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Short communication

Evaluation of apoptosis and necrosis induced by statins using fluorescence-enhanced flow cytometry

Noriko Yasuda, Sumio Matzno, Chihiro Iwano, Mayumi Nishikata, Kenji Matsuyama *

School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien, Kyuban-cho, Nishinomiya, Hyogo 663-8179, Japan

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Abstract

The purpose of this study was to evaluate the apoptosis and necrosis induced by five kinds of statins in IM-9 human lymphoblasts with fluorescence-enhanced flow cytometry using avidin–biotin complex. IM-9 human lymphoblasts (2×10^4 cells/cm^2) were seeded into tissue culture plates and incubated with five kinds of statins. Statin-treated cells were first incubated with biotin-annexin V, followed by addition of avidin-FITC and propidium iodide, and then subjected to flow cytometry. The fluorescence intensity was enhanced using an avidin–biotin complex system, resulting in successful separate determination of the statin-induced apoptosis and necrosis by flow cytometry, which enabled us to quantitatively evaluate the statin-induced cell damage. Flow cytometric analysis results in the intensity of statin-induced apoptosis in IM-9 cells as follows: atorvastatin ≒ cerivastatin > fluvastatin ≒ simvastatin > pravastatin. The intensity of statin-induced necrosis in IM-9 cells was expressed as follows: atorvastatin ≒ cerivastatin > fluvastatin ≒ simvastatin > pravastatin. The total damage of IM-9 cells induced by five kinds of statins were expressed as the sum of both percentages of apoptosis and necrosis as follows: atorvastatin ≒ cerivastatin > fluvastatin ≒ simvastatin > pravastatin. Our studies show that fluorescence enhancement with avidin–biotin complex is useful for the identification and quantitation of annexin-positive apoptosis cells and thus, the fluorescence-enhanced flow cytometry was shown to be applicable for screening of statins as new anti-leukemia agents.

Keywords: Apoptosis; Necrosis; Flow cytometry; Statins; Lymphoblasts

1. Introduction

3-Hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are a class of drugs that inhibits the rate-limiting step of the mevalonate pathway [1], which is essential for the synthesis of various compounds, including cholesterol. Statins, i.e., cerivastatin, atorvastatin, fluvastatin, simvastatin, and pravastatin have been widely used for patients with type II hyperlipoproteinemia. Statins have also proven effective for prevention of cardiovascular disease, and shown to be efficacious in reducing cardiovascular morbidity and mortality in primary and secondary prevention clinical trials [2–4].

However, the risk of myopathy, a prominent and severe adverse effect, has been reported for statins. Clinical signs include diffuse myalgia, muscle tenderness and elevation of blood creatinine phosphokinase concentrations [5]. Also, combination therapy with cerivastatin and gemfibrozil can cause rhabdomyolysis [6–8]. On August 8, 2001, Bayer Pharmaceutical Division voluntarily withdrew cerivastatin from the US market because of reports of fatal rhabdomyolysis [9].

Our previous in vivo studies using urethane-anaesthetized rats [10] revealed the extent of rhabdomyolysis induced by various statins, and in vitro studies using L6 rat skeletal myoblasts [11] showed that cellular apoptosis could be determined using phase-contrast and fluorescein microscopic observation with Hoechst 33342 staining. Skeletal cytotoxicity was ranked as cerivastatin > fluvastatin > simvastatin > atorvastatin > pravastatin. However, the detailed mechanism of myopathy and drug interaction between

* Corresponding author. Tel.: +81 798 45 9954; fax: +81 798 41 5150.
E-mail address: matsuya@mwu.mukogawa-u.ac.jp (K. Matsuyama).

