Determinants of sensitivity to lovastatin-induced apoptosis in multiple myeloma

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Abstract

Statins, commonly used to treat hypercholesterolemia, have been shown to trigger tumor-specific apoptosis in certain cancers, including multiple myeloma (MM), a plasma cell malignancy with poor prognosis. In this article, we show that of a panel of 17 genetically distinct MM cell lines, half were sensitive to statin-induced apoptosis and, despite pharmacodynamic evidence of drug uptake and activity, the remainder were insensitive. Sensitive cells were rescued from lovastatin-induced apoptosis by mevalonate, geranylgeranyl PPI, and partially by farnesyl PPI, highlighting the importance of isoprenylation. Expression profiling revealed that Rho GTPase mRNAs were differentially expressed upon lovastatin exposure in sensitive cells, yet ectopic expression of constitutively active Rho or Ras proteins was insufficient to alter sensitivity to lovastatin-induced apoptosis. This suggests that sensitivity involves more than one isoprenylated protein and that statins trigger apoptosis by blocking many signaling cascades, directly or indirectly deregulated by the oncogenic lesions of the tumor cell. Indeed, clustering on the basis of genetic abnormalities was shown to be significantly associated with sensitivity (P = 0.003). These results suggest that statins may be a useful molecular targeted therapy in the treatment of a subset of MM. [Mol Cancer Ther 2007;6(6):1886–97]

Introduction

Statins are a family of drugs commonly used to control hypercholesterolemia that have recently been shown to induce apoptosis in a variety of tumor types, including multiple myeloma (MM; refs. 1–4). These compounds have the potential to be moved rapidly into the clinic as they have a long history of safety when used appropriately. Moreover, evidence shows that statins trigger tumor-specific apoptosis and do not cause collateral damage to nontransformed cells (5, 6). This has led to the hypothesis that the genetic lesions that cause disease also sensitize tumor cells to statin-induced apoptosis. Studies also show that statin induction of apoptosis is due to inhibition of the rate-limiting enzyme of the mevalonate pathway, hydroxymethylglutaryl CoA reductase (HMG-CoA reductase; ref. 1). The downstream molecular mechanism of statin-induced apoptosis in tumor cells involves the depletion of geranylgeranyl PPI (GGPP), an end-product of the mevalonate pathway and a substrate used in protein isoprenylation (1).

Approximately 0.5% to 2% of cellular protein is post-translationally modified by the addition of GGPP or farnesy1 PPI (FPP) moieties to COOH-terminal motifs, broadly termed CAAX boxes, to ensure proper localization at cellular membranes (7, 8). Of the known isoprenylated proteins, Rho and Ras GTPases are critical in promoting survival, proliferation, transformation, cell cycle control, and the regulation of cytoskeleton formation (9). Indeed, it has recently been shown that statin exposure leads to the mislocalization of several Ras and Rho GTPases from the membrane (10–15) and that the expression of many of these GTPases has also been shown to be up-regulated in response to statin exposure (14, 16, 17). These observations have been made in a wide range of cell types, presumably as part of a feedback response to replace functionally depleted isoprenylated proteins in statin-treated cells. For this reason, a role for the Ras and Rho proteins in mediating statin-induced apoptosis has been proposed (1, 4, 11–14, 16–19). It is thought that altering the activity of these proteins by statin treatment might affect tumor cell survival; however, this hypothesis has not yet been addressed experimentally.

MM is a plasma cell malignancy with a median survival time of 5 to 10 years despite the use of high-dose chemotherapy and autologous stem cell transplants (20). As such, there is an urgent need for the advancement of molecular diagnostics and treatment options in MM patient
care. Approximately half of all patients diagnosed with MM possess an immunoglobulin translocation involving juxtaposition of IgH (14q32) to known oncogenes (21). These include fibroblast growth factor receptor 3 (FGFR3/MM with a SET domain (MMSET), c-myc, mafB, and cyclins D1 and D3. Disease progression is characterized by the acquisition of mutations in FGFR3, N-Ras, K-Ras, or p53, and by subsequent secondary translocations that deregulate c-myc and inactivate PTEN or pRb (22–27). Prognosis for patients with IgH translocations, in particular those involving FGFR3/MMSET, is poor and novel therapeutic options are urgently required (28).

To advance statins as anticancer agents, it is crucial to understand the molecular mechanism and delineate markers that identify the subset of tumors that are sensitive to statin-induced apoptosis. To address this issue in MM, we assayed a panel of cell lines and showed that about half were sensitive, whereas the remainder were insensitive to statin-induced apoptosis. A comparison of these two subsets of cells within the same tumor type by expression profiling identified mRNAs that encode pivotal isoprenylated proteins whose expression levels were specifically altered in sensitive cells exposed to statins. We then directly addressed the functional role of these isoprenylated proteins, as well as several others, belonging to the Ras and Rho GTPase families. Interestingly, the regulation of statin sensitivity could not be assigned to any one protein, suggesting that disruption of several signaling pathways controlled by isoprenylated proteins is essential or responsible for the apoptotic response to statin exposure. Given that the identity of all isoprenylated proteins remains unclear, we assessed the utility of etiologic genetic abnormalities to serve as markers of sensitivity and suggest that this approach has merit to further define the determinants of sensitivity to statin-induced apoptosis.

