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Cytomorphological Alterations Induced by Simvastatin in Graffi Tumor Cells

Ani Georgieva[1], Reneta Toshkova[1], Veselina Uzunova[2], Rumiana Tzoneva[2]*

[1] *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[2] *Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria*

***** Corresponding author e-mail: georgieva_any@abv.bg

Statins are a group of lipid-lowering drugs widely used for treatment of hypercholesterolemia and known to reduce the risk of cardiovascular diseases. Recently, the statins have been gaining an increasing scientific interest due to their anti-inflammatory, immuno-modulatory, neuroprotective and anticancer effects. The present study aims to assess the antitumor activity of simvastatin on Graffi myeloid tumor cells. The effect of simvastatin on the viability of the tumor cells was studied by MTT assay. The alterations in the tumor cell morphology induced by the statin were analyzed by fluorescent microscopy after staining with acridine orange/ethidium bromide, DAPI and annexin V/propidium iodide. The results of the MTT test showed a significant and dose-dependent reduction of the viability of simvastatin-treated cells. Fluorescent microscopic analysis of Graffi myeloid cells exposed to simvastatin revealed morphological alterations typical for the apoptosis. These findings are in accordance with previous data indicating significant *in vitro* antitumor and proapoptotic activity of simvastatin and suggests that it is a promising drug candidate for cancer treatment.

Key words: statins, simvastatin, tumor, antitumor activity, apoptosis

Introduction

Statins are effective and safe cholesterol-lowering drugs, widely used for the treatment of dyslipidemia [1]. The statin family consists of eight members, some of which (mevastatine, lovastatin, simvastatin, pravastatin) are natural products derived from *Penicillium* and *Aspergillus* filamentous fungi and others (fluvastatin, rosuvastatin, atorvastatin, and pitavastatin) are chemically synthesized [6, 21]. The introduction of the statins into medical practice led to a significant reduction of morbidity and mortality associated with cardiovascular diseases [19, 21]. The molecular mechanism by which statins reduce cholesterol is well understood. It is known that statins decrease cholesterol concentrations by blocking the enzyme 3-hydroxy-3-methylglytaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA into mevalonate that is the rate-limiting step in the cholesterol biosynthesis pathway [8, 18].

Besides the cholesterol-lowering properties, statins exhibit many other beneficial pharmacological activities including anti-inflammatory and immuno-modulatory, neuroprotective and anticancer effects [9, 10]. The increasing interest in statins as potential anticancer agents has originated from several studies demonstrating a decreased cancer incidence in patients treated with statins [1, 10, 16, 17]. Consequently, a wide variety of experimental studies, as well as some clinical trials, has confirmed the noticeable antitumor effect of statins [3, 7, 13, 20]. Statins have been reported to exert antineoplastic activity by inhibition of tumor cell growth, induction of apoptosis, suppression of angiogenesis, repression of metastatic potential and stimulation of antitumor immunity [2, 17]. The exact molecular mechanism by which these drugs elicit their antitumor activity still remains unclear. It has been suggested that the changes in lipid metabolism initiated by the statins lead to inhibition of intracellular proliferative and survival signaling pathways and induction of apoptosis [2, 15].

The present study aims to assess the antiproliferative and proapoptotic activity of simvastatin on cell cultures derived from Graffi myeloid tumor in hamsters (GMTH). The tumor was originally induced by Graffi murine leukemia virus and adapted to grow in a solid form after subcutaneous inoculation in Syrian golden hamsters. GMTH is a transplantable, rapidly growing tumor with very aggressive behavior that never show a spontaneous regression. This experimental tumor and the cell cultures derived from it are reliable model systems for studying the antitumor effects of natural and synthetic chemical substances, showing precise and reproducible results [22].

Materials and Methods

Simvastatin. A stock solution of simvastatin (Sigma-Alrich) in DMSO was prepared and stored at 4ºC. Serial dilutions of the stock solution with cell culture growth medium were made and solutions with concentrations ranging from 1 to 100 μM were used in the experiments.

Cell cultures and cultivation. Cell cultures of Graffi myeloid cells were isolated from the solid tumor tissue of Syrian golden hamsters under aseptic conditions. The cells were grown as monolayers in 25cm^2 tissue culture flasks in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cultures were maintained in an incubator at 37ºC in a humidified atmosphere with 5% CO_2.

MTT-dye reduction assay. The *in vitro* antitumor activity of simvastatin was studied on Graffi myeloid tumor cells using the standard MTT-dye reduction assay, described by Mosmann [14]. Tumor cells in a density of 1×10^5 cells/mL RPMI-1640 containing 10% FBS were plated (100 μL/well) in 96-well microplates and allowed to adhere for 24 h. The cells were then treated with simvastatin at concentrations of 0.8, 1.6, 3.2, 6.4, 12.5 25, 50 and 100 μM for 24, and 48 h. Untreated tumor cells were used as negative controls. After simvastatin treatment, the culture medium was discarded and 100 μL of MTT solution with a concentration of 0.5 mg/mL were added to each well. The plates were incubated for 3h at 37.5°C in a humidified atmosphere and 5% CO_2. Formazan crystals were dissolved by adding 100 μL/well of an absolute ethanol/DMSO (1:1 v/v) solution and the absorption was measured using a microplate reader (TECAN, Sunrise TM, Groedig/Salzburg, Austria) at 540 nm.

