EXPERIMENTAL STUDY

Lovastatin-induced apoptosis in thyroid cells: involvement of cytochrome *c* and lamin B

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Abstract

Objective: The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, lovastatin, induces apoptosis in the thyroid cell line TAD-2 and in proliferating normal human thyroid cells in culture, through a p53-independent mechanism involving caspase-3-like proteases. The combination of lovastatin with other anti-neoplastic drugs potentiates chemotherapy of tumors. This drug has been suggested for the chemotherapy of tumors and is potentially useful in the treatment of thyroid proliferative diseases. Based on this premise, we analyzed in more detail the role of some molecular effectors and the role of the caspase family proteases in the lovastatin-induced apoptotic pathway in TAD-2 cells.

Methods: TAD-2 cells were treated with lovastatin to induce apoptosis, and expression of p53, Bcl-2, Bcl-XL and Bax was analyzed by Western blot. Caspase activation was evaluated by the assay of enzymatic activity with chromogenic peptides and Western blot. Nuclear, cytosolic and mitochondrial fractions were prepared by differential centrifugation and the presence of cytochrome *c* and lamin B was evaluated by Western blot.

Results: p53, Bcl-2, Bcl-XL and Bax protein expression were unchanged during apoptosis. Cytochrome *c* was released from mitochondria into the cytosol, a pivotal event in the activation of caspase-3. Caspase-3 and -6 but not caspase-2 were activated, and proteolysis of PARP and lamin B, a caspase-6 substrate located in the inner nuclear membrane, was demonstrated by Western blot. The nuclear localization of lamin B was also inhibited by lovastatin.

Conclusions: These data demonstrate that, in TAD-2 thyroid cells, lovastatin induces lamin B proteolysis and inhibits its nuclear localization and induces cytochrome *c* release from mitochondria into the cytosol.

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Introduction

In a number of cancer cells, in rat fibroblasts, in human prostate cells and in the thyroid cell line TAD-2, lovastatin induces apoptosis through the blocking of geranylated and/or farnesylated compounds (1–4). Recently, Agarwal and co-workers (5) demonstrated that pretreatment with lovastatin significantly increases apoptosis induced by 5-fluorouracil and cisplatin in a number of colon cancer cell lines, suggesting that lovastatin, alone or in combination with other drugs, could improve the chemotherapy outcome of aggressive tumors and it has been suggested for the therapy of prostate hyperplasia. Lovastatin prevents the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) to mevalonate, the precursor of isopentenyl

pyrophosphate then converted into geranyl pyrophosphate, farnesol pyrophosphate (FPP) and all-trans-geranylgeranyl pyrophosphate (GGPP) (6). FPP and GGPP are transferred to various proteins, including the small GTP-binding proteins of the Ras superfamily Rho, Raf, Rab, Rac and Rap, involved in important cellular functions such as proliferation, cell adhesion and motility (2, 7, 8). Independent studies have demonstrated that while in the prostate cancer cells LNCaP lovastatin induces caspase-7 but not caspase-3 activation, caspase-3 is activated in Ha-ras-transformed NIH/3T3 fibroblasts and in Ki-ras-transformed rat kidney cells (9–11). Caspases are cysteine proteases which play a pivotal role in apoptotic cell death (12–14). These enzymes comprise a multi-gene family with 14 mammalian members identified thus far. On the basis of their substrate specificity, the caspases can be

subdivided into three groups: group I (caspases-1, -4, -5 and -13), group II (caspases-2, -3 and -7) and group III (caspases-6, -8, -9 and -10) (15, 16). During apoptosis, caspases can participate in the death pathway by simultaneous or sequential activation, defining different apoptotic phases. Death receptors such as CD95/FAS or tumor necrosis factor α (TNF)R1 receptors that recruit caspase-8 through FADD and TRADD adapters can directly activate apical caspases (17, 18). These caspases of the initiation phase can drive the activation of the caspases of the effector phase, during which multiple caspases are recruited in the destruction of cellular structures.

As a logical extension of our recent studies, we investigated the role of the caspase family proteases in the lovastatin-induced apoptotic pathway in TAD-2 cells, demonstrating that caspase-3 and caspase-6 but not caspase-2 are activated and providing evidence that lovastatin inhibits the nuclear localization of lamin B and induces cytochrome *c* release into the cytosol.

