antiproliferative, proapoptotic, antiinvasive, and radiosensitizing properties [16,17]. However, as we previously reported in the B16F10 murine melanoma model, statins inhibit Rho GTPases and modify protein expression on tumor membranes in a manner favoring a T-cell–dependent tumor-specific immune response. Indeed, statins induced an overexpression of interferon-γ–induced major histocompatibility complex class I antigens and expression of CD80 and CD86 costimulatory molecules [18]. We chose the B16F10 melanoma model for its spontaneous weak expression of membrane FasL [19] to study the effect of statins and other inhibitors of Rho proteins on FasL expression.

Rho GTPases form a subgroup of the Ras superfamily of GTP binding proteins that regulate a wide spectrum of cellular functions. Activated Rho GTPases interact with intracellular target proteins or effectors to trigger a wide variety of cellular responses, including the reorganization of the actin cytoskeleton, cell cycle progression, cell death, adhesion, metastasis, and gene transcription [20–28]. Rho proteins are posttranslationally prenylated by mevalonate-derived isoprenoid compounds, such as farnesylpyrophosphate and geranylgeranylpyrophosphate on the C-terminal end of the protein. The attachment of such isoprenoid residues is necessary for their anchorage to cell membranes and full functionality [29]. This isoprenylation can be inhibited by several inhibitors of the mevalonate pathway such as the statins or by isoprenyl transferase inhibitors such as farnesyl transferase inhibitor (FTI) or geranylgeranyl transferase inhibitor (GGTI).

Here we demonstrate, in the B16F10 tumor model, that RhoA proteins downregulate membrane FasL expression and, consequently, the possibility of increasing *in vitro* this expression by pharmacological treatments with RhoA inhibitors such as statins. Moreover, B16F10 melanoma cells overexpressing membrane FasL after such treatments were able to induce *in vitro* the apoptosis of cocultivated Fas-sensitive B lymphocytes.

Materials and Methods

Cell Lines

The murine melanoma cell line B16F10 and the murine B cell lymphoma A20 were maintained by serial passages in complete culture medium composed of RPMI 1640, 1% L-glutamine, 1% penicillin/streptomycin, and 10% heat-inactivated fetal calf serum (Gibco BRL, Invitrogen, Cergy-Pontoise, France).

In Vitro *Treatment of Tumor Cells*

Tumor cells were treated *in vitro* by addition of different components to the complete culture medium. C3 exotoxin was produced in the laboratory and used at 10 or 20 μg/ml. FTI-277 was used at 10 or 20 μM (Calbiochem, San Diego, CA). GGTI-298 was used at 10 or 20 μM (Calbiochem). We used 5 μM atorvastatin and 5 mM mevalonolactone (Sigma, St. Louis, MO), 0.5 μM of the p160ROCK inhibitor H1152 (Alexis, Lausen, Switzerland), and 100 μM of the caspase inhibitor Z-VAD-Fmk (Alexis). Anti-Fas (Jo-2) (50 μg/ml) (BD Biosciences, San Jose, CA) was used to induce A20 lymphoma apoptosis and an Armenian hamster IgG2 (50 μg/ml) (BD Biosciences) was used as a control. Anti-FasL antibody (50 μg/ml) (c178) (Santa Cruz Biotechnology, Santa Cruz, CA) was used to block Fas/FasL interaction and a rabbit IgG (50 μg/ml) was used as a control.

Flow Cytometry Analysis

FasL and Fas membrane expressions were analyzed using phycoerythrin- or fluorescein isothiocyanate-conjugated specific antibodies (BD Biosciences). Apoptosis of Fas-sensitive A20 lymphoma cells was detected by cell cycle analysis, according to the protocol of Vindelov and Christensen [30], and with a fluorescein isothiocyanate-conjugated anti–active caspase-3 antibody (BD Biosciences) after permeabilization and fixation of the tumor cells with the Fix and Perm kit (BD Biosciences). All stainings were performed according to the manufacturer's instructions and were analyzed on a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ).

RhoAV14 Overexpression

The coding region of RhoAV14 was subcloned into a pSG5 vector. An empty pSG5 vector was used as a control. B16F10 cells, 1×10^5, were transfected with 2 μg of pSG5-RhoAV14 (B16F10RhoAV14) construct or empty vector (B16F10Mock) using Jet PEI (Qbiogen, Illkirch, France).

Small Interfering RNA (siRNA) Treatment

SiRNA against RhoA were designed using the criteria developed by Simpson et al. [31], synthesized as synthetic oligonucleotides (Eurogentec, Angers, France), and annealed to form a short double-stranded RNA with a 3′-dithymidine overhang: siRNA RhoA, 5′–GAA GUC AAG CAU UUC UGU CdTdT–3. As a siRNA control, we used a nonspecific control provided by Pharmacon Research (Attica, Greece). Transfections of siRNA (20 nM) were performed using Oligofectamine and Opti-MEM media (Invitrogen) according to the manufacturer's instructions on cells at 30% to 50% confluency in 60-mm cell culture dishes followed by a 72-hour incubation period before experiments.

Immunoblot Analysis of Proteins

Cells were lysed in radioimmune precipitation buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM dithiothreitol, 10 mM *p*-nitrophenyl phosphate, 2 mM Na_3VO_4, 20 mM NaF, and 1× protease inhibitor mixture) for 30 minutes on ice, and cleared by centrifugation (14,000 rpm for 10 minutes). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Proteins were resolved on SDS-PAGE with the appropriate acrylamide concentration (10%). Detections were made using antibodies against FasL (c178), RhoA (119), Rap1a (121), and unprenylated Rap1a (C-17) (Santa Cruz Biotechnology); Hdj2 antibody (KA2A5.6) was obtained from NeoMarkers (Interchim, Montluçon, France) and antiactin was from Chemicon (Billerica, MA).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Detection of FasL mRNA Transcripts

The expression of FasL mRNA was analyzed by RT-PCR. Total RNA was isolated using RNA easy kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. One microgram of total RNA was used for first-strand cDNA synthesis in iScript cDNA Synthesis Kit (Bio-Rad, Marnes la Coquette, France). Then, RT-PCR was conducted as previously described [19]; briefly, FasL cDNA were amplified by 35 cycles of PCR using the intron-spanning primers described by Ryan et al. [19]: forward 5′–CGGTG-GTATITITCATGGTTCTGG–3′ and reverse 5′–CTTGTGGTTTAGGGGCTGGTT-GTT–3′ (380) and compared to β-actin. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

RhoA Activity Assay

RhoA activity was assayed using the RhoA activation assay of Ren and Schwartz [32]. Briefly, the Rho binding domain of rothekin (TRBD), an effector of Rho proteins that selectively binds to the GTP-loaded form, was expressed as a recombinant fusion with glutathione *S*-transferase (GST) in *Escherichia coli* and purified through binding to glutathione–Sepharose beads. At various times after exposure, cells were lysed by scraping on ice and by vigorous mixing in lysis buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 10 mM dithiothreitol, 10 mM *p*-nitrophenyl phosphate, 2 mM Na_3VO_4, 20 mM NaF, and 1× protease inhibitor mixture (Sigma)). After preclearing by centrifugation (12,500 rpm for 5 minutes), the lysates were combined with 30 μl GST–TRBD beads and rotated for 45 minutes at 4°C. An aliquot from each lysate was removed as a control for equivalent input into the assay. Beads were washed twice with ice-cold wash buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, and 1% Triton X-100). Bound proteins were eluted from the beads with SDS-PAGE sample buffer at 95°C. RhoA proteins were analyzed by immunoblot.