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statins and fibrates remains to be clarified. Regarding acute myeloid leukemic (AML) cell blasts, several studies [12–14] have shown apoptosis induced by statins, i.e., lovastatin. Soehnlein et al. [15] also reported that atorvastatin significantly enhanced apoptosis of human endothelial cells. However, statin-induced apoptosis in acute lymphocytic leukemic (ALL) cell blasts has not been reported in vitro. In this study, we chose IM-9 human lymphoblasts (IM-9) to evaluate statin-induced cell damage using flow cytometric analysis. Since IM-9 is a floating cell, it is better suited to flow cytometric analysis than adhesive cells, such as L6 rat skeletal myoblasts. Here, we tried to quantitatively evaluate statin-induced cell damage using flow cytometric analysis with the aim of developing a new therapy of leukemia using statins. The possibility of using statin against leukemia is also discussed.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate-conjugated annexin V (FITC-annexin V) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). Statins, i.e., atorvastatin, cerivastatin, fluvastatin, simvastatin, pravastatin were kindly supplied by Sankyo Res. Labs (Tokyo, Japan). Biotin-conjugated annexin V (biotin-annexin V) was obtained from Caltag Laboratories (Burlingame, CA). Avidin-conjugated FITC (avidin-FITC) was from EY laboratories (San Mateo, CA). Fetal bovine serum (FBS) and RPMI 1640 were purchased from ICN Biochemicals Inc. (Costa Mesa, CA). Fibronectin (human) was from Asahi Techno Glass Corporation (Tokyo, Japan). All other materials were from Nacalai Tesque (Kyoto, Japan).

2.2. Cell culture

IM-9 human lymphoblast cells were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). IM-9 cells were grown in RPMI 1640 medium with 10% FBS, 0.2% bicarbonate, 50 IU/ml penicillin G and 50 μg/ml streptomycin, and IM-9 cell of less than 20 passages, was used in the experiment.

L6 rat skeletal myoblasts that subcloned by limiting dilution in our laboratory [11] were routinely maintained in the growth medium (alpha-medium essential medium (α-MEM) containing 50 IU/ml penicillin G, 50 μg/ml streptomycin and 10% FBS) and less than 20 passages were used in the experiments.

2.3. Microscopic observation

IM-9 cells (2.6×10^5 cells/cm^2) were grown on cover slips coated with fibronectin (10 μg/ml) at 37 °C for 2 h. Cells were incubated with statins for 16 h, and fixed with 2% glutaraldehyde for 5 min. The glass was then washed twice with PBS and stained by the following procedures. In procedure A, cells on the glass were incubated with 10 μl of reaction buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.5) containing 10 μg/ml FITC-annexin V and 10 μg/ml PI for 10 min under dark condition. In procedure B, to enhance the fluorescence intensity, cells on the glass were incubated in 10 μl of binding buffer (pH 7.5) composed of 140 mM NaCl, 2.5 mM $CaCl_2$, and 10 mM HEPES, and then 1 μl of biotin-annexin V was added. The mixture was incubated for 10 min in the dark. After washing with phosphate buffered saline (PBS), the cells on the glass were soaked in 10 μl of binding buffer. Next, 1 μl of avidin-FITC and 1 μl of PI were added, and the mixture was incubated for 10 min in the dark. Cells were observed using a fluorescent microscope with a blue filter at an excitation wavelength of 490 nm and an emission one of 530 nm.

L6 myoblasts were seeded on the cover glass at concentration of 2.6×10^5 cells/cm^2 and cultured at 37 °C, 5% CO_2 in air. Twenty-four hours after incubation, mononuclear cells were washed twice with Hanks' balanced salt solution and the medium was replaced with differentiation medium (α-MEM supplemented with 0.1% bovin serum albumin, 10 μM insulin, 5 μM transferring and 10 nM sodium selenite). Clofibrate (100 μM) was simultaneously added into differentiation medium. Two hours after differentiation with drug, cells were fixed with 1% glutaraldehyde for 10 min, stained with above procedure A and B, and then observed using fluorescein microscope.

2.4. Flow cytometric analysis

Cells (2.0×10^4 cells/cm^2) were incubated with each statin for 16 h. After washing with PBS, cells were suspended in 10 μl of binding buffer (pH 7.5), and 1 μl of biotin-annexin V was added. The mixture was then incubated for 10 min in the dark. After washing with PBS, the cells were resuspended in 10 μl of binding buffer (pH 7.5). Next, 1 μl of avidin-FITC and 1 μl of PI were added, and the mixture was incubated for 10 min in the dark. The samples suspended in 500 μl of PBS were subjected to flow cytometric assay. Flow cytometric profiles were determined with a FACSCalibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA) with an argon laser at an excitation wavelength of 488 nm.

