Changes of Apolipoprotein M Gene Expression During the Cell Differentiation and Apoptosis Induced by Simvastatin in Combination with All-*Trans* Retinoic Acid in Human Promyelocytic Leukemia Cell Line NB4

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Abstract We examined the effect of simvastatin (SV) alone and in combination with all-*trans* retinoic acid (ATRA) on proliferation, differentiation, apoptosis and apolipoprotein M (apoM) expression in the human promyelocytic leukemia cell line NB4. The NB4 cells were incubated with 10 μ M Simvastatin (10SV) and 0.5 μ M ATRA alone or in combination, taking NB4 cells without any treatment as normal controls. The cells of different groups were collected at 24, 48 and 72 h post-incubation for further detection. Their morphological changes were observed after Wright stain. MTT method was used to assay the growth inhibition rate and flow cytometry to detect CD11b expression level and the early stage

apoptosis ratio. Real-time quantitative reverse transcriptase-polymerase chain reaction was used to detect the apoM gene expression levels. As expected 0.5 μ M ATRA did not affect proliferation or apoptosis, strongly induced differentiation and decreased apoM expression. 10SV inhibited proliferation, increased apoptosis, induced differentiation and increased apoM expression in a time-dependent manner. The addition of ATRA to SV did not increase the effect of SV on proliferation and apoptosis, but increased the effect of SV on differentiation. And completely abrogated the effect of SV on apoM expression. Together these results show that SV has anti-leukemic properties by itself and that combined therapy may have a place in the current anti-leukemic arsenal.

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Introduction

Simvastatin (SV) as a pharmacologic inhibitor of 3-hydroxy-3-methylglutaryl-CoA(HMG-CoA) reductase, is widely used in the treatment of hypercholesterolemia in humans. Nowadays there are evidences that statins exhibit other more activities in addition to reducing cholesterol levels. Notably, extensive work over the years has established that these agents also exhibit important antiinflammatory, proapoptotic, and antineoplastic properties [1–4], raising the possibility that they may ultimately find a place in the prevention and/or treatment of patients with inflammatory or malignant disorders. Various statins have been previously shown to exert several beneficial anti-neoplastic properties, including antiproliferative effects on tumor cells, inhibition of tumor growth, induce of cell

differentiation and apoptosis, and inhibition of angiogenesis and metastasis of malignant cells [5]. Atorvastatin and fluvastatin were found to be potent inducers of cell differentiation and apoptosis of the acute promyelocytic leukemia (APL) cell line NB4. Cytotoxic potency against HL-60 was as follows; simvastatin (SV) > atorvastatin > cerivastatin > fluvastatin. Interestingly, HL-60-R2, an all-*trans* retinoic acid (ATRA)-resistant HL-60 variant, was twice as sensitive to SV than HL-60 [6]. These studies establish that statins exhibit potent antileukemic properties in vitro and raise the possibility that combinations of simvastatins with ATRA may be an effective approach to overcome the development of ATRA resistance by the leukemic cells [7].

Apolipoprotein M (apoM) as one of the lipocalin family member, was first identified and characterized in 1999 [8]. It is mainly synthesized in the liver, and to a smaller amounts, in the kidney [8]. It has been well documented that human apoM is exclusively expressed in the hepatocytes in liver and tubular epithelial cell in kidney, and small amounts were also found in fetal liver and fetal kidney in most investigations [9]. Recently, Luo [10] found that apoM could also be expressed in human colorectal tissues besides liver and kidney using the real-time RT-PCR and immunohistochemistry technique. ApoM mRNA levels in the colorectal cancer tissues were significantly increased in the patients with lymphnode metastasis. However, there were no reports on the apoM gene expression profiles in hematopoietic tissues or hematopoietic malignancies.

Use of ATRA with other chemotherapeutic agents in the treatment of APL has been shown to cause the differentiation of abnormally granulated specific blast cells into mature granulocytes by acting on the t (15; 17) fusion gene product. The complete remission rate is increased and survival time is prolonged in APL patients who receive chemotherapy plus ATRA, whereas ATRA syndrome and other ATRA-related adverse effects including hypercholesterolemia may be observed especially during induction phase of the treatment [11]. We previously discovered obvious hyperlipidemia during the differentiation of NB4 cells induced by ATRA [12]. Theoretically, we suppose that simvastatin in combination with ATRA exhibited not only synergistic anti-leukemic effects on APLs but also abrogation of hypercholesterolemia in APLs induced by ATRA through regulating lipid synthesis and metabolism. Therefore, according to other report that simvastatin manifested the most cytotoxic potency against HL-60 cells, in the present study, we focus on the changes in the apoM gene expression levels during the cell differentiation and apoptosis induced by ATRA plus simvastatin, and then we discuss the possible anti-leukemia mechanism of simvastatin.