Visualization of Actin Cytoskeleton By Fluorescence Microscopy

At day 0, B16F10 cells were seeded onto glass coverslips in six-well plates to obtain 60% confluence on day 2. On day 1, the cells were treated for 24 hours with 0.5 μM H1152. On day 2, cells were fixed with 3% paraformaldehyde/PBS for 20 minutes and then permeabilized with 0.1% Triton X-100/PBS for 5 minutes. To visualize the actin fibers, the coverslips were incubated with tetramethylrhodamine isothiocyanate-labeled phalloidin (Molecular Probes, Invitrogen) for 30 minutes at room temperature. The cells were viewed on a Zeiss Axiophot microscope (Zeiss, New York, NY) and pictures were taken with a Princeton Camera (Princeton, Scientific Instruments, Monmouth Junction, NJ).

Cocultures

A20 cells, 1×10^5, were cocultivated in complete culture medium with 1 to 3×10^5 B16F10 melanoma cells or with 1×10^5 B16F10-transfected or inhibitor-treated cells. The percentage (%) of A20 cells in the sub–G_1-peak (% subG_1) and the percentage of A20 cells expressing the caspase-3 active form (% active caspase-3) was analyzed by flow cytometry after 24 and 72 hours of coculture, respectively.

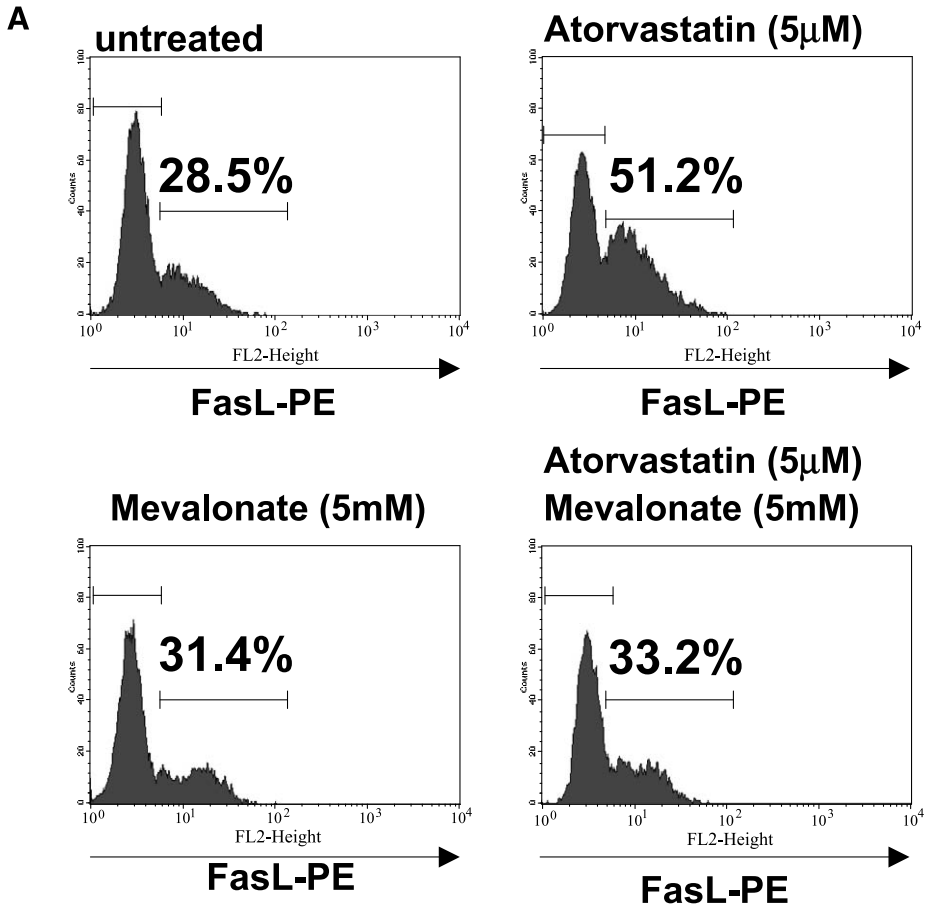
Statistical Analysis

All experiments were performed three or six times. Results are expressed as mean ± SEM and were analyzed by Student's *t* test (differences were considered significant at a P value of $< .05$).

Results

Atorvastatin Upregulates FasL Expression on the B16F10 Cell Membrane

We previously described that several pharmacological inhibitors of protein prenylation could modulate melanoma membrane expression of molecules implicated in the immune response [18]. In the mevalonate pathway, statins are inhibitors of 3-hydroxy-3-methylgutaryl CoA (HMG-CoA) reductase and thus of protein prenylation. We tested the role of atorvastatin on FasL expression in melanoma cells using the weakly FasL-positive murine melanoma cell line B16F10 [19]. Membrane and total FasL expressions were measured by cytofluorometric and Western blot analysis, respectively. Treatment of B16F10 cells for 48 hours with atorvastatin (5 μM) enhanced the percentage of melanoma cells expressing FasL on the membrane (Figure 1 *A*) compared to the untreated control. This effect was completely reversed by mevalonate (5 mM), the first product of HMG-CoA reductase (Figure 1 *A*). The analysis of three independent experiments confirmed that statin treatment statistically increases the percentage of FasL$^+$ B16F10 cells ($P < .05$) (Figure 1 *B*). To determine whether total FasL protein is also altered by statin treatment, we treated B16F10 cells for 48 hours with atorvastatin and/or mevalonate and analyzed whole cell lysates for FasL expression using Western blot. Results showed no difference in total FasL expression between statin-treated and control cells (Figure 1 *C*), showing that statin activity is restricted to membrane FasL localization.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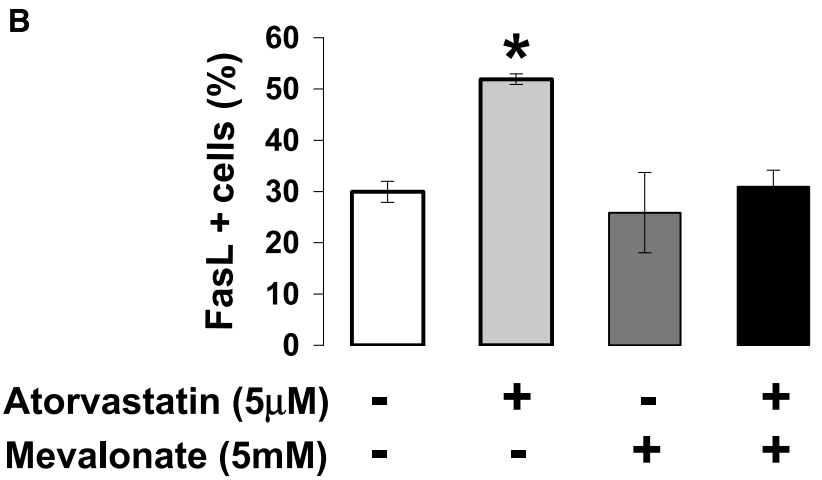
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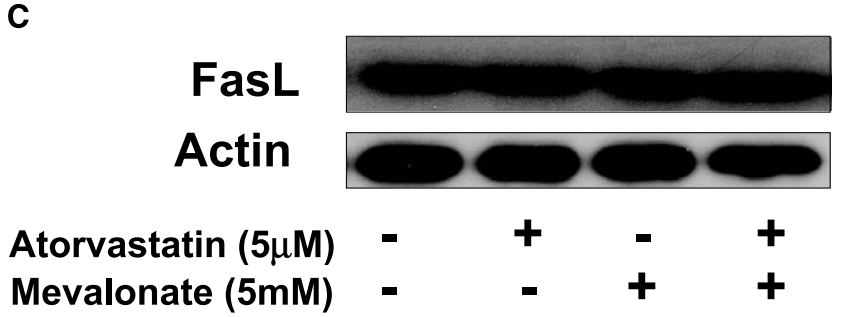
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Overall, these results show that statin increases membrane FasL expression in the murine B16F10 melanoma model.

