Statins activate the mitochondrial pathway of apoptosis in human lymphoblasts and myeloma cells

Paola Cafforio, Franco Dammacco, Angela Gernone and Franco Silvestris*

Department of Internal Medicine and Oncology (DIMO), University of Bari, P.zza Giulio Cesare, 11 - 70124 Bari, Italy
*To whom correspondence should be addressed. Tel./Fax: +39 080 5478771; Email: f.silvestris@dimo.uniba.it

Although statins are lipid-lowering drugs that block cholesterol biosynthesis, they exert immunomodulatory, anti-inflammatory, anti-angiogenic and anti-proliferative functions by reducing the isoprenylation of proteins involved in cell signal transduction such as Ras and RhoA. In this study, we provide evidence that several natural (lovastatin, simvastatin and pravastatin) and synthetic (cerivastatin and atorvastatin) statins exert a cytotoxic effect on human T, B and myeloma tumor cells by promoting their apoptosis. Dissimilar susceptibility to apoptosis has been detected in these lines, presumably in relation to the altered expression of proteins involved in the regulation of cellular signals. Cerivastatin promptly activated the cell death even in doxorubicin resistant cell lines such as MCC-2, whereas pravastatin, a hydrophilic compound, failed to induce any effect on either proliferation or apoptosis. The statin-induced apoptotic pathway in these cell lines was presumably regulated by altered prenylation of either Ras or RhoA, as measured by the defective membrane localization of these small GTPases. In addition the cell proliferation was rescued by both farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), whereas no effect was obtained with squalene, a direct precursor of cholesterol. Statins primed apoptosis through its intrinsic pathway involving the mitochondria. In fact, we observed the reduction of mitochondrial membrane potential and the cytosolic release of the second mitochondria-derived activator of caspases (Smac/DIABLO). The apoptotic pathway was caspase-dependent since caspases 9, 3 and 8 were efficiently activated. These results support the potential use of statins in association with conventional treatment as apoptosis-triggering agents in these tumors.

Introduction

Statins are cholesterol-lowering drugs (1) that exert pleiotropic functions by preventing the synthesis of mevalonic acid, the precursor of non-steroidal isoprenoid compounds that play a key role in a number of cellular processes (2); indeed, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) act as lipophilic anchors on the cell membrane for both attachment and biological activity of small GTP-binding proteins from the GTPase family (3,4). In order to play their role in cell signal transduction, these proteins, including Ras and RhoA, translocate from the cytoplasm to the membrane after their isoprenylation with FPP and GGPP, respectively (5). Other functional proteins involved in both the cell cycle and the proliferation are activated by isoprenylation mechanism(s) during the progression of cholesterol biosynthesis.

Clinical studies have demonstrated that statins reduce the risk of both acute and chronic rejection in renal and cardiac transplant recipients by modulating a number of lymphocyte functions (6,7). In vitro they suppress both T and B cell responses (8,9), reduce the expression of class II major histocompatibility complex molecules on antigen presenting cells (10), inhibit chemokine synthesis in peripheral lymphocytes (11), modulate several inflammatory responses and downregulate the production of inflammatory cytokines by endothelial cells (12). Definite anti-proliferative and cytotoxic effects have been ascribed to statins in promoting the in vitro lysis of human smooth muscle cells (13), and rabbit (14), rat (15) and human (16) myeloblasts. The possible occurrence of rhabdomyolysis in patients chronically treated with several statins (17), however, is in line with the cytotoxic effect detected in vitro. The mechanism potentially involved in suppression of the cell cycle acts by blocking cell proliferation in the G0/G1 phase by a low expression of Ki67, a proliferative marker, and by an increase in the protein tyrosine phosphorylation, which regulates the intracellular signal transduction leading to apoptosis (18).

Additional evidence suggests that both lovastatin and cerivastatin exert a cytostatic effect on mesothelioma (19), glioma (20), neoplastic thyroid (21), acute myeloid leukemia (22) and multiple myeloma cells (23) by directly promoting apoptosis. It seems likely that defective isoprenylation of proteins involved in the cell cycle, namely Ras, Rac and Rho A, results in their inappropriate localization and functions and triggers apoptosis (19). Some tumors downregulate the Bcl-2 mRNA and related protein, thus promoting an apparent susceptibility to statin-induced apoptosis (24). However, this effect is inducible in highly proliferating tumor cells (25), whereas a number of cell lines are insensitive to statins in vitro (26). In addition, it has been shown that apoptosis in response to stimulation by lovastatin is caspase-3-dependent (21) and induces both cytochrome c release and PARP cleavage (27). However, the molecular mechanisms activated by statins in enhancing apoptosis remain unclear.

