0013-7227/99/$03.00/0
Endocrinology
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Vol. 140, No. 2
Printed in U.S.A.

Prenyltransferase Inhibitors Induce Apoptosis in Proliferating Thyroid Cells through a p53-Independent, CrmA-Sensitive, and Caspase-3-Like Protease-Dependent Mechanism*

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ABSTRACT

The inhibitors of protein prenylation have been proposed for chemotherapy of tumors. Lovastatin, a 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase inhibitor, displays proapoptotic activity in tumor cells blocking the synthesis of isoprenoids compounds. To test whether HMG-CoA reductase inhibition can induce apoptosis in proliferating thyroid cells, we studied the effects of lovastatin in normal and neoplastic thyroid cells and in primary cultures from normal human thyroids. In an immortalized human thyroid cell line (TAD-2) and in neoplastic cells, lovastatin induced cell rounding within 24 h of treatment. After 48 h the cells were detached from the plate and underwent apoptosis, as evidenced by DNA fragmentation. Morphological changes and apoptosis did not occur in serum-starved quiescent TAD-2 cells or in primary cultures of normal thyrocytes. Mevalonate, the product of the HMG-CoA reductase enzymatic activity, and the protein synthesis inhibitor cycloheximide completely blocked the effects of lovastatin in a dose-dependent fashion. The geranylgeranyl transferase GGTI-298 inhibitor mimicked the effects of lovastatin on cell morphology and induced cell death, whereas the farnesyl transferase inhibitor FTI-277 was less effective to induce both cell rounding and apoptosis. Resistance to lovastatin-induced apoptosis by expression of the viral serpine CrmA and by the peptide inhibitor of caspases, Z-DEVD-fmk, demonstrated the involvement of CrmA-sensitive, caspase-3-like proteases. Inhibition of endogenous p53 activity did not affect the sensitivity of thyroid cells to lovastatin, demonstrating that this type of apoptosis is p53 independent.

We conclude that lovastatin is a potent inducer of apoptosis in proliferating thyroid cells through inhibition of protein prenylation. This type of apoptosis requires protein synthesis, is CrmA sensitive and caspase-3-like protease dependent, and is independent from p53. (*Endocrinology* **140:** 698–704, 1999)

PROGRAMMED cell death or apoptosis is a process by which cells activate their own suicide. Apoptosis occurs in a variety of physiological and pathological situations. Cytokines (1–3), growth factors, and hormone depletion determine apoptosis in a number of tissues. Also denied extracellular adhesion and serum withdrawal induce programmed cell death in endothelial and thyroid cells (4–6). Besides these stimuli, anticancer drugs can induce apoptosis by triggering biochemical events that are part of the apoptotic machinery (7). In view of potential pharmacological interventions, it is important to identify the molecular events of the apoptotic pathway and to determine how and at which site a drug is connected to the signal transduction pathway leading to cell death. Some of the genes involved in the regulation of apoptosis have been identified. Among these, the p53 gene has emerged as key regulator of cell death controlling the RNA transcription of pro- and antiapoptotic genes (8–10). Recently, inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase have been reported to induce apoptosis in a number of cells (11–14). Lovastatin, a member of this group of drugs, prevents the reduction of HMG-CoA to mevalonate, the precursor of isopentenyl pyrophosphate then converted to geranyl pyrophosphate, farnesol pyrophosphate, and all-*trans*-geranylgeranyl pyrophosphate. Farnesol pyrophosphate and all-*trans*-geranylgeranyl pyrophosphate are transferred by farnesyl and geranylgeranyl transferase to various small GTP-binding proteins of the Ras superfamily, such as Rho, Raf, Rab, Rac, and Rap (15), that are involved in important cellular functions, such as proliferation, cell adhesion, and motility. In the absence of prenyl modification the small GTP-binding proteins are not able to form complexes with their target proteins impairing their function. In prostate cancer cells and in rat fibroblast cells, HMG-CoA reductase inhibitors induce cell cycle arrest or apoptosis through the block of geranylated and/or farnesylated compounds (14, 16). Interestingly, a number of Ras-transformed cells, including rat thyroid cells, are much more sensitive to HMG-CoA inhibitors than their normal counterparts, suggesting that these drugs may have a therapeutic potential (16, 17) (Bifulco *et al.*, submitted). Activating mutations of *ras* and other oncogenes are frequent in thyroid tumors, and *ras* mutation has

Received June 24, 1998.
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* This work was supported in part Consiglio Nazionale della Ricerche Comitato Nazionale Biotecnologie e Biologia Molecolare (to M.V.), Ministero dell'Università e della Ricerca Scientifica (to G.R.), and Associazione Italiana per la Ricerca sul Cancro (to G.F. and M.B.).

also been found in thyroid nodular hyperplasia (18–21). Thus, these thyroid diseases are potentially susceptible to therapeutic intervention by HMG-CoA inhibitors. In this study we tested whether the HMG-CoA inhibitor lovastatin can induce apoptosis in human thyroid cells. We conclude that lovastatin is a potent inducer of apoptosis in proliferating thyroid cells through inhibition of protein prenylation. This type of apoptosis requires protein synthesis and caspases and is independent from p53.

Materials and Methods

Cells, plasmid constructs, and transfections

Cell cultures from normal thyroids and from a papillary carcinoma (P6) were prepared as previously described (22) and cultured in a 5% CO_2 atmosphere at 37 C in Ham's F-12 medium supplemented with 10% FCS. The TAD-2 cell line, obtained by simian virus 40 infection of human fetal thyroid cells, was a gift from Dr. T. F. Davies, Mount Sinai Hospital (New York, NY). Papillary thyroid carcinoma cell line TPC-1 was donated by Dr. M. Nagao, National Cancer Center (Tokyo, Japan). Cells were cultured in a 5% CO_2 atmosphere at 37 C in DMEM supplemented with 10% FCS. Medium was changed every 3–4 days. Cells were detached by 0.5 mM EDTA in calcium- and magnesium-free PBS with 0.05% trypsin. TAD-2 cells were transfected by calcium phosphate precipitation with pRSVneo alone to generate TADneo control cells or with pBKRSVcrmA (containing the pox virus antigen CrmA (gift from N. Boudreau, Lawrence Berkeley Laboratories, Berkeley, CA) to generate TADcrmA cells or pLTRp53cG containing the temperature-sensitive, dominant negative p53 gene mutated at codon 135 (gift from Dr. A. Levin, Princeton University, Princeton, NJ) to generate TAD53 cell clones. Stably transfected cells were selected and grown with G-418 sulfate (Life Technologies, Gaithersburg, MD).