2.5. Data analysis

Fluorescent microscope images in Fig. 1 were splitted into RGB channels using GIMP 1.2 software (http://www.gimp.org/) and then relative intensity of green fluorescence was analyzed with Scion Imae 1.62 software (Scion Corporation, http://www.scioncorp.com/). Data were expressed as mean ± S.D. Statistical significance was assessed with Student's *T*-test (in Fig. 1) and Dunnett's multiple comparison method (Table 2) adjusted with randomized block design. A *p*-value of lower than 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Enhancement of fluorescence intensity

On initiation of apoptosis, most mammalian cell types translocate phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface [16]. We used fluorescent-conjugated annexin V that bound translocate PS from the inner face of the plasma membrane to the cell surface after initiating apoptosis [16,17]. According to their method, IM-9 cells treated with 30 μM of atorvastatin were stained with FITC-annexin V and PI. As shown in Fig. 1 (panel A), photograph of IM-9 lymphoblasts was indistinct using a fluorescein microscope. We tried a method using biotin-annexin V, avidin-FITC and PI (panel B). The method successfully enhanced fluorescence intensity in IM-9 by three-fold from 34,000 ± 13,000 in panel A to 105,000 ± 36,000 in panel B ($p < 0.05$), which enabled us to divide the early stage apoptotic cells from those of late stage, induced by the same concentration of atorvastatin. Similarly the fluorescence enhancement was also two-fold from 63,000 ± 29,000 in panel C to 128,000 ± 48,000 in panel D ($p < 0.05$) on the detection of apoptotic L6 rat skeletal myoblasts. Both observations suggest that the alternative method using avidin–biotin complex markedly improved the detection and identification of early apoptosis and necrotic cell populations.

3.2. Assessment of statin-induced apoptosis and necrosis as numerical values using flow cytometry

For quantitative analysis of early and late stages of apoptosis together with necrosis by flow cytometry, the intensity of the fluorescence is the most important factor. We tried to compare the current method with that of Koopman et al. [17] to quantitatively determine both apoptosis and necrosis in IM-9 cells by flow cytometry. Fig. 2 shows the result from flow cytometric analysis when cells were treated with control (A), 30 μM of cerivastatin (B) and simvastatin (C). As shown in Fig. 2, the fluorescence intensity using FITC-annexin V and PI was too weak to detect the early stage of apoptosis (lower-right) and late stage of apoptosis (upper-right).

On the other hand, Fig. 3 shows the result from flow cytometric analysis when cells were treated with 30 μM of statins (atorvastatin, cerivastatin, fluvastatin, simvastatin) or 3 mM of pravastatin for 16 h, followed by staining with