Materials and methods

Cell culture and chemicals

Immortalized human fetal thyroid cells (TAD-2) were generously donated by Dr T F Davies, Mount Sinai (New York, NY, USA) and cultured in a 5% CO₂ atmosphere at 37 °C, in RPMI medium supplemented with 10% fetal calf serum (FCS). Medium was changed every 3–4 days. A stock solution of 2 mM lovastatin (a gift from Dr A W Alberts, Merck, Sharp and Dohme Institute) was prepared in dimethyl sulfoxide and stored at –20 °C.

Estimation of apoptotic cells

Analysis of DNA fragmentation in cells undergoing apoptosis was performed as described previously in the same cell line (19) and the percent of apoptotic cells was determined by flow cytometry. Briefly, cytofluorimetric estimation of apoptosis was performed as follows: floating and trypsinized adherent cells were collected and washed in cold phosphate-buffered saline (PBS). Cells were fixed in 70% cold ethanol for 30 min. Ethanol was removed by two PBS washes and cells were incubated in PBS, 50 µg/ml propidium iodide, 10 µg/ml ribonuclease A, deoxyribonuclease-free for 1–3 h at 4 °C. Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA, USA). The percent of apoptotic hypodiploid cells was calculated by dividing the number of cells displaying red fluorescence lower than the G₀–G₁ diploid peak by the total number of cells $\times$ 100.

Antibodies and Western blot analysis

Mouse monoclonal antibodies to p53, caspase-2 -3 and -7 were purchased from Transduction Laboratories

(Lexington, KY, USA), rabbit polyclonal antibodies to the caspase-6 prodomain fragment were from Biosource (Camarillo, CA, USA) and mouse monoclonal antibody to Bcl-2 and rabbit polyclonal antibodies to Bcl-X and Bax were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies to lamin B were from Calbiochem (La Jolla, CA, USA), and mouse monoclonal antibodies to poly(ADP-ribose)polymerase (PARP) and to cytochrome *c* were from Alexis (Laufelfingen, Switzerland). Cells were washed in cold PBS and lysed for 10 min at 4 °C with 1 ml lysis buffer (50 mM Tris, pH 7.4, 0.5% NP40 and 0.01% SDS) containing protease inhibitors. Lysates from floating cells and from adherent cells collected by scraping were centrifuged at 12 000 *g* for 15 min at 4 °C. The protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA) and 50 µg total protein from each sample was boiled for 5 min in Laemmli sample buffer (125 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 1% p-mercaptoethanol and 0.006% bromophenol blue). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Acrylamide concentration was 12% for p53, Bcl-XL and PARP, and 15% for Bcl-2, Bax, caspases and cytochrome *c*. Membranes were blocked by 5% non-fat dry milk, 1% ovalbumin, 5% FCS and 7.5% glycine and, after three washes, incubated for 1 h at 4 °C with 0.5 µg/ml mouse monoclonal or rabbit polyclonal primary antibodies in PBS. After three washes, filters were incubated for 1 h at 4 °C with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Bio-Rad) diluted 1:2000 in PBS and Tween-20. After a final wash, protein bands were detected by an enhanced chemiluminescence system (Amersham International plc, Amersham, Bucks, UK).

Assay of enzymatic activity

The enzymatic activity of caspases was determined using a spectrophotometric assay in which the cleavage of the chromogenic peptides Ac-DEVD-pNA, Ac-VEID-pNA and Ac-YVAD-pNA (Alexis) was monitored. Floating and adherent cells from a confluent 10 cm Petri dish were washed in cold PBS, then resuspended in 0.4 ml extraction buffer (25 mM HEPES, pH 7.4, 0.1% Triton X-100, 10% glycerol, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml pepstatin). Twenty microliters of extract were incubated for 1 h at 37 °C with or without 100 µM non-chromogenic peptides, then equimolar amounts of p-nitroanilide-conjugated peptides were added and the reaction was incubated for 3 h at 37 °C. The release of p-nitroanilide was measured at 405 nm in a microtiter plate reader. Enzymatic activity was plotted as differential absorbance in the presence or absence of non-chromogenic peptides.