Drugs

A 2-mM lovastatin (gift from Dr. A. W. Alberts, Merk, Sharp, and Dohme Institute, Rahway, NJ) stock solution was prepared in dimethylsulfoxide and stored at −20 C. The synthetic peptide Ac-YVAD-cmk (Ac-Tyr-Val-Ala-Asp-cmk), an inhibitor primarily of caspase-1 and the caspase-3 inhibitor Z-DEVD-fmk [Z-Asp(Ome)-Val-Asp(OMe)-fmk] were purchased from Calbiochem (La Jolla, CA). Mevalonate was obtained from Sigma Chemical Co. (St. Louis, MO). Geranylgeranyl transferase I (GGTI-298), and farnesyl transferase I (FTI-277) inhibitors were gifts from Dr. S. M. Sebti, University of South Florida (Tampa, FL). Etoposide was purchased from Sigma Chemical Co.

DNA electrophoresis

Suspended cells collected by centrifugation were washed in PBS, lysed in 300 μl 0.5% Triton X-100, 5 mM Tris buffer (pH 7.4), and 20 mM EDTA for 20 min at 4 C and centrifuged at 13,000 rpm for 30 min. Centrifugation-resistant low mol wt DNA was extracted with phenol/chloroform, precipitated with ethanol, and incubated with 0.5 μg/ml ribonuclease A, deoxyribonuclease-free for 30 min at 37 C. DNA with loading buffer were electrophoresed in 1% agarose and 1 μg/ml bromide at 50 V in 45 mM Tris-borate and visualized by UV.

Apoptosis measurements

Cytofluorometric estimation of apoptosis was performed as follows. Floating cells and adherent cells obtained by trypsin/EDTA were collected, washed in cold PBS, and fixed in 70% cold ethanol for 30 min. Ethanol was removed by a PBS wash, and cells were incubated in PBS, 50 μg/ml propidium iodide, and 10 μg/ml ribonuclease A, deoxyribonuclease free overnight at 4 C. Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson and Co., Mountain View, CA). The percentage of apoptotic cells was calculated by dividing the number of cells displaying red fluorescence lower than the G0-G1 diploid peak by the total number of collected cells × 100.

Statistics

Results are presented as the mean ± SD from three to five independent experiments. Student's *t* test for paired samples was performed where indicated. Differences with $P < 0.05$ were considered statistically significant.

Results

Lovastatin induces change in cell shape and apoptosis in proliferating TAD-2, PTC-1, and P6 cells, but not in quiescent cells

Thyroid cell lines and thyroid cells obtained from normal glands cultured *in vitro* were treated with variable lovastatin concentrations for several days. In proliferating TAD-2 cells, the treatment with 5 μM lovastatin induced a dramatic change in cell morphology (Fig. 1). After 24 h of treatment, cell shape changed from flat to round, but most cells remained adherent. By 48 h, about 80% of the cells were detached from the plate and floated in the medium. Flow cytometric analysis of cells stained with propidium iodide did not display a significant number of hypodiploid cells after 24 h of treatment with 5 μM lovastatin, whereas after 48 h about 65% of the cells were hypodiploid (Fig. 2). Condensed and fragmented nuclei, detected by acridine staining, cell blebbing, and decreased cell size (not shown), together with DNA fragmentation, analyzed by agarose gel electrophoresis (Fig. 2, *lower panel*), demonstrated a massive apoptotic process. TAD-2 cells were also cultured for 24 h in FCS-containing medium to allow adhesion and spreading, then the cells were induced to quiescence by serum withdrawal. PTC-1 and P6 cells showed identical changes in cell morphology (not shown). Quiescent TAD-2 cells obtained by 3 days of serum starvation before lovastatin treatment and cultured in serum-free medium as well as primary cultures of normal thyrocytes used after 7–10 days of culture did not show changes in shape, plate detachment, or morphological features of apoptosis up to 7 days of 5 μM lovastatin treatment (not shown). Lovastatin induced a dose-dependent effect on proliferating thyroid cells. Apoptosis, estimated by

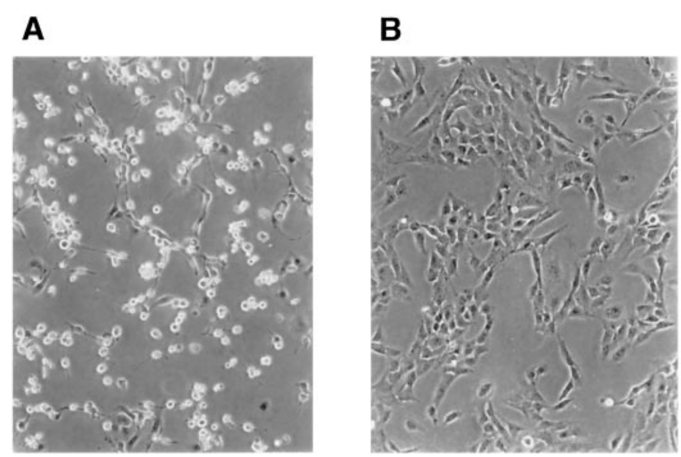

FIG. 1. Phase contrast photomicrographs of lovastatin-induced changes in cellular morphology. TAD-2 cells were cultured for 24 h in plastic plates in FCS-containing medium, and then 5 μM lovastatin was added to the medium. After 24 h of treatment, the cells were still attached to the plates, whereas cell shape changed from flat to round (A). B, Untreated cells. Magnification, ×100.