Inhibition of Protein Geranylgeranylation by GGTI-298 Increases FasL Expression on the B16F10 Cell Membrane

The mevalonate-derived isoprenoid compounds farnesylpyrophosphate and geranylgeranylpyrophosphate are transferred on target proteins such as Rho by the specific enzymes farnesyl transferase and geranylgeranyl transferase. These posttranslational modifications enable the functional activity of isoprenylated Rho proteins. However, this isoprenylation can be inhibited by the specific inhibitors FTI and GGTI, leading to loss of anchorage and activity of the Rho proteins. We therefore used either FTI-277 or GGTI-298 to test whether inhibition of protein isoprenylation also induces an increase in membrane FasL expression. Firstly, we ensured that these two prenyl transferase inhibitors prevented protein isoprenylation with weak toxicity using our culture conditions. Indeed as expected, FTI-277 treatment at 10 μM inhibited the exclusive farnesylation of Hdj2 (Figure 2 *C*), but had no effect on the exclusive geranylgeranylation of Rap1a [34]. On the contrary, GGTI-298 at 10 μM prevented the prenylation of Rap1a but not of Hdj2 (Figure 2 *F*). We then treated the B16F10 cells for 48 hours with FTI-277 or GGTI-298 at 10 or 20 μM before cytofluorometry analysis of membrane FasL expression. As shown in Figure 2, *D* and *E*, GGTI-298, but not FTI-277 (Figure 2, *A* and *B*), increased the percentage of B16F10 cells expressing membrane FasL to a similar extent as that noted with atorvastatin (Figure 1 *A*). We obtained similar results in three independent experiments (Figure 2, *B* and *E*), confirming a statistical increase in $FasL^+$ B16F10 cells after GGTI-298 treatment ($P < .01$).

Together, these results show that geranylgeranyl transferase inhibition enhances membrane FasL expression in B16F10 melanoma cells.

RhoA/ROCK Pathway Negatively Regulates Membrane FasL Expression in B16F10 Melanoma Cells

As indicated previously, GGTI-298 efficiently prevents geranylgeranylation of several proteins, including the Rho GTPases [34,35]. We therefore tested the hypothesis that RhoA, the most characterized Rho GTPase, is involved in the regulation of FasL. We firstly ensured that GGTI-298 treatment impaired RhoA activation in the B16F10 cells in our culture conditions. Indeed, using a GST Rho binding domain pull-down assay [36], we detected a strong inhibition of the GTP-bound RhoA in the B16F10 cells treated with 10 μM GGTI-298 compared to untreated cells (Figure 3 *A*). This result suggests that GGTI-298 could enhance membrane FasL expression through the inhibition of Rho protein activation. The ADP-ribosyl transferase C3 toxin of *Clostridium botulinum* can also inhibit the activation of Rho proteins [37]. We therefore checked the occurrence of ADP ribosylation of RhoA proteins in our culture conditions (Figure 3 *B*) and tested the effects of this toxin on membrane FasL expression. As shown in Figure 3 *C*, treatment of B16F10 cells with C3 exotoxin significantly increased ($P < .01$) the percentage of membrane $FasL^+$ cells in a dose-dependent manner. Together, these results suggest that Rho proteins negatively regulate membrane FasL expression in these melanoma cells. To confirm the involvement of RhoA proteins in the regulation of FasL expression, we analyzed the effect of both RhoA-specific siRNA and an expression vector for constitutively active RhoA (RhoAV14). As shown in Figure 3 *D*, the transfection of RhoA-specific siRNA simultaneously resulted in a strong inhibition of RhoA expression as detected by Western blot and an upregulation of membrane FasL expression as detected by cytofluorometry. Both detections were made 72 hours after the transfection. This increase in $FasL^+$ melanoma cells was statistically significant with a $P < .01$. Furthermore, these transfected cells were simultaneously used to detect the total FasL expression by Western blot and the FasL mRNA level by RT-PCR. As also illustrated in Figure 3 *D*, the transfection of RhoA-specific siRNA affected neither total FasL protein expression nor FasL mRNA level. Conversely, transfection of the pSG5-RhoAV14 vector led to a simultaneous increase in RhoA expression and a statistically significant ($P < .05$) decrease in B16F10 cells expressing membrane FasL.

Together, results from these two experiments show that RhoA negatively regulates membrane FasL expression on these melanoma cells. The critical role of RhoA in the regulation of FasL expression prompted us to investigate downstream effectors of RhoA with possible involvement in this regulation. One such candidate effector molecule is the multidomain protein p160ROCK containing a serine/threonine kinase domain, a pleckstrin homology domain, cysteine-rich regions, and an amphipathic alpha-helical region. The kinase activity of p160ROCK is required to regulate events downstream of RhoA and particularly actin polymerization. We investigated the implication of p160ROCK in FasL membrane expression using the specific p160ROCK inhibitor H1152. As shown in Figure 4 *A*, a 24-hour treatment with 0.5 μM H1152 decreased stress–fiber formation, suggesting that, in our culture conditions, this treatment efficiently inhibited p160ROCK activity. In these same conditions, we therefore tested the effect of H1152 on membrane FasL expression. As shown in Figure 4 *B*, B16F10 cells treated with 0.5 μM H1152 showed an overexpression of membrane FasL compared to untreated cells. This indicates an implication of the RhoA effector protein p160ROCK in the RhoA-dependent regulation of membrane FasL expression.

Altogether, these data support the proposition that the RhoA/ROCK pathway is specifically involved in the control of FasL expression on B16F10 cell membranes.

Figure 1. *Statins upregulate FasL expression on B16F10 cell membrane. B16F10 melanoma cells were incubated with atorvastatin (5 μM), mevalonate (5 mM), or both for 48 hours. Cells were harvested and FasL was immunodetected by flow cytometry using a phycoerythrin-conjugated murine FasL monoclonal antibody. The percentage of FasL-positive cells ($FasL^+$ cells (%)) is indicated (A). This percentage of FasL-positive cells was also analyzed in three independent experiments (B). A statistically significant increase ($P < .05$) is illustrated by an asterisk (*). Similarly treated B16F10 cells were lysed and immunoblotted with murine anti-FasL antibody for total FasL protein detection. Data from one representative of three independent experiments are shown (C).*