Materials and Methods

Reagents

Simvastatin was obtained as a sodium salt from Merck Chemical Ltd. (German) and dissolved in DMSO to obtain a 1 mM stock solution kept in -20°C and later diluted in media prior to use in culture. ATRA was purchased from Sigma Ltd. (USA), and stock concentrations of 10 mM were made in 99.5 % ethanol and kept in -20°C away from light.

Cell Culture and Treatment

The human promyelocytic leukemia cell line NB4 (kindly gifted by Jiangsu Institute of Hematology, Suzhou, China) was cultured in RPMI 1640 (Gibco Ltd, Paisley, UK) supplemented with 10 % heat-inactivated fetal calf serum (Gibco Ltd. USA), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in a humidified 5 % CO_2 -atmosphere at 37°C . Exponentially growing cells were used for all experiments. ATRA was diluted with RPMI-1640 medium such that the final concentration was 0.5 μM . Simvastatin was diluted with RPMI-1640 medium at the final concentration 10 μM for further treatment. The number of cells was determined by counting in a Burkner chamber and the final NB4 cell concentration was 2×10^5 cells/ml. NB4 cells were seeded at 2×10^5 cells/ml in 6-well plastic plates and treated with 10SV, ATRA and ATRA + 10SV respectively for a total treatment of 72 h, taking untreated NB4 cells as normal controls. Cells of different groups 24, 48, and 72 h post-incubation were collected for further detection.

MTT Assay

Cell proliferation was assessed using a methyl thiazolyl tetrazolium (MTT) assay. Briefly NB4 cells of different groups in the presence or absence of simvastatins and ATRA were seeded in 96 well flatbottomed plates (100 μl /well, Falcon) at a final concentration of 2×10^5 cells/ml for the time indicated. 24, 48 and 72 h post-incubation, NB4 cells were incubated with 5 mg/ml MTT for 4 h at 37°C and the medium was removed, cells were solubilized in dimethyl sulfoxide, and absorbance was measured at 570 nm. All samples were run in triplicate. Background absorbance was corrected by subtracting the absorbance values from the media alone (control) wells. Cell growth inhibition rate was calculated according to the formula as following: cell growth inhibition rate (%) = $(1 - (\text{Absorbance of experimental group} - \text{Absorbance of blank group}) / (\text{Absorbance negative group} - \text{Absorbance of blank group})) \%$.

Fig. 1 The NB4 cell inhibition rates of 24, 48 and 72 h post-incubation treated with ATRA and/or simvastatin. The results displayed that the NB4 cell growth inhibition rates gradually increased ($P = 0.011$) when treated with 10 μ M SV. ATRA stands for all-*trans* retinoid acid, 10SV stands for 10 μ M simvastatin; ATRA + 10SV stands for all-*trans* retinoid acid in combination with 10 μ M simvastatin

Observation of Morphologic Changes of NB4 Cells

NB4 cells (2×10^5 /ml) of different groups were harvested 24, 48 and 72 h post-incubation, washed once in PBS, centrifuged at 1000 rpm on glass slides in a cytospin apparatus, and then fixed and subsequently stained with Wright–Giemsa solution (Nanjing, China). NB4 cell morphology was observed by microscope.

Flow Cytometric Analysis of Cell Differentiation and Cell Apoptosis

Following 24, 48 and 72 h of incubation with simvastatin and/or ATRA, NB4 cells of different groups were collected and transferred to 5 ml plastic tubes, washed twice with cold PBS, stained with annexin V-FITC and PI according to the manufacturer's instruction (Roche Diagnostics, USA), and analyzed by FCM (Becton Dickinson). CellQuest software

Fig. 3 The CD11b expression levels of NB4 cells treated with simvastatin and/or ATRA 24, 48 and 72 h post-incubation. Statistic analysis showed that 0.5 μ M ATRA as well as 10 μ M SV obviously increased the CD11b expression levels of NB4 cells time dependently, moreover combination of the two drugs manifested synergistic effect on CD11b expression (CI 0.83). ATRA stands for all-*trans* retinoid acid, 10SV stands for 10 μ M simvastatin; ATRA + 10SV stands for all-*trans* retinoid acid in combination with 10 μ M simvastatin

(Becton Dickinson) was used for the acquisition and analysis of data. Samples were run in duplicate with 10,000 events counted per sample. Viable cells included cells that were negative for both annexin and PI. The apoptotic rate was expressed as the mean of three independent experiments. Cell differentiation was determined by flow cytometric analysis after staining with the anti-CD11b monoclonal antibody. The anti-CD11b monoclonal antibody and a matched isotype control were purchased from BD Biosciences.