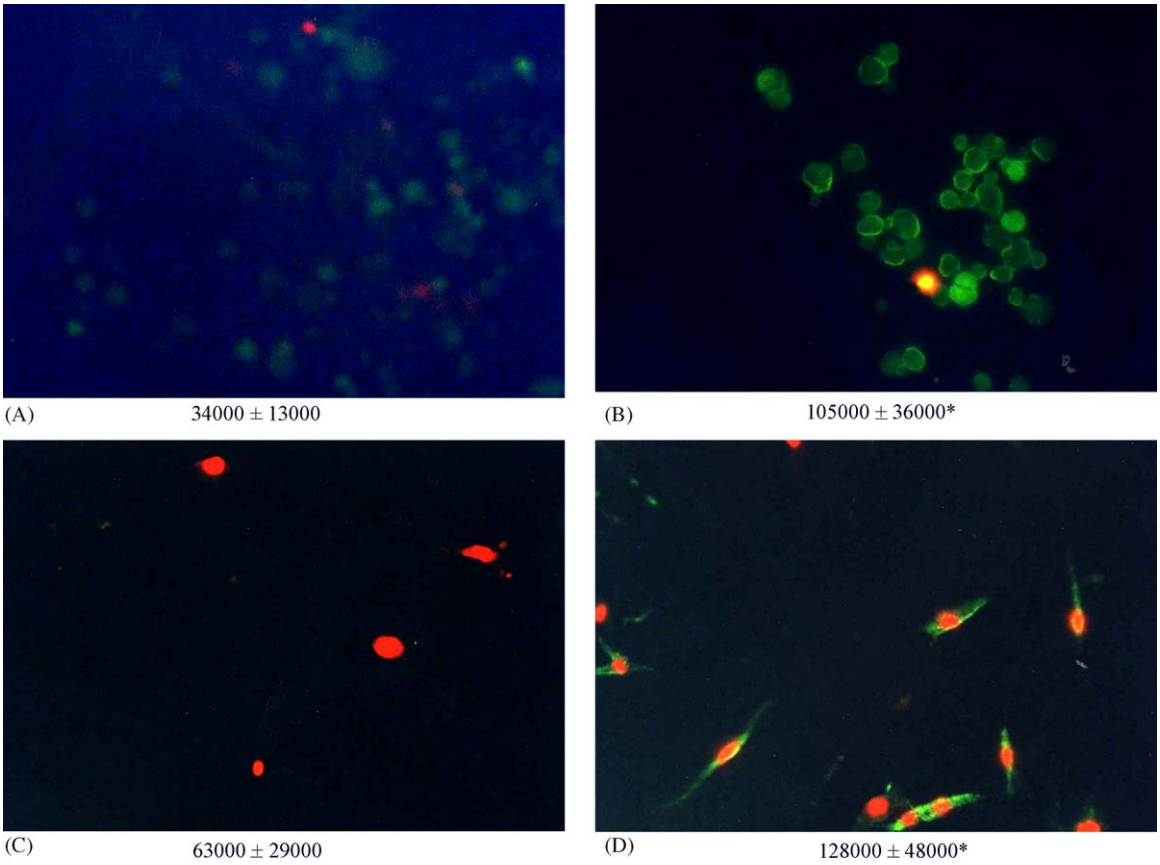

Fig. 1. Fluorescence was improved by avidin–biotin complex in IM-9 lymphoblasts from A to B and L6 myoblasts from C to D, respectively. IM-9 cells (panel A and B) were grown on the cover glasses coated with fibronectin (10 μg/ml) at 37 °C for 2 h. Cells were incubated with 30 μM of atorvastatin for 16 h. L6 myoblasts (panel C and D) were incubated with 100 μM of clofibrate for 2 h. Each cell was stained with FITC-annexin V (panel A and C) or avidin–biotin complex (panel B and D). Early apoptotic cells were shown in green color fluorescence and necrotic cells as red color fluorescence by PI. Relative intensity was shown at the bottom of each photo as mean ± S.D. *$p < 0.05$ vs. FITC-annexinV.