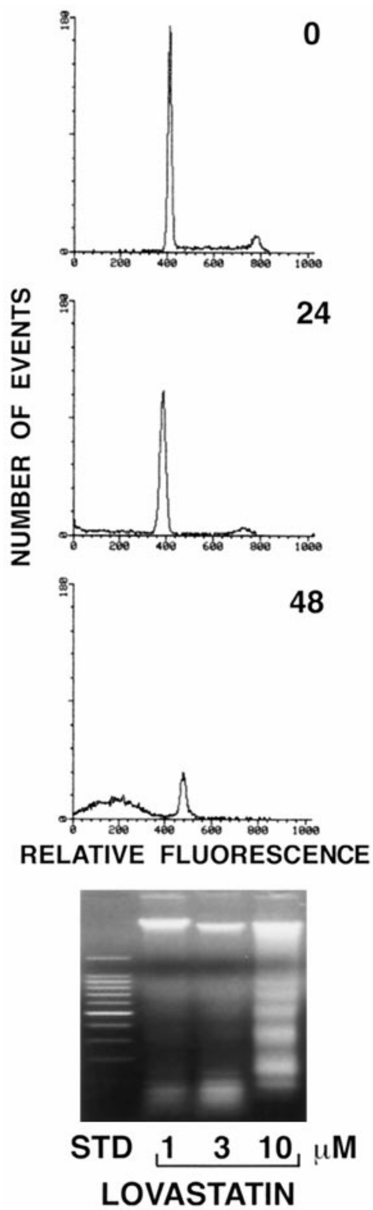

FIG. 2. Flow cytometric and DNA electrophoresis analysis of thyroid cells treated with lovastatin. *Upper panels*, TAD-2 cells were treated with 10 μM lovastatin for 0, 24, or 48 h, then floating and adherent cells were collected, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Apoptotic cells with hypodiploid DNA content were gated and counted. *Lower panel*, Gel electrophoresis analysis of low mol wt DNA from TAD-2 cells cultured for 48 h in the presence of 1, 3, and 10 μM lovastatin. Centrifugation-resistant, low mol wt DNA was extracted from suspended and adherent cells, electrophoresed in 1% agarose and 1 μg/ml propidium bromide in Tris-borate buffer, and visualized by UV. Parallel increases in the lovastatin concentration and low mol wt DNA with characteristic apoptotic internucleosomal fragmentation was evident. STD, Markers of DNA mol wt.

flow cytometric analysis after 48 h of treatment, showed a dose dependence affecting more than 78% of TAD-2, PTC-1 and P6 cells cultured with 10 μM lovastatin, whereas no effect was observed in thyroid primary cultures or in TAD-2 cells induced to quiescence by 3 days of serum starvation (Fig. 3).

The effect of mevalonate, the isoprenoid pathway intermediary metabolite inhibited by lovastatin, was studied in lovastatin-treated cells. TAD-2 cells were incubated with 5 μM lovastatin in the presence of increasing concentrations of mevalonate. After 48 h of culture, cells were observed by phase contrast microscope and collected, and the percentage of apoptotic cells was determined by flow cytometry (Fig. 4). Both changes in cell shape and apoptosis induced by lovastatin were completely prevented by mevalonate in a dose-dependent fashion.

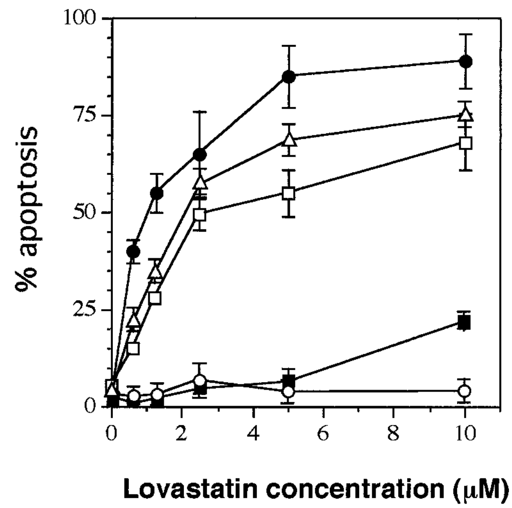

FIG. 3. Dose response of lovastatin-induced apoptosis. TAD-2 cells (*full circles*), TPC-1 (*open squares*), P6 (*open triangles*) and primary thyrocytes (*full squares*) were cultured in FCS-containing medium. TAD-2 cells were also cultured for 24 h in FCS-containing medium, then serum was withdrawn (*open circles*). After 3 days, cells were treated with lovastatin at different doses for 48 h, stained with propidium iodide, and analyzed by flow cytometry. The percentage of apoptotic cells was determined by gating the cells with hypodiploid DNA content. Results are presented as the mean ± SD from four independent experiments.

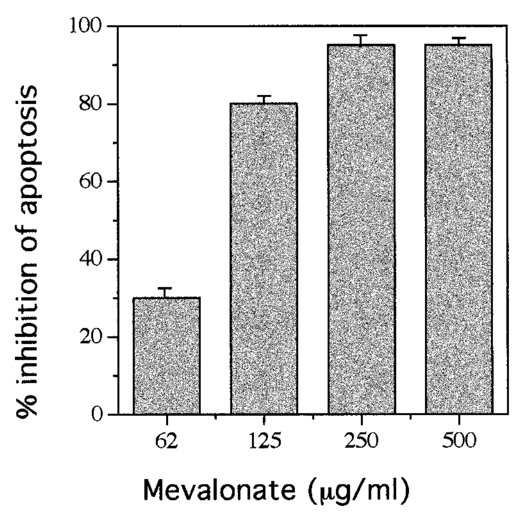

FIG. 4. Prevention of lovastatin-induced apoptosis by mevalonate. Cells were treated with 5 μM lovastatin and mevalonate at different doses for 48 h and stained with propidium iodide, and the percentage of apoptotic cells was determined by flow cytometry. Results are reported as the percent inhibition of apoptosis from three separate experiments.