Preparation of cytosolic and mitochondrial fractions

Untreated and lovastatin-treated TAD-2 cells were resuspended in 20 mM Hepes, pH 7.5, 10 mM EDTA, 1 mM DTT, 300 mM sucrose and protease inhibitors. After several passages through a fine needle, intact cells and nuclei were removed by centrifugation at 1000 *g* for 10 min and the supernatant was centrifuged at 10 000 *g* for 30 min. The pellet fraction, containing mitochondria, and the supernatant, containing the cytosol, were analyzed by Western blot for the presence of cytochrome *c*. The presence of mitochondria in the three fractions was evaluated by Western blot using a polyclonal antibody for the mitochondrial voltage-dependent anion-selective channel protein 1 (VDAC-1) (Santa Cruz). VDAC-1 was abundant in the mitochondrial fraction, whereas minimal VDAC-1 contamination was present only in the nuclear fraction (not shown).

Results

Expression of p53, Bcl-2, Bax and Bcl-XL is not regulated by lovastatin

TAD-2 cells were treated with 20 μ M lovastatin for different times and induction of cell death was evaluated by flow cytometry determining the number of hypodiploid DNA cells, the hallmark of late cell death (Fig. 1A). The level of expression of different pro- and anti-apoptotic proteins was determined by Western blot analysis (Fig. 1B). In a previous study, we demonstrated that lovastatin induces apoptosis in thyroid cells through a p53-independent mechanism (4). Western

Figure 1 (A) Estimation of apoptosis by flow cytometry in TAD-2 cells treated with 20 μ M lovastatin. At various time-intervals, adherent and floating cells were harvested and apoptosis was determined by flow cytometry. (B) Western blot analysis of p53, Bcl-2, Bcl-XL and Bax expression in TAD-2 cells treated with 20 μ M lovastatin for different times. Fifty micrograms of each sample were loaded on the gel. Acrylamide concentration was 12% for p53 and Bcl-XL and 15% for Bcl-2 and Bax. Representative immunoblots of each protein are shown.

blot analysis confirmed this finding, showing that p53 was constant in cells undergoing apoptosis induced by lovastatin. The anti-apoptotic protein Bcl-XL was also unchanged. A slight increased expression of Bcl-2 and a decreased expression of Bax were detected after 48 h of treatment during the late phase of apoptosis, these variations were likely due to translational or post-translational events rather than to transcriptional regulation by p53. The molecular weights of p53 and Bcl-2 did not shift, indicating that phosphorylation of these two proteins had not occurred. These results demonstrated that lovastatin-induced apoptosis is independent from variations in the levels of p53, proteins under p53 transcriptional control (Bcl-2 and Bax) and Bcl-XL, and that apoptosis is also independent from their phosphorylation.

Activation of caspase-3 and caspase-6

To investigate the role of individual caspase members, we determined the cysteine protease activity of caspase-3-like (group II caspases) and caspase-1-like (group I) proteases and caspase-6 (a member of group III) by cleavage of chromogenic peptides (Fig. 2A). Activation of proteases with DEVD and VEID (caspase-6 substrate) cleavage activity was first detected after 16 h of lovastatin treatment, and became clearly evident by 24 h. By 48 h, caspase-3-like proteases displayed the maximum enzymatic activity while the activity of caspase-6 decreased. YVAD cleavage was undetectable during 24 h of lovastatin treatment and a minor caspase-1-like protease activity was present only by 48 h. Caspase-2, -3 and -7 are members of group II proteases with DEVD cleavage activity. Activation of caspase-7 was previously demonstrated in the prostate cancer cell line LNCaP (9). To answer the question as to whether caspase-2 and -3 are activated during apoptosis induced by lovastatin, cellular extracts were analyzed by Western blot for the presence of these three caspases. The 32 kDa procaspase-3 was abundant in TAD-2 cells and the active 17 kDa caspase-3 fragment was not present in untreated cells nor after 12 h of lovastatin treatment (Fig. 2B). After 12 h, generation of the active 17 kDa caspase-3 fragment was observed and increased thereafter. Western blots with anti-caspase-2 antibodies showed the 37 kDa band corresponding to procaspase-2 inactive enzyme but failed to show the p20 fragment, thus suggesting that caspase-2 is not involved in lovastatin-induced apoptosis (Fig. 2C). A Western blot for caspase-6 was also performed (Fig. 2D). A decrease of the 34 kDa procaspase-6 during lovastatin treatment was evident by 24 h. Western blot did not show the small fragments generated by caspase-6 cleavage because the antibody used recognizes only the small prodomain fragment that is also cleaved during activation.

