Atorvastatin prevents RhoC isoprenylation, invasion, and metastasis in human melanoma cells

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Abstract
Melanoma is a deadly cancer due to its propensity to metastasize. Pharmacological inhibition of cell motility may benefit patients with cutaneous melanoma by preventing metastasis to internal organs. The Rho GTPases are signaling molecules that drive metastasis by controlling cell motility. We found RhoC to be expressed in clinical melanoma specimens and hypothesized that inhibiting its activation might prevent metastasis. Some Rho proteins, such as RhoC, depend on posttranslational geranylgeranylation for biological activity. We investigated the effect that Atorvastatin, a 3-hydroxy 3-methylglutaryl CoA (HMG-CoA) reductase inhibitor that prevents Rho geranylgeranylation, had on subcellular localization and activity of Rho proteins as well as the metastatic ability of melanoma cells. Atorvastatin inhibited Rho activation and reverted the metastatic phenotype of human melanoma cells in vitro. Moreover, Atorvastatin, at plasma levels comparable to those used to treat hypercholesterolemia, inhibited in vivo metastasis of melanoma cells overexpressing RhoC. These results support further examination of statins for primary prophylaxis of melanoma metastasis. (Mol Cancer Ther. 2003;2:941 – 948)

Introduction
Metastatic melanoma is a highly aggressive, often fatal malignancy, which exhibits resistance to all current therapeutic approaches. At the time of diagnosis, about 20% of melanoma patients already have metastatic disease. Once metastasis has occurred, the overall median survival is only 6–9 months (1). The recent elevation in the incidence of melanoma (2) has highlighted the need for novel approaches targeting the molecular basis of melanoma metastasis.

The Rho family of small GTPases coordinates many aspects of cell motility through reorganization of the actin cytoskeleton and changes in gene transcription (3). High-throughput screens for transcriptionally regulated targets in the metastatic process have revealed that RhoC overexpression dramatically increases the metastatic potential of melanoma in vivo (4). Similar effects of RhoC overexpression have been described in breast cancer (5), and RhoC overexpression correlates with nodal metastasis in pancreatic carcinoma (6).

The Rho proteins are dependent on posttranslational isoprenylation for their biological function (7, 8), and RhoC and RhoA depend specifically on geranylgeranylation (9). We sought to explore clinically tolerable therapies that would block or attenuate Rho GTPase signaling with the goal of primary pharmaceutical prophylaxis of metastasis in patients at elevated risk for metastatic melanoma.

The statin class of drugs is an attractive candidate to meet this need. By inhibiting 3-hydroxy 3-methylglutaryl CoA (HMG-CoA) reductase, statins prevent the conversion of HMG-CoA to mevalonate, a precursor in the cholesterol biosynthetic pathway. Inhibition of mevalonate synthesis leads to dramatic reductions in both cholesterol and its isoprenoid precursors; farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Reduction of isoprenoid synthesis may mediate some of the extrahepatic effects of statins by inhibiting the function of proteins dependent on posttranslational isoprenylation. Statins are currently among the most widely prescribed drugs, due to their potent cholesterol-lowering properties. It has recently been suggested that extrahepatic effects of statins might play a potentially beneficial role in cancer therapy (10). Furthermore, two large clinical trials have demonstrated antineoplastic effects of statins in general (11) and in melanoma in particular (12).

In this study, we describe the effects of Atorvastatin on melanoma cytoskeletal structure, invasive ability, and metastatic potential. We chose to study Atorvastatin, because of its favorable safety profiles relative to other drugs in its class (13). We demonstrate that RhoC protein is expressed in melanoma and that Rho-mediated phenotypic features can be targeted by statin therapy. Atorvastatin-induced changes in RhoC localization correlate with reorganization of the actin cytoskeleton.
Furthermore, well-characterized Rho responsive elements are effectively blocked by statin therapy. Finally, animals tolerated Atorvastatin treatment without obvious toxicity and showed markedly reduced pulmonary melanoma metastases.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed on 10 melanoma using a polyclonal anti-RhoC antibody (14). Five of these samples were primary melanomas of the skin and the other 5 were metastatic melanomas to regional lymph nodes. The slides were microwave pretreated for antigen retrieval using 10 mM citrate buffer (pH 6.0) for 10 min. The antibody was titered and used at 1:400 dilution overnight at room temperature. The detection reaction was performed per instruction of the DAKO Envision + Peroxidase Rabbit kit with DAB kit (DAKO Cytomation, Carpinteria, CA). Sections were then scored for immunoreactivity using a four-tiered system: score 1 (no staining); score 2 (weak); score 3 (moderate); and score 4 (strong).