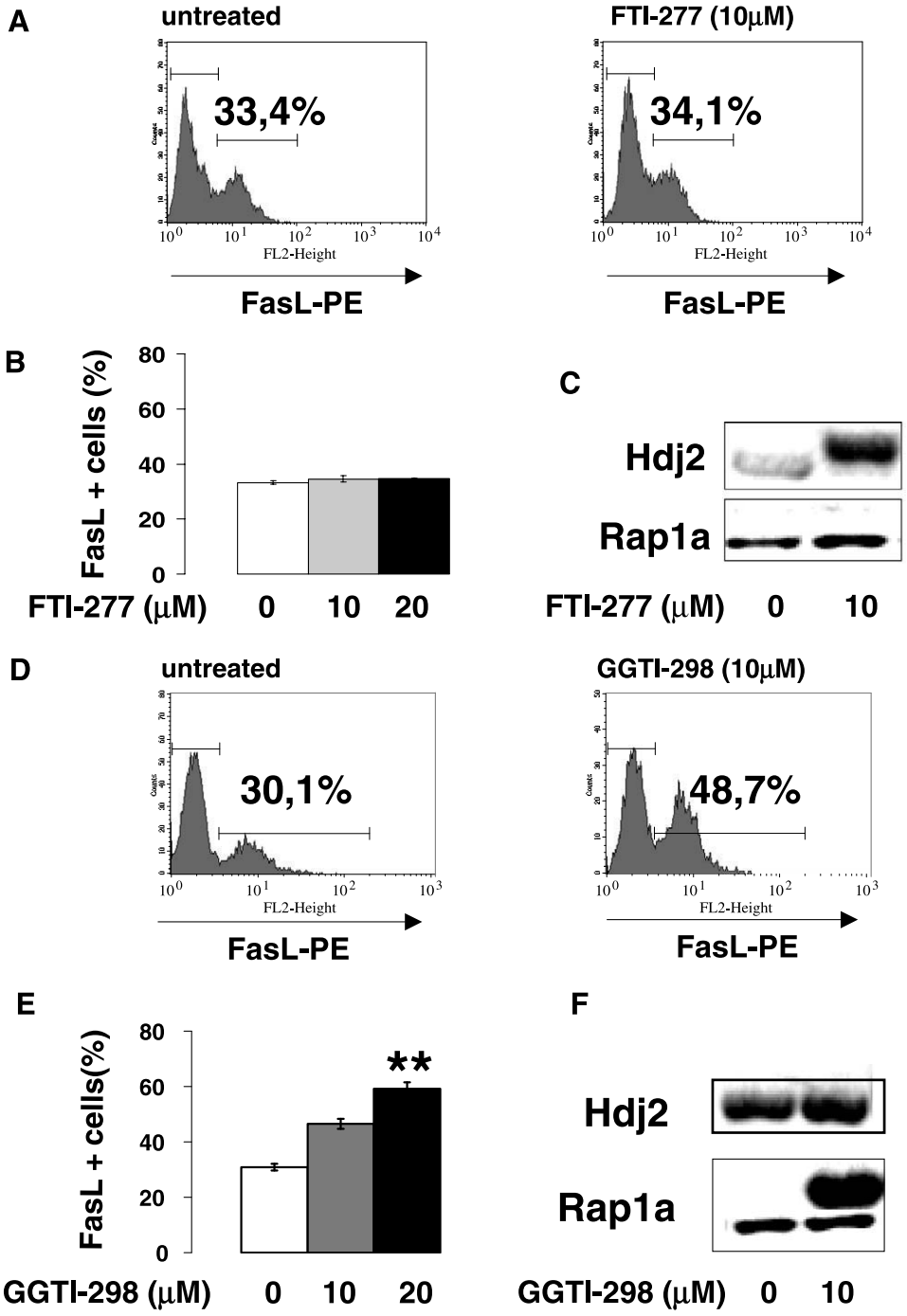

Figure 2. *Inhibition of protein geranylgeranylation by GGTI-298 increases FasL expression on B16F10 cell membrane. B16F10 cells were incubated with FTI-277 (10 and 20 μM) or GGTI-298 (10 and 20 μM) for 48 hours. Cells were harvested and FasL was immunodetected by flow cytometry. The percentage of FasL-positive cells is indicated (A and D). Histograms illustrating this percentage of FasL$^+$ cells analyzed by flow cytometry from three independent experiments, using increasing doses of FTI-277 (0, 10, and 20 μM) (B) or GGTI-298 (0, 10, and 20 μM) (E). A statistically significant increase ($P < .01$) is illustrated by double asterisks (**). Similarly treated B16F10 cells were also analyzed and immunoblotted with Rap1a or Hdj2 antibody to confirm farnesyl transferase inhibition (C) and geranylgeranyl transferase inhibition (F) obtained by FTI-277 (10 μM) or GGTI-298 (10 μM) treatment, respectively.*

Apoptosis of A20 Lymphoma Cells Induced by Coculture with B16F10 Melanoma Cells

To study the *in vitro* biologic function of membrane-expressed FasL, we tested the capacity of B16F10 cells to induce the apoptosis of Fas-positive murine A20 lymphoma cells. First, we checked the sensitivity of A20 cells to apoptosis by the Fas/FasL pathway in our culture conditions. We treated the A20 cells with an anti-Fas antibody (clone Jo-2) (50 μg/ml) and measured the occurrence of apoptosis by cytofluorometric analysis of the cell cycle and expression of active caspase-3. As expected, treatment of A20 cells with anti-Fas antibody increased the percentage of cells expressing the active caspase-3 form and the percentage of cells in the sub–G_1-peak, compared to cells treated with the IgG2