Acridine orange/ethidium bromide double staining. Apoptotic cell morphology was assessed using acridine orange and ethidium bromide double staining according to standard procedures [11] with a minor modification [22]. Briefly, Graffi cells cultured on 13-mm-diameter cover glasses in 24-well plates were treated with 25μM simvastatin.

After 24 h of incubation, the glass coverslips were washed with phosphate buffer saline (PBS), equal volumes of fluorescent dyes AO (10 µg/mL) and EtBr (10 µg/mL) were added and the cells were immediately examined under fluorescent microscope (Leica DM 500B, Wetzlar, Germany).

DAPI staining. The alterations in the nuclear morphology of the Graffi cells induced by simvastatin were studied by fluorescent microscopy after staining with DNA binding dye 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI). The tumor cells were seeded on glass coverslips in 24-well tissue culture plates at a concentration of 1×10^5/well, incubated overnight at 37°C and 5% CO_2 and then treated with 25µM simvastatin for 24 hours. The culture medium was poured off and the coverslips were fixed with methanol. The cells were incubated for 15 minutes in 1 µg/mL DAPI in methanol in the dark, mounted with glycerol on microscope slides and examined under a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany).

Annexin V and pripidium iodide (PI) double staining. Apoptosis-inducing ability of simvastatin was assessed using annexin V Apoptosis Detection Kit: sc-4252 AK (Santa Cruz Biotechnology, Inc. USA) according to manufacturer's instructions. Briefly, glass cover slips were placed on the bottom of each well in 24-well plates. Graffi cells were seeded on glass coverslips (1×10^5 cells/well), incubated overnight and treated with 25µM simvastatin for 24 h. The culture medium was removed, the glass coverslips were washed twice with PBS (pH 7.4) and fixed with 3% paraformaldehyde. The cells were washed with distilled water and incubated in a solution containing 1 µg/ml PI and 1 µg/ml annexin V-FITC for 10 min, in the dark, at room temperature. After staining the coverslips were mounted with glycerol on slides and analyzed using fluorescence microscope Leica DM 5000B.

Statistical analysis. Statistical analysis was performed by One-way ANOVA followed by Bonferroni's post hoc test (GraphPad Prism software package). $p<0.05$ was accepted as the lowest level of statistical significance. All results are presented as a mean ± SD.

Results and Discussion

The *in vitro* antiproliferative activity of simvastatin on the Graffi myeloid tumor cells was evaluated by MTT test after 24 and 48 hours of exposure. The results from the assay showed that the tested statin induce a concentration-dependent decrease of the tumor cell growth and viability (**Fig. 1**).

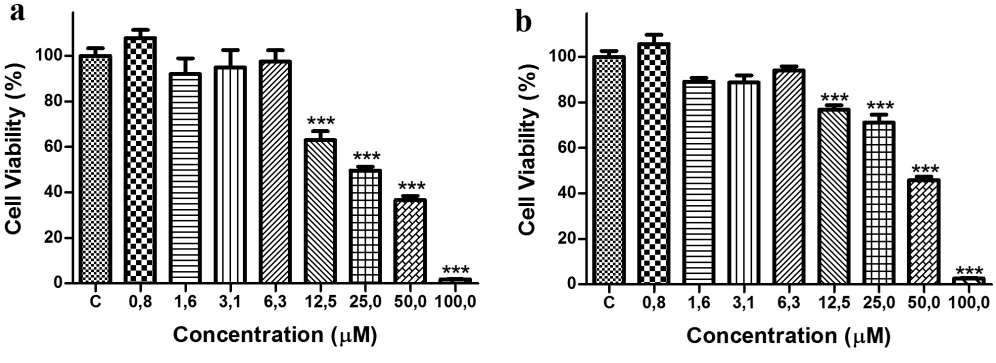

Fig. 1. Antiproliferative effect of simvastatin on Graffi myeloid tumor cells after 24 (a) and 48 (b) hours of treatment. MTT-dye reduction assay; ***$p<0.001$, compared to the untreated control.

As evident from the presented data, statistically significant reduction of the cell viability of simvastatin-treated cells ($p < 0.001$) as compared to the untreated control was established at all concentrations higher than 10 μM. The inhibition of the tumor cell growth was more clearly expressed after 24h as compared to 48h exposure. The IC_{50} values of simvastatin, determined after 24 and 48 hours of treatment were 24.67 μM and 36.90 μM, respectively.

Fluorescent microscopy of acridine orange/ethidium bromide stained cells showed that simvastatin induced cytopathic alterations such as shrinkage, rounding up and detachment of the cells and loss of monolayer integrity. In contrast to the untreated cell that were uniformly green stained (**Fig. 2a**), some of the simvastatin-treated cells showed granular yellow-green fluorescence or orange staining (**Fig. 2b**) that are characteristic for the early and late apoptosis, respectively.