Materials and Methods

Cell Culture

All cell lines were assayed as asynchronously growing cells and cultured in a humidified incubator at 37°C and 5% CO2. KHM11 and U266 were cultured in Iscove’s MEM and penicillin/streptomycin. KHM11 was also supplemented with 10% human serum; U266 was supplemented with 10% fetal bovine serum. ANBL-6 and OCI MY7 were grown in RPMI 1640 and supplemented with 10% human serum. ANBL-6 was further supplemented with 10% human serum; OCI MY7 was supplemented with 1 mmol/L pyruvate, 50 mmol/L HEPES, and 10% fetal bovine serum. Human serum was obtained following informed consent according to institutional guidelines. All other cells were cultured in RPMI 1640 and supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Compound Preparation

Lovastatin powder was a gift from Apotex Corp. and was activated as described previously (5). GGTT-298 and FTI-277 were obtained from Calbiochem and dissolved in DMSO. 3,4-Dimethylthiazolyl-2,2,5-diphenyl tetrazolium bromide, squalene, isopentenyladenine, dolichol, cholesterol, mevalonate, GGPP, and FPP were purchased from Sigma.

Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling

Cells (10^5) were seeded at a density of 2.5 × 10^5/mL and exposed to lovastatin for 48 h, harvested, and fixed with 4% formaldehyde for 15 min on ice. Cells were washed in cold PBS and stored at −20°C in 70% ethanol. Samples were labeled with FITC using the terminal deoxynucleotidyl transferase–mediated nick-end labeling method. Cells were incubated with biotin-dUTP (Roche) and 12.5 units terminal deoxynucleotidyl transferase enzyme (Roche) for 45 min at 37°C. Samples were washed, spun (1,000 rpm, 5 min), and resuspended in 200 μL 1:1,000 FITC-conjugated avidin (Sigma; in 4× SSC, 5% skim milk powder, 0.05% Tween 20) for 1 h or 1:200 Alexa Fluor 633–conjugated streptavidin (Molecular Probes; in 4× SSC, 0.05% Tween 20). Samples were washed and stained with 1 μg/mL propidium iodide (Sigma) or 10 μmol/L Hoechst 33342 (Molecular Probes) and DNase-free RNase (Roche) for 30 min. Cells (10^5) were analyzed for FITC-positive cells by flow cytometry.

Antibodies and Immunoblot Analysis

Cells (10^5) were washed in cold PBS and lysed [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPI, 1 mmol/L 2-β-glycerophosphate, 1 mmol/L Na2VO4, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride]. Blots were probed with anti–poly(ADP)ribose polymerase (PARP) antibody (Cell Signaling Technology; 1:1,000 dilution), anti–N-Ras, anti–K-Ras, anti-RhoA, anti–RhoB, and anti–Cdc42 antibodies (Santa Cruz Biotechnology; 1:500 dilution), anti-Rac1 (Cytoskeleton; 1:500 dilution), or anti-actin (Sigma; 1:3,000 dilution), and anti-rabbit or mouse peroxidase-conjugated secondary IgG antibodies (Amersham Biosciences; 1:10,000 dilution). Enhanced chemiluminescence (New England Biolabs) was used for detection on film from Kodak or Interscience Bioflex MSI (VWR).

Expression Profiling

Cells (10^5) were treated with ethanol or 20 μmol/L lovastatin. To avoid detecting apoptotic effects, kinetics of lovastatin-induced apoptosis were evaluated. Sensitive cells were exposed to 20 μmol/L lovastatin for between 3 and 48 h and assessed for PARP cleavage compared with solvent control. PARP cleavage was evident by 18 to 24 h in KMS11 and by 30 to 48 h in H929 (data not shown). Therefore, we exposed KMS11 cells to 20 μmol/L lovastatin for 15 h and H929 cells to 20 μmol/L lovastatin for 27 h before harvesting for expression profiling. −3 h before apoptosis. For time-matched controls, insensitive cells were exposed to 20 μmol/L lovastatin for 15 h (LP1 and ANBL-6) or 27 h (U266).

RNA was extracted with TRIzol reagent (Invitrogen) and 80 μg was used to synthesize cDNA with SuperScript II (Invitrogen) then stored at −20°C. Ten micrograms of
control and treated cDNA were labeled with either Cy3 or Cy5 and hybridized at 42°C overnight onto single-spotted 19.2 k human cDNA arrays (UHN Microarray Centre; version 6). Arrays were washed, scanned using a Packard Scanner, and quantified using ImageQuant (BioDiscovery, v1.1). Samples were hybridized six independent times.

Quantified array data was background corrected with a convolution of exponential and normal distributions (29). A 50-iteration LOESS smoother was applied across print-tip groups to remove spatial variation across each array (30); then, quantile normalization was used to bring all arrays to a common distribution (31). Each array was manually investigated for spatial or distributional inhomogeneities. A linear model was then fitted to estimate the expression levels for each RNA sample (32). A contrast-matrix was used to extract the lovastatin-induced effects in each cell line and model-based t tests were applied to each contrast. Following an empirical Bayes moderation of SE (29), a false-discovery rate adjustment for multiple testing was applied (33). Each spot on the array was then re-annotated in a custom-designed IBM DB2 database. Re-annotation used a novel poll-based cluster-assignment algorithm. Briefly, the sequence representing each spot was BLASTed at low stringency against the human expressed sequence tags database. The resulting set of BLAST results was then "polled" to identify the UniGene cluster (34) most representative of the initial sequence. A significance threshold of P = 0.01 was used for all cell lines. Analysis was done with the Limma package (v2.3.3; ref. 29) for the R statistical language (v2.0.1).