In this study, we evaluated the apoptogenic effect of several natural (lovastatin, simvastatin and pravastatin) and synthetic (cerivastatin and atorvastatin) statins on both T, B and myeloma cell lines.

Materials and methods

Cell cultures

Jurkat and CEM T leukemic cells and both IM9 and U266 cell lines were obtained from American Type Cell Collection (ATCC), whereas MCC-2...
myeloma cells were established in our laboratory (28). The cells were cultured in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin and 2 mM l-glutamine (Life Technologies Ltd, Paisley, UK). Both lovastatin and simvasta-

fin were used in this study and were purchased from Merck Sharp & Dohme (Whitehouse Station, NJ) and resuspended in ethanol, whereas pravastatin (Bristol Myers Squibb, Epirone) was purchased from Sigma-Aldrich (Germany) and resuspended in methanol.

For dose–response experiments, 1 × 10^5 cells of each cell line were incubated for 24 h in 96-well plates with increasing concentrations (0.1–100 μM) of the relative statin in its active form (29). The respective solvents were used in control tests. Cerivastatin was also tested at lower concentration, up to 1 nM for 36 h, to verify its effect at concentration comparable to that induced in vivo on circulating cells. Additional time-dependent experiments were performed by incubating each cell line with 20 μM of each statin for 4, 8, 12 and 24 h. In addition, several instances cerivastatin was used in proliferation experiments in combination with doxorubicin in MCC-2 cells, a doxorubicin resistant cell line (28) to evaluate a possible synergic effect. Briefly, cells were incubated overnight with 10 nM cerivastatin plus 1 μg/ml doxorubicin and then measured in their proliferative rate. Finally, to assess the role of either defective isoprenylation or inhibited cholesterol synthesis in influencing the cell cycle, several cerivastatin-treated cultures were co-incubated with increasing concentrations of squalene (SQ; 10, 50, 100 and 200 μM), mevalonic acid (MA; 10, 50 and 100 μM), GGPP and FPP (1, 5 and 10 μM) (Sigma Chemical Co., St Louis, MO), as previously described (22).

### Cell Proliferation and Apoptosis Assays

Cell proliferative rate was determined by measuring [3H]thymidine uptake. Briefly, cells were incubated overnight in the presence of 1 μCi of [3H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) and subsequently evaluated with a β-counter (Beckman, Palo Alto, CA). Proliferation was expressed as mean values of both [3H]thymidine uptake and inhibition of proliferation (30). Both viability and proliferative rates were also assessed by the MTT colorimetric method (Promega, Madison, WI) using an aqueous soluble tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-2,5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] in combination with an electron coupling reagent (phenazine ethosulfate). The MTS tetrazolium compound was metabolized by functional mitochondria in a solubilized formazan salt after 1 h of incubation at 37°C. The colorimetric reaction was measured with a 96-well plate reader (Benchmark, Bio Rad Labs, Hercules, CA) at 490 nm and the absorbance values reflected the mitochondrial activity of live cells. Each assay was conducted in triplicate.

Apoptosis was investigated by propidium iodide (PI) staining, as previously described (31) and by the FITC-conjugated-Annexin-V method in a FACSScan cytofluorimeter, using the Cell Quest program (Becton Dickinson, Mountain View, CA). The TUNEL technique for **in situ** cell death detection (Roche-Boehringer, Indianapolis, IN) using the PE-conjugated nucleotide mixture was also employed. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution. Then, the samples were incubated for 1 h at 37°C in a humidified chamber with the nucleotide mixture and terminal deoxynucleotidyl transferase and then the fluorescent nucleotide polymers, labeled to DNA strand breaks, were quantified by flow cytometry. The data were calculated as the mean values from three to five independent experiments.