Cells, Chemicals, and Plasmids

A375M cells, a kind gift from Dr. R. Hynes (Massachusetts Institute of Technology), SK-Mel 128, CHL, and WM-166-4 (ATCC, Manassas, VA) were maintained in DMEM (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% pen-strep (Life Technologies). Atorvastatin (Pfizer, New York, NY) was obtained in tablet form (20 mg) from the UCLA pharmacy, dissolved in DMSO for cell culture work, and resuspended in PBS for animal administration. GGPP was purchased from Sigma (St. Louis, MO) and used at 10 μM. The geranylgeranyltransferase inhibitor GTTI-2133 (Calbiochem, San Diego, CA) was dissolved in DMSO.

For RhoC transfection experiments, A375M cells were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA). pcDNA 3.1 HA-RhoC was purchased from Dr. David Michaelson (NYU School of Medicine, New York, NY). pGFP-RhoC was constructed by excising the cDNA from pcDNA 3.1 HA-RhoC with HindIII and EcoRI digesting pEGFP-C3 (Clontech) cloning it into the HindIII and SalI-digested pEGFP-C3 plasmid (Clontech, Palo Alto, CA) in frame with the GFP cDNA.

A375M-Fluc cells were established by recombinant lentiviral transduction. Briefly, the firefly luciferase cDNA (Fluc) along with a downstream EMCV-IRES sequence was subcloned into pCS-CG lentivector using the NheI and Eco47III sites upstream of the GFP gene resulting in pCS-CG-Fluc-I-GFP. Recombinant lentivirus was produced by standard calcium phosphate cotransfection of 293T cells along with pΔVPR and pSV40 (15). A375M melanoma cells (1 x 10⁶) were seeded in 10-cm plates and incubated with lentiviral supernatants filtered from virus-producing cultures in the presence of Polybren (8 μg/ml). Stable cell pools were selected by fluorescence-activated cell sorting (FACS) for GFP fluorescence.

Invasion Assays

Invasion assays were conducted in Matrigel chambers (Clontech) exactly as described (7). At 48 h, cells were incubated with calcien-AM (Molecular Probes, Eugene, OR) and visualized under a fluorescence microscope.

Serum Response Factor Luciferase Assays

A375M cells were cotransfected with 0.3 μg of the reporter plasmid pSRF-firefly luciferase (Stratagene, La Jolla, CA), 0.2 μg of pCMV-β-Gal, and 0.5 μg of pcDNA-HA-RhoC, 0.5 μg of pcDNA-HA-V12-H-Ras, or 0.5 μg of pEGFP C3 (Clontech) using Lipofectamine2000 (Invitrogen). Twenty-four hours later, cells were harvested, washed with PBS, and lysed in 250 μl of lysis buffer (Promega, Madison, WI). Luciferase values were obtained by analyzing 10 μl of lysate according to the protocol provided in the Luciferase Assay Kit (Promega) in a Lumat LB 9501 luminometer (10-s count). Transfection efficiency was normalized by OPNNG assay of β-Gal activity. Relative luciferase activity is represented as (firefly luciferase value/β-Gal value).

Fluorescent Microscopy

For phalloidin staining, A375M cells were plated on 1 glass coverslips and treated with Atorvastatin or DMSO for 36 h, at which time they were fixed in 2% paraformaldehyde, and incubated with Texas Red Phalloidin (Molecular Probes) at a 1:333 dilution for 30 min at room temperature. Coverslips were washed three times in PBS and mounted. For GFP-RhoC microscopy, A375M cells were transfected with pGFP-RhoC and dbl plasmids, treated and imaged exactly as described (7). Confocal microscopy was performed using a Leica confocal microscope (Leica, Bannockburn, IL).

Cell Fractionation

Rho protein levels were assessed in cytosolic and membranous fractions as described (16). Briefly, A375M cells were transfected with pcDNA3.1 HA-RhoC and treated as indicated for 24 h, at which time cells were lysed in 1% Triton X-114 (previously enriched as described) (Sigma), in PBS on ice. Lysates were vortexed and incubated on ice for 15 min, cleared by centrifugation at 4°C and partitioned into membranous and cytosolic fractions by incubation at 37°C followed by centrifugation at room temperature.