**Real-time Reverse Transcription-PCR**

Cells (10⁶) were treated with ethanol (solvent control) or 20 μM/L lovastatin as described for expression profiling. RNA was harvested from cells using TRIzol reagent (Invitrogen) and cDNA was synthesized from 1 μg of RNA with SuperScript II (Invitrogen). Probe and primer sets were TaqMan Gene Expression Assays (Applied Biosystems) for glyceraldehyde-3-phosphate dehydrogenase (Hs00269660_s1), RhoA (Hs00357608_m1), and RhoB (Hs00269660_s1). Real-time PCR acquisition and analysis was done on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Three independent experiments were conducted in triplicate and the expression of RhoA and RhoB relative to glyceraldehyde-3-phosphate dehydrogenase was determined.

**Generation of Constructs**

V12K-Ras was obtained from Dr. Ming Tsao (Ontario Cancer Institute, Toronto, ON, Canada) and Q61N-Ras was obtained from Dr. Robert Kay (Terry Fox Institute, Vancouver, BC, Canada). Flag-tagged V14RhoA, V14RhoB, V12Rac1, and V12Cd42 were obtained from Dr. Anne Ridley (Ludwig Cancer Institute, London, United Kingdom). Bcl-2 and Bcl-xL cDNAs were obtained from Dr. David Andrews (McMaster University, Hamilton, ON, Canada). All constructs were subcloned into the pBabeM-NiresGFP vector obtained from Dr. Garry Nolan (Stanford University, Stanford, CA). The myristoylated sequence was inserted into the pBabeMNiresGFP vector using Quick-change Site Directed Mutagenesis Kit (Stratagene). All clones were verified by sequencing.

**Retrovirus Construction, Production, and Infection**

Cell lines expressing the ecotropic receptor (LP1-EcoR and KMS11-EcoR) were generated and subsequently infected by spin infection with the appropriate green fluorescent protein (GFP) bicistronic retroviral constructs as previously described (35). Cell populations containing >95% GFP-positive cells were used for subsequent experiments.

**GTPase Pull Downs**

GTPase pull downs were done as per the manufacturer’s instructions (Upstate Cell Signaling), for the following substrates, Pak-1 PBD agarose, Rhotekin RBD agarose, and Raf RBD agarose. For RhoB pull downs, the following changes were made: 2 × 10⁷ cells were used; the incubation with beads was carried out for 2.5 h followed by three washes and centrifugation at 3,000 rpm for 30 s.

**Genetic Abnormality Clustering Analysis**

Cell lines were grouped into two clusters using an unsupervised pattern-recognition algorithm, k-means clustering, iterated 100,000 times to ensure the global minimum was reached (R, v2.2.0). Statistical significance was determined with the hypergeometric distribution.

**Results**

**MM Cell Lines Display Differing Degrees of Sensitivity to Statin-Induced Apoptosis**

Several recent reports on a limited number of cell lines indicate that MM cells are sensitive to statin-induced apoptosis (1–4). We expand on these results by assessing a substantially larger set of MM lines for their sensitivity to undergo apoptosis in response to statins. Our previous work has shown that statins trigger apoptosis in a manner that is both time- and dose-dependent and that cells derived from tumor types that are sensitive to statins in vitro can show a response at achievable therapeutic concentrations in vivo (5, 36–38). For example, cell lines derived from acute myelogenous leukemia will undergo apoptosis by the same mechanism of action in response to either 20 μM/L lovastatin for 48 h or 1 μmol/L lovastatin for 4 days (39). Moreover, comparing responsiveness to statins between cell lines can distinguish tumor cell types that are sensitive to statin-induced apoptosis (acute myelogenous leukemia) from those that are insensitive (acute lymphocytic leukemia; ref. 5), and here we apply this approach to further evaluate MM.

Following treatment, cells were harvested and assayed for apoptosis by using terminal dUTP nick-end labeling

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6 P.C. Boutros et al., in preparation.

7 http://www.stanford.edu/group/nolan/protocols/pro_optimiz.html
(TUNEL) or immunoblotting for PARP cleavage (Fig. 1A, B, and D). Cell lines were classified as sensitive if evidence of both TUNEL-positive staining (>10%) and PARP cleavage was observed. By this approach, 8 of the 17 cell lines tested were shown to be sensitive to lovastatin-induced apoptosis: KMS11, OPM2, KHM11, H929, OCI MY7, 8226, KMM1, and MM-S1 (shaded in Fig. 1D). Those considered insensitive to lovastatin-induced apoptosis include MM.1-144, EJM, LP1, OCI MY5, ARK, SKMM1, ANBL-6, U266, and JJN3.

To determine if the absence of lovastatin-induced apoptosis was due to poor drug uptake, we monitored the processing of Rap1, a protein known to be exclusively geranylgeranylated (40). Unprocessed Rap1 was evident in all cell lines after lovastatin exposure (Fig. 1C and data not shown), indicating that although lovastatin uptake and activity was present in all cell lines examined, only a subset were sensitive to lovastatin-induced apoptosis (Fig. 1D). Because our panel of MM cell lines contains cells both sensitive and insensitive to lovastatin-induced apoptosis, we had the opportunity to examine the molecular mechanism and determinants of sensitivity between these cells, within a similar tumor type.

