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Synergistic Induction of Apoptosis by HMG-CoA Reductase Inhibitor and Histone Deacetylases Inhibitor in HeLa Cells

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Abstract

HMG-CoA reductase inhibitors and histone deacetylases (HDACs) inhibitors have been shown to induce apoptosis in a variety of cells, which could potentially be used as an anti-cancer therapy in addition to the designated applications. In the present study, we explored the possible synergistic pro-apoptotic effects and the underlying mechanisms when the two classes of inhibitors were combined. Exposure of HeLa cells to the combined treatment of mevastatin (an inhibitor of HMG CoA reductase) and trichostatin A (TSA) (an inhibitor of HDACs) synergistically induced apoptosis. Mevastatin treatment transcriptionally and translationally up-regulated RhoA expression in the cells by negative feedback mechanism. While TSA enhanced mevastatin-induced RhoA up-regulation, more importantly, it also accelerated mevastatin-mediated depletion of membrane-bound (geranylgeranylated) RhoA. Moreover, TSA treatment down-regulated protein geranylgeranyl transferase-I (GGTase-I) β subunit expression, which is one of the key enzymes for protein geranylgeranylation. Taken together, TSA down-regulated GGTase-I β expression, hence enhanced the statin-induced depletion of geranylgeranylated RhoA, which could be an important mechanism for the synergistic induction of the apoptosis.

Keywords

HMG-CoA; statin; TSA; apoptosis; RhoA

INTRODUCTION

The HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase is a key rate-limiting enzyme in the biosynthesis of mevalonate pathway. Inhibition of HMG-CoA reductase by statins not only reduces cholesterol biosynthesis, but also decreases the biosynthesis of important isoprenoids, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which was nicely reviewed by Winter-Vann and Fritz [1,2]. FPP and GGPP are involved in posttranslational modification of a variety of proteins including Ras and Rho GTP-binding proteins, which play crucial roles in normal cell functions.

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FPP can be elongated to GGPP by GGPP synthase (GGPS) [3]. When cells are treated with statins, GGPP synthesis is inhibited due to the blockage of biosynthesis of mevalonic acid and its derivatives, which in turn result in a reduction of protein geranylgeranylation. Consequently, the activities of many proteins that posttranslationally require geranylgeranyl modification will be inhibited. The HMG-CoA reductase inhibitors or statins, therefore, have pleiotropic biological and physiological effects on cell functions. Notably, statins have been shown to induce apoptosis in many cell lines [4–11].

Geranylgeranylation of proteins is catalyzed by protein geranylgeranyl transferase-I (GGTase-I) using GGPP as the substrate, which is an important maturation process for many proteins and their attachments to membrane [12]. Blockage of protein geranylgeranylation is believed to be the major mechanism for statin induction of apoptosis [4–11]. Therefore, targeting at protein geranylgeranylation could have an important role in anti-carcinogenesis [1,2,13].

Inhibitors of histone deacetylases (HDACs) have also been demonstrated to induce cell growth arrest and apoptosis in a variety of tumor cells [14–18]. One of the HDACs inhibitors SAHA is currently in clinical trials for patients with hematological or solid tumors [19,20]. However, some tumor cells remain resistant to conventional chemotherapeutic agents or a single anticancer agent. It is well known that many signal pathways become aberrant in carcinogenesis. Tumor cells can easily develop resistance to anticancer agents if only a single agent is applied. Therefore, it may be clinically important to find effective combination of anticancer agents to fight cancers. Several agents have already been tested together with HDACs inhibitors and certain synergistic interactions have been reported [21–24]. It occurred to us whether combination of HMG-CoA inhibitors and HDACs inhibitors could have more anticancer effects; if yes, what the possible mechanisms are. In the present study, we investigated the pro-apoptotic effects of the combined treatment with HMG-CoA inhibitor mevastatin and HDACs inhibitor TSA on HeLa cells, and explored the possible mechanisms underlying their pro-apoptotic effects individually or synergistically.

MATERIALS AND METHODS

Reagents

Trichostatin A (TSA), mevastatin (compactin) and geranylgeranylpyrophosphate (GGPP) were purchased from Sigma (St. Louis, MO). TSA and mevastatin were dissolved in dimethyl sulfoxide (Me₂SO). Mevastatin was chemically activated by alkaline hydrolysis prior to use as described previously [26]. Antibodies used in Western blot were mouse monoclonal antibody to RhoA (Santa Cruz, CA) and rabbit polyclonal antibody to β actin (Sigma).

Cell Culture

HeLa (human cervical epithelial cancer cell line) was cultured in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum at 37°C with 5% CO₂. For cell treatment, the reagents were added in culture media. The same volume of solvent was added in culture media as the corresponding controls.

Assessment of Cell Death

Nonviable cells were determined by trypan blue exclusion assay. In all trypan blue exclusion assays, 625–2000 cells were counted and data were calculated as the means of at least three independent experiments. The number of cells (blue cells) was expressed as a percentage of the total cell number [27].

Quantitative Real-time RT-PCR

Total RNA was extracted from the cells with Trizol (Invitrogen) according to the manufacturer's protocol. The RNA solution was treated with 1 U RNase-free DNase I (1 U/ μ l, Promega) in a final volume 20 μ l at 37°C for 15 min to remove the trace amount of genomic DNA. The DNase I was inactivated by incubating at 75°C for 10 min. Reverse-transcription and real-time PCR were performed as described previously [28]. The primers used in this study are as follows. human RhoA: sense/antisense 5'-TGGAAGACATGCTTGCTCAT-3'/5'-GCCTCAGGCGATCATAATCTTC-3'; human GGTase-I β subunit: sense/antisense 5'-GCTGGATTTCTTACGGGATCG-3'/5'-CAGCCCGGAGAGTGCAAAA-3'; human geranylgeranyl diphosphate synthase 1 (GGPS1): sense/antisense, 5'-CCAGGTAAACAAGTGAGAACCAA-3'/5'-CGTCGGAGTTTTGAGTTTCT-3'; human housekeeping gene β -actin, sense/antisense, 5'-CTGGAACGGTGAAGGTGACA-3'/5'-AAGGGACTTCCTGTAACAATGCA-3'.

Phase Separation of Geranylgeranylated and Non-geranylgeranylated RhoA

Geranylgeranylated (membrane-bound) and non-geranylgeranylated RhoA were separated by the Triton X-114 partition method as described previously with minor modifications [29]. Briefly, cells were washed once in ice-cold phosphate-buffered saline (PBS) and lysed in a lysis buffer containing 50 mM Tris pH 7.6, 1 mM dithiothreitol (DTT), 2 mM EGTA, 2 mM Vanadate, 1 mM PMSF and protease inhibitor cocktail (Sigma, St. Louis, MO). Triton X-114 (10% w/v) was added to the lysates to make a final concentration of 1% (v/v). Membrane-bound proteins were extracted by incubating the lysates on ice for 30 min and briefly vortexing every 5 min, followed with centrifugation of 10,000 g at 4°C for 10 min. The supernatants were collected for incubation at 37°C for 10 min and centrifuged at 15,000 g for 2 min at room temperature (RT) to separate the aqueous phase (upper phase, containing non-membrane-bound proteins) from the detergent phase (lower phase, containing membrane-bound proteins). Both aqueous and detergent phases were re-extracted once by adding adequate volume of Triton X-114 or the lysis buffer, respectively. The aqueous and detergent phases were pooled respectively and proteins were precipitated by 10% trichloroacetic acid (TCA) and protein concentration was determined by Bradford method (Sigma).

Statistic Analyses

All quantitative data are presented as the means $\pm$ SD of three separate experiments. Statistical differences were analyzed by one-way ANOVA for three or more group comparisons, and the independent Student's *t*-test for two group comparisons (SPSS v15.0, SPSS Inc, Chicago, IL). Two-tailed *P* < 0.05 was regarded as statistically significant.

RESULTS

Effects of HMG-CoA Inhibitