ORIGINAL ARTICLE

Cell-Cycle Gene Expression in Lovastatin-Induced Medulloblastoma Apoptosis

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ABSTRACT: ***Background:*** 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a key rate-limiting enzyme in the mevalonate pathway, which generates precursors both for cholesterol biosynthesis and for the production of nonsteroidal mevalonate derivatives that are involved in a number of growth-regulatory processes. We have reported that lovastatin, a competitive inhibitor of HMG-CoA reductase, not only inhibits medulloblastoma proliferation *in vitro*, but also induces near-complete cell death via apoptosis. The mechanism of this phenomenon is unclear. Possible involvement of changes in expression of certain cell-cycle related genes led us to study some of them in more detail. ***Methods:*** Medulloblastoma cell lines were exposed *in vitro* to lovastatin, and the effects of gene expression changes were studied using RT-PCR, antisense oligonucleotide, DNA electrophoresis and Western blotting analysis. ***Results:*** 1) Levels of total *Ras* gene mRNA and individual *Ras* gene mRNA are stable in lovastatin treatment in all examined medulloblastoma cell lines. 2) Blocking *c-myc* gene over-expression does not enhance medulloblastoma cell sensitivity to lovastatin. 3) Following lovastatin treatment, *p16* expression exhibits no change, but pronounced increases of $p27^{KIP1}$ protein are observed in all examined cell lines. Lovastatin induces pronounced increases of $p21^{WAF1}$ protein only in Daoy and UW228, but not in D283 Med and D341 Med. 4) Following lovastatin treatment, increased p53 protein is detected only in D341 Med, and bax protein is unchanged in all cell lines. ***Conclusions:*** Lovastatin-induced growth inhibition and apoptosis in medulloblastoma are not dependent on the regulation of *Ras* and *c-myc* gene expression, but may be mediated by $p27^{KIP1}$ gene expression. Lovastatin-induced apoptosis in medulloblastoma is probably p53 independent, but *p53* and $p21^{WAF1}$ gene expression may also mediate anti-proliferative effects of lovastatin on specific medulloblastoma cell lines.

RÉSUMÉ: Apoptose induite par la lovastatine: **expression génique du cycle cellulaire dans des lignées cellulaires de médulloblastome.** ***Introduction***: La 3-hydroxy-3-méthylglutaryl-coenzyme A (HGM-CoA) réductase est un enzyme limitant clé de la voie du mévalonate qui génère des précurseurs tant pour la biosynthèse du cholestérol que pour la production de dérivés non stéroïdiens du mévalonate qui sont impliqués dans certains processus régulateurs de la croissance. Nous avons rapporté que la lovastatine, un inhibiteur non compétitif de l'HMG-CoA réductase, inhibe la prolifération du médulloblastome in vitro et induit également la mort cellulaire presque complète via l'apoptose. Le mécanisme sous-jacent à ce phénomène n'est pas clair. La possibilité que des changements dans l'expression de certains gènes du cycle cellulaire soient impliqués nous a incités à en étudier quelques-uns de plus près. ***Méthodes***: Des lignées cellulaires de médulloblastome ont été exposées in vitro à la lovastatine et les effets des changements dans l'expression génique ont été étudiés au moyen de RT-PCR, d'oligonucléotides antisenses, de l'électrophorèse de l'ADN et du buvardage western. ***Résultats***: 1) Les niveaux d'ARNm de tous les gènes Ras et des gènes Ras pris individuellement sont stables dans toutes les lignées cellulaires de médulloblastome étudiées après traitement par la lovastatine. 2) Le fait de bloquer la surexpression du gène c-myc n'augmente pas la sensibilité des cellules de médulloblastome à la lovastatine. 3) Suite au traitement par la lovastatine, l'expression de p16 ne change pas, mais on observe des augmentations considérables de la protéine p27KIP1 dans toutes les lignées cellulaires examinées. La lovastatine provoque des augmentations marquées de la protéine p21WAF1 dans le lignées Daoy et UW228, mais pas dans D283 Med et D341 Med. 4) Suite au traitement par la lovastatine, une augmentation de la protéine p53 est détectée seulement dans D341 Med et la protéine Bax demeure inchangée dans toutes les lignées cellulaires. ***Conclusions***: L'inhibition de la croissance et l'apoptose induites par la lovastatine dans le médulloblastome ne sont pas dépendantes de la régulation de l'expression des gènes Ras et c-myc, mais elles pourraient être médiées par l'expression du gène p27KIP1. L'apoptose induite par la lovastatine dans le médulloblastome est probablement indépendante de p53, mais l'expression des gènes p53 et p21WAF1 peut également médier des effets antiprolifératifs de la lovastatine dans des lignées cellulaires spécifiques de médulloblastome.

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Medulloblastoma, a primitive neuroectodermal tumor of cerebellum, accounts for about 20% of childhood intracranial tumors.[1] The prognosis of patients with medulloblastoma is unpredictable and only 50-70% survive after five years.[2,3] Radiation therapy is standard for medulloblastoma, but is not administered to patients less than three years old due to the

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deleterious effects on intellectual development.[4,5] Some success with adjuvant chemotherapy[2,6] has been balanced by dose-limiting toxicity and induction of drug resistance genes.[7,8] Significantly, the mortality of recurrent medulloblastoma approaches 100%,[9] emphasizing the need for effective treatment strategies.

Lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, blocks the mevalonate pathway, decreasing cholesterol biosynthesis as well as the production of nonsteroidal mevalonate derivatives.[10] Lovastatin has profound cellular effects, including inhibition of proliferation and induction of apoptosis,[11-19] and has been used as a potential anticancer drug in clinical studies.[20,21] We earlier found that lovastatin interrupts the mitotic cycle, leading to growth inhibition and the induction of apoptosis in medulloblastoma cells.[13] We also demonstrated that the inhibition of mevalonate production and protein farnesylation is critical for lovastatin-induced medulloblastoma apoptosis.[22] However, the sensitivity of cell lines to lovastatin varies[13] for uncertain reasons, and the regulation of gene expression in lovastatin-induced growth inhibition and apoptosis is not fully understood.

The mevalonate pathway generates nonsteroidal derivatives such as geranyl and farnesyl pyrophosphate, which are required for the isoprenylation of many membrane-bound proteins like the Ras protein super family.[23] Lovastatin impairs post-translational isoprenylation of these small G-proteins, rendering such proteins unable to anchor to membranes. In yeast, the mevalonate pathway directly regulates Ras levels by controlling *Ras* mRNA abundance.[24] Our previous results and others showed that isoprenylation inhibition increases total Ras protein levels.[14,25] However, the *Ras* gene family is complex, and contains *N-Ras, K-Ras* and *H-Ras*; furthermore, the *K-Ras* gene produces two K-Ras proteins via a single alternatively spliced transcript by which either exon 4a or 4b is spliced onto exon 3 during mRNA processing.[26] The regulation of expression of these *Ras* genes in lovastatin-induced apoptosis is not clarified.

In medulloblastoma, lovastatin 'resistant' cell lines, D283 Med and D341 Med,[13] over express *c-myc*.[27,28] c-myc normally participates in G1 to S transition, although *c-myc* gene over expression induces apoptosis when growth factor or serum is deprived.[29-31] It is interesting that lovastatin dramatically reduces *c-myc* gene expression via E2F-1 modulation.[32] We sought to determine whether *c-myc* gene over expression accounts for cell 'resistance' to lovastatin in D283 Med and D341 Med.

The expression and activity of cell cycle checkpoint proteins, particularly cyclins and cyclin-dependent kinases (Cdk), regulate transition through the cell cycle. Cyclins and Cdks in turn are regulated by the family of Cdk inhibitor proteins, such as p16, $p21^{WAF1}$ and $p27^{KIP1}$. $p21^{WAF1}$ and $p27^{KIP1}$ appear to function as broad specificity inhibitors of cyclin/CDK complexes. $p21^{WAF1}$ induction may be either p53-dependent or -independent, and $p21^{WAF1}$ expression induces growth inhibition and terminal differentiation.[33-35] $p27^{KIP1}$ shares partial homology with $p21^{WAF1}$, acts as a cyclin E/CDK2 inhibitor, and is thought to mediate general anti-proliferative effects in response to certain growth inhibitory signals.[36] The effects of HMG-CoA reductase inhibitors on $p21^{WAF1}$ and $p27^{KIP1}$ expression have been studied in different laboratories with conflicting results.[32,37-43]

Under certain conditions, inhibitors of macromolecule synthesis retard the process of apoptosis;[44] thus, it has been suggested that the process of apoptosis is under some form of genetic control. Several genes that are involved in the regulation of apoptosis have been identified thus far, such as *p53* and *bax*. p53 phosphoprotein is a transcription factor,[45] which functions to promote differentiation and apoptosis in certain cellular contexts[46] and acts as a cell cycle regulator which can induce cell cycle arrest in G1 phase.[47] Baxα, the principle product of *bax* gene, binds to Bcl-2 and promotes apoptosis.[48] p53 is a direct transcriptional activator of the human *bax* gene;[49] thus, bax expression serves as a marker of p53-dependent apoptosis.[50-52]

In this study, we investigate gene expression in lovastatin-induced growth inhibition and apoptosis in medulloblastoma cells, and report that 1) lovastatin has no effect on *Ras* gene expression; 2) *c-myc* gene over expression does not account for relative resistance of certain cell lines to lovastatin; 3) $p21^{WAF1}$ and possibly $p27^{KIP1}$, but not p16, contribute to lovastatin-induced growth inhibition and apoptosis; 4) lovastatin-induced apoptosis is probably p53 independent.

Material and methods

Unless stated otherwise, reagents and PCR primers were obtained from Gibco BRL, Gaithersburg, MD, while RT-PCR reagents were from Perkin Elmer, Branchburg, NJ.

Cell lines and culture conditions

Medulloblastoma cell lines Daoy, D283 Med and D341 Med were all obtained from the ATCC (American type culture collection), and UW228[53] was a gift from Dr. J.R. Silber. These cells were cultured in DMEM (Dulbecco's modified Eagle's medium)/F12 nutrient mixture supplemented with 10% fetal calf serum, L-glutamine and antibiotics in a humidified atmosphere of 5% CO_2 at 37ºC.