### Detection of both Ras and Rho on cerivastatin-treated MCC-2 cells

Standard western blot analysis was adopted. Briefly, MCC-2 cells were cultured in 6-well plates with or without 1 μM cerivastatin for 12 h and the membrane lysates were prepared as described by Stirewalt et al. (32) and electrophoresed at 30 μA/ml in a 12% SDS-PAGE. Following the protein transfer to the Immobilon Transfer membranes (Millipore, Billerica, MA, USA), these were incubated for 2 h with anti-p-Ras or anti-Rho primary monoclonal antibodies (Becton Dickinson, Mountain View, CA). The membranes were then washed and incubated with a alkaline phosphatase conjugated anti-mouse IgG antibody (Sigma). Antibodies reactivity on the membranes was visualized using enhanced chemiluminescence (Amersham, Amersham, UK).

For each cell sample, Ras and Rho signal intensities were compared with actin signals used as intra-assay controls for sample lysates quality and loading.

### Assay for caspase activation

Activation of caspases was investigated to elucidate the apoptosis pathway induced by the statins. Caspases 8 and 9 were evaluated using specific cell permeable caspase inhibitors labeled with FAM (InterGen Company, Purchase, NY) in association with the colorimetric assay (BioVision Research Products, Mountain View, CA). Cells were first treated for 24 h with 20 μM of each statin and then incubated for 1 h at 37°C with FAM–LETD–FMK which irreversibly binds the active form of caspase-8, whereas FAM–LEHD–FMK was adopted for caspase-9. Finally, the cells were incubated with PI at isotonic concentration to promote the DNA staining of apoptotic cells. The samples were then analyzed in triplicate by flow cytometry for caspase quantitation and the colorimetric assay confirmed in parallel the activation of single caspases.

To assess the apoptotic potential of each statin, we carried out several inhibition experiments. Cell lines were incubated for 24 h with statins in the presence of LEHD–FMK and LETD–FMK (5 μM, Alexis Corporation, San Diego, CA) and their apoptosis measured by PI staining of nuclei. In addition, the intracellular content of active caspase-3 was investigated by incubating 0.5 × 10⁵ cells with the PE-conjugated anti-human active caspase-3 rabbit antibody (Pharmingen, San Diego, CA) and subsequent analysis in the FACScan.

### Mitochondrial apoptotic pathway

The mitochondrial pathway of apoptosis was evaluated with the Mitocapture™ mitochondrial apoptosis detection kit (MBL, Nagoya, Japan). This fluorescence-based method reveals the changes in the membrane potential of mitochondria. Mitocapture is a cationic dye showing different fluorescence in healthy and apoptotic cells. Healthy cells show accumulation of Mitocapture in the mitochondria, producing a bright red fluorescence, whereas in the apoptotic cells the dye remains in the cytoplasm in its monomeric form, leading to green fluorescence. Therefore, cells treated with statins were incubated with Mitocapture for 20 min at 37°C in a 5% CO₂ incubator and subsequently analyzed by flow cytometry using FL1 and FL2 fluorescence channels. Each cell preparation was measured in triplicate.

The expression of cytosolic Smac/DIABLO, a second mitochondria-derived activator of caspases enveloped in the cytochrome C/Apaf-1/caspase-9 apoptotic pathway (33), was also analyzed. The anti-human Smac/DIABLO mAb (Alexis Corporation) was added to each cell line and, after 30 min incubation at 4°C, the activator was revealed by a second FITC-conjugated antibody. The cell preparations were analyzed under a fluorescence microscopy to detect the cellular localization of Smac/DIABLO.

### Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney U-test to compare groups of data and the results were expressed as mean values ± SD from three to five independent experiments.

### Results

#### Statins inhibit cell proliferation

Figure 1 shows the suppressive effect on both B and myeloma (upper section) and T (lower section) cell lines after incubation with each statin at 20 μM for 24 h. The proliferation rate was inhibited by all statins, except pravastatin. We observed a significant (P < 0.01) reduction of [3H]thymidine uptake in all lines with the exception of CEM. MCC-2 cells showed the highest sensitivity to statins, since the basal c.p.m. values dropped to <50% with lovastatin and ~25% with cerivastatin. The cerivastatin induced a greater effect in responsive cell lines (63.7 ± 10.7%), whereas a lower sensitivity was detected with lovastatin (40.2 ± 6.8%), atorvastatin (33.2 ± 8.9%) and simvastatin (24.0 ± 6.5%). By contrast, pravastatin showed no effect on any cell line.