Immunoblot Analysis

Protein expression was assessed by immunoblot analysis of cell lysates. Cytosolic and membranous protein pools of Rho proteins were analyzed by electrophoretic separation on SDS-12% polyacrylamide gels followed by transfer onto nitrocellulose membranes. The membranes were blocked with PBST (PBS + 0.1% Tween 20) containing 5% milk and immunoblotted for HA-RhoC by incubation with anti-HA antisera (Covance, Princeton, NJ) at 1:4000 dilution, or for RhoA by incubation with anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 in PBST-5% milk for 1 h at room temperature. Total cellular lysates processed similarly were blotted with anti-p21Waf1/Cip1 (Cell Signal, Beverly, MA) at 1:1000, anti-p27kipl (F-8 clone, Santa Cruz Biotechnology) at 1:500 and anti-actin (Sigma) at 1:5000 in

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PBST-5% milk for 1 h at room temperature. The blots were washed three times for 20 min with PBST at room temperature, exposed to horseradish peroxidase-conjugated sheep anti-rabbit/mouse IgG (Promega) at a 1:5000 dilution for 1 h at room temperature, and washed with PBST three times for 20 min at room temperature. Protein expression was visualized with ECL detection reagents (Amersham, Piscataway, NJ) followed by exposure to X-ray film.

**Animal Studies**

All animal studies were approved by the UCLA Animal Research Committee. Eight-week-old Beige SCID mice were given injections of 1 x 10^6 A375M-Fluc cells s.c. or 7 x 10^5 A375M-Fluc cells via the lateral tail vein on day 0. Mice were treated with 10 mg/kg Atorvastatin or PBS by gavage QD for the first 14 days following injection (n = 4 per group). Mice were imaged by i.p. injection of 100 µl of D-luciferin (30 mg/ml) (Xenogen, Alameda, CA) and imaged using a cooled CCD camera (IVIS). Imaging data were quantified as described (17) by averaging the region of interest (ROI) from the maximal photon emitting exposure.

**Results**

**RhoC Is Expressed in Melanoma**

To evaluate both the degree and the prevalence of RhoC expression in melanoma, and its relationship to invasive ability, we stained several melanoma tissues and melanoma cell line lysates with RhoC-specific antiserum (14). The primary melanomas had a lower mean staining intensity than the metastatic tumors (mean score 2, SD 1, versus mean score 3.2, SD 1, P = 0.004). Fig. 1, A and B show a representative primary/metastasis pair. Similarly, all melanoma cell lines tested expressed higher levels of RhoC than normal human melanocytes grown in culture (Fig. 1C).

**Atorvastatin Alters A375M Morphology and Rho Localization**

We employed the A375M line in our studies to examine the effects of Atorvastatin therapy on melanoma. This cell line was isolated from an amelanotic melanoma, and subsequently selected for high metastatic ability in vivo (4). When grown in the absence of Atorvastatin, A375M cells exhibited prominent stress fibers and extensive polymerization of F-actin and focal adhesions (not shown), phenotypes characteristically dependent on Rho signaling (18) (Fig. 2). In contrast, incubation of A375M cells with Atorvastatin leads to F-actin depolymerization and disassembly of stress fibers, along with other morphological changes appreciated elsewhere in response to statin treatment (19).

The localizations of RhoA and RhoC are thought to correlate with their activation state (9). To address the role Rho proteins play in the observed cytoskeletal rearrangements, we examined the effects of statin treatment on the subcellular localization of RhoA and RhoC. RhoA predominately resided in membrane fractions in control cells. Atorvastatin treatment lead to both up-regulation of endogenous RhoA protein as has been reported previously (20) and Rho exclusion from the membrane compartment. Atorvastatin treatment of A375M cells expressing an HA-tagged RhoC construct similarly excluded RhoC from the membrane fraction (Fig. 3A). The addition of excess GGPP partially reversed membrane exclusion. Similar effects were observed by transfecting a GFP-RhoC construct into A375M cells along with the dbl oncogene. As seen in Fig. 3B, GFP-RhoC was found primarily in the plasma and intracellular membranes in control cells, whereas treatment of these cells with Atorvastatin resulted in a diffuse, cytosolic distribution of GFP-RhoC, consistent with a failure of proper protein isoprenylation.
Atorvastatin Treatment Attenuates RhoC Signaling

Previous work in fibroblasts has demonstrated an essential role for Rho in cellular proliferation by showing inhibition of cyclin-dependent kinase inhibitor (CDKI) transcription in response to Ras-Raf-ERK signaling (21). We treated A375M cells with Atorvastatin and found that both p21^{WAF1/CIP1} and p27^{KIP1} protein levels were elevated after treatment (Fig. 4A). This elevation was partially reversed by addition of GGPP, suggesting possible involvement of RhoA or RhoC. To further

Figure 2. Atorvastatin reverts stress fiber formation. The RhoC-expressing A375M cells were plated and treated with DMSO (A) or 3 µM Atorvastatin (B) for 24 h. Stress fibers are highlighted with arrows.