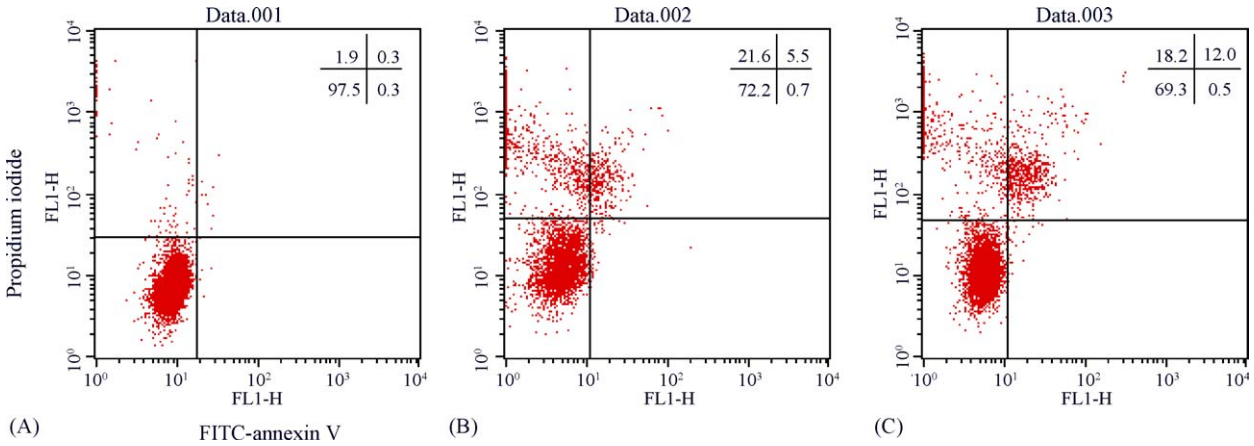

Fig. 2. Flow cytometric analysis of statin-induced apoptosis in IM-9. Cells were treated with 30 μM of statins for 16 h and stained with FITC-annexinV and PI: (A) vehicle (0.3% dimethylsulfoxide), (B) cerivastatin and (C) simvastatin. The percentages of alive cells, early stage of apoptosis, late stage of apoptosis and necrosis were expressed in the upper right quadrant.

biotin-annexin V, avidin-FITC and PI. In contrast to the former experiment, the method using biotin-annexin V, avidin-FITC and PI enhanced the fluorescence intensity, resulting in successfully dividing both the early stage of apoptosis (lower-right) and the late stage of apoptosis (upper-right) in IM-9 cells. This enabled us to quantify the cell damage using flow cytometry (Fig. 3).

Next we examined the concentration- and time-dependent of atorvastatin-induced apoptosis. Table 1A shows that atorvastatin dose-dependently induced apoptosis from 2.8%