We also measured the dose-dependent effect of this suppression, which is expressed as percent value in Figure 2. All statins induced a parallel increment of their effect at increasing concentrations (0.1–100 μM). However, cerivastatin showed a greater anti-proliferative effect, since at 1 μM it induced ~50% inhibition at 24 h of incubation (compared with the median value of 15% for the other statins) and reached its maximum effect at 10 μM whereas the other statins attained the same at ~100 μM. However, its inhibitory effect on cell proliferation was also significant at lower doses up to 50 nM (P < 0.02) after 36 h of incubation (Figure 3A) whereas at 1 nM which is its approximate concentration in peripheral blood of treated patients, the anti-proliferative effect was not significant (P > 0.5) though present. Finally, the combination of cerivastatin at suboptimal dose (10 nM) with doxorubicin after overnight
downregulated to ~80% the proliferative extent of MCC-2 cells, thus suggesting a possible synergic effect with doxorubicin (Figure 3B).

Evaluation of the time-dependence of cellular suppression by statins demonstrated that cerivastatin at 20 μM induced an extent of suppression ≥50% after 8 h of incubation, whereas the effect of the other statins was evident only after 12 h (data not shown). Finally, the inhibition of the proliferative rate paralleled the decrease of Ki67 expression, whereas Bcl-2 was unaffected (data not shown).

The MTT was also adopted to investigate the effect of statins on mitochondrial functions (34). As shown in Table I, the decreased OD reflected the reduction of enzymatic activity thus confirming the anti-proliferative effect of statins and suggesting that they are able to derange the mitochondrial function. However, the addition of MA, GGPP and FPP to cerivastatin-treated cultures resulted in the recovery of both viability and proliferation: complete recovery was obtained with 50 μM MA and 10 μM GGPP and 10 μM FPP (Figure 4). No effect was obtained by adding squalene to the statin-treated cells indicating that the reconstitution of cholesterol did not inhibit apoptosis. These results showed that statins interfere with the cellular functions which block the isoprenylation of several proteins, whereas the addition of intermediates of this pathway restores the viability.

Fig. 1. Effect of statins on cell proliferation. Cell lines were variably suppressed in their proliferative extent after incubation with 20 μM statins for 24 h, as indicated by [3H]thymidine uptake according to the statin used. All statins (except pravastatin) decreased their proliferation rate, as shown by a significant reduction of c.p.m. MCC-2 myeloma cells showed high sensitivity to the suppressive effect, whereas CEM leukemic cells were apparently refractory. However, cerivastatin induced the highest inhibition. The results are expressed in c.p.m. as mean ± SD from three experiments.

Fig. 2. Dose-dependent suppression of lymphoid cells by statins. Increasing concentrations of each statin (0.1–100 μM) induced a progressive inhibition of cell proliferation detected by decrease of [3H]thymidine uptake after 24 h of incubation. A general inhibition of ~50% was induced by 20 μM of each statin. However, a higher effect was displayed by cerivastatin, which significantly suppressed (P < 0.01) proliferation at 1 μM in all lines except CEM, in keeping with its greater inhibitory effect.

Statins promote apoptosis

Next, we measured apoptosis in statin-treated cells to see whether defective proliferation was related to the induction of apoptosis or to cell cycle blockade in G0/G1 phase. Figure 5 shows the results obtained with the three methods employed to measure the statin-induced apoptosis. PI staining revealed a considerable apoptotic effect on MCC2, IM9, U266 and Jurkat cells. These cell preparations displayed a greater effect with
cerivastatin at 20 μM, whereas pravastatin was inert. In particular, an average of 70.5 ± 12.9% cells showed an increased subdiploid content of DNA after treatment with cerivastatin, whereas both lovastatin and atorvastatin induced 51.7 ± 10.8% of apoptosis. Finally, apoptosis in the presence of simvastatin was ~33.2 ± 7.9%. Both annexin-V-FITC and TUNEL analyses confirmed the increased apoptosis in MCC-2, IM9, U266 and Jurkat cell lines, whereas CEM cells were refractory (data not shown). By contrast, MCC2 myeloma cells promptly underwent apoptosis, as shown by a mean value of 81.2 ± 10.8% of TUNEL+ cells.