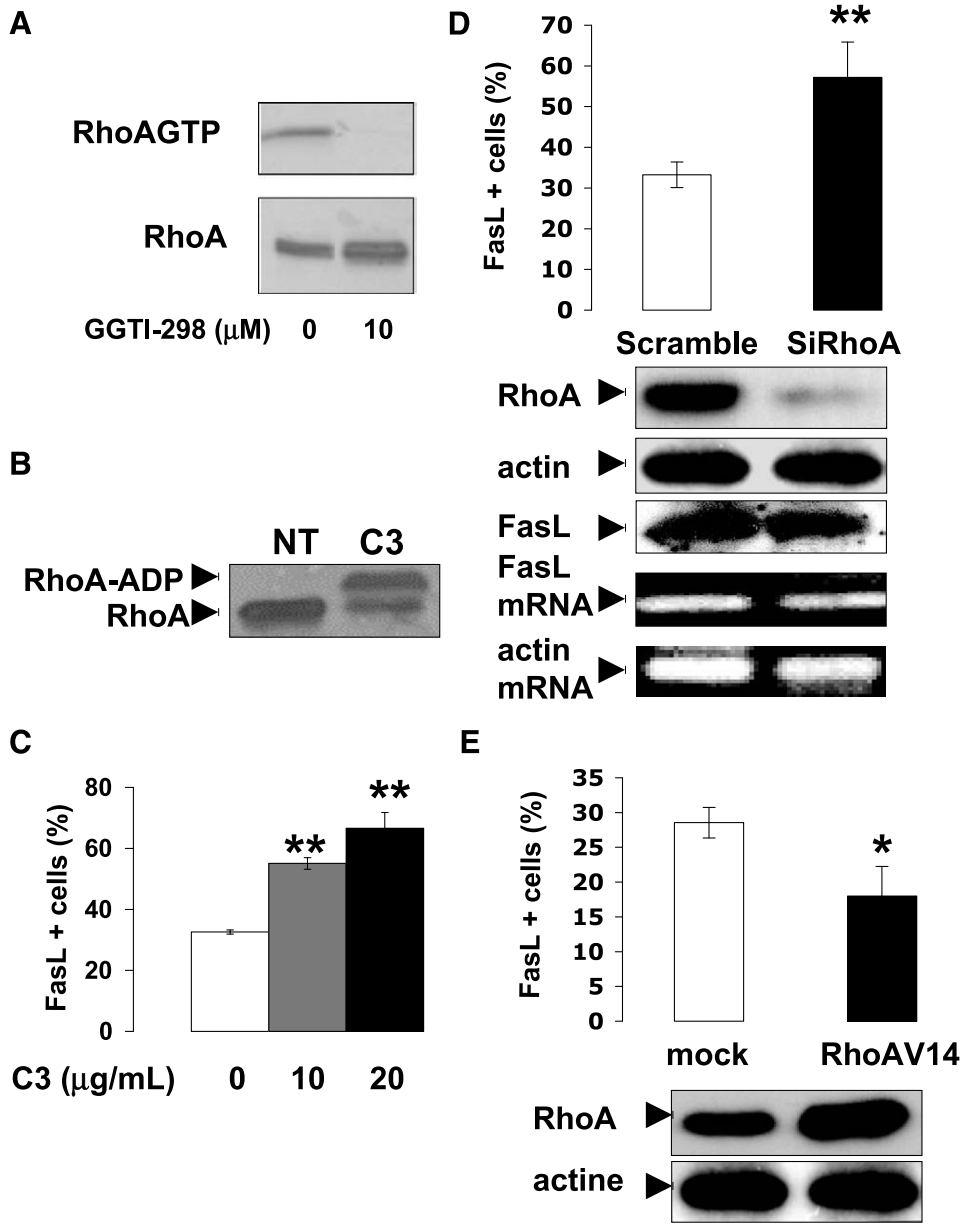

Figure 3. *RhoA negatively regulates membrane FasL expression on B16F10 melanoma cells. (A) B16F10 cells were incubated with GGTI-298 (10 μM) for 48 hours and RhoA activity was analyzed by TRBD assay as described in the Materials and Methods section. (B) B16F10 cells were incubated with C3 exoenzyme (10 μg/ml) for 48 hours. Untreated and treated cells were lysed and immunoblotted with anti-RhoA antibody to test RhoA-ADP ribosylation as described in the Materials and Methods section. (C) B16F10 cells were treated with C3 exoenzyme (10 and 20 μg/ml). Cells were harvested and FasL was immunodetected by flow cytometry. The percentage of FasL-positive cells (FasL$^+$ cells (%)) was analyzed; data shown are mean values of three independent and reproducible experiments. A statistically significant increase ($P < .01$) is illustrated by double asterisks (**). (D) B16F10 cells were transfected either with scramble siRNA or with RhoA-specific siRNA (SiRhoA) and analyzed after 72 hours to evaluate by cytofluorometry the percentage of FasL-positive cells. The scramble siRNA contains a disorganized sequence of the same nucleotides and is therefore unable to cut the target RNA sequence. Again, data shown are mean values of three independent and reproducible experiments. A statistically significant increase ($P < .01$) is illustrated by double asterisks (**). Similarly transfected cells were also lysed and immunoblotted with either anti-RhoA antibody or anti-FasL antibody and compared to actin expression to check the efficiency of the RhoA-specific siRNA and the total FasL protein expression. RNA of these transfected cells were also used to compare their FasL mRNA levels by RT-PCR, as described in the Materials and Methods section. (E) B16F10 cells were also transfected with a pSG5 empty vector (Mock) or with a pSG5-RhoAV14 vector encoding for the active form of RhoA (RhoAV14). After 72 hours, membrane-expressed FasL was immunodetected by flow cytometry. The percentages of FasL-positive cells are shown as mean values of three independent and reproducible experiments. A statistically significant decrease ($P < .05$) is illustrated by an asterisk (*). These cells were also lysed and immunoblotted with anti-RhoA antibody to test the efficiency of the transfected pSG5-RhoAV14 vector to increase RhoA expression in B16F10 cells.*

control antibody (Figure W1). To test the ability of B16F10 cells to induce lymphocyte apoptosis, we cocultured A20 lymphoma cells with a growing number of B16F10 cells for 72 hours and measured the occurrence of apoptosis as previously described. We detected a B16F10 dose-dependent increase in A20 cells in the sub−G_1-peak (Figure W2). Moreover, as shown in Figure 5 *A*, the percentage of A20 cells expressing the active form of caspase-3 strongly increased (8-fold increase) following 24 hours of A20 and B16F10 coculture. We obtained similar results in three

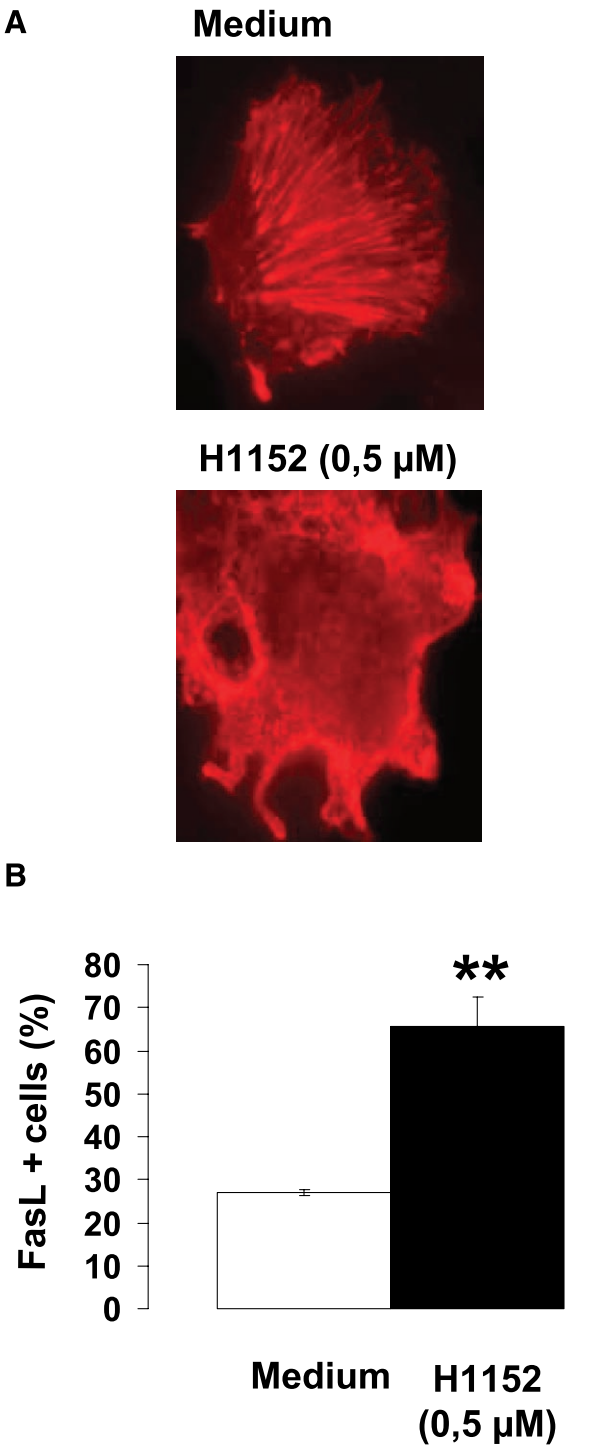

Figure 4. *Inhibition of p160ROCK increases FasL expression on B16F10 cell membrane. (A) B16F10 cells were seeded onto glass coverslips in six-well plates to obtain 60% confluence on day 2. On day 1, cells were treated with 0.5 μM H1152 for 24 hours. After treatment, actin fibers were visualized by tetramethylrhodamine isothiocyanate-labeled phaloidin. Cells were viewed under a Zeiss Axiophot microscope (× 630), and pictures taken with a Princeton Camera. (B) B16F10 cells were incubated with 0.5 μM H1152 for 24 hours. Cells were harvested and FasL was immunodetected by flow cytometry. Histograms illustrating the percentage of FasL$^+$ cells analyzed by flow cytometry from three independent experiments. A statistically significant increase (P < .01) is illustrated by double asterisks (**).*

independent experiments (Figure 5 *B*), confirming statistically significant increases in A20 cells expressing the active form of caspase-3 after coculture with growing ratios of B16F10 to A20 cells. To confirm the implication of the Fas signaling pathway and Fas/FasL interaction in these events, we added either a caspase inhibitor (Z-VAD-Fmk) or a blocking anti-FasL antibody (c178) to the coculture medium. The results presented on Figure 5 *C* show that both the caspase inhibitor and the FasL blocking antibody inhibited A20 apoptosis induced by B16F10 coculture, as illustrated by the decrease in the active form of caspase-3, not observed in the control culture conditions.