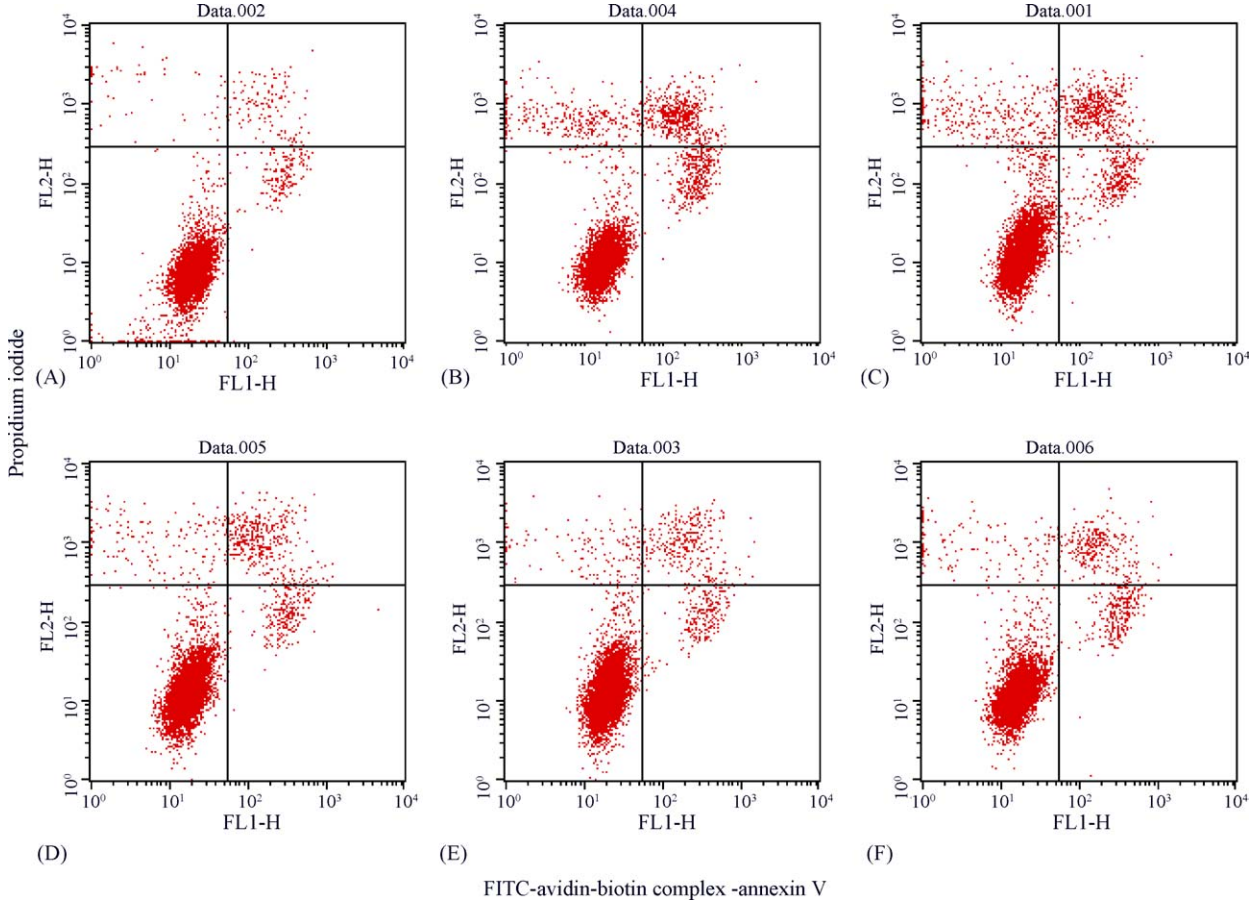

Fig. 3. Flow cytometric analysis of statin-induced apoptosis in IM-9 using avidin–biotin complex. Cells were treated with various statins for 16 h, followed by staining with biotin-annexin V, avidin-FITC and PI: (A) vehicle, (B) atorvastatin (30 μM), (C) cerivastatin (30 μM), (D) fluvastatin (30 μM), (E) simvastatin (30 μM) and (F) pravastatin (3 mM).

Table 1
Concentration (A) and time (B) dependent manner of apoptosis induced in atrorvastatin-treated IM-9 cells

Concentration (μM)	Apoptosis (%)
Part (A)[a]	
0	2.8
3	4.8
10	8.5
30	21.7
Time (h)	**Apoptosis (%)**
Part (B)[b]	
Control	1.9
8	4.6
16	21.7
24	64.8

[a] Cells were treated with atorvastatin for 16 h, followed by staining with biotin-annexin V, avidin-FITC and PI. Apoptosis ratio was expressed as the sum of both percentage of early and late stage of apoptosis.
[b] Cells were treated with 30 μM of atorvastatin for 8, 16 and 24 h, followed by staining with biotin-annexin V, avidin-FITC and PI. Apoptosis ratio was expressed as the sum of both percentage of early and late stage of apoptosis.

(control) 0 to 21.7% (30 μM). Time dependency was also examined. The apoptosis caused by 30 μM of atorvastatin was generated with the increasing time of incubation (Table 1B). Preliminary study (data not shown) indicated that "toxic" statins (i.e., cerivastatin) caused severe cell damage, recognized as necrosis, at 24 h, thus, we decided to use 16 h treated lymphocytes for the following evaluation.

Finally, under our optimal condition as mentioned above, we determined the intensity of statin-induced apoptosis in IM-9 cells as follows (Table 2): atorvastatin ≒ cerivastatin > fluvastatin ≒ simvastatin > pravastatin. The intensity of statin-induced necrosis was expressed as follows: atorvastatin ≒ cerivastatin > fluvastatin ≒ simvastatin > pravastatin. The total cell damage induced by five kinds of statins was expressed as the sum of both percentages of apoptosis and necrosis, and ranked as follows: atorvastatin ≒ cerivastatin > fluvastatin ≒ simvastatin > pravastatin (Table 1). On the other hand, our previous report (11) showed that skeletal cytotoxicity of statins in L6 cells was ranked as cerivastatin > fluvastatin > simvastatin > atorvastatin >pravastatin. As to the different result, it may be reasonable to consider the different susceptibility between IM-9 and L6.