Primers used in RT-PCR

Based on the cDNA sequences of human *K-Ras, H-Ras, N-Ras* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, primers were designed and synthesized. To detect total Ras mRNA levels, a common sequence for K-Ras4A, K-Ras4B, H-Ras and N-Ras mRNA was used for primers. The antisense primer for total Ras mRNA was 5'-AATTTGCTC TCTGTAGTGGT-3', corresponding to a consensus bridging exons 2 and 3 in all *Ras* genes (primer RT1); the sense primer was 5'-TGACGGAATATAAACTGGTG-3' corresponding to a sequence located at the beginning of the first exon of all *Ras* genes (primer F1). The expected product was 299 bp. The primer RT1 was used in combination with sense primer 5'-GGAGATAGGCATGCTGAAA-3' (primer F2) for K-Ras mRNA (K-Ras4A and K-Ras4B). The expected product was 319 bp. RT1 combining with sense primer 5'-GATCTTGAG GTTATTGCTG-3' (primer F3) were used for N-Ras mRNA, producing a 326 bp product. RT1 and sense primer 5'-TAGGTCAGGAGAACCTGTA-3' (primer F4) was used for H-Ras mRNA, producing a 353 bp product. As an internal standard, GAPDH was used with the antisense primer 5'-CTCAGTGTA GCCCAGGATGC-3' (primer RT2) and the sense primer 5'-ACCACCATGGAGAAGGCTGG-3' (primer F5). The expected product was 528 bp.

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Antisense and sense c-myc oligonucleotides

Based on the cDNA sequence of *c-myc*, the oligonucleotide 5'-AAC GTT GAG GGG CAT-3' is complementary to the translation initiation site of c-myc mRNA. This was used as a *c-myc*-specific antisense inhibitor of translation. A sense oligonucleotide 5'-ATG CCC CTC AAC GTT-3' with a secondary structure identical to that of the antisense oligonucleotide served as a control. The antisense oligonucleotide used in this experiment has been successfully used previously by other groups for specific inhibition of *c-myc* expression.[54-58] To increase stability of oligonucleotides, both antisense and sense oligonucleotides were modified with phosphothioate.

Lovastatin administration

Cells were grown to sub-confluency in flasks, then treated with lovastatin (kindly provided by W.L. Henckler, Merck Research Laboratories, Rahway, NJ, USA). Lovastatin was prepared as previously described.[11] For attached cell lines, media was aspirated and replenished with fresh medium containing varying concentrations of lovastatin. For suspended or partially attached cell lines, floating cells were aspirated, spun and resuspended in medium, then returned to the flasks containing varying concentrations of lovastatin. Vehicle treatment was used as control.

RT-PCR analysis of Ras mRNA levels

After cellular RNA was extracted from lovastatin-treated medulloblastoma cells using the acid guanidinium-phenol-chloroform method with the TriZol reagent according to the manufacturer's instructions, relative levels of *Ras* mRNA were measured using a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method. The levels of GAPDH mRNA were measured in parallel as internal control. For cDNA generation, total cellular RNA (1 μg) was carried in 20 μl reaction containing 1 x PCR buffer with 5 mM $MgCl_2$, 1 mM each of deoxynucleotide triphosphates (dNTP), 20 units RNase inhibitor, 50 units of MuLV reverse transcriptase and 50 pMol of random hexamer oligodeoxynucleotides. After pre-incubation at 21°C for 10 minutes, the reaction was performed for 15 minutes at 42°C, followed by 99°C for five minutes on Perkin-Elmer GeneAmp PCR System 2400. For cDNA amplification, PCR was performed on the above PCR System in 50 μl of reaction containing 2 μl of cDNA products as template DNA, 1 x PCR buffer with 2 mM of $MgCl_2$, additional 50 μM of each dNTP, 1.25 units of Ampli Taq Gold™ DNA polymerase, 20 pMol of GAPDH primers and 50 pMol of Ras primers. Following pre-incubation at 95°C for nine minutes, amplification proceeded for serial cycles (30 to 41) consisting of 94°C for 30 sec, 49°C for 20 sec, 72°C for 30 sec, to obtain data within the linear range of the assay. After the last cycle, samples were incubated for seven minutes at 72°C to extend incomplete products. An aliquot of each PCR product was size-fractionated by electrophoresis in 1.4% agarose gel.

Antisense c-myc treatment of medulloblastoma cells

After medulloblastoma cells were grown to sub-confluency in flasks, they were treated for 12 hours with 25 μM antisense oligonucleotide or sense oligonucleotide. Following this treatment, medium was removed, floating cells were collected and resuspended in medium, then returned to the flasks containing 20 μM lovastatin plus 25 μM antisense oligonucleotide or sense oligonucleotide. After treatment at varying time points, cells were harvested for DNA analysis.

DNA assays

Antisense *c-myc* treated cells were harvested at the indicated time points. DNA was extracted and assessed by electrophoreses as previously described.[13]

Western blotting

Western blotting was performed as previously described.[14] Briefly, cells were treated with lovastatin and harvested. Cell pellets were washed four times with ice cold phosphate buffered saline, lysed in RIPA-NP40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 1% Doc, 0.1% SDS, 2.5 mM EDTA, 1 mM PMSF) according to a modification of a previously described method.[59] Protein concentrations were determined by BioRad (Hercules, CA) DC protein assay according to the manufacturer's instructions. Lysates were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. For each blot, protein was equally loaded across all lanes as follows: c-myc, 30 μg; p16, 40 μg; p21, 20 μg; p27, 25 μg; p53,30 μg; and bax, 50 μg.