**Mevalonate and GGPP Abrogate Lovastatin-Induced Apoptosis in MM Cells**

Mevalonate, the product of the reaction catalyzed by HMG-CoA reductase, is the precursor to metabolic pathways leading to essential cellular end products such as cholesterol, isopentenyladenine, dolichol, ubiquinone, GGPP, and FPP. We and others have shown that of these downstream end-products, GGPP most consistently reverses statin-induced apoptosis in sensitive tumor cells (1, 11, 16, 41). To evaluate the mechanism of lovastatin-induced apoptosis in MM, four sensitive MM cell lines (KMS11, OPM2, H929 and OCI MY7) were screened by TUNEL assay (Fig. 2A) and immunoblotting for PARP cleavage (Fig. 2B). Co-incubation with mevalonate fully reversed lovastatin-induced apoptosis in sensitive MM cells (Supplementary Table S1). Eight supplementary material for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

To further investigate the role of protein isoprenylation in lovastatin-induced apoptosis of MM cells, we assessed the effects of geranylgeranyl transferase and farnesyl transferase inhibitors (GGTIs and FTIs, respectively). Both the TUNEL assay (Fig. 2C) and the presence of cleaved PARP (data not shown) were used to assess apoptosis. Our results showed that 20 μmol/L GGTI-298, but not 20 μmol/L FTI-277, induced apoptosis comparably with 20 μmol/L lovastatin. Thus, the analysis of sensitive MM cell lines has revealed that the loss of GGPP, and FPP to a lesser extent, play an important role in lovastatin-induced apoptosis in MM cells.

**Rho GTPases Are Differentially Expressed in MM Cells Sensitive to Statin-Induced Apoptosis**

Having established a lovastatin-sensitive subset of MM cells that undergo apoptosis through a mechanism that involves isoprenylated proteins, we were able to investigate the mechanism of statin-induced apoptosis by comparing and contrasting to the lovastatin-insensitive subset of MM cells. Because direct analysis of all cellular isoprenylated proteins is not technically feasible at this time, we profiled the mRNA expression changes in response to statins with the idea that proteins whose loss-of-function contributed to apoptosis would be up-regulated at the level of mRNA expression as part of a cellular feedback response. Two sensitive MM cell lines (KMS11 and H929) and three relatively insensitive MM cell lines (LP1, ANBL-6, and U266) were profiled on cDNA microarrays after exposure to vehicle control or lovastatin. To ensure that we did not capture apoptotic effects, we evaluated the kinetics of lovastatin-induced apoptosis (data not shown) and assayed mRNA expression at time points ~3 h before apoptosis.

Interestingly, a substantially smaller number of transcripts were differentially expressed in response to lovastatin in sensitive cell lines compared with those that are sensitive (Fig. 3A; Supplementary Table S1). There were also no common differentially expressed transcripts among the three insensitive MM cell lines profiled. Only isopentenyl-diphosphate δ isomerase was found to be commonly up-regulated in insensitive lines (LP1 and ANBL-6), but was also found to be up-regulated in the sensitive KMS11 cells (Supplementary Table S1).

Within the sensitive MM cell lines, 21 genes were significantly up-regulated and only one gene was significantly down-regulated in both lines (Table I). The 21 differentially expressed genes were grouped using GOstat (42) to determine statistically overrepresented gene ontology groups (P < 0.1). One such group is the cytoskeleton gene ontology group (including KRT8, MYOSC, KIFAP3, VIM, TMSB4X, and ADD3), suggesting a role for cytoskeletal structure in MM cell lines sensitive to lovastatin-induced apoptosis. Furthermore, RhoB was significantly up-regulated in response to lovastatin in both sensitive cell lines and RhoA was
Determinants of Statin Sensitivity

A

KMS11 - Sensitive

![Graph showing FITC-dUTP and Propidium Iodide for ethanol and lovastatin]

OCI MY5 - Insensitive

![Graph showing FITC-dUTP and Propidium Iodide for ethanol and lovastatin]

B

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<thead>
<tr>
<th></th>
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</tr>
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<td>- +</td>
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- PARP
- p85
- actin

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- U
- P
- Rap1
- actin

D

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<td>JJN3</td>
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also up-regulated in one line (H929). Differential expression of RhoA and RhoB was confirmed using real-time PCR (Fig. 3B). The differential expression in response to lovastatin indicates a potential role for the Rho GTPases in determining sensitivity to lovastatin-induced apoptosis in MM.

Ectopic Expression of Isoprenylated Proteins Does Not Modulate Sensitivity to Statin-Induced Apoptosis

The Rho GTPases are isoprenylated proteins differentially expressed in response to lovastatin within sensitive MM cells, and have previously been implicated in statin-induced apoptosis in tumor cells, including MM (4, 11, 18). Together, these data prompted us to assess whether members of this family could functionally modulate sensitivity to lovastatin-induced apoptosis in MM cells.