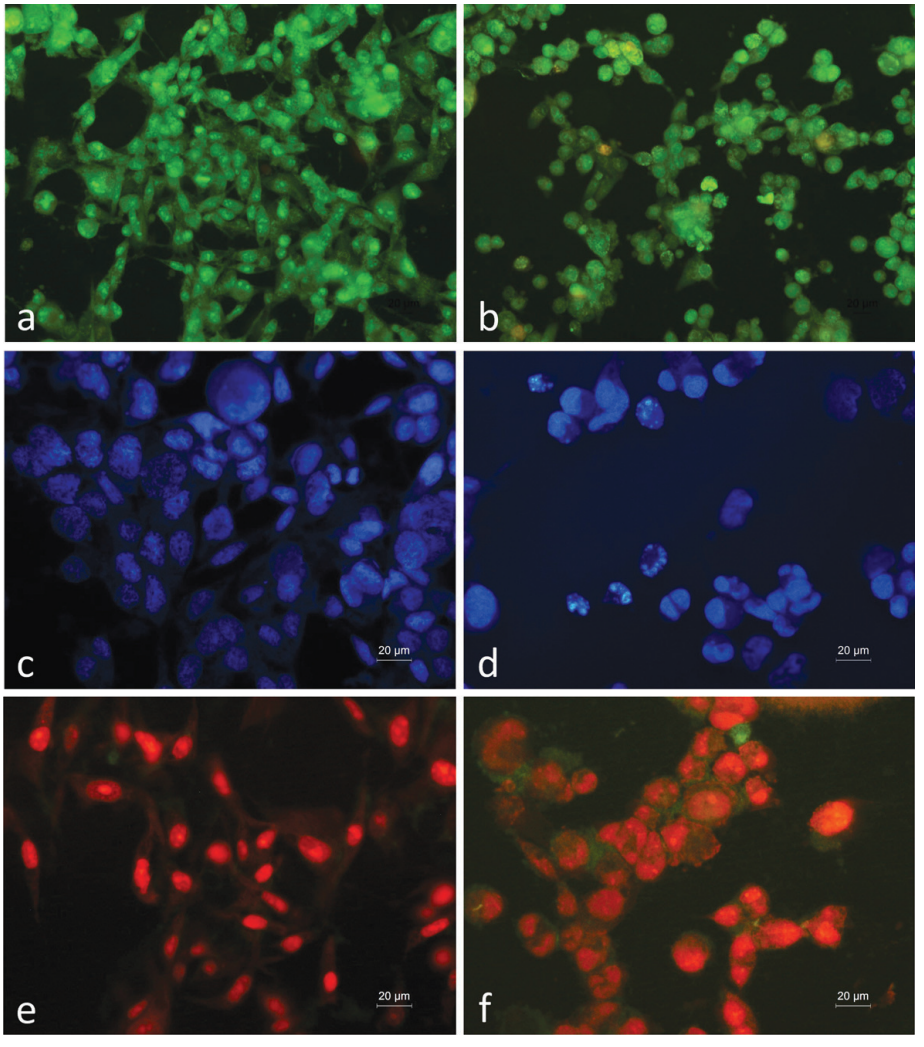

Fig. 2. Fluorescent microscopy images of the morphological alterations induced by simvastatin in Graffi myeloid tumor cells.
a-, c-, d- control cell cultures; b- ,d-, f- cell cultures exposed to 25 μM simvastatin for 24h; a-, b- Acridine orange/ethidium bromide staining**;** c-,d- DAPI staining; e-, f- Annexin V-FITC and propidium iodide staining.

Simvastatin-induced alterations in the nuclear morphology of the tumor cells were analyzed after DAPI staining. The nuclei of the control cells were homogenously stained with DAPI (**Fig. 2c**), whereas cancer cells treated with simvastatin showed an altered nuclear staining pattern with condensed chromatin, fragmented nuclei and apoptotic bodies (**Fig. 2d**) that are hallmarks of the apoptotic cell death.

The apoptosis-inducing ability of simvastatin was confirmed by Annexin V-FITC/PI staining. One of the earlier events of apoptosis includes translocation of membrane phosphatidylserine from the inner side of the plasma membrane to the surface. The green fluorescence observed in simvastatin-treated cells (**Fig. 2f**), but not in controls (**Fig. 2e**) indicates the binding of FITC-labeled Annexin V to the exposed phosphatidylserine and is an evidence for apoptosis.

A number of reports have shown that simvastanin inhibits proliferation and induces apoptosis in various cancer cell types including leukemic cells [23], breast [13], cervical [5], prostate [13], colon [4, 13], liver [13], pancreatic [13], renal [7], lung [12, 13] and skin [13] cancer cells. Our results complement and confirm the previous findings and indicate that this statin exhibits pronounced antineoplastic effect against myeloid tumor cells.

Conclusion

Simvastatin induced a significant and dose-dependent reduction of cell viability of Graffi myeloid tumor cells. Apoptotic cell death is one of the main mechanisms of the antineoplastic activity of simvastatin. Presented results indicate that simvastatin is a promising drug candidate for treatment of hematologic malignances.

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