Membranes were blocked in Tris-buffered saline containing 5% skim milk and 0.05% Tween-20 (TBST) overnight at 4°C prior to incubation with antibodies against p53 (DAKO Diagnostics Canada Inc., Mississauga, ON), or $p27^{KIP1}$ (DAKO Diagnostics Canada Inc., Mississauga, ON), or $p21^{WAF1}$ (Ab-1) (Oncogene Science Diagnostics, Cambridge, MA), or c-myc (Ab-1) (Oncogene Science Diagnostics, Cambridge, MA), or bax (Oncogene Science Diagnostics, Cambridge, MA), or p16 (Pharmingen Canada, Mississauga, ON) in 0.5% skim milk-TBST. Development was accomplished using a biotinylated secondary antibody (Vectastain ABC-AP Reagent; Vector, Burlingame, CA) and BCIP/NBT according to the manufacturer's instructions.

Densitometry

Experiments were run in duplicate, and informative bands were quantified twice using a gel documentation system (Gel Doc 2000, Bio-Rad Laboratories, Mississauga, ON). Western blotting for *c-myc* yielded the expected 65 kDa band, as well as an inconsistent shadow band at 76 kDa. The latter was not quantified since it may be nonspecific, but it tended to reflect the changes observed in the 65 kDa band.

Results

Lovastatin does not affect *Ras* gene expression

Ras mRNA levels were analyzed by RT-PCR in cell lines treated with lovastatin. At time points of 20 μM of lovastatin sufficient to induce apoptosis,[13,14,22] the total Ras mRNA levels showed constitutive levels without any demonstrable change (Figure 1). Furthermore, with varying concentrations of lovastatin treatment, total Ras mRNA levels failed to show any consistent change, compared with control cells (data not shown). This finding indicates that *Ras* gene transcription or total mRNA is stable despite the blockage of Ras protein isoprenylation. However, it does not exclude the possibility that particular *Ras*

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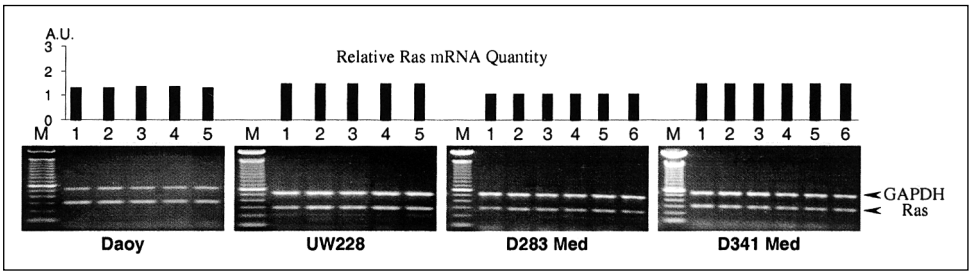

Figure 1: *RT-PCR for total Ras mRNA in medulloblastoma cells treated with 20 μM of lovastatin for indicated time intervals. The relative* Ras *expression was normalized to* GAPDH *expression after densitometric analysis of the bands. (Columns: means of two different experiments, each measured twice). GAPDH: 528 bp; Ras: 299 bp. Time intervals for lanes: 1) time 0; 2) 6 hr; 3) 12 hr; 4) 24 hr; 5) 48 hr in D283 Med and D341 Med, or untreated x 24 hr in Daoy and UW228; 6) untreated x 48 hr in D283 Med and D341 Med. M: Marker. A.U.: arbitrary units.*

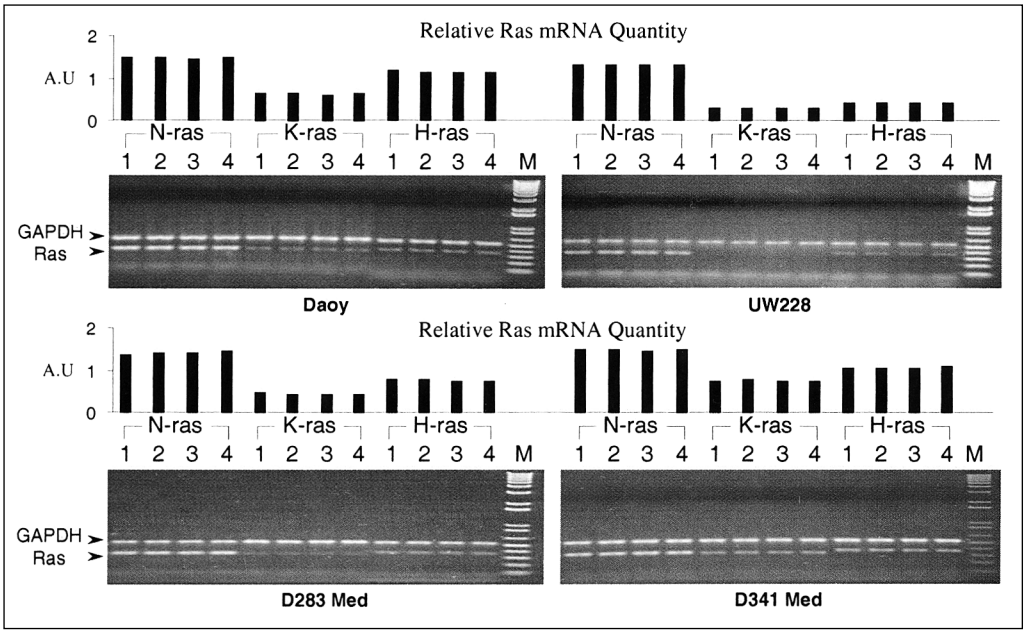

Figure 2: *RT-PCR for N-, K- and H-Ras mRNA in medulloblastoma cells treated with 20 μM of lovastatin. The relative* Ras *expression was normalized to* GAPDH *expression after densitometric analysis of the bands. GAPDH: 528 bp; N-Ras: 326 bp; K-Ras: 319 bp; H-Ras: 354 bp. Lanes: in Daoy and UW228: 1) lovastatin x 12 hr; 2) lovastatin x 24 hr; 3) vehicle x 24 hr; 4) untreated x 24 hr. Lanes in D283 Med and D341 Med: 1) lovastatin x 24 hr; 2) lovastatin x 48 hr; 3) vehicle x 48 hr; 4) untreated x 48 hr. M: Marker. A.U.: arbitrary units.*