A series of activated Rho family members were introduced into both sensitive and insensitive MM cells. Constitutively activated forms of RhoA (V14RhoA), RhoB (V14RhoB), Rac1 (V12Rac1), and Cdc42 (V12Cdc42) were retrovirally and stably expressed in lovastatin-insensitive LP1 cells. We also introduced myristoylated and constitutively activated forms of RhoA (mV14RhoA), RhoB (mV14RhoB), Rac1 (mV12Rac1), or Cdc42 (mV12Cdc42) into the lovastatin-sensitive KMS11 line. The myristoylation signal sequence was added in-frame to the NH2 terminus of these constructs to ensure the activated molecules would be localized to the membrane despite inhibition of isoprenylation through lovastatin-mediated depletion of FPP and GGPP (43). Because lovastatin-induced apoptosis has been previously shown to be abrogated by Bcl-2 expression (39), the antiapoptotic Bcl-2 and Bcl-xL were also introduced into KMS11 cells as positive controls.

Cells expressing the various activated Rho family members were first analyzed for constitutive activation by Rhotekin (for RhoA and RhoB) and Pak-1 (for Rac1 and Cdc42) precipitations of the GTP-bound forms (Fig. 4A and B). Subsequently, the LP1 cells expressing activated Rho GTPases were treated with 20 μmol/L lovastatin for 48 h and apoptosis was measured using TUNEL staining and immunoblotting for PARP cleavage. There was no significant increase observed in the percentage of cells staining TUNEL (apoptotic) positive (Fig. 4C) or the presence of PARP cleavage (data not shown).

To determine if expression of the Rho family members could rescue KMS11 cells from lovastatin-induced apoptosis, cells expressing the different myristoylated and activated Rho GTPases were exposed to 2.5 or 5 μmol/L lovastatin for 24 and 48 h. Low doses were chosen because, although even the potently antiapoptotic Bcl-2 only delays statin-induced apoptosis (39), we wanted to ensure that even weak or partial rescues would be detectable. Although Bcl-2 and Bcl-xL performed as robust positive controls, myristoylated and constitutively activated Rho family constructs did not significantly delay the apoptotic response (Fig. 4D; data not shown). Taken together, ectopic expression of constitutively active RhoA, RhoB, Rac1, and Cdc42 did not modulate sensitivity to lovastatin-induced apoptosis in MM cells.

Of the various molecular abnormalities associated with MM etiology, mutations in either K-Ras or N-Ras are quite common (22, 26, 27). Furthermore, the disruption of Ras signaling has also been suggested to be important in statin-induced apoptosis of MM cells (4, 10). Therefore, we next retrovirally introduced constitutively active K-Ras (V12) or N-Ras (Q61) into the lovastatin-insensitive LP1 cell line and constitutively active myristoylated K-Ras (V12) or N-Ras (Q61) into lovastatin-sensitive KMS11 cells. Activation of the Ras constructs was verified by precipitating the GTP-bound forms with the G-protein binding domain of Raf (44; Fig. 5A and B).

These cells were then exposed to lovastatin as described above and analyzed for apoptosis by TUNEL staining (Fig. 5C and D) and PARP cleavage (data not shown). As observed with the Rho constructs, ectopic expression of the Ras constructs did not significantly change the apoptotic response to lovastatin in either the LP1 or KMS11 cells, despite robustly decreased apoptosis in KMS11 cells expressing Bcl-2 and Bcl-xL.

Contrary to widely held notion and published opinion, our results clearly indicate that constitutive activation of individual Rho (RhoA, RhoB, Rac1, and Cdc42) and Ras family members (K-Ras or N-Ras) does not modulate lovastatin-induced apoptosis in MM. These data suggest that either another isoprenylated protein is essential to lovastatin-induced apoptosis, or that the apoptotic response depends on blocking the isoprenylation of more than one protein. Thus, a more global genetic approach to distinguish between sensitive and insensitive cells was required.

MM Sensitivity to Statin-Induced Apoptosis Correlates with Particular Molecular Markers of MM Disease and Progression

To further identify molecular markers of sensitivity to statin-induced apoptosis, we focused on the classic

Figure 1. Lovastatin induces apoptosis in a subset of MM cell lines. Cells were exposed to ethanol (solvent control) or 20 μmol/L lovastatin for 48 h. A, representative profiles assessing apoptosis, using TUNEL staining. The percentage of apoptotic cells [TUNEL positive; top right quadrant of the profile] are representative of a minimum of three independent experiments. Eight of 17 MM cell lines show an increase in the TUNEL-positive population upon lovastatin exposure (D). B, cells were harvested, lysed, and immunoblotted for the detection of cleaved PARP (p85), and actin as a loading control. PARP cleavage is observed in some, but not all, MM cell lines (D). C, processed (P) and unprocessed (U) Rap1 was detected by immunoblotting in representative groups of both sensitive and insensitive MM cell lines after exposure to lovastatin; actin was detected as a loading control. Rap1 becomes unprocessed in all MM cell lines, thus, the lack of an apoptotic response in insensitive MM cells is not due to poor drug uptake. D, sensitivity to lovastatin-induced apoptosis determined by TUNEL (>10%) and PARP cleavage (+); shaded data, cell lines sensitive to lovastatin-induced apoptosis. Each cell line was assayed in a minimum of three independent experiments and results are mean ± SE. Each cell line was assayed in a minimum of three independent experiments.
molecular features of MM disease etiology. These include either activating mutations of N-Ras, K-Ras or FGFR3, or chromosome translocations of c-myc (8q24), mafB (20q11), FGFR3/MMSET (4p16), cyclin D1 (11q13), cyclin D3 (6p21), c-mafB (16q23), or N-myc (1p36; Fig. 6; refs. 22, 24, 45–47). First, as four of the five cell lines harboring t(4;14) were sensitive, we determined whether single genetic abnormalities could be associated with lovastatin sensitivity. No single genetic abnormality was associated with sensitivity to lovastatin-induced apoptosis (Fisher’s test, data not shown).