Detection of ApoM Gene Expression by Real-Time Quantitative RT-PCR

Following 24, 48 and 72 h of incubation simvastatin and/or ATRA, NB4 cells of different groups were collected. Total RNA was extracted from NB4 cells, using TRIzol

Fig. 2 The changes of NB4 cells treated with ATRA and/or simvastatin at different concentrations 48 h post-incubation (stained by Wright-Giemsa, $\times 1000$). **a** Normal control NB4 cells; **b** in NB4

cells treated with simvastatin at 10 μ M, apoptotic body formation with cytoplasm increase were observed; **c** petal-like nuclei were shown in NB4 cells treated with ATRA + simvastatin at 10 μ M

Fig. 4 CD11b expression levels of NB4 cells treated with simvastatin and/or ATRA 48 h post-incubation detected by FCM

one step procedure according to manufacturer's instructions (Gibco Ltd.) and dissolved in diethylpyrocarbonate (DEPC)-treated water. Reverse transcription was performed using random hexamer primers 100 ng for total RNA (2 µg/40 µL) and 100 U of reverse transcriptase (Promega Ltd). The cDNA was stored at -20°C or immediately used for real-time quantitative polymerase chain reaction (PCR).

The PCR primer sets were designed to amplify the apoM and GAPDH gene (house keeping gene) according to the information of GenBank, which are shown in previous [10]. Real time RT-PCR analyses were performed using LightCycler fluorescence detection system (Roche, Mannheim, Germany). The composition of PCR reaction mixture for apoM was as the follows: 10 × TaqMan PCR buffer 2.5 µl, 25 mM MgCl_2 2.5 µl, 10 mM dNTP 0.5 µl,

5 U/µl Taq DNA Polymerase 0.25 µl (TaKaRa Ltd, China), 100 µM primers and probe 0.1 µl respectively, cDNA 2 µl (resemble to 100 ng RNA). Sterilized water was added to a final volume of 25 µl. The PCR was initiated at 95°C for 5 min to inactivate the UNG enzyme and heat start, then running for 40 cycles at the following condition: denaturation at 95°C for 10 s and annealing/extension at 58°C for 45 s. All sample analysis was performed in duplicate. apoM copy numbers were calculated as the average. The PCR reaction mixture and the procedure for GAPDH gene were the same to apoM gene.

The copy value of apoM obtained by RQ-RT-PCR was normalized with respect to the number of GAPDH. Normalized apoM expression (apoM_N) was represented as a ratio of apoM to GAPDH levels times 10^4 in each individual sample.

Fig. 5 The Annexin V expression levels of NB4 cells treated with simvastatin and/or ATRA 24, 48 and 72 h post-incubation. ATRA did not increase the NB4 cell apoptosis. The Annexin V expression levels of NB4 cells increased time-dependently when treated with simvastatin, yet combination of the two drugs exhibited no synergistic apoptotic effects on NB4 cells. ATRA stands for all-*trans* retinoid acid, 10SV stands for 10 μ M simvastatin; ATRA + 10SV stands for all-*trans* retinoid acid in combination with 10 μ M simvastatin

Statistical Methods

Statistical analysis were performed with SPSS software (version 16.0). All experiments were performed three times in each individual sample and the results were presented as the mean value of the three. One-way analysis of variance (ANOVA) and paired *t* test were used to compare the means of different groups. *P* values < 0.05 were considered to be significant. The effects of combination were estimated using the CalcuSyn software, which was developed based on the median-effect method. CI < 0.9 indicates synergy; CI 0.9–1.1 is additive; and CI > 1.1 means antagonism.

Results

Changes of NB4 Cell Proliferation Inhibition Treated by Simvastatin and/or ATRA

Statistic analysis of MTT results displayed that ATRA did not inhibit the NB4 cell proliferation, however simvastatin significantly inhibited NB4 cell proliferation in time depend manner ($P = 0.011$). Furthermore the addition of ATRA to SV did not increase the effect of SV on proliferation ($P = 0.082$), indicating that simvastatin potentially inhibit the NB4 cell proliferation however ATRA in addition to simvastatin displayed neither additive nor synergistic NB4 cell proliferation inhibition effects (shown as Fig. 1).