Table I. Results of MTT test expressed as mean value of OD ± SD in three different experiments

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Basal value</th>
<th>Lovastatin</th>
<th>Simvastatin</th>
<th>Pravastatin</th>
<th>Cerivastatin</th>
<th>Atorvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>U266</td>
<td>1.7 ± 0.321</td>
<td>1.12 ± 0.13</td>
<td>1.392 ± 0.1</td>
<td>1.71 ± 0.46</td>
<td>0.672 ± 0.2</td>
<td>1.239 ± 0.1</td>
</tr>
<tr>
<td>IM9</td>
<td>1.5 ± 0.154</td>
<td>1.0 ± 0.15</td>
<td>1.24 ± 0.34</td>
<td>1.58 ± 0.39</td>
<td>0.57 ± 0.17</td>
<td>1.105 ± 0.2</td>
</tr>
<tr>
<td>MCC-2</td>
<td>1.8 ± 0.42</td>
<td>1.08 ± 0.09</td>
<td>1.368 ± 0.2</td>
<td>1.85 ± 0.36</td>
<td>0.65 ± 0.14</td>
<td>1.206 ± 0.1</td>
</tr>
<tr>
<td>JURKAT</td>
<td>1.95 ± 0.37</td>
<td>1.27 ± 0.2</td>
<td>1.58 ± 0.38</td>
<td>2.0 ± 0.475</td>
<td>0.752 ± 0.1</td>
<td>1.406 ± 0.3</td>
</tr>
<tr>
<td>CEM</td>
<td>1.77 ± 0.28</td>
<td>1.72 ± 0.4</td>
<td>1.745 ± 0.5</td>
<td>1.78 ± 0.33</td>
<td>1.735 ± 0.3</td>
<td>1.79 ± 0.29</td>
</tr>
</tbody>
</table>

The absorbance of soluble colored formazan produced by mitochondria was measured at 490 nm after 24 h incubation with different statins at 20 μM. The clear-cut decrease of OD obtained with cerivastatin, lovastatin, atorvastatin and simvastatin corroborated their anti-proliferative effect and pointed to alteration of mitochondrial functions.

**Fig. 3.** Effect of cerivastatin at lower doses. (A) MTT assay in presence of cerivastatin showed 70% of inhibition of proliferation using decreasing amounts of the statins up to 50 nM after 36 h of incubation, whereas 10 nM induced an inhibition of 32%. (B) Cerivastatin at 10 nM in combination with doxorubicin induced in MCC-2 resistant cells 62% of proliferation inhibition after overnight incubation thus suggesting the occurrence of a synergic effect restoring the cell sensitivity to the doxorubicin.

Cerivastatin promotes both Rho and Ras delocalization from membrane to cytosol

To demonstrate whether or not statins inhibit the functions of both Ras or Rho, by suppressing their isoprenylation, we measured by standard western-blot assay the presence of both small GTPases in the membrane lysates of MCC-2 cells incubated for 12 h with or without cerivastatin. The membrane localization of both Rho and Ras proteins was significantly decreased in cerivastatin-treated cells compared with untreated cells (Figure 6), thus suggesting that both Ras and Rho pathways were inhibited by statins in our experimental model. In contrast, actin, a membrane-associated structural protein which is not isoprenylated was well detected in the membrane cell lysates and its levels remained intact after the cerivastatin treatment.

The apoptotic pathway is mediated by caspases

We assessed both colorimetric and cytofluorimetric methods to test the activation of caspases 8, 9 and 3. Caspase 8 initiates death receptor-induced apoptosis whereas caspase 9 acts through mitochondrial damage and caspase 3 is the downstream effector caspase. We found a general activation of caspases which paralleled the biological activity of statins in those cell lines. Cerivastatin induced the maximum activation (up to a 4-fold increase) (Figure 7). Cytofluorimetry of the apoptotic PI+ cell populations confirmed the results obtained with colorimetric assay. In fact, 90.7 ± 5.8% of apoptotic cells were labeled by FAM-conjugated caspase-9 inhibitor and 87.3 ± 4.8% by the caspase-8 inhibitor indicating, that
both caspases are involved in statin-induced apoptosis. Figure 8 shows representative plots of active caspase accumulation in cerivastatin-treated MCC-2 cells. A total of 92.5% of apoptotic cells were colored by caspase-9 inhibitor and 91.8% by caspase-8 inhibitor. Finally, the activation of effector caspase 3 was revealed by a specific polyclonal antibody in >60% of cells and confirmed its involvement in statin-induced apoptosis.