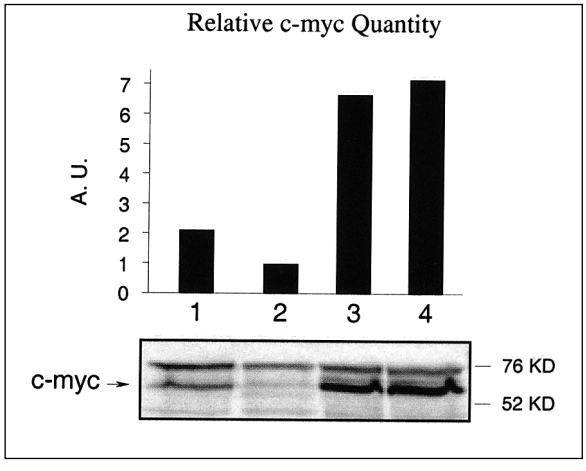

Figure 3: *Western blotting for c-myc expression in medulloblastoma cells. 30 μg of cell lysates of each cell line were loaded. The relative expression in each cell line was normalized to the level of c-myc in UW228 after densitometric analysis of the bands. Lane 1: Daoy; Lane 2: UW228; Lane 3: D283 Med; Lane 4: D341 Med. A.U.: arbitrary units.*

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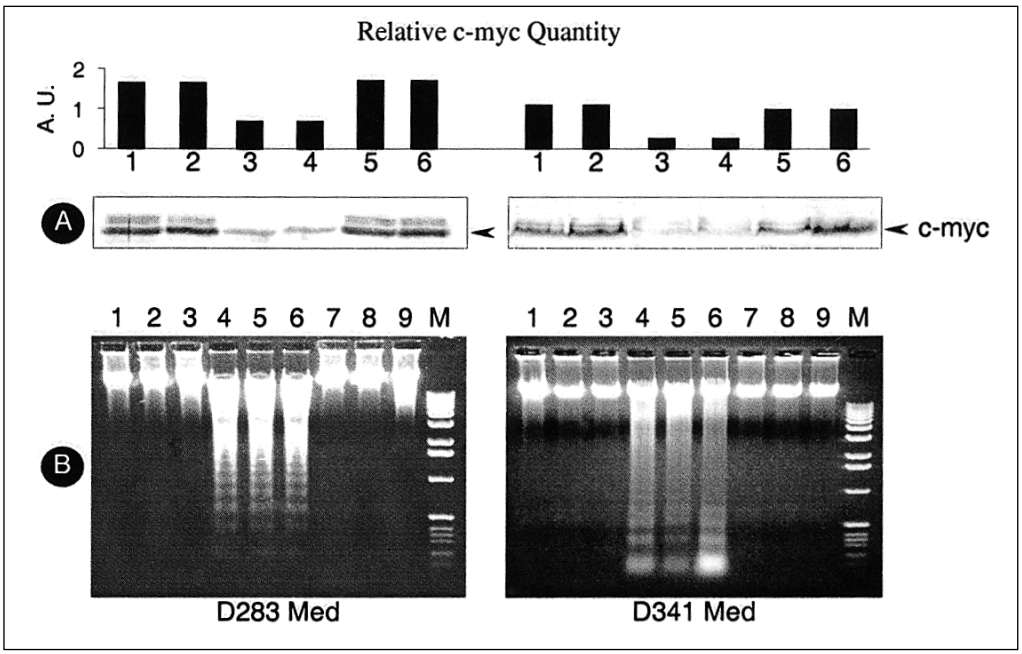

Figure 4: *c-myc expression and lovastatin-induced apoptosis. Left pattern represents D283 Med, right pattern is for D341 Med. All test cell lines were treated with 20 μM lovastatin, 25 μM* c-myc *antisense or sense oligonucleotides for the stated time interval. A: Western blotting for C-myc. The relative level of c-myc protein of D283 Med and D341 Med cells in each treatment was normalized to the level of c-myc in untreated cells after densitometric analysis of the bands. B: Agarose gels of DNA laddering. Lanes in A show 48 hr treatment of D283 and 60 hr treatment of D341 Med, which are (1) Untreated; (2) sense* c-myc*; (3) antisense* c-myc*; (4) lovastatin plus antisense* c-myc*; (5) lovastatin plus sense* c-myc*; (6) Lovastatin. Lanes in B : (1) lovastatin plus antisense* c-myc*, D283 Med x 48 hr, D341 Med x 60 hr; (2) lovastatin plus sense* c-myc*, D283 Med x 48 hr, D341 Med x 60 hr; (3) lovastatin, D283 Med x 48 hr, D341 Med x 60 hr ; (4) lovastatin plus antisense* c-myc*, D283 Med x 72 hr, D341 Med x 84 hr; (5) lovastatin plus sense* c-myc*, D283 Med x 72 hr, D341 Med x 84 hr; (6) lovastatin, D283 Med x 72 hr, D341 Med x 84 hr; (7) antisense* c-myc*, D283 Med x 72 hr, D341 Med x 84 hr; (8) sense* c-myc*, D283 Med x 72 hr, D341 Med x 84 hr; (9) Untreated D283 Med x72 hr, D341 Med x 84 hr. M: Marker. A.U.: arbitrary units.*

genes may have been differentially regulated by mevalonate derivatives.