Morphological Changes of NB4 Cell Treated by Simvastatin and/or ATRA

NB4 cells stained by Wright–Giemsa solution exhibited karyorrhexis, petal-like nuclei, and apoptotic body formation with cytoplasm increase 24 and 48 h post-incubation treated with simvastatin, and 72 h post-incubation with simvastatin, most of the NB4 cells manifested karyorrhexis. As for those treated with simvastatin in addition of ATRA, obvious karyorrhexis were observed under the microscopy (shown in Fig. 2).

NB4 Cell Differentiation and Apoptosis Treated with Simvastatin and/or ATRA

The results showed that 0.5 μ M ATRA as well as 10 μ M SV obviously increased the CD11b(BD Biosciences PharMingen) expression levels time dependently ($P < 0.01$, $P = 0.014$), yet interestingly the CD11b expression levels of NB4 cells treated with ATRA in combination with SV were significantly higher than those treated with ATRA or simvastatin alone ($P = 0.025$, $P < 0.01$), indicating that both simvastatin and ATRA potentially induce NB4 cell differentiation, moreover combination of the two drugs manifested synergistic differentiation effect (CI 0.83) (shown as Figs. 3, 4).

Treated with 10 μ M simvastatin, the Annexin V expression levels of NB4 cells increased time dependently ($P < 0.01$). However, there were no statistic differences for the Annexin V expression levels of NB4 cells prior to, and 24, 48 and 72 h after exposure to 0.5 μ M ATRA, suggesting that ATRA did not increase the NB4 cell apoptosis. Moreover the Annexin V expression levels of NB4 cells treated with simvastatin in combination with ATRA were of no statistic differences with those treated with simvastatin alone, indicating that simvastatin potentially promote NB4 cell apoptosis, yet combination of the two drugs exhibited no synergistic apoptotic effects on NB4 cells (shown as Figs. 5, 6).

ApoM Gene Expression Levels of NB4 Cells Treated with Simvastatin and/or ATRA

ApoM gene expression levels were detected by real-time quantitative RT-PCR. Statistic analysis showed that apoM gene expression levels gradually decreased in NB4 cells induced by ATRA ($P < 0.001$). On the contrary, treated with simvastatin alone, apoM gene expression levels increased gradually by a time-dependent manner ($P = 0.01$). Interestingly the apoM gene expression levels of NB4 cells treated with 10 μ M SV in combination with 0.5 μ M ATRA after 48 h, 72 h-incubation were significantly decreased than those treated with SV alone (25.21 ± 1.23 vs.

Fig. 6 Annexin V expression levels of NB4 cells treated with simvastatin and/or ATRA 48 h post-incubation detected by FCM

48.21 ± 2.36 , $P < 0.01$; 27.66 ± 3.03 vs. 89.14 ± 8.26 , $P < 0.01$), indicating that ATRA might abrogate the adaptive defensive apoM gene expression elevation induced by simvastatin ($P < 0.01$) (shown as Table 1).

Discussion

Since the introduction of ATRA in the treatment and optimization of the ATRA-based regimens for APL, the complete remission rate was raised up to 90–95 % and 5-year disease free survival (DFS) to 74 %. The important mechanism of successful treatment of APL by ATRA is that ATRA cause the differentiation of abnormally granulated specific blast cells into mature granulocytes by acting on the t (15; 17) fusion gene product [13]. Atorvastatin and fluvastatin were found to be potent inducers of cell differentiation and apoptosis of the NB4 cell line [7]. Our present results showed that simvastatin not only

induced NB4 cell differentiation but also synergistically induced NB4 cell differentiation with ATRA, moreover, simvastatin inhibited NB4 cell proliferation and promoted cell apoptosis time-dependently, as were of the same to the other investigators. As regard to the underlying proapoptotic dose of simvastatin, there was a report that combining tipifarnib and simvastatin at a dose of 5 and 50 μM exhibited synergistic apoptosis effect in KG1 and TF-1 cells [14]. Our results showed that combination of 10 μM simvastatin with ATRA synergistically induced NB4 cell differentiation, yet manifested no synergistic effects on NB4 cell proliferation or apoptosis, probably due to simvastatin exhibited no cell growth inhibition or cell apoptosis effects on mature granulocytes derived from the NB4 cells induced by ATRA. Meanwhile 10 μM simvastatin exhibited the obvious anti-leukemic effect avoiding dose-related side effects of simvastatin.