Effect of inhibition of protein synthesis on lovastatin-induced apoptosis

The role of protein synthesis in lovastatin-induced apoptosis was determined by treating TAD-2 cells for 48 h with 5 μM lovastatin or in the presence of different concentrations of cycloheximide. The apoptotic effect of lovastatin, as determined by flow cytometry, was reduced by low doses of cycloheximide and was completely abolished at 0.25–0.50 μg/ml of the protein synthesis inhibitor (Fig. 5). Also changes in cell morphology were affected by cycloheximide, as cells remained flat and adherent. Cell death and changes in shape from flat to round were inhibited by cycloheximide in a parallel, dose-dependent fashion.

Effect of inhibitors of protein prenylation on TAD-2 cells

To determine whether inhibition of protein prenylation was responsible of lovastatin-induced apoptosis, the effects of farnesyl (FTI-277) and geranylgeranyl (GGTI-298) protein transferase inhibitors were determined (Fig. 6). After 24 h, at the lower concentration (12.5 μM) only GGTI-298 induced cell rounding in less than 30% of the cells. The totality of the cells treated with 25 μM FTI-277 were adherent and round. At 50 μM GGTI-298, all of the cells were floating in the medium. FTI-277 was much less effective in inducing changes in cell shape, as at 50 μM only 50% of the cells were round, whereas floating cells were not observed. This experiment demonstrated that dose-dependent apoptosis was induced by both GGTI-298 and FTI-277, although GGTI-298 was twice as effective as FTI-277.

Inhibition of cysteine protease activity suppresses lovastatin-induced apoptosis

To determine whether the cysteine proteases of the caspases family were implicated in the lovastatin-induced apoptosis of thyroid cells, TAD-2 cells were transfected with a vector encoding the cowpox virus protein CrmA that specifically inhibits the enzymatic activity of some caspases. Different TADcrmA stable transfectants were generated by

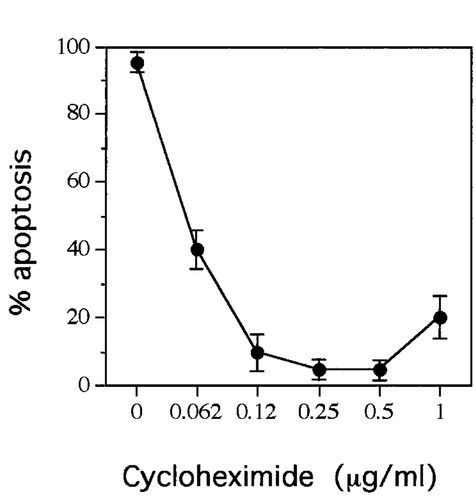

FIG. 5. Effect of cycloheximide on lovastatin-induced cell death. TAD-2 cells were treated for 48 h with 5 μM lovastatin alone or with different concentrations of cycloheximide. Results are reported as the percent apoptosis from three separate experiments.

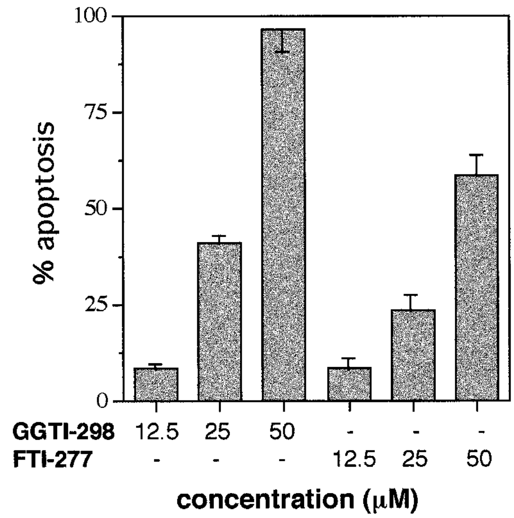

FIG. 6. Induction of apoptosis by prenyltransferase inhibitors. TAD-2 cells were cultured for 48 h in the presence of different molar concentrations of GGTI-298 or FTI-277, and the percentage of apoptotic cells was determined by flow cytometry. Results are reported as the mean of three independent experiments.

cotransfection of the expression vectors pBKRSVcrmA and pRSVneo and were selected by G-418 resistance. The intracellular expression of CrmA did not produce a significant effect on the proliferation of TAD cell mutants, as determined by comparing the proliferation curves of TADcrmA and TADneo (carrying only pRSVneo) clones (Fig. 7A). Resistance of TADcrmA mutants to apoptosis was assayed by comparing the sensitivities of TADcrmA and TADneo cells to the apoptotic antineoplastic drug etoposide. Both TADcrmA mutants analyzed displayed a marked resistance to apoptosis induced by 24 h treatment with 70 μg/ml etoposide (Fig. 8A). Then, resistance of TADcrmA mutants to apoptosis induced by lovastatin was determined (Fig. 8B). TADcrmA and TADneo cells were cultured in the presence of 0.75–10 μM lovastatin for 48 h, and apoptosis was estimated by flow cytometric analysis. Apoptosis of TADcrmA cells was strongly decreased compared with that of control cells, thus demonstrating the involvement of CrmA-sensitive caspases.