To address this question, relative levels of N-Ras, K-Ras and H-Ras mRNA were assessed with RT-PCR in lovastatin-treated cells. No appreciable change of individual Ras mRNA was observed after lovastatin treatment compared to control (Figure 2). *N-Ras* and *H-Ras* gene expression was similar when cell lines were compared to one another; however, *K-Ras* gene expression was low in each cell line, and was difficult to detect in UW228 and D283 Med. Although *N-Ras* gene expression appeared higher than *K-Ras* and *H-Ras* expression, this comparison may be inaccurate due to differences in conditions and primer characteristics among these experiments.

c-myc over expression does not account for medulloblastoma resistance to lovastatin

As previously reported, D283 Med and D341 Med were relatively resistant to lovastatin, compared to UW228 and Daoy.[13] In attempting to explain this difference, note was made of differences in the reported genetic backgrounds between the different cell lines. *c-myc* gene is over expressed in D283 Med and D341 Med.[27,28] Western blotting confirmed that *c-myc* is relatively highly expressed in D283 Med and D341 Med, compared to sensitive cell lines Daoy and UW228 (Figure 3), raising the possibility that *c-myc* gene over expression may confer relative resistance to lovastatin.

Following the treatment with 25 μM of *c-myc* antisense oligonucleotides on D283 Med and D341 Med, *c-myc* gene expression was markedly blocked (Figure 4A lanes 3 and 4), compared to its expression in cells without antisense *c-myc* treatment (Figure 4A lanes 1 and 6) and *c-myc* sense oligonucleotides treated cells (Figure 4A lanes 2 and 5). However, blocking *c-myc* gene expression did not increase the sensitivity of D283 Med and D341 Med to lovastatin. Apoptotic DNA fragmentation occurred after lovastatin treatment 72 hours for D283 Med and 84 hr for D341 Med; similarly, DNA laddering was seen when 25 μM of *c-myc* antisense or sense oligonucleotides accompanied 20 μM lovastatin of D283 Med for 72 hr and D341 Med for 84 hr (Figure 4B lanes 4, 5 and 6). Blocking *c-myc* expression with *c-myc* antisense oligonucleotides did not shorten the time required for lovastatin-induced DNA fragmentation (Figure 4B lane 1), compared to lovastatin alone (Figure 4B lane 3). *c-myc* sense oligonucleotide had no effect (Figure 4B lane 2). Like untreated control cells (Figure 4B lane 9), no DNA laddering was evident after administration of *c-myc* antisense and sense oligonucleotides

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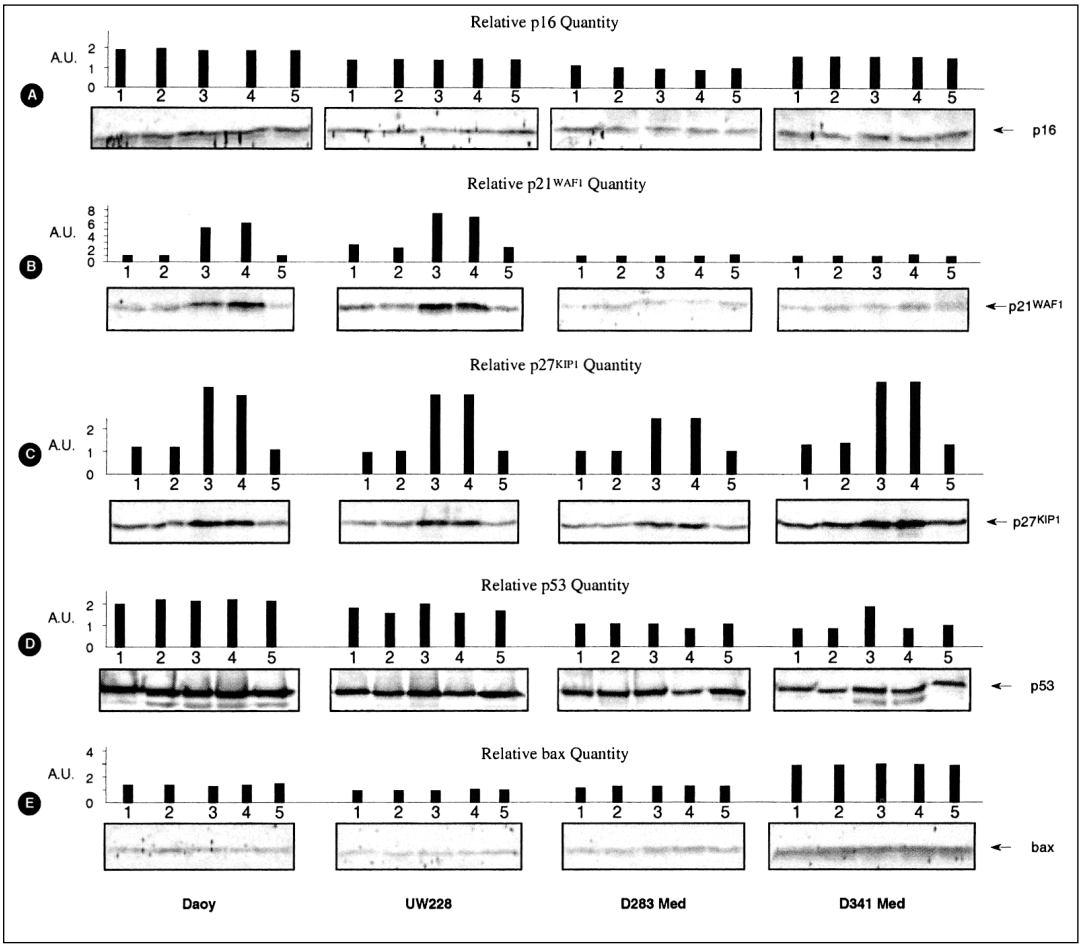