To verify whether both caspases 8 and 9 activated the apoptotic pathway in the presence of statins, cerivastatin MCC-2-treated cells were incubated with specific inhibitors for 24 h and their apoptosis was then measured. The caspase-9 inhibitor LEHD–FMK dramatically reduced apoptosis from 68.4 to 15.4%, whereas the caspase-8 inhibitor LETD–FMK had virtually no effect (Figure 9). These results suggest that caspase 9...
is the potential initiator, whereas caspase-8 is subsequently transformed by caspase 3 into its active form.

Mitochondrial damage is primed by statins
We also used Mitocapture™ to determine whether statins induce a decrease in the mitochondrial membrane potential and lead to functional perturbation. Figure 10 (upper panel) shows the effect of cerivastatin on the potential of MCC-2 and Jurkat cell lines, and had no effect on CEM cells. Mitochondria were functional in ~90% of MCC-2 cells cultured in complete medium. However, this value was dramatically reduced by 10 μM cerivastatin for 24 h (26.0% of FL2 positive cells) and the specific dye remained in its monomeric form (green fluorescence in 65.6% of cells). There was a similar disruption (40.2%) of the potential on Jurkat cells. As expected, no effect was exerted on CEM cell mitochondria.

To evaluate the direct involvement of mitochondria in statin-induced apoptosis, we demonstrated the cytosolic release of Smac/DIABLO, namely a second mitochondrial activator of caspases released into the cytosol from mitochondria along with the cytochrome c in the intrinsic apoptosis pathway. We evaluated the cellular localization of human Smac/DIABLO in both statin-treated and untreated cells and observed a localized perinuclear (mitochondrial) fluorescence in viable cells, whereas a uniform cytoplasmic fluorescence was detected in apoptotic cells incubated with statins. Figure 10 (lower panel) shows a representative pattern of Smac/DIABLO expression in both untreated (Figure 10A) and cerivastatin-treated MCC-2 cells (Figure 10B).

Discussion
In addition to lowering the lipid levels, statins exert pleiotropic effects which include immunomodulatory, anti-inflammatory, anti-angiogenic and anti-proliferative functions. A number of cell types and tissues are sensitive to statins because they inhibit the synthesis of squalene and cholesterol and reduce the isoprenyl metabolites such as mevalonate, FPP and GGPP needed to activate the cell proteins. These include Ras, Rho and other small GTPases normally involved in the cell cycle and proliferation pathways. In this study, we demonstrated that several natural and synthetic statins exert an apoptotic effect on human lymphoblastoid and myeloma tumor cells. This phenomenon is directly related to HMG-CoA reductase inhibition that blocks the synthesis of isoprenylated small GTPases since cell viability is completely rescued by isoprenoid compounds (FPP, GGPP and MA), but not by squalene, an intermediate of cholesterol synthesis. Moreover, the apoptotic pathway engaged by statins is mediated by mitochondria activation, rather than by the death receptors, and both caspases 3 and 9 appear to be directly involved and activated within the intrinsic pathway of apoptosis. Based on the evidence that isoprenoid intermediates rescued the cell proliferation completely, we can speculate that the defect of isoprenylation of small GTP-binding proteins is essential in regulating both apoptosis and proliferation pathways including
PI-3K, serine-threonine kinases, NF-kB, SAPK-JNK and Ras-MAPK (35). The altered signals result in the collapse of the mitochondrial transmembrane potential and subsequent apoptosis initiation. Recently, van de Donk (36) demonstrated that the inhibition of geranyl-geranylation in myeloma cells was associated with reduced Mcl-1 mitochondrial protein expression which in turn primes the apoptotic cascade, but whether one or more of the mentioned GTP-binding proteins are enrolled in the apoptotic pathway is under investigation.