Because several proteases are the targets of CrmA, more specific caspase inhibitors were also used to determine whether caspase-1-like and/or caspase-3-like proteases are involved in lovastatin-induced apoptosis. A total of 2×10^5 TAD-2 cells/well were plated in 24-well plates. After 24 h, the medium was replaced with fresh medium containing 5 μM lovastatin and different concentrations of cell-permeable caspases inhibitors, Ac-YVAD-cmk and Z-DEVD-fmk. The inhibitors were replenished at 24 h, and at 48 h the cells were collected, and apoptosis was measured by FACS analysis (Fig. 9). Change in cell morphology from flat to round were not affected by either peptide, and by 48 h, the majority of the cells was detached from the plate. The peptide Z-DEVD-fmk strongly inhibited the apoptosis induced by lovastatin, and even at a concentration as low as 1.5 μM, 18% inhibition of apoptosis was still observed. At similar concentrations, apoptosis was unaffected by the addition of the peptide

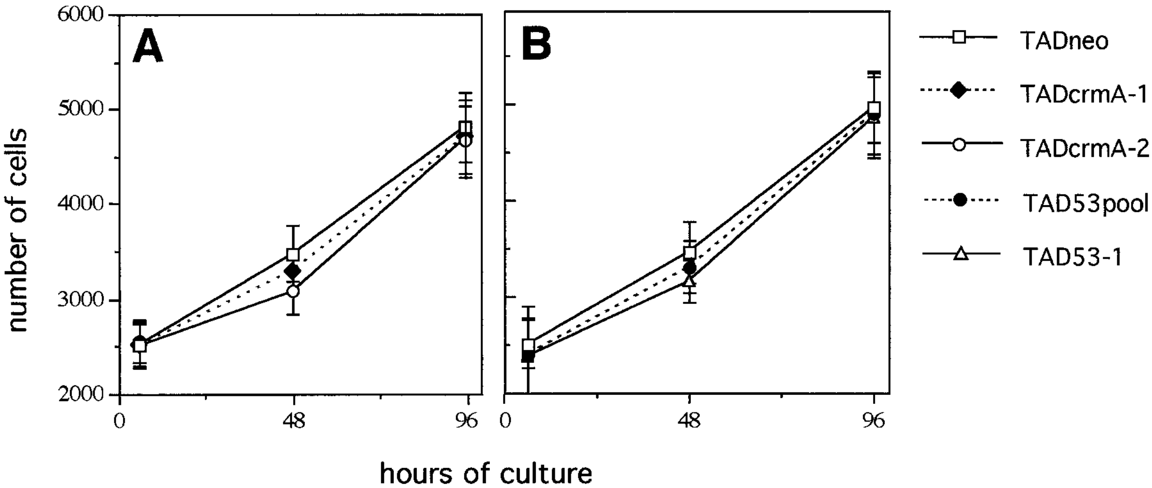

FIG. 7. Time course of proliferation of TAD cell mutants. A total of 3×10^3 cells/well were seeded in 24-well plates and cultured at 37 C (A) or 39 C (B). After 6 h, the plates were washed to remove floating cells, and adherent cells were cultured in fresh medium. At the indicated time, the cell number of triplicate wells was determined.

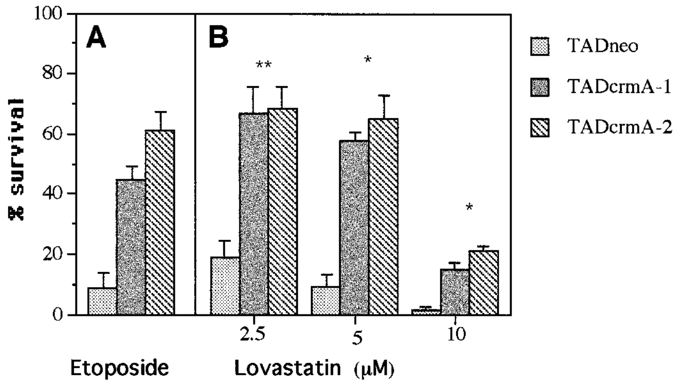

FIG. 8. Resistance of TADcrmA mutants to lovastatin-induced apoptosis. TADneo, TADcrmA-1, and TADcrmA-2 cell clones were cultured for 24 h in the presence of 70 μg/ml etoposide, and apoptosis was estimated by flow cytometric analysis (A). The same clones were treated with different concentrations of lovastatin for 48 h (B). Results are reported as the percentage of surviving cells: 100 × (total − hypodiploid cells)/total. Results are presented as the mean ± SD from five independent experiments. Differences in sensitivity to lovastatin of the TADcrmA mutants *vs.* TADneo cells were statistically significant (*, $P < 0.01$; **, $P < 0.05$).

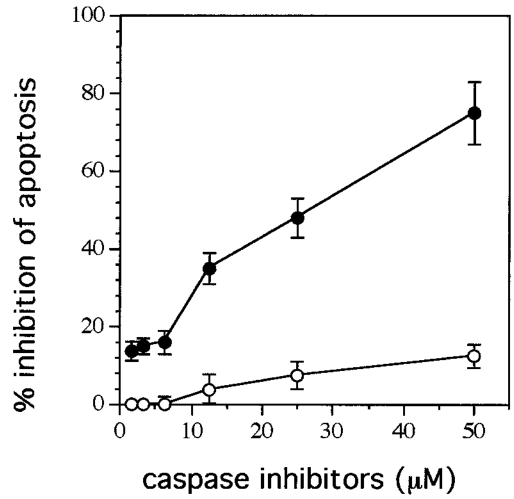

FIG. 9. Inhibition of lovastatin-induced apoptosis by caspase inhibitors. TAD-2 cells were plated in 24-well plates. After 24 h, the medium was replaced with fresh medium containing 5 μM lovastatin and the caspase inhibitors Ac-YVAD-cmk (*open circles*) and Z-DEVD-fmk (*full circles*). The inhibitors were replenished at 24 h, and at 48 h, the cells were collected, and apoptosis was measured by FACS analysis. Results are reported as the percent inhibition of apoptosis from three separate experiments.

Ac-YVAD-cmk. Only at much higher concentrations did Ac-YVAD-cmk display a weak inhibitory effect. This peptide is a potent inhibitor of caspase-1 and -4, but it also inhibits caspase-3 and -7 at higher concentrations. Thus, the modest inhibitory effect observed only at higher concentrations suggests that the caspase-1 subfamily is not involved in the apoptotic pathway triggered by lovastatin.