Figure 5: *Western blotting for lovastatin-induced changes of p16, p21^{WAF1}, p27^{KIP1} and p53 as well as bax Expression. A: p16 protein. B: p21^{WAF1} protein. C: p27^{KIP1} protein. D: p53 protein. E: bax protein. Protein expression was detected after 20 μM lovastatin treatment for varying intervals. The relative level of p16, p21^{WAF1}, p27^{KIP1} and p53 as well as bax protein of lovastatin-treated medulloblastoma cells was normalized to their level in vehicle treated cells after densitometric analysis of the bands. Time intervals for lanes: (1) Untreated x 24 hr; (2) Untreated, x 36 hr in Daoy and UW228, x 72 hr in D283 Med and D341 Med; (3) Lovastatin, x 24 in Daoy and UW228, x 48 hr in D283 Med and D341 Med; (4) Lovastatin, x 36 hr in Daoy and UW228, x 72 hr in D283 Med and D341 Med; (5) Vehicle of lovastatin, x 36 hr in Daoy and UW228, x 72 hr in D283 Med and D341 Med. A.U.: arbitrary units.*

alone (Figure 4B lanes 7 and 8). Lovastatin treatment had no effect on the level of *c-myc* protein (Figure 4A lane 1 and 6).

Cdk inhibitor regulation of lovastatin-induced growth inhibition and apoptosis

Following 20 μM of lovastatin treatment, p16 expression exhibited no change by Western blotting (Figure 5), indicating that p16 was not responsible for lovastatin-induced growth inhibition and apoptosis. In contrast, Western blotting showed that 20 μM lovastatin induced pronounced increases of p21^{WAF1} expression in Daoy and UW228 while p21^{WAF1} protein was stable in lovastatin-treated D283 Med and D341 Med (Figure 5). These results suggested a role for p21^{WAF1} over expression in lovastatin-induced growth inhibition and apoptosis in Daoy and UW228. Furthermore, 20 μM lovastatin resulted in pronounced increases of p27^{KIP1} protein in all cell lines (Figure 5). These results suggest a role for p27^{KIP1} in lovastatin-induced growth inhibition and apoptosis, possibly adding to the effects of increased p21^{WAF1} expression in Daoy and UW228.

p53 and bax expression in lovastatin-induced apoptosis

Increased p53 expression was detected by Western blotting after administration of 20 μM lovastatin in D341 Med (48 hr). In contrast, no change was detected in either Daoy, UW228 or D283 Med treated with 20 μM lovastatin (Figure 5). However, p53 levels were reduced to normal, even to below baseline, after longer treatments with lovastatin (36 hr for Daoy and UW228, 72 hr for D283 Med and D341 Med). p53 levels were stable in untreated controls and vehicle treated controls. Results varied among cell lines, and the timing of changes mitigated against a consistent effect. Despite the increase in p53 expression following lovastatin treatments in D341 Med, Western blotting

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failed to show changes in *bax* expression in any cell line (Figure 5), suggesting that lovastatin-induced apoptosis is probably p53 independent.

Discussion

Lovastatin has, in recent years, gained more attention as an anticancer agent. We have already shown its anti-proliferative effects on medulloblastoma.[13,14,22] In this study, we have attempted to disentangle the complex molecular regulatory mechanisms of these effects. We investigated: 1) *Ras* gene expression in lovastatin-induced growth inhibition and apoptosis; 2) the participation of c-myc over expression in relative lovastatin resistance of two medulloblastoma cell lines; 3) the contribution of Cdk inhibitors to lovastatin-induced growth inhibition and apoptosis; and 4) the role of p53 in lovastatin-induced growth inhibition and apoptosis.

Ras gene expression in lovastatin-induced growth inhibition and apoptosis

Inhibition of HMG-CoA reductase by lovastatin depletes mevalonate production, and then alters the post-translational processing of CAAX-containing small G-proteins including Ras protein.[60,61] We reported that Ras protein levels were increased upon lovastatin treatment,[14] postulating that this was due to *Ras* gene over expression, since the mevalonate pathway directly regulates Ras levels by controlling Ras mRNA abundance.[24] However, in this report, semiquantative RT-PCR for N-, K- and H-Ras revealed that lovastatin does not affect either total *Ras* gene or specific *Ras* gene expression in medulloblastoma. Thus, changes in *Ras* gene expression are not implicated in lovastatin-induced medulloblastoma apoptosis; rather, the lovastatin-induced increase of Ras protein level is probably due to the regulation of translation and post-translational degradation.[25] Expression of N-Ras and K-Ras may account for some Ras processing despite farnesylation inhibition,[14] because they may be geranylgeranylated when protein farnesylation is inhibited.[62,63]