We next used an unbiased pattern recognition algorithm to determine if lovastatin sensitivity is associated with multiple genetic abnormalities. Cell lines grouped into two clusters using k-means clustering (48) were tested for the enrichment of cells with similar sensitivity to lovastatin-induced apoptosis. The first cluster is primarily composed of cell lines that are sensitive to lovastatin-induced apoptosis (five of seven), whereas the second cluster is primarily composed of cells that are insensitive (7 of 10). The enrichment for sensitive and insensitive cell lines within clusters 1 and 2, respectively, was statistically significant ($P = 0.003$) as determined by the hypergeometric distribution. Therefore, we have identified that genetic abnormalities can be used to differentiate sensitive MM cells from those that are insensitive. These results further suggest that molecular markers of drug sensitivity will be defined by the various genetic abnormalities underlying tumor formation and progression.

**Discussion**

The statin family of drugs has been shown to induce tumor-specific apoptosis, yet, the molecular mechanism and determinants of sensitivity remain unclear. In this report, a large panel of MM cells was assessed and, while approximately half are sensitive to lovastatin-induced apoptosis, the other half are insensitive despite evidence of drug uptake. These intriguing results expand upon previous studies (1–4), which identified smaller numbers of MM cell lines as sensitive. It is of interest to note that cell lines derived from most tumor types generally tend to be either primarily sensitive or insensitive (5,36). As such, our MM panel is uniquely composed of both sensitive and insensitive cell lines. We have exploited this cohort to provide insight into the molecular mechanisms underlying sensitivity to statin-induced apoptosis.

Reversal of lovastatin-induced apoptosis by mevalonate was notably consistent in the sensitive cells, confirming that HMG-CoA reductase is the cellular target of lovastatin. Lovastatin-induced apoptosis was also reversed by the addition of GGPP, and partially by FPP. Although FPP lies upstream of GGPP in the mevalonate pathway, the addition of FPP would be unable to restore protein geranylgeranylation because a second molecule, isopentenyl PPy, is required for the conversion of FPP to GGPP. As isopentenyl PPy is also depleted by statin exposure, it would be unavailable to the statin-treated cell (49, 50).
These results, as well as the ability of GGTI-298 to induce apoptosis comparably with statins in sensitive tumor lines, build on previous reports (1, 11, 16, 41) to clearly indicate the importance of protein geranylgeranylation in statin-induced apoptosis.

Further insight into the mechanism of statin sensitivity was derived from our mRNA expression analysis of MM cells treated with or without lovastatin. In our study, typical apoptosis-related transcripts were not altered in sensitive MM cells, confirming that mRNA expression was assayed before traditional apoptotic events, a feature that may have confounded a previous microarray study using fluvastatin-treated H929 cells (51). Furthermore, transcripts differentially expressed in response to lovastatin in MM do not overlap with those differentially expressed in breast cancer cells exposed to cerivastatin (52).

As breast cancer cells typically undergo classic growth arrest upon statin treatment, regulators of cell proliferation (such as cyclin D1, c-myc, p21\textsuperscript{WAF1}, and p19\textsuperscript{INK4d}) are differentially expressed. The observation that these transcripts were not differentially expressed in our study indicates that cells sensitive to statin-induced apoptosis may either not receive, or cannot carry out, classic growth arrest signals.

mRNAs that were up-regulated in sensitive MM cells include transcripts coding for Rho GTPases and cytoskeletal proteins, such as intermediate filaments. Disruption of intermediate filaments may be in part due to lovastatin-induced abrogation of Rho GTPase activity, which resulted in the up-regulation of Rho GTPase expression. Up-regulation of isoprenylated proteins in response to HMG-CoA reductase inhibition has been shown in other sensitive cell types (14, 16, 17). These results, combined with other studies indicating the importance of geranylgeranylated proteins in statin-induced apoptosis (1, 4, 10–15, 18, 19, 53), prompted us to directly address the ability of individual Rho GTPases to modulate sensitivity to lovastatin.

Surprisingly, ectopic expression of constitutively active RhoA, RhoB, Rac1, or Cdc42 individually did not alter sensitivity to statin-induced apoptosis in MM cells. Another study also showed that introduction of a dominant-negative Rac1 reduced proliferation but did not induce apoptosis in MM cells (18). Furthermore, knockdown of RhoA or RhoC reduced proliferation in breast cell lines (54). Taken together, these studies suggest that the Rho family members may be important for proliferation but not survival in these cell types.

Activating mutations in K-Ras and N-Ras have been shown to be common in MM etiology (22, 26, 27). To determine whether these isoprenylated proteins play a role in statin-induced apoptosis individually, constitutively active K-Ras and N-Ras were ectopically expressed in MM cells. Sensitivity to statin-induced apoptosis in these cells was again unaltered, in agreement with recent evidence suggesting activating mutations of Ras in acute myeloid leukemia do not correlate with sensitivity to statin-induced apoptosis (55).