Figure 2 Activation of proteases by lovastatin. (A) Ac-DEVD-pNA, Ac-VEID-pNA and Ac-YVAD-pNA were incubated with lysates of cells treated with lovastatin for the time indicated. Enzymatic activity is expressed as chromogenic activity and reported as optical density (O.D.) $\pm$ s.d. Results indicate the activation of caspase-3-like and caspase-6 proteases, while no caspase-1-like proteases were observed. (B, C and D) Western blot analysis of lovastatin-induced activation of caspases. Cell lysates were collected after treatment with 20 μ M lovastatin for the time indicated. Fifty micrograms of each sample were loaded on the gel. (B) Caspase-3; (C) caspase-2; (D) caspase-6.

Proteolytic substrates for caspases during apoptosis

Although, in most cases, the responsible enzyme cannot be established, the proteolysis of some substrates can indicate the activation of a group of caspases. PARP, an enzyme substrate of caspase-3 involved in genome functions, was investigated by Western blot during lovastatin-induced apoptosis to verify the proteolytic activity of caspase-3 (Fig. 3A). A faint band corresponding to the 85 kDa fragment generated by PARP proteolysis was present by 12 h of

treatment, and became more evident in the following hour. The cleavage of lamin B, a substrate of caspase-6, was then investigated by Western blot (Fig. 3B). Extensive cleavage of lamin B was evident as early as after 12 h, indicating that caspase-6 activation is an early apoptotic event. The early appearance of proteolytic products of caspase-6 as compared with active caspase fragments could be explained by the accumulation of the enzymatic products that amplify and render the Western blot more sensitive.

Figure 3 Proteolysis of caspase substrates. Lysates of cells treated with 20 μ M lovastatin for the time indicated were loaded on a 12% acrylamide gel. (A) A faint band corresponding to the 85 kDa fragment generated by PARP proteolysis was present by 12 h and increased in the following hours, and (B) extensive cleavage of lamin B was evident as early as after 12 h.

Lovastatin induces cytochrome *c* release into the cytosol and cytosolic localization of lamin B

Mitochondria play a pivotal role in several apoptotic processes. Cytochrome *c* can be released from the mitochondria into the cytosol where it binds Apaf-1 which then activates caspase-9 which, in turn, activates caspase-3. To determine whether this pathway is activated by lovastatin, we examined whether lovastatin induced release of cytochrome *c* into the cytosol. Cells treated with lovastatin for 24 h were collected and fractionated into cytosolic, mitochondrial and nuclear fractions. The presence of cytochrome *c* in these fractions was detected by Western blot analysis using an anti-cytochrome *c* monoclonal antibody. As shown in Fig. 4A, cytochrome *c* was detected in the cytosol after 24 h of lovastatin treatment with a concomitant decrease of cytochrome *c* in the mitochondrial fraction. Lamin B was then investigated by Western blot (Fig. 4B). In untreated cells, intact lamin B was present only in the nuclear fraction, whereas it was absent from the cytosolic fraction. In lovastatin-treated cells, both full-size lamin B and its fragment

Figure 4 Cytochrome *c* release from mitochondria, and proteolysis and localization of lamin B. TAD-2 cells were untreated or treated with 20 μ M lovastatin (lova) for 24 h. Cells were harvested and cytosolic and mitochondrial fractions were prepared. (A) The presence of cytochrome *c* was determined by Western blot analysis with anti-cytochrome *c* monoclonal antibody. Cytochrome *c* was released from mitochondria into the cytosol. w, whole lysate; cy, cytosolic fraction; mt, mitochondrial fraction. (B) Localization of lamin B. Cells were treated as above and then cytosolic and nuclear fractions were prepared and analyzed by Western blot with anti-lamin B antibody. Full-size lamin B and its cleavage fragment were present in the cytosolic fraction from lovastatin-treated cells. nu, nuclear fraction.

were also present in the cytosolic fraction, indicating that inhibition of prenylation partially inhibited the nuclear localization of lamin B.