Collectively, these results show that membrane FasL on B16F10 melanoma cells induces apoptosis of A20 lymphoma cells by the Fas signaling pathway in coculture experiments.

Transfection of B16F10 Cells with RhoA-Specific siRNA or Activated RhoA Modifies Their Efficiency to Induce Apoptosis of Cocultivated Cells

Having shown that RhoA protein negatively regulates membrane FasL expression in B16F10 cells and that in coculture these cells induce apoptosis of Fas-sensitive A20 cells by the Fas signaling pathway, we now wished to check if modulating the level of membrane FasL on B16F10 cells could modify the induction of apoptosis in cocultivated A20 cells. We therefore cocultivated A20 cells with B16F10 melanoma cells previously transfected with either the pSG5 empty vector (B16F10Mock) or the RhoAV14 pSG5 vector (B16F10RhoAV14), which decrease FasL expression as shown in Figure 3 *E*. We then analyzed apoptosis through caspase-3 activity. As shown in Figure 6 *A*, the level of A20 apoptosis significantly decreased ($P < .01$) when A20 cells were cocultivated with B16F10RhoAV14 cells compared to B16F10Mock. Conversely, A20 cells were also cocultivated with B16F10 cells previously transfected with control (scramble) or RhoA-specific siRNA and the level of the active form of caspase-3 was evaluated. As shown in Figure 6 *B*, we observed an increase in the level of apoptosis in A20 cells on cocultivation with SiRhoA-transfected B16F10 melanoma cells (B16F10SiRhoA) as compared with B16F10 cells transfected with the scramble plasmid (B16F10Sc). Moreover, the addition of a blocking anti-FasL antibody (c178) in the culture medium inhibited the increase in caspase-3 activity observed in these A20 cells compared to control culture conditions (IgG control). These data suggest that the increase in A20 apoptosis is associated with an increase in available membrane FasL molecules.

Finally, we tested the implication of p160ROCK molecules in this apoptosis induction mechanism. We cocultivated A20 cells with B16F10 cells either untreated or previously pretreated with the p160ROCK inhibitor (H1152, 0.5 μM). As shown in Figure 6 *C*, H1152 pretreatment significantly ($P < .05$) enhanced the capacity of B16F10 cells to induce apoptosis, as illustrated by the increase in the active form of caspase-3. Moreover, addition of the blocking anti-FasL antibody (c178) in the culture medium significantly ($P < .01$) inhibited this effect.

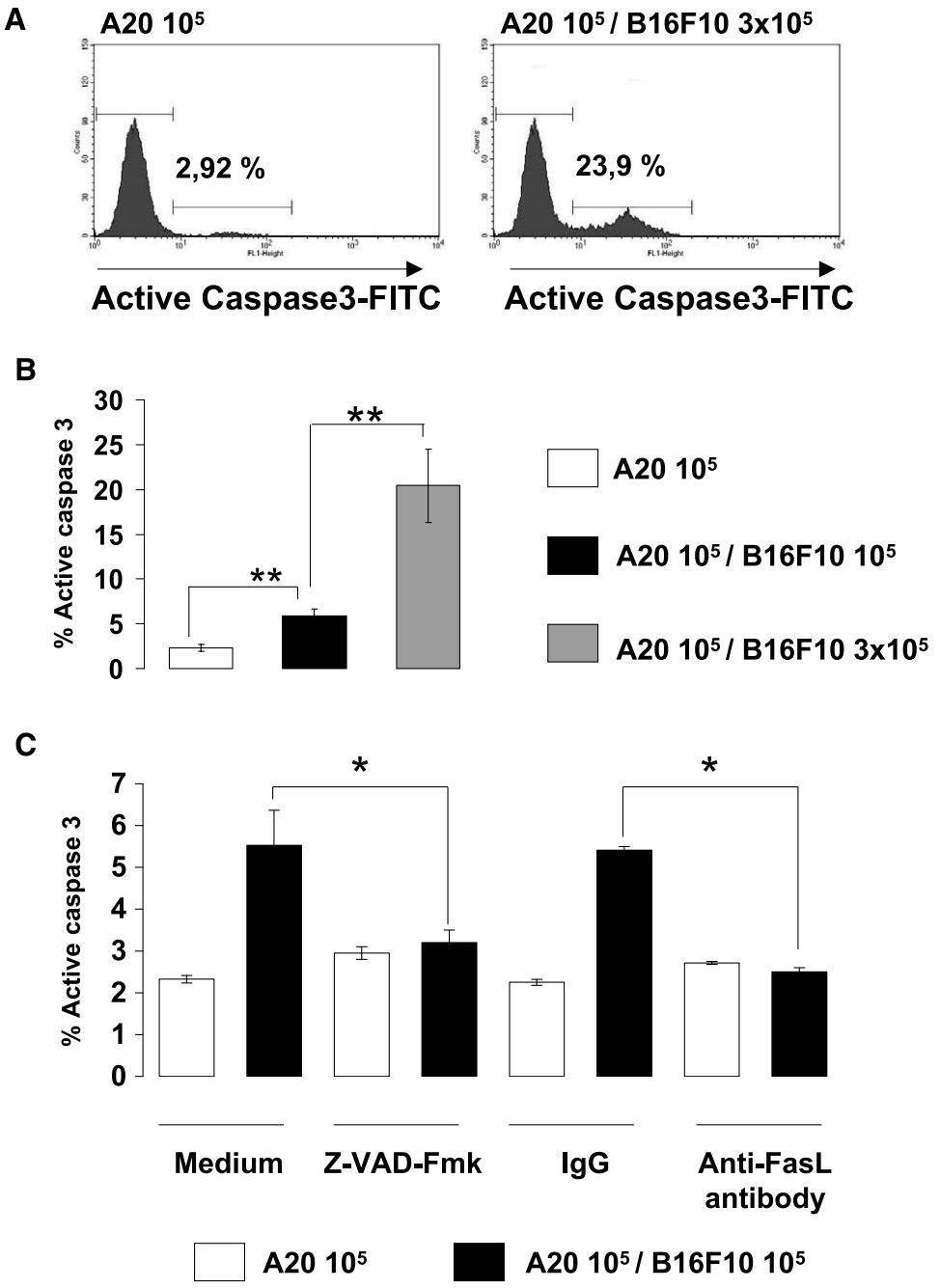

Figure 5. *Apoptosis induced by Fas/FasL pathway of A20 lymphoma cocultivated with B16F10 melanoma cells. (A) A total of 1 × 10^5 A20 cells were cultivated alone or with 3 × 10^5 of B16F10 melanoma cells for 24 hours and the expression of the active form of caspase-3 was evaluated by cytofluorometry with a specific antibody in permeabilized A20 cells. Data shown are from one representative of three independent and reproducible experiments. (B) A total of 1 × 10^5 A20 cells were cultivated alone or with increasing numbers of B16F10 melanoma cells (1 × 10^5; 3 × 10^5) for 24 hours and expression of the active form caspase-3 was evaluated by cytofluorometry. Data shown are mean values of three independent experiments. A statistically significant increase (P < .01) is illustrated by double asterisks (**). (C) A total of 1 × 10^5 A20 cells were also cultivated for 24 hours alone or with 1 × 10^5 B16F10 cells, in the absence (medium) or the presence of 100 μM caspase inhibitor Z-VAD-Fmk (Z-VAD-Fmk). Medium corresponds to the normal culture medium containing 1% DMSO to be in the same conditions as those required by the Z-VAD-Fmk inhibitor. Coculture experiments were also carried out with blocking anti-FasL antibody (c178) at 50 μg/ml (anti-FasL antibody) or matched isotype control (IgG). IgG corresponds to the normal culture medium containing 50 μg/ml of a rabbit IgG used as control for the anti-FasL antibody. Data shown are mean values of three independent experiments. Statistically significant decreases (P < .05) induced by the inhibitor or the blocking antibody are illustrated by an asterisk (*).*

Altogether, these data demonstrate that membrane FasL molecules are responsible for the apoptosis induced by B16F10 melanoma cells in A20 lymphoma target cells. Furthermore, as RhoA/ROCK negatively regulates membrane FasL expression on these melanoma cells, their inhibition increased the apoptosis of A20 cells, whereas the overexpression of activated RhoA reduced A20 apoptosis. RhoA or p160ROCK may therefore represent interesting target molecules to modulate the antitumor immune response through membrane FasL expression.