It was reported in 1978 that AML cells in vivo take up exceptionally large amounts of low-density lipoprotein

Table 1 ApoM gene expression levels of NB4 cells treated with simvastatin and/or ATRA (apoM $\times 10^4$ /GAPDH)

Group	24 h	48 h	72 h
Control	29.62 $\pm$ 2.13	27.31 $\pm$ 2.96	27.35 $\pm$ 2.78
ATRA	26.53 $\pm$ 1.91	17.48 $\pm$ 1.44 **##	11.11 $\pm$ 2.23 **##
10SV	28.69 $\pm$ 2.06	48.21 $\pm$ 2.36 **##	89.14 $\pm$ 8.26 **##
10SV + ATRA	26.82 $\pm$ 1.92	25.21 $\pm$ 1.23 \$\$	27.66 $\pm$ 3.03 \$\$

Table 1 showed that apoM gene expression levels gradually decreased in NB4 cells induced by ATRA. Treated with simvastatin alone, apoM gene expression levels of NB4 cells increased gradually by a time-dependent manner. Combination of simvastatin and ATRA displayed neither obvious increase nor significant decrease of apoM expression levels

SV (simvastatin), apoM expression level was calculated as apoM $\times 10^4$ /GAPDH, Values represent mean $\pm$ S.D. (n = 3)

** $P < 0.01$ versus 24 h post-incubation

$P < 0.01$ versus control group

\$\$ $P < 0.01$ versus 10SV group

(LDL) [15], and since then numerous other tumors have been shown to be avid for LDL [16]. Leukemic cells, in contrast to normal cells, can meet increased cholesterol requirements by either elevated LDL receptor activity or increased cholesterol synthesis, which is of potential interest for diagnosis and specific treatment of leukemia [17]. AML cells (NB4 and HL-60 cells) which were treated with equitoxic doses of chemotherapy or radiation showed significantly higher cholesterol levels 24 h after γ -irradiation or after 24 h of treatments with daunorubicin (DNR; 0.05 μ M for NB4, 0.01 μ M for HL-60) or cytarabine (*araC*; 0.5 μ M for NB4, 0.25 μ M for HL-60), and blocking these acute cholesterol responses selectively sensitizes AML cells to therapeutics [18]. Further research reported that the levels of mRNAs encoding the cholesterol synthesis-regulating enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and the cholesterol-importing low-density lipoprotein (LDL) receptor were both increased by DNR or *ara-C* treatments in almost three-fourths of cultured AML samples [19]. All the above results provided the evidence of decreasing the intracellular cholesterol might sensitizes AML cells to chemotherapy, indicating simvastatin may find a place in AML therapy.

Apolipoprotein M is one of the latest discovered lipoprotein-associated plasma protein that is mainly synthesized in the liver, and to a smaller amounts, in the kidney [8]. However, the synthesis pathway of apoM in the liver and the physiological functions of apoM expression in the tubular epithelial cell in kidney are not clarified at present. Recent investigation has demonstrated that apoM may participate in the high density lipoprotein (HDL) related biological activities as an important component of HDL particle on the protection of endothelial cells [20]. Our present results showed that apoM gene expression levels gradually decreased in NB4 cells induced by ATRA. However, treated with simvastatin alone, apoM gene expression levels of NB4 cells increased gradually by a time-

dependent manner. Combination of simvastatin and ATRA displayed neither obvious increase nor significant decrease of apoM expression levels, indicating that ATRA might abrogate the adaptive defensive apoM gene expression elevation induced by simvastatin. The mechanism of apoM gene expression decrease in NB4 cells induced by ATRA as well as the relationship of NB4 cell differentiation and apoptosis induced by simvastatin and apoM metabolism need to be further investigated.

Taking together, simvastatin potentially inhibit the NB4 cell proliferation, induce cell differentiation and apoptosis, as well as increased the apoM expression time-dependently, moreover, synergistically induce NB4 cell differentiation when combining with ATRA, while ATRA might abrogate the adaptive defensive apoM gene expression elevation induced by simvastatin, providing theoretical evidence for coadministration of simvastatin and ATRA as a new approach in treating APL, avoiding the hyperlipidemia caused by ATRA. The more detailed mechanism of simvastatin acting as an anti-leukemic drug needs to be further investigated.

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