In agreement with previous studies (21,22), we found a remarkable suppression of proliferation by cerivastatin at variable doses. This lipophilic statin induced at 1 μM a proliferative suppression as high as 50% of the baseline value after 24 h of incubation. In addition, we tested the cerivastatin at lower doses and its effect was evident up to 10 nM after 36 h of incubation thus confirming the ability of this statin to induce apoptosis even at levels comparable with those obtained in vivo in treated patients (37,38). However, since the effect is dose- and time-dependent, lower concentrations of cerivastatin used for a longer time would also be effective as reported by other authors. Although several studies describe lovastatin as a promising drug in cancer therapy (19,20,23,26,27,29), in our experiments its apoptogenic concentration was as high as 20–100 μM and these doses in vivo induce a consistent drug-related toxicity. Since this effect is time- and dose-dependent, we can speculate that lower doses of these drugs may also be functional in longer incubations. In all proliferation and apoptosis experiments, pravastatin had no effect on the viability of each cell line even at higher concentrations (data not shown) thus suggesting that such an inert effect is probably dependent on its hydrophilicity, as reported by others (35). In fact, pravastatin is the only statin which is hydrophilic enough to cross the cell membranes of nearly all tissues and only liver cells exert active transport mechanisms for the uptake of this drug (39,40). However, the different efficacy of each statin was directly related to their relative affinity for HMG-CoA reductase, as reported (41).

The treatment with statins affected the T and B lymphoblasts and the myeloma cells. Interestingly, MCC-2, a plasma cell line refractory to doxorubicin, obtained from a patient with aggressive myeloma unresponsive to chemotherapy (28), was particularly sensitive to these drugs. Moreover, suboptimal doses of cerivastatin used in combination with doxorubicin restored the MCC-2 cell sensitivity to this anti-neoplastic drug thus suggesting a synergic effect of the statin. In contrast, CEM cells showed no susceptibility. Their incubation with higher doses of each statin for 24 h had no effect on cell survival and proliferation. Previous studies had demonstrated a similar response in tumor cells from patients with acute myeloid leukemia, which remained apoptosis-resistant to the highest concentration of lovastatin or cerivastatin (22).

The main effect of statins is to deplete the cells of mevalonic acid and thereby prevent the synthesis of downstream products such as cholesterol, heme A and dolichol. In addition, mevalonic acid acts as a precursor to lipid moieties covalently attached to isoprenylated proteins as small GTPases. In our hands, statins and particularly cerivastatin, was able to inhibit both Ras and Rho prenylation and prevent their localization within the inner plasma membrane. This effect also down-regulates a number of cellular functions essential for normal

---

Fig. 10. Upper panel: Mitocapture™ staining of statin-treated cells. The cationic dye Mitocapture was used to test the reduction of mitochondrial membrane potential. In viable cells it aggregates in mitochondria and produces a red fluorescence (FL2), whereas in cells with altered membranes it remains in the cytoplasm (green fluorescence; FL1) in monomeric form. R1 region represents the cell population with disrupted membrane potential. In MCC-2 and Jurkat cells treated with cerivastatin the dye was present as green fluorescence in 65.6 and 40.2% of cells, respectively. No effect was induced by cerivastatin on CEM cell mitochondria. Lower panel: Cellular localization of Smac/DIABLO. Smac/DIABLO, a second mitochondria-derived activator of caspase, was detectable as a localized perinuclear (mitochondrial) fluorescence in control cells (A), whereas it occurred in cerivastatin-treated MCC-2 cells as diffuse cytoplasmic fluorescence (B). (Magnification 100×.)
cell homeostasis such as proliferation and viability. In fact, the membrane translocation allows Ras to interact with factors facilitating the binding to GTP. Ras-GTP stimulates downstream signaling molecules, including phosphoinoside-3 (PI3) kinase directly implicated in cell survival and MAPK involved in cell proliferation and activates NFκB signal (37). In this context, we completed preliminary experiments investigating the activity of NFκB, constitutively activated in both MCC-2 and Jurkat cells, after treatment with statins and found a relative decrease in its activation (data not shown). However, further studies are required to assess the inhibitory potential of statins on NFκB as well as MAPK and STAT3.