Figure 3. Atorvastatin abrogates membrane association of Rho proteins. A, A375M cells expressing HA-tagged RhoC were treated with either DMSO, 3 µM Atorvastatin, or 3 µM Atorvastatin + 10 µM GGPP. After 24 h, membrane-associated (Mem) and cytosolic (Sol) fractions were collected and immunoblotted for either anti-RhoA (top blot), anti-HA (middle blot), or β Catenin as a loading control (bottom blot). B, A375M cells were cotransfected with pEGFP-RhoC and pCMV-dbl, treated with either DMSO or 3 µM Atorvastatin (Atorv) for 24 h, and examined live by confocal microscopy.

Figure 4. Rho signaling is attenuated by Atorvastatin. A, A375M cells were treated with either DMSO, 3 µM Atorvastatin, or 3 µM Atorvastatin + 10 µM GGPP for 24 h, lysed, and blotted for levels of p27^{KIP1} (top blot), p21^{WAF1/CIP1} (middle blot), or actin (bottom blot). B, A375M cells transfected with either pEGFP-C1 (GFP), pcDNA V12 Ras (V12 Ras), or pcDNA RhoC, and the pSRF reporter. pcDNA RhoC-transfected cells were treated with DMSO (wtRhoC), 3 µM Atorvastatin (3 µM Atorv), or 1.5 µM geranylgeranyl transferase inhibitor (1.5 µM GTI). Luciferase values were quantitated 24 h later, and were normalized for transfection efficiency by LacZ activity. Error bars, ± SD.
examine the effects of statin treatment on Rho-mediated transcriptional regulation, we performed SRF reporter experiments. The SRF is widely known to be activated by Rho (22) and it has been shown that this activation requires isoprenylation (7). Cotransfection of A375M cells with RhoC and a synthetic SRF reporter leads to a roughly 6-fold induction of transcriptional activity in the presence of serum (Fig. 4B). Treatment of A375M cells with Atorvastatin inhibited RhoC-augmented transcription from SRF. A specific geranylgeranyl transferase inhibitor (GGTI-2133) produced a similar effect, indicating that inhibition of geranylgeranylation is most likely responsible for the reduced transcriptional activity of treated cells.

**Atorvastatin Inhibits Invasion of Melanoma Cells**

Previous work has defined a role for Rho proteins in the invasion of tumor cells (23). To measure the effect of Atorvastatin on melanoma cell invasion, we performed Matrigel chamber assays with escalating dosages of Atorvastatin in four cell lines derived from human melanomas. We found that Atorvastatin inhibited invasion of all lines tested in a dose-dependent manner. Of note, 3 μM Atorvastatin dramatically reduced the invasive potential of A375M cells (Fig. 5) without causing toxicity (7). These results suggest that statin treatment may block metastasis by specifically affecting invasiveness and not simply inhibiting cell growth.
Atorvastatin Does Not Affect Cell Growth in Vivo

Depletion of mevalonate has many consequences in the cell. To examine the extent to which Atorvastatin affected the growth kinetics of A375M-Fluc cells, we performed s.c. tumor growth studies in SCID mice. Oral treatment of mice with Atorvastatin did not appreciably affect A375M proliferation in the s.c. compartment (Fig. 6).

Atorvastatin Inhibits Metastasis in Vivo

RhoC augments the metastatic capacity of A375 cells (4). Because Atorvastatin inhibited Rho signaling in these cells, we examined the effect of oral Atorvastatin on the metastatic ability of these cells in a tail vein injection metastasis model. We employed a tail vein injection model as a first step in understanding of the effects of statins on the extravasation, establishment, and micrometastasis formation of hematogenously seeded melanoma cells. The use of bioluminescent imaging allowed repeated quantification of tumor load as described (15, 24). We observed that although similar numbers of A375M-Fluc cells reached the mouse lungs after injection, Atorvastatin dramatically inhibited colonization and formation of metastatic lesions (Fig. 7). Imaging results were verified by necropsy.

Discussion

Metastasis is the direct cause of mortality in most cancer patients. Therefore, efforts to understand the metastatic process are of tremendous clinical importance. Due to its intrinsically high metastatic rate, melanoma represents an attractive model in which to study metastasis. While several novel approaches are being pursued to target cancer cell growth, relatively few focus specifically on preventing metastasis with drugs that can be safely administered on a long-term basis. Because the majority of cancer patients eventually succumb to metastatic disease, agents that prevent or significantly delay metastasis, without excessive collateral toxicity to other organs, offer tremendous potential clinical benefit (25).