Lovastatin-induced apoptosis is p53 independent

The p53 tumor suppressor gene is crucial in some, but not all, forms of apoptosis. To determine whether p53 is required for apoptosis to occur in thyroid cells treated with lovastatin, we transfected TAD-2 cells with a vector encoding a p53 mutant (p53cG) that displays a dominant negative effect at 37–39 C and wild-type activity at 32 C. Several TADp53cG stable cell clones and a pool of clones (TAD53-p) were generated by G-418 selection of TAD-2 cells cotransfected with pLTRp53cG and pRSVneo expression vectors. The proliferations of TADp53cG and TADneo mutants at 39 C were comparable, as determined by proliferation curves (Fig. 7B). At 39 C, the TADp53cG mutant TAD53–1 and TAD53-p were resistant to the p53-dependent apoptotic drug etoposide, indicating inactivation of endogenous p53 activity (Fig. 10A). TAD53–1, TAD53-p, and TADneo cells were cultured in the presence of 0.75–10 μM lovastatin for 48 h, and apoptosis was estimated by flow cytometric analysis (Fig. 10B). Student's *t* test, paired analysis, did not show a statistically significant difference in sensitivity to lovastatin between the clones, thus demonstrating that p53 is not required in this type of drug-induced apoptosis.

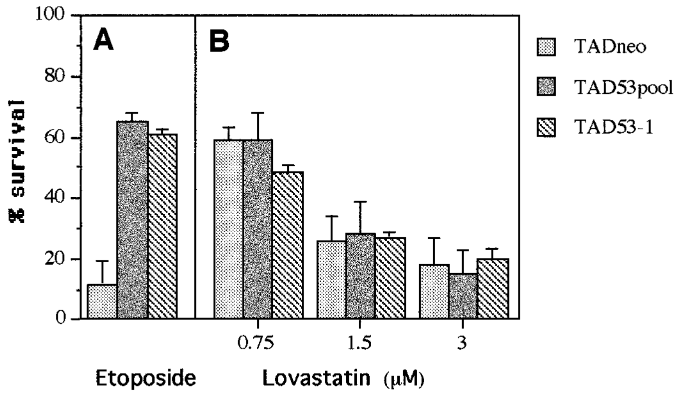

FIG. 10. Involvement of p53 in lovastatin-induced apoptosis. TADneo, TAD53-pool, and TAD53–1 cells were cultured for 24 h in the presence of 70 μg/ml etoposide, and apoptosis was estimated by flow cytometric analysis (A). Results are reported as the percentage of surviving cells. The same cells were treated with different concentrations of lovastatin for 48 h (B). Results are presented as the mean ± SD from five independent experiments. Differences in sensitivity to lovastatin of TADp53 *vs.* TADneo cells were not statistically significant.

Discussion

It has been suggested that activation of the apoptosis pathway by prenylation inhibitors results from a reduction of prenylated signaling proteins that contribute to cell growth. We have previously shown that impaired formation of isoprenylated proteins is also important for cytoskeletal organization in the rat thyroid cell line FRTL-5, inducing dramatic morphological changes (23). Targets of prenylation inhibitors are farnesylated small GTP-binding proteins of the Ras superfamily, including Rab, Raf, and Rac; some of these proteins, such as the actin cytoskeleton regulator Rho, are also geranylgeranylated. Different groups reported evidence of Rho involvement in apoptosis induced by HMG-CoA reductase inhibitors. Lebowitz and co-workers showed that apoptosis induced by farnesyl transferase inhibitors was blocked in cells expressing a myristylated and activated form of RhoB (16). Then, Ghosh and co-workers demonstrated that lovastatin treatment of mesangial cells caused RhoA accumulation in the cytosol, and by time lapse cinematography, the same group showed that lovastatin did not cause cell rounding, but, rather, prevented rounded up cells undergoing mitosis from spreading back again, thus inducing cell death (17). In agreement with this hypothesis, DNA electrophoresis and flow cytometry demonstrated that in TAD-2 cells DNA fragmentation followed cell rounding and detachment, taking place after 48 h of lovastatin treatment (Figs. 1 and 2).

Although prenylation of signaling proteins also occurs in normal untransformed cells, tumor cells and active proliferating cells have been reported to be more sensitive to prenylation inhibitors (11). Thus, concentrations of the drugs sufficient to induce apoptosis in the latter cells can be well tolerated by their normal nontransformed or low proliferating counterparts. In this study, we demonstrate that lovastatin is a potent apoptotic factor in proliferating thyroid cells, whereas it has no effect on TAD-2 cells induced to quiescence by serum starvation or on thyroid primary cultures, whose proliferation in culture is poor. Lovastatin, by inhibiting HMG-CoA, impairs the synthesis of all of the end products of the isoprenoid pathway. Although FTI-277 was less effective than GGTI-298 in inducing cell death, the results of the experiments shown in Fig. 6 suggest that both farnesylated and geranylgeranylated proteins are involved in apoptotic pathways. Apoptosis has been described to be differentially affected by protein synthesis inhibition and by the transcription regulatory activity of p53, depending on the cell system. Some types of apoptosis do not require macromolecular synthesis, and entry into the apoptosis pathway does not always involve p53 transcriptional activity. This type of apoptosis involves interaction of death domains and activation of proteases of the caspase cascade already in place. Fas/APO-1/CD95- and tumor necrosis factor receptor-1-induced cell death are examples of this type of apoptosis (24). Through protein synthesis inhibition, DNA synthesis and proliferation also can be inhibited by cycloheximide (25). TAD-2 cell proliferation, cell rounding, and detachment were completely blocked by cycloheximide, thus suggesting that the ensuing inhibition of apoptosis was not caused by impaired synthesis of apoptotic proteins but, rather, by inhibition of cell cycle progression.