Moreover, constitutive or mutational activation of Ras signaling is common in some tumors (42,43). In particular, the activating mutations Ras occur in ~40% of newly diagnosed MM patients and in 64–70% of patients with progressing disease (44). The presence of Ras mutations at diagnosis is also associated with a poor response to chemotherapy and a short survival in these patients (23). Since the farnesylation of Ras by farnesyltransferase (Ftase) is a critical step for Ras functional activity, the inhibitors of Ftase have been proposed as potential anti-cancer agents to specifically inhibit oncogenic Ras signaling and Ras-dependent cell transformation by inducing apoptosis of drug-resistant IL-6-producing myeloma cells (45). Similar to Ras, membrane Rho GTPases act as regulated GDP/GTP switches by several extracellular stimuli that activate G protein-coupled receptors, receptor tyrosine kinases, integrins and other cell surface receptors. Once activated, each Rho GTPase interacts with a wide spectrum of functionally different downstream effectors to initiate cytoplasmic signaling pathways and to regulate cell cycle progression (42). In addition, Rho GTPases may contribute to Ras regulation of cell cycle, as suggested by Pruitt and Der. (42).

Finally, we found that both FPP and GGPP restored proliferation, confirming that farnesylated and geranyl-geranylated molecules are affected by statins such as Ras and Rho GTPases, as described by other authors (36). Different mechanisms can be suspected to explain the unresponsiveness in CEM cells. First, the deregulation of HMG-CoA reductase may be suspected, since naive cells drastically increase its amount to overcome acute depletion of endogenously synthesized sterol and non-sterol compounds in response to treatment with statins (24,46). In fact, in agreement with other authors (47), we found the upregulation of mRNA of HMG-CoA reductase in CEM cells (data not shown). Second, the constitutive activation of Ras/mitogen-activated protein kinase (MAPK) signaling in CEM proliferation can be also postulated (43).

Here, we demonstrated that statins induce apoptosis by directly involving the mitochondrial pathway. To support this observation, we documented in cerivastatin-treated cells the reduction of mitochondrial membrane potential as well as the cytosolic translocation of Smac/DIABLO (33) and the activation of both caspases 9 and 3. A number of apoptotic stimuli, including anti-cancer agents, induce the intrinsic pathway of apoptosis by triggering the loss of mitochondrial membrane integrity, resulting in the release from the mitochondria of multiple death-promoting molecules, including cytochrome c, apoptosis-inducing factor, endonuclease G, and Smac/DIABLO. Cytochrome c and ATP bind Apaf-1 in cytosol, allowing the recruitment of pro-caspases 9 and 3 into an apoptosisome, which in turn activates both caspases and leads to apoptosis.

In statin-induced apoptosis we observed the cytosolic translocation of Smac/DIABLO, which is normally assembled within the mitochondrial membrane. This protein is released by mitochondria during apoptosis and directly interacts with IAP proteins by blocking their inhibitory effects on activation of both caspases 9 and 3 (33). In our experiments, the inhibitor of caspase 9 blocked apoptosis. This suggests that active caspase 9 primes caspase 3 activation, whereas the final caspase 8 cleavage is presumably devoted to amplifying the death signals. These results are in agreement with other reports, showing that caspase 8 acts in the mitochondrial pathway as an amplifier of executioner caspases (48,49).

It has been demonstrated that statins in hypercholesterolemic patients reduce the recurrence of tumors by providing an oncoprotective effect (24) and a number of clinical trials using statins are in progress in advanced solid tumors (50) and in patients with acute myeloid leukemia (29,51). Most importantly, some authors demonstrated that statins can trigger apoptosis in a tumor-specific manner (24). In fact, primary myeloid, B leukemic and myeloma cells undergo apoptosis with statins, whereas their normal counterpart is partially or completely resistant to statin effects (9,22,23). Based on our data demonstrating the suppression of T, B lymphoblastoid and myeloma cells, we postulate that statins, in addition to the prevention and treatment of coronary heart disease, could be useful in combination with traditional chemotherapeutic agents such as doxorubicin since, based on our observation, they would sensitize resistant tumor cells to apoptosis.

Acknowledgements

This work was supported by FIRB 2001 and PRIN 2003 grants from the Ministry for Education, the Universities and Research (MIUR), Rome, and by an AIRC (Associazione Italiana per la Ricerca sul Cancro) research grant, Milan, Italy.

References

Statin activate apoptosis in lymphoblasts and myeloma cells