Mevalonate is an important component of a biochemical pathway complex and its products and intermediates are vital for a variety of key cellular functions, including membrane integrity, cell signaling, protein synthesis, and cell cycle progression [1]. The regulation of mevalonate synthesis is complex, involving multiple feedback mechanisms in which the endproducts of this pathway can regulate the activity of HMG-CoA reductase, the rate-limiting enzyme of this metabolic pathway [18]. The endproducts of the mevalonate pathway include sterols, mainly cholesterol, involved in membrane structure and steroid production. However, several alternative intermediates also play an important roles in the energy metabolism and signal transductions: ubiquinone, involved in electron transport, and farnesyl and geranylgeranyl isoprenoids involved covalent binding of proteins, such as ras or rho to membranes [19]. Ras and rho small G proteins play crucial roles in the cell growth and differentiation. For their activation and signal response, they must be anchored to the plasma membrane through farnesyl and geranylgeranyl residues that covalently bind to the C-terminus of ras and rho proteins, respectively. The geranylgeranylation of the rho protein is essential for their activation since it has been reported that the activity of rho proteins requires their attachment to the inner leaflet of the plasma membrane. Moreover, rho proteins were shown to be translocated with geranylgeranylpyrophosphate, which were derived from mevalonate [20]. Our study showed that five kinds of statins gave rise to both apoptosis and necrosis in IM-9 cells. Regarding the cell damage caused by statins, Dimitroulakos et al. [12] reported that the majority of AML cell lines (6/7) and primary cell cultures (13/22) showed significant sensitivity to lovastatin-induced apoptosis, similar to the apoptotic response in human neuroblastoma cells. They also evaluated the apoptotic response of AML and ALL cells and of retinoic acid responsive and non-responsive leukemias, respectively, to lovastatin. Lishner et al. [13] clarified that simvastatin has a major antiproliferative effect on AML blasts in vitro. These reports suggest the possibility of using statins for treatment of AML. Considering these reports described above and our

Table 2
Evaluation of statins-induced apoptosis and necrosis using flow cytometry

	Concentration	Apoptosis	Necrosis	Total damage
Control		2.13 ± 0.62	2.13 ± 1.36	4.25 ± 1.28
Atorvastatin	30 μM	8.38 ± 4.88**	6.36 ± 1.43**	14.76 ± 5.59**
Cerivastatin	30 μM	6.07 ± 2.19**	7.32 ± 2.58**	13.40 ± 3.18**
Fluvastatin	30 μM	5.38 ± 2.40*	3.75 ± 1.41	9.10 ± 2.24*
Simvastatin	30 μM	5.10 ± 1.91*	3.63 ± 1.39	8.74 ± 2.69*
Pravastatin	3 mM	4.49 ± 2.95	3.21 ± 0.62	7.70 ± 2.94

IM-9 cells were incubated with various statins for 16 h, followed by staining with biotin-annexinV, avidin-FITC and PI. As control sample, cells were treated with dimethylsulfoxide (0.3%) instead of various statins solution. Apoptosis induced by a series of statins was expressed as the sum of both percentage of early and late stage of apoptosis. Total cell damage induced by statins was expressed as the sum of both percentage of apoptosis and necrosis.
Values represent mean ± S.D. ($n = 6$).
* $p < 0.05$ vs. control (Dunnett's multiple comparison).
** $p < 0.01$ vs. control (Dunnett's multiple comparison).

present study, statins is suggested to be a potent agent for the treatment of leukemia. For most potent therapy of leukemia we are now screening a series of statins, together with or without the ordinal anticancer agent, i.e., tretinoin using flow cytometry.

4. Conclusions

Fluorescein enhancement using avidin–biotin complex strongly improved the apoptotic cell detection and this can be useful for quantitative analysis of apoptosis induced by drugs. In further studies, a series of statin-derivatives are being synthesized as novel anticancer agents for the treatment of leukemia, with quantitative screening of the potency of these compounds by our method using fluorescence-enhanced flow cytometry.

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