These results clearly show that it is highly unlikely that any one of the isoprenylated proteins addressed here are of singular importance to statin-induced apoptosis of MM cells, as has been commonly proposed in the past based purely on their inactivation by statin-induced
mislocalization. This lead to the conclusion that either the one isoprenylated protein responsible for inducing statin-induced apoptosis was not evaluated, or, perhaps more likely, apoptosis depends on the loss of multiple isoprenylated proteins. This is supported by the ability of GGTIs to kill tumors in vivo (56) and efforts to identify other isoprenylated proteins may further inform us of the mechanism of action.

MM is a complex disease involving multiple, well-defined abnormalities (22, 24, 45–47). To successfully implement statins in patient care, we have exploited these defined abnormalities to understand the molecular markers conferring sensitivity to statin-induced apoptosis. For example, four of five cell lines harboring the t(4;14) translocation of FGFR3/MMSET were determined to be sensitive to lovastatin-induced apoptosis. Furthermore, Otsuki et al. (2, 57) assayed a panel of MM cell lines characterized to express FGFR3 and has shown them to be largely sensitive to apoptosis induced by simvastatin.

### Table 1. Commonly differentially expressed genes (P < 0.01) determined by GOSTAT in sensitive MM cell lines (KMS11 and H929) exposed to lovastatin

<table>
<thead>
<tr>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isoprenylated proteins</strong></td>
<td><strong>Metabolism</strong></td>
</tr>
<tr>
<td>Hs.502876 Ras homologue gene family, member B</td>
<td>Hs.76244 Spermidine synthase</td>
</tr>
<tr>
<td><strong>Signaling</strong></td>
<td><strong>Metabolism</strong></td>
</tr>
<tr>
<td>Hs.175343 Phosphoinositide-3-kinase, class 2, a polypeptide</td>
<td>Hs.501012 Adducin3</td>
</tr>
<tr>
<td>Hs.404321 Glycyl-tRNA synthetase</td>
<td>Hs.187199 Metastasis-associated lung adenocarcinoma transcript 1</td>
</tr>
<tr>
<td>Hs.75069 Serine hydroxymethyltransferase 2 (mitochondrial)</td>
<td>Hs.502756 AHNAK nucleoprotein (desmoyokin)</td>
</tr>
<tr>
<td><strong>Transcription</strong></td>
<td><strong>Metabolism</strong></td>
</tr>
<tr>
<td>Hs.522074 A Sleep inducing peptide, immunoreactor</td>
<td>Hs.76244 Spermidine synthase</td>
</tr>
<tr>
<td>Hs.49647 Activating transcription factor 4</td>
<td><strong>Cytoskeleton</strong></td>
</tr>
<tr>
<td>Hs.553317 Vimentin</td>
<td>Hs.533317 Vimentin</td>
</tr>
<tr>
<td>Hs.533782 Keratin 8</td>
<td>Hs.522584 Thymosin, β 4, X-linked</td>
</tr>
<tr>
<td>Hs.433442 Kinesin-associated protein 3</td>
<td>Hs.487036 Myosin VC</td>
</tr>
<tr>
<td>Hs.496478</td>
<td>Hs.501012 Adducin3</td>
</tr>
<tr>
<td>Hs.75069</td>
<td><strong>Other</strong></td>
</tr>
<tr>
<td><strong>Cytoskeleton</strong></td>
<td>Hs.187199 Metastasis-associated lung adenocarcinoma transcript 1</td>
</tr>
<tr>
<td>Hs.533317</td>
<td>Hs.502756 AHNAK nucleoprotein (desmoyokin)</td>
</tr>
<tr>
<td>Hs.533782</td>
<td>Hs.113314 Similar to hypothetical protein LOC192734</td>
</tr>
<tr>
<td>Hs.433442</td>
<td>Hs.344165 Ubiquitin-conjugating enzyme E2H</td>
</tr>
<tr>
<td>Hs.487036</td>
<td>Hs.475506 IQ motif and Sec7 domain 1</td>
</tr>
<tr>
<td>Hs.501012</td>
<td>Hs.448738 Ferritin, heavy polypeptide 1</td>
</tr>
<tr>
<td>Hs.496478</td>
<td>Hs.33756 Similar to Kruppel-like factor 7 (ubiquitous); ubiquitous</td>
</tr>
<tr>
<td><strong>Transcription</strong></td>
<td>Hypothetical LOC388969</td>
</tr>
<tr>
<td>Hs.516159</td>
<td>Hs.516159 Hypothetical LOC388969</td>
</tr>
<tr>
<td>Hs.529948</td>
<td>Hs.529948 MDN1, midasin homologue (yeast)</td>
</tr>
<tr>
<td><strong>Down-regulated</strong></td>
<td><strong>Transcription</strong></td>
</tr>
<tr>
<td>Hs.529948</td>
<td>Hs.516159 Hypothetical LOC388969</td>
</tr>
<tr>
<td>Hs.344165</td>
<td>Hs.529948 MDN1, midasin homologue (yeast)</td>
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**Determinants of Statin Sensitivity**

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Although these results suggest an association between t(4;14) and sensitivity to statin-induced apoptosis, the translocation alone was not significantly associated with sensitivity (data not shown). It is possible that the size of the MM cell line panel used in this study was not large enough to detect an association between a single genetic abnormality andLovastatin sensitivity. Patients with this translocation have a particularly poor prognosis with no clinical benefit of high-dose chemotherapy (28), creating enormous demand for novel treatment options. Further study will determine whether statin treatment can benefit these patients.