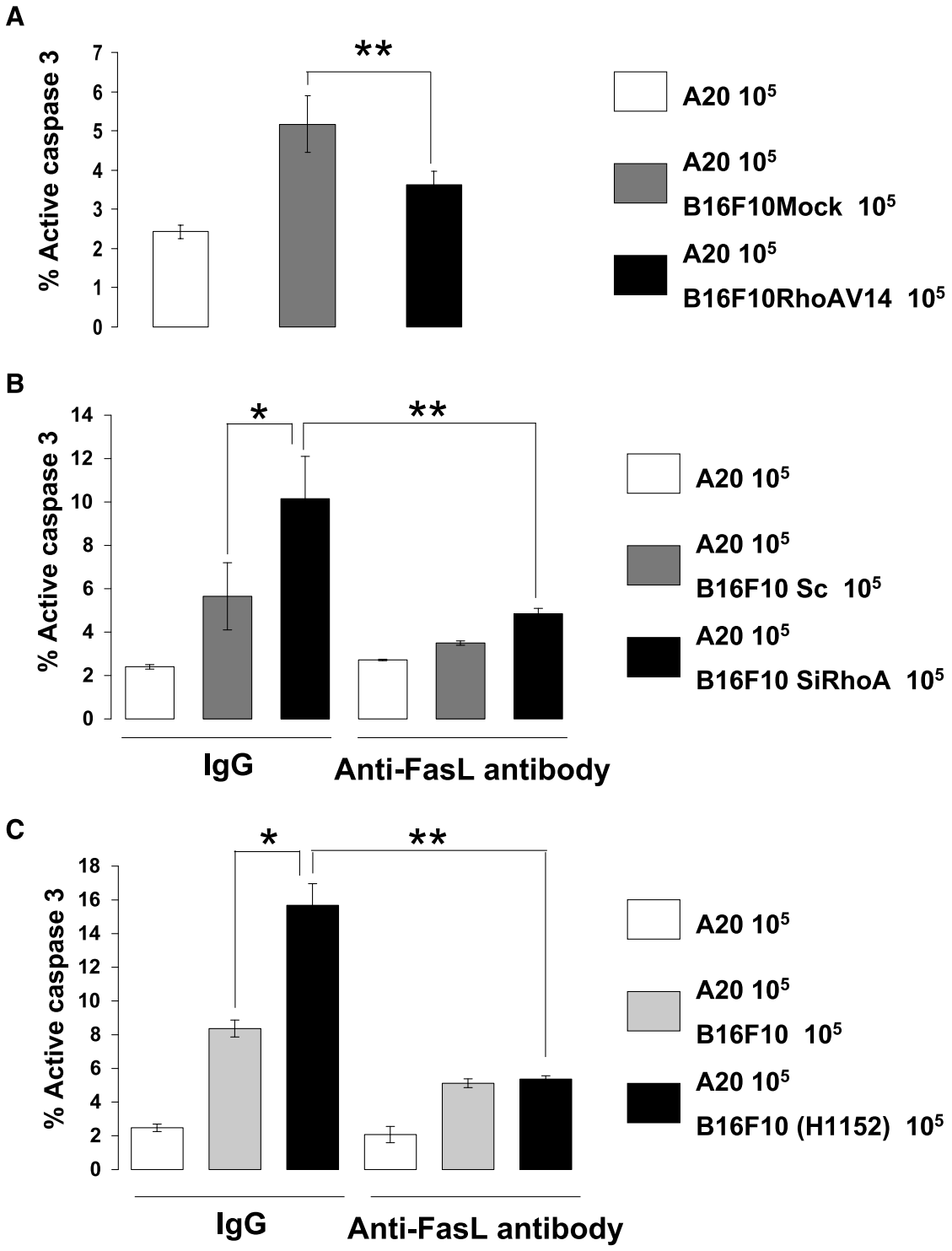

Figure 6. *RhoA negatively regulates FasL-induced apoptosis of A20 cells cocultivated with B16F10 melanoma cells. (A) A20 lymphoma cells (1×10^5) were cultivated for 24 hours, either alone or with 1×10^5 B16F10 cells, transfected with either pSG5 empty vector (B16F10Mock) or pSG5-RhoAV14 vector (B16F10RhoAV14). The percentage of A20 cells expressing the active form of caspase-3 (% active caspase-3) was analyzed by flow cytometry. (B) A20 lymphoma cells (1×10^5) were also cocultivated for 24 hours either alone or with 1×10^5 B16F10 cells transfected with a control siRNA (B16F10Sc) or a RhoA-specific siRNA (B16F10SiRhoA). (C) A20 cells (1×10^5) were also cocultivated for 24 hours either alone or with 1×10^5 B16F10 cells treated or not by ROCK inhibitor (0.5 μM H1152). These cultures (B) and (C) were made both in a control medium containing an IgG isotype control (IgG) and in a medium containing a blocking anti-FasL antibody at 50 μg/ml (antibody anti-FasL). The percentage of A20 cells expressing the active form of caspase-3 was analyzed by flow cytometry. Data shown are from one experiment representative of three. Statistically significant increases or decreases of A20 cells expressing the active form of caspase-3 are illustrated by an asterisk (*) ($P < .05$) or double asterisks (**) ($P < .01$).*