Discussion

We have previously shown that lovastatin activates an apoptotic pathway dependent on protein synthesis (4). However, in the same study, we demonstrated that the anti-oncogene p53, a transcriptional factor that plays a pivotal role in apoptosis by regulating some Bcl-2 family proteins, was not required. Some, but not all, kinds of apoptosis require protein synthesis while some others, such as that activated by FAS or by TNFR1, can

be accelerated by protein synthesis inhibitors (17). Variation of the overall protein expression is not the only mechanism by which the Bcl-2 checkpoint can be reset and post-translational mechanisms, such as Bcl-2 inactivation by serine phosphorylation, also appear to be involved in response to some apoptotic stimuli (20, 21). Western blot analysis confirmed that both variations of protein expression as well as post-transcriptional modification of p53, Bcl-2, Bax and Bcl-XL did not occur during lovastatin-induced apoptosis. Thus, since the inhibition of protein synthesis completely abolished lovastatin apoptosis, alternative regulatory mechanisms of apoptotic proteins, or generation of intermediate metabolites by the lovastatin inhibition of pathways that control cell life, must be considered (4).

The analysis of caspase activation by lovastatin in different cell systems suggested cell line specificity. A study by Marcelli and co-workers (9) reported that in the prostate cancer cell line LNCaP, within the members of the group II caspases, caspase-7 was activated by lovastatin, while caspase-3 was not. In cell lines of different origin (Ha-Ras-transformed NIH/3T3 fibroblasts, Ki-ras-transformed rat kidney cells and human thyroid cells TAD-2), caspase-3 was activated during lovastatin-induced apoptosis (10, 11). This discrepancy could be due either to a tissue specificity of the lovastatin activation pathway or to some peculiarity of the LNCaP cell line. Indeed, specificity of some apoptotic pathways has already been documented in some cell lines that display caspase activation pathways with unique features; for instance in MCF-7 cells the lack of caspase-3 is overcome by caspase-7 (22).

Cytochrome *c* resides in the inter-membrane space of the mitochondria and is involved in energy production. During some types of apoptosis, cytochrome *c* is released into the cytosol where it binds Apaf-1 and activates caspase-9 which, in turn, activates caspase-3. As in v-Ki-ras-transformed rat kidney cells (11), our work demonstrates that cytochrome *c* is released by lovastatin.

The present study identifies additional potential loci, besides mitochondria, from which apoptotic signals can be generated. Correct localization of lamin B in the nuclear membrane and its interaction with proteins of the inner nuclear membrane is required for several cellular functions including DNA replication and chromatin organization. Lamin B, besides its role in apoptosis as caspase-6 substrate, can be involved in activation and/or propagation of apoptosis. Lamin B phosphorylation by protein kinase C- δ (PKC- δ) alters its interaction with proteins of the inner nuclear membrane and contributes to induce apoptosis as demonstrated by the observation that inhibition of PKC- δ delays apoptosis (23). Inhibition of lamin B prenylation by lovastatin induces its incomplete incorporation into the nuclear envelope in TAD-2 cells and

might result in alteration of the nuclear membrane. This event might lead to nuclear damage with DNA degradation and generation of apoptotic signals. The cytochrome *c* release from mitochondria and lamin B cleavage induced by lovastatin are the terminal events of a process that leads to apoptosis, and the question of which up-stream events are promoted by lovastatin remains unanswered. HMG-CoA reductase is a pivotal enzyme in the metabolism of cholesterol and isoprenylated proteins, and it is involved in important cellular functions. In the absence of prenyl modification, the small GTP-binding proteins of the Ras superfamily are not able to form complexes with their target proteins, impairing their function and leading to cell growth arrest. Lovastatin reduces DNA synthesis but does not impair cell viability in the rat thyroid cells PC13 and FRTL-5 (24). However, protein synthesis is differentially modulated by lovastatin in these two cell lines, demonstrating a cell type-dependent response to the inhibition of HMG-CoA reductase. Thus, the results from human thyroid cells in primary culture and from human immortalized cell lines are not in contrast with the results obtained from rat cells. The results from the human cells suggest that lovastatin or other more effective prenylation inhibitors may have therapeutic potential in the treatment of thyroid proliferative disorders.

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