Because cancer arises from combinations of mutations, we reasoned thatlovastatin sensitivity may, in turn, be more strongly associated with multiple genetic abnormalities than with a single abnormality. Interestingly, clustering analysis identified a signature of genetic abnormalities significantly enriching for sensitive cell lines (P = 0.003). The clustering analysis has been uniquely applied to abnormalities underlying MM disease onset and progression, but is strictly analogous to earlier work with mRNA (58) and micro-RNA (59) expression. This novel genetic clustering approach has the potential for predictive power and, as with all clustered profiles (60), will require further validation in a clinical setting to determine its utility in predicting tumor sensitivity to statin-induced apoptosis. Taken together, sensitivity toLovastatin-induced apoptosis seems to be highly associated with a particular profile of genetic abnormalities that results in the onset and progression of MM.

Most conventional treatment options available to MM patients only minimally improve outcome. This underscores the urgent requirement for novel treatment options, and we propose that statins are ideal candidates to fulfill this need. To date, they have been shown to synergize with clinical therapies currently used to treat MM patients.

**Figure 4.** Rho GTPase family members do not modulate sensitivity to statin-induced apoptosis. Activated Rho GTPase family members (V14RhoA, V14RhoB, V12Rac1, and V12Cdc42) were introduced into LP1, an insensitive MM cell line. Myristoylated and activated forms (mV14RhoA, mV14RhoB, mV12Rac1, and mV12Cdc42) were introduced into sensitive KMS11 cells, as were Bcl-2 – and Bcl-xL – positive controls. **A** and **B,** constitutive activation of the Rho constructs in LP1 and KMS11 was assessed by precipitating the active, GTP-bound form and subsequent immunoblotting. More of the active, GTP-bound form was detected in cells expressing the Rho constructs (lane 3) than in the parental cells (lane 1), or those expressing GFP alone (lane 2), given an equal amount of input protein (lanes 4–6). **C,** parental LP1 cells and those expressing the ecotropic receptor (EcoR) alone, GFP, or the activated Rho constructs were exposed to 20 μmol/L lovastatin for 48 h and were assessed for apoptosis by TUNEL. KMS11 cells were included as a positive control for lovastatin-induced apoptosis. **Columns,** mean percentage of TUNEL-positive cells in three independent experiments; bars, SE. None of the activated Rho constructs were able to increase sensitivity to lovastatin-induced apoptosis in LP1 cells. **D,** parental KMS11 cells and those expressing the ecotropic receptor alone, GFP, Bcl-2, Bcl-xL, or the activated and myristoylated Rho GTPases were exposed to 2.5 μmol/L lovastatin for 24 and 48 h and assessed for apoptosis by TUNEL. **Columns,** mean percentage of TUNEL-positive cells in three independent experiments; bars, SE. Although Bcl-2 and Bcl-xL inhibited lovastatin-induced apoptosis, the myristoylated and activated Rho constructs were unable to do so in KMS11 cells.

**Figure 5.** Ras GTPase family members do not modulate sensitivity to statin-induced apoptosis. Activated Ras GTPase family members (V12K-Ras or Q61N-Ras) were introduced into LP1, an insensitive MM cell line. Myristoylated and activated forms (mV12K-Ras or mQ61N-Ras) were introduced into sensitive KMS11 cells, as were Bcl-2 and Bcl-xL. **A** and **B,** constitutive activation of the Ras constructs was assessed by precipitating the GTP-bound form and subsequent immunoblotting. More of the active, GTP-bound form was detected in cells expressing the Ras constructs (lane 3) than in the parental cells (lane 1), or those expressing GFP alone (lane 2), given an equal amount of input protein (lanes 4–6). **C,** parental LP1 cells and those expressing the ecotropic receptor alone, GFP, or the activated Ras constructs were exposed to 20 μmol/LLovastatin for 48 h and were assessed for apoptosis by TUNEL. KMS11 cells were included as a positive control forLovastatin-induced apoptosis. **Columns,** mean percentage of TUNEL-positive cells in three independent experiments; bars, SE. The activated Ras GTPases were unable to increase sensitivity toLovastatin-induced apoptosis in LP1 cells. **D,** parental KMS11 cells and those expressing the ecotropic receptor alone, GFP, Bcl-2, Bcl-xL, or the activated and myristoylated Ras constructs were exposed to 2.5 μmol/LLovastatin for 24 and 48 h and assessed for apoptosis by TUNEL. **Columns,** mean percentage of TUNEL-positive cells in three independent experiments; bars, SE. Although Bcl-2 and Bcl-xL inhibitedLovastatin-induced apoptosis, the activated Ras constructs were unable to do so in KMS11 cells.
including dexamethasone, all-trans retinoic acid, melphalan, and doxorubicin (1, 2, 19, 61). Furthermore, because they can be rapidly introduced into clinical practice, we believe that statins have potential as a viable therapeutic option for MM patients.

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Determinants of sensitivity to lovastatin-induced apoptosis in multiple myeloma

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