The Rho family of small GTPases may play an essential role in the dissemination of both melanoma and carcinomas (3). However, specific information on the expression patterns of RhoC in clinical melanoma samples has been lacking. We report here that RhoC is expressed in the vast majority of clinical melanoma samples, indicating that this molecule may be a potential target in preventing metastatic disease.
The statin class of drugs inhibits HMG-CoA reductase thereby depleting cellular stores of both mevalonate and isoprenoids. Our previous work demonstrated that isoprenylation is essential for Rho-mediated invasion of melanoma cells (7). We hypothesized that Atorvastatin could alter endogenous Rho signaling in melanoma cells and that this alteration might decrease metastatic potential in vivo.

Atorvastatin disrupted stress fibers in A375M cells, suggesting that Rho signaling is affected by statin treatment. Indeed, we found that Atorvastatin both altered the subcellular localization of Rho proteins and inhibited the downstream pathways leading from GTP-bound Rho to cytoskeletal rearrangement and changes in gene transcription. These results are in agreement with other studies using pancreatic carcinoma cells (26) or mammary carcinoma cells (27), in which statin treatment was found to interfere with RhoA membrane localization.

In prior work, we showed that Rho overexpression can increase the invasive ability of melanoma cells and that this phenomenon depends on isoprenylation (7). In the current study, we similarly showed that statin treatment prevented the invasion of melanoma cells in a Matrigel invasion assay. These two observations indicate that Rho proteins, when properly isoprenylated, have the ability to increase melanoma invasion. Furthermore, prevention of geranylgeranylation is a potentially exploitable treatment modality. Potent and specific geranylgeranyl transferase inhibitors have been developed for this purpose but have led to substantial in vivo toxicity, primarily due to lethal myelosuppressive effects (28).

Treatment of tumor-bearing animals with HMG-CoA reductase inhibitors decreases pulmonary colonization of breast carcinoma (27) and rat lymphoma (29) cells as assessed by counting of tumor nodules after animal sacrifice. Many studies have suggested that statins exert anti-neoplastic effect at least in part through the inhibition of cellular proliferation. In support of this theory, statins cause a G1 arrest and eventually apoptosis in NIH 3T3 cells transformed by an activated ras allele (30). However, using in vivo imaging, we found that s.c. implanted tumors proliferate at similar rates in both treated and untreated animals. In contrast, treatment with Atorvastatin prevented colonization of the pulmonary stroma with hematogenously seeded melanoma cells.

These results suggest that Atorvastatin treatment, at the dosages employed in this study, exerted an anti-metastatic effect due to inhibition of adherence, extravasation, seeding, or colonization of the lung beds rather than through an overt anti-proliferative mechanism. Whereas the current study focused specifically on Atorvastatin, other statins also hold promise for metastasis prophylaxis and deserve investigation as well.

Our results are in accordance with the observations of Clark et al. (4) who found the RhoC-overexpressing A375M cells employed herein to have similar in vitro growth kinetics as the parental A375P cells from which they were derived even though their in vivo metastatic potential was greatly augmented. In similar studies, van Golen et al. (5) observed no appreciable difference in the growth rates of inflammatory breast cancer cell lines expressing RhoC over controls even though RhoC lines behaved more aggressively in vivo. Taken together, these results suggest that, at levels routinely prescribed for hypercholesterolemia (31, 32), statins may have specific anti-metastatic effects. This implies that the potential clinical benefit of statin therapy need not correlate with the ability to kill cancer cells per se, just as the adaptive genetic changes leading to invasion and metastasis need not necessarily provide proliferative advantage.

In summary, we show that RhoC is widely expressed in human melanoma. The expression levels of RhoA or RhoC negatively correlate with survival in patients with colorectal and breast (33) carcinoma. Our results indicate that Atorvastatin inhibits the in vitro invasion of melanoma cells via negative modulation of geranylgeranylation. Furthermore, we demonstrate that Atorvastatin specifically inhibits in vivo metastasis rather than proliferation. Taken together, our results suggest that Atorvastatin, at dosages currently employed for hypercholesterolemia, may hold promise for the management of patients at high risk for melanoma metastasis and recurrence. These findings support the prospective analysis of the statin family of drugs in the primary pharmaceutical prophylaxis of melanoma metastasis.

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References


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