Proteases of the caspase family are implicated in mammalian apoptosis and constitute a protease cascade (for review, see Ref. 26). Eleven proteins homologous to interleukin-1β-converting enzyme have been identified and are classified into three subfamilies: caspase-1-, caspase-2-, and caspase-3-like proteases (27). The caspase-1-like subfamily includes caspase-1 (28), -4 (29), and -5 (30). Although they are clearly involved in procytokine activation, their roles in apoptosis are still uncertain. Our results demonstrate that lovastatin-induced apoptosis in TAD-2 cells is antagonized by CrmA, a cowpox virus-derived protein. This viral serpine protein is able to prevent apoptosis induced by different factors, such as Fas ligand, serum withdrawal, and denied extracellular matrix adhesion, whereas it is ineffective at blocking apoptosis induced by glucocorticoids, ionizing radiations, and some DNA-damaging agents (31–35). CrmA inhibits the caspase-1-like subfamily as well as other caspases, such as caspase-8 and -9, whereas *in vitro* it binds other caspases (caspase-3, -6, and -7) too weakly to directly affect their activity *in vivo* (36–38). Synthetic peptide inhibitors that mimic cleavage sites of the caspases can be used for *in vitro* and *in vivo* analysis of protease activity. Although YVAD-cmk is a potent inhibitor of caspase-1-like enzymes and poorly inhibits caspase-3 and -7, DEVD-fmk is a potent inhibitor of the caspase-3-like proteases. Although these peptide inhibitors do not block individual caspases and thus do not allow definitive identification of each protease involved, they can clarify which caspase subfamilies are involved in a specific apoptotic pathway. The results of the experiments with peptide inhibitors demonstrate that caspase-3-like proteases are involved in the apoptotic pathway triggered by lovastatin and that CrmA-sensitive caspases outside the caspase-1 subfamily are also involved. The activation of caspases can occur in parallel pathways as well as sequentially. The exact order of caspase activation is still controversial, but it is current opinion that some procaspases (procaspase-3 and -7) are the target of apical caspases, such as the CrmA-sensitive caspase-8 and caspase-9, and procaspase-6 is a substrate of caspase-3 (39–42). Proteolytic activation of caspase-7 has been demonstrated in a prostate cancer cell line (LNCaP) undergoing lovastatin-induced apoptosis (43), and involvement of caspase-3 in a different cell system was reported during revision of the present manuscript (44). Our knowledge of the pathway of caspase activation is largely incomplete, and it is unknown whether this pathway is specific depending on cell type or apoptotic stimulus; thus, sequential activation of caspases in thyroid cells treated with lovastatin must be demonstrated.

In conclusion, we have shown that lovastatin is a powerful inducer of apoptosis in proliferating thyroid cells in culture, suggesting that lovastatin or other more effective prenylation inhibitors may have therapeutic potential not only in prostate cancer or prostatic hyperplasia, but also in thyroid proliferative diseases. A complete understanding of the molecular mechanisms regulating the apoptotic pathway triggered by prenylation inhibitors is needed to develop new, more effective treatments.

Acknowledgments

We thank Dr. T. F. Davies for the kind gift of the TAD-2 cell line, Dr. N. Boudreau for the plasmid pBKRSVcrmA, and Dr. A. Levine for the plasmid pLTRp53cG.