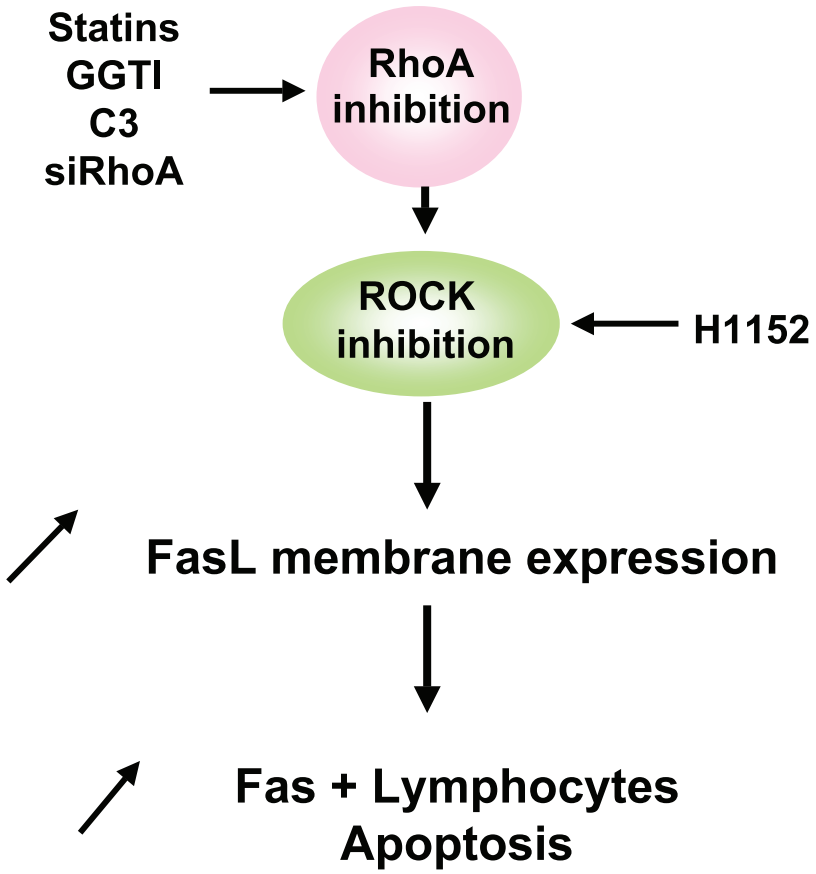

Figure 7. *Schematic representation of the effects of RhoA/ROCK signaling pathway inhibition on membrane FasL expression and lymphocytes apoptosis.* In vitro *treatments of B16F10 melanoma cells with inhibitors of the RhoA/ROCK pathway increase the membrane FasL expression on these treated tumor cells. This (over)expression promoted the apoptosis of Fas-sensitive lymphocytes.*

Discussion

Malignant cells possess several strategies to escape immune surveillance such as low-level expression of target antigens recognized by cytotoxic T lymphocytes [38], a decrease or loss of the human leukocyte antigen class I or class II molecule expression required for antigen presentation [39,40], and suppression of immune cell function by secreting factors such as IL-10 or transforming growth factor–β [41]. Interestingly, some tumor cells have also developed another strategy named the Fas-counterattack mechanism [42], whereby loss of Fas expression or function and the aberrant expression of FasL by tumor cells contribute to their evasion of host immune surveillance by triggering apoptosis of Fas-positive antitumor effector lymphocytes [43,44].

FasL expression has been described on some tumor models [19]; however, few studies have focused on the regulation of expression levels. In this *in vitro* study, we have shown that expression of FasL on the membranes of B16F10 murine melanoma cells [19] is strongly increased by treatment with the HMG-CoA reductase inhibitor atorvastatin. However, we observed no change in the level of total FasL protein, indicating that atorvastatin interferes with membrane localization of FasL, either modifying its stability or trafficking to the cell surface. The upregulation of membrane FasL expression induced by atorvastatin is reversed by coincubation with mevalonate, suggesting the involvement of isoprenoid compounds from the cholesterol pathway downstream of mevalonate. An inhibitor of protein geranylgeranylation (GGTI-298) but not of protein farnesylation (FTI-277) can also upregulate FasL expression, supporting the role of a protein modified by geranylgeranylation in this regulation. We also observed an upregulation of membrane FasL in the presence of *C. botulinum* C3 exotoxin, a selective inhibitor of Rho protein activation. Overall, these results indicate a close relationship between the upregulation of membrane FasL and the Rho inhibition. Transfection experiments with either a vector expressing an active form of RhoA (RhoAV14) or siRNA against RhoA confirmed a role of this small G protein in the control of FasL expression. Inhibition of RhoA by siRNA increased FasL expression in B16F10 cells, whereas constitutively active RhoA decreased it. These data show that RhoA negatively regulates membrane FasL expression in B16F10 cells.

Previous studies have shown that the small GTPases RhoA or Rac1 regulate transcription of FasL in different cell lines such as Jurkat leukemia cells or NIH-3T3 fibroblast [45,46]. However, to our knowledge, this study is the first to implicate RhoA in the negative regulation of membrane FasL expression, showing no correlation with the total level of FasL protein. Rho GTPases are involved in different cellular mechanisms such as the regulation of transcription, cell motility, adhesion, or membrane trafficking. Bossi and Griffiths [47]

demonstrated the storage of newly synthesized FasL in specialized secretory lysosomes in $CD4^+$ and $CD8^+$ T lymphocytes and natural killer cells and showed that polarized degranulation controls the delivery of FasL to the cell surface. More recently, studies in tumor cells and, more particularly, melanoma cells have demonstrated the storage of preformed FasL in lysosomal microvesicles, able to traffic to the cell surface [48,49]. These observations could explain why RhoA negatively regulates membrane FasL expression. Indeed, Nishimura et al. [50] demonstrated that, by regulating both cytoskeletal microtubule organization and the actin cytoskeleton, the RhoA/ROCK–mediated signaling pathway is involved in the intracellular membrane dynamics of lysosomes. In this model, RhoA/ROCK pathway regulates the traffic and the localization of lysosomes in the cytoplasm and the cell membrane, possibly explaining the modulation of membrane-expressed FasL without modification of the total level of this ligand. The upregulation of membrane FasL expression in B16F10 cells treated with either the ROCK inhibitor HT115 or RhoA SiRNA strongly support the hypothesis that an active RhoA/ROCK pathway disrupts vesicle trafficking to the cell surface thereby decreasing membrane FasL expression.

The biologic significance of FasL expressed on tumor cells, still needs to be elucidated. *In vitro*, membrane-expressed FasL favor tumor immune evasion through apoptosis of cocultivated lymphocytes. Here, we demonstrated that FasL-expressing B16F10 cells can induce apoptosis of Fas-sensitive A20 B lymphoma cells. Growing numbers of B16F10 melanoma cells cocultured with A20 cells induce increased A20 cell death, associated with an upregulation of the active form of caspase-3. Hetz et al. [51] demonstrated the association of upregulated caspase-3 activity and Fas/FasL–dependent A20 apoptosis and identified this specific caspase as a marker of apoptosis in this lymphoma cell. The increased caspase-3 activation seen in A20 cocultivated with B16F10 must be dependent on the binding of FasL expressed on B16F10 membrane with the Fas molecule on A20 cells. This hypothesis is further supported by the fact that B16F10 cells transfected with constitutively active RhoA, which decreases membrane FasL expression, induce A20 apoptosis less efficiently than B16F10 cells transfected with the empty vector. Conversely, the *in vivo* significance of FasL molecules expressed on tumor cells remains ambiguous. On the one hand, downregulation of FasL expression in colon cancer cells was recently shown to result in an increased antitumor immune response and decreased tumor formation *in vivo*, providing functional evidence in favor of the *Fas counterattack* as a mechanism of tumor immune evasion [52,53]. Conversely, several studies have suggested that FasL expressed on tumor cells did not favor *in vivo* tumor evasion [54]. Interestingly, Wada et al. [14] demonstrated that the level of FasL expression on B16F10 cells is crucial for their *in vivo* behavior. Indeed, B16F10 cells expressing a high level of FasL induce tumor rejection associated with an inflammatory response, whereas B16F10 expressing a low level of FasL induce enhanced tumor growth by a counter-attack mechanism without activating the protective inflammatory response.

Our study in B16F10 melanoma cells provides evidence that *in vitro* lymphocyte apoptosis, induced through membrane-expressed FasL molecules, may be negatively regulated by the RhoA/ROCK pathway, as summarized in Figure 7. According to the enhanced tumor rejection of B16F10 cells expressing high levels of FasL [14], inhibitors of the RhoA/ROCK pathway could be interesting pharmacological agents to reduce tumor growth. This work, therefore, provides further support for our previous findings suggesting an involvement of Rho GTPases in mechanisms by which tumors either escape or modulate the antitumor immune response [18]. Our works identify RhoA protein as a new target for therapeutic strategies on melanoma treatment.

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