Participation of c-myc over expression in relative lovastatin resistance

Expression of the myc transcription factor is important for cell proliferation;[64] myc has also been implicated in the induction of apoptosis under certain conditions that cause growth inhibition, such as growth factor and serum deprivation.[29-31] A number of reports have focused on the oncogenic activity of myc proteins. Myc interacts with the retinoblastoma protein (pRB) and is able to override pRB-induced cell cycle arrest. *myc* over expression results in uncontrolled cell proliferation.[65] Since cell proliferation is induced when *myc* is expressed in the presence of certain growth promoting factors, and our culture media are supplemented with fetal bovine serum, we postulated that *c-myc* over expression in D283 Med and D341 Med confer relative resistance to lovastatin-induced apoptosis. However, this does not appear to be the case, since blocking *c-myc* expression with antisense oligonucleotides did not affect sensitivity of these cell lines to lovastatin. This is consistent with data showing that the effect of lovastatin on neuroblastoma appears to be independent of the level of *N-myc* expression.[66] Our data show that *c-myc* protein level is stable in lovastatin treatment, indicating lovastatin has no effect on *c-myc* gene expression, in contrast to recently published data showing that lovastatin down-regulates *c-myc* gene expression in the human prostate carcinoma cell PC-3.[32] This is likely cell type dependent. Further evidence of the relationship between lovastatin and *c-myc* gene expression remains to be addressed.

Contribution of Cdk inhibitors to lovastatin-induced growth inhibition and apoptosis

Recent studies have tried to explore how Cdk inhibitors and *p53* expression relate to lovastatin-induced growth inhibition and apoptosis,[18,32,37-43,67,68] but the results are varied. We previously demonstrated cell-cycle arrest following lovastatin treatment of medulloblastoma cell lines.[13] To study these proteins in lovastatin treatment of medulloblastoma, we investigated p16, $p21^{WAF1}$, $p27^{KIP1}$, and p53 as well as bax expression. Our studies demonstrated that the accumulation of $p21^{WAF1}$and $p27^{KIP1}$ proteins, but not p16, accompany lovastatin treatment, correlating with growth inhibition and apoptosis, consistent with several reports from other investigators.[37-43] However, $p21^{WAF1}$ protein is increased only in Daoy and UW228, but not in D283 Med or D341 Med with lovastatin treatment, suggesting that $p21^{WAF1}$ gene over expression mediates the antiproliferative effects of lovastatin in a cell type dependent manner. This may explain why some reports showed that lovastatin induces the increase of $p21^{WAF1}$ protein,[39-42] while others did not.[32,69] Since $p27^{KIP1}$ protein is inducible and increased in all test cell lines treated with lovastatin, $p27^{KIP1}$ is likely a major player to mediate growth inhibition and apoptosis in lovastatin treatment, consistent with other reports.[37-40,43]

Role of p53 in lovastatin-induced growth inhibition and apoptosis

Despite the increase in p53 expression following lovastatin treatments in D341 Med, the level of bax protein in all examined cell lines was stable during lovastatin treatment. This is not consistent with one report that p53 and bax were induced in lovastatin-induced neuronal apoptosis,[70] but consistent with a recent report that lovastatin-induced thyroid cell apoptosis is not accompanied by p53 or bax induction.[18] Since *bax* gene has been identified as a p53-immediate early response gene,[49] the expression of which serves as a useful marker for p53-dependent apoptosis,[50-52] lovastatin-induced apoptosis in medulloblastoma cells is probably p53-independent. To support this view, lovastatin does induce apoptosis in other tumor cell lines and primary tumors through p53-independent pathways.[39,42,68,71]

p53 can also induce apoptosis without initiating *de novo* protein or RNA synthesis,[72,73] and both wild-type and mutant p53 can promote exit from ionizing-radiation induced G2 arrest resulting in apoptosis.[74,75] Direct protein-protein interactions involving p53 could represent the nontransactivitional mechanism for p53 regulation of apoptosis. It is therefore possible that this, or an alternative p53-dependent mechanism, may be involved in lovastatin-induced apoptosis in medulloblastoma. Whether lovastatin pretreatment of medulloblastoma may enhance sensitivity to tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), as has previously been documented for certain systemic cancers,[76] remains to be determined.

p53 protein may contribute to growth inhibition and apoptosis

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through inducing *p21^{WAF1}* expression.[33-35] In D341 Med cell line, the induction of both p53 and p21^{WAF1} protein accompanied lovastatin treatment, raising the possibility that p53 may regulate lovastatin-induced growth inhibition in this case through the regulation of *p21^{WAF1}* gene expression. In Daoy cell line, p21^{WAF1} protein, but not p53, was induced in lovastatin treatment, possibly due to the high level of background of p53. However, since *p21^{WAF1}* gene expression could also be p53-independent, for example, in TGF-β induced cell cycle arrest,[33-35] it is possible this is the case in Daoy.

Collectively, the data presented here demonstrate that lovastatin-induced growth inhibition and apoptosis in medulloblastoma are not dependent on the regulation of *Ras* and *c-myc* gene expression, but may be mediated by *p27^{KIP1}* gene expression. Lovastatin-induced apoptosis in medulloblastoma is probably p53 independent, but *p53* and *p21^{WAF1}* gene expression may also mediate antiproliferative effects of lovastatin on specific medulloblastoma cell lines.

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