References

1. **Cohen JJ** 1993 Apoptosis. Immunol Today 14:126–130
2. **Ellis RE, Yuan JY, Horvitz HR** 1991 Mechanisms and functions of cell death. Annu Rev Cell Biol 7:663–698
3. **Yamaguchi Y, Suda T, Ohta S, Tominaga K, Miura Y, Kasahara T** 1991 Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. Blood 78:2542–2547
4. **Dremier S, Golstein J, Mosselmans R, Dumont JE, Galand P, Robaye B** 1994 Apoptosis in dog thyroid cells. Biochem Biophys Res Commun 200:52–58
5. **Di Jeso B, Ulianich L, Racioppi L, D'Armiento F, Feliciello A, Pacifico F, Consiglio E, Formisano S** 1995 Serum withdrawal induces apoptotic cell death in Ki-ras transformed but not in normal differentiated thyroid cells. Biochem Biophys Res Commun 214:819–824
6. **Vitale M, Di Matola T, Fenzi GF, Illario M, Rossi G** 1998 Fibronectin is required to prevent thyroid cell apoptosis through an integrin-mediated adhesion mechanism. J Clin Endocrinol Metab 83:3673–3080
7. **Lowe SW, Ruley HE, Jacks T, Housman DE** 1993 p53-dependent apoptosis mudulates the cytotoxicity of anticancer agents. Cell 74:957–967
8. **Miyashita T, Reed JC** 1995 Tumor suppressor p53 is a direct transcriptional activator of human bax gene. Cell 80:293–299
9. **Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW, Vogelstein B** 1992 Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256:827–830
10. **Liebermann DA, Hoffman B, Steinman RA** 1995 Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. Oncogene 11:199–210
11. **Jones KD, Couldwell WT, Hinton DR, Su Y, He S, Anker L, Law RE** 1994 Lovastatin induces growth inhibition and apoptosis in human malignant glioma cells. Biochem Biophys Res Commun 205:1681–1687
12. **Sebti SM, Hamilton AD** 1997 Inhibition of Ras prenylation: a novel approach to cancer chemotherapy. Pharmacol Ther 74:103–114
13. **Lerner EC, Hamilton AD, Sebti SM** 1997 Inhibition of Ras prenylation: a signaling target for novel anti-cancer drug design. Anticancer Drug Des. 12:229–238
14. **Padayatty SJ, Marcelli M, Shao TC, Cunningham GR** 1997 Lovastatin induced apoptosis in prostate stromal cells. J Clin Endocrinol Metab 82:1434–1439
15. **Sun J, Qian Y, Hamilton AD, Sebti SM** 1998 Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-Ras prenylation but each alone is sufficient to suppress human tumor growth in nude mouse xenografts. Oncogene 16:1467–1473
16. **Lebowitz PF, Sakamuro D, Prendergast GC** 1997 Farnesyl transferase inhibitors induce apoptosis of Ras-transformed cells denied substratum attachment. Cancer Res 57:708–713
17. **Ghosh PM, Mott GE, Ghosh-Choudhury N, Radnik RA, Stapleton ML, Ghidoni JJ, Kreisberg JI** 1997 Lovastatin induces apoptosis by inhibiting mitotic and post-mitotic events in cultured mesangial cells. Biochim Biophys Acta 1359:13–24
18. **Fagin JA** 1992 Genetic basis of endocrine disease. III. Molecular defects in thyroid gland neoplasia. J Clin Endocrinol Metab 75:1398–1400
19. **Wynford-Thomas D** 1993 Molecular basis of epithelial tumorigenesis: the thyroid model. Crit Rev Oncogen 4:1–23
20. **Santoro M, Grieco M, Melillo RM, Fusco A, Vecchio G** 1995 Molecular defects in thyroid carcinomas: role of the RET oncogene in thyroid neoplastic transformation. Eur J Endocrinol 133:513–522
21. **Suarez HG, du Villard JA, Severino M, Caillou B, Schlumberger M, Tubiana M, Parmentier C, Monier R** 1990 Presence of mutations in all three ras genes in human thyroid tumors. Oncogene 5:565–570
22. **Vitale M, Illario M, Di Matola T, Casamassima A, Fenzi GF, Rossi G** 1997 Integrin binding to immobilized collagen and fibronectin stimulates the proliferation of human thyroid cells in culture. Endocrinology 138:1642–1648
23. **Bifulco M, Laezza C, Aloj SM, Garbi C** 1993 Mevalonate controls cytoskeleton organization and cell morphology in thyroid epithelial cells. J Cell Physiol 155:340–348
24. **Nagata S, Golstein P** 1995 The Fas death factor. Science 267:1449–1456
25. **Stimac E, Housman D, Huberman JA** 1977 Effects of inhibition of protein synthesis on DNA replication in cultured mammalian cells. J Mol Biol 115:485–511
26. **Salvesen GS, Dixit VM** 1997 Caspases: intracellular signaling by proteolysis. Cell 91:443–446
27. **Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J** 1996 Human ICE/CED-3 protease nomenclature. Cell 87:171
28. **Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Ellison KO, Ayala YM, Casano FJ, Chin J, Ding GJ-F, Egger LA, Saffney E, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin T-T, Lee TD, Shively JE, MacCross M, Mumford RA, Schidt JA, Tocci MJ** 1992 A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature 356:768–774
29. **Faucheu C, Diu A, Chan AW, Blanchet AM, Miossec C, Herve F, Collard-Dutilleul V, Gu Y, Aldape RA, Lippke JA, Rocher C, Su MSS, Livingstone DJ, Hercend T, Lalanne J-L** 1995 A novel human protease similar to the interleukin-1β converting enzyme induces apoptosis in transfected cells. EMBO J 14:1914–1922
30. **Munday NA, Vaillancourt JP, Ali A, Casano FJ, Miller DK, Molineaux SM, Yamin TT, Yu VL, Nicholson DW** 1995 Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. J Biol Chem 270:15870–15876
31. **Tewari M, Dixit VM** 1995 Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. J Biol Chem 270:3255–3260
32. **Gagliardini V, Fernandez PA, Lee RK, Drexler HC, Rotello RJ, Fishman MC, Yuan J** 1944 Prevention of vertebrate neuronal death by the *crm*A gene. Science 263:826–828
33. **Frisch SM, Vuori K, Kelaita D, Sicks S** 1996 A role for Jun-N-terminal kinase in anoikis; suppression by *bcl-2* and *crm*A. J Cell Biol 135:1377–1382
34. **Geley S, Hartmann BL, Kapelari K, Egle A, Villunger A, Heidacher D, Greil R, Auer B, Kofler R** 1997 The interleukin 1β-converting enzyme inhibitor CrmA prevents Apo1/Fas- but not glucocorticoid-induced poly(ADP-ribose) polymerase cleavage and apoptosis in lymphoblastic leukemia cells. FEBS Lett 402:36–40
35. **Datta R, Kojima H, Banach D, Bump NJ, Talanian RV, Alnemri ES, Weichselbaum RR, Wong WW, Kufe DW** 1997 Activation of a CrmA-insensitive, p35-sensitive pathway in ionizing radiation-induced apoptosis.J Biol Chem 272:1965–1969
36. **Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS, Pickup DJ** 1992 Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. Cell 69:597–604
37. **Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM, Salvesen GS** 1997 Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J Biol Chem 272:7797–7800
38. **Kamada S, Washida M, Hasegawa J, Kusano H, Funahashi Y, Tsujimoto Y** 1997 Involvement of caspase-4(-like) protease in Fas-mediated apoptotic pathway. Oncogene 15:285–290
39. **Villa P, Kaufmann SH, Earnshaw WC** 1997 Caspases and caspase inhibitors. Trends Biochem Sci 22:388–393
40. **Armstrong RC, Aja T, Xiang J, Gaur S, Krebs JF, Hoang K, Bai X, Korsmeyer SJ, Karanewsky DS, Fritz LC, Tomaselli KJ** 1996 Fas-induced activation of the cell death-related protease CPP32 Is inhibited by Bcl-2 and by ICE family protease inhibitors. J Biol Chem 271:16850–16855
41. **Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, Okazaki T, Yamamoto K, Sasada M** 1998 Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. J Exp Med 187:587–600
42. **Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES** 1998 Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell 1:949–957
43. **Marcelli M, Cunningham GR, Haidacher SJ, Padayatty SJ, Sturgis L, Kagan C, Denner L** 1998 Caspase-7 is activated during lovastatin-induced apoptosis of the prostate cancer cell line LNCaP. Cancer Res 58:76–83
44. **Chang MY, Jan MS, Won SJ, Liu HS** 1998 Ha-*ras*Val12 oncogene increases susceptibility of NIH/3T3 cells to lovastatin. Biochem Biophys Res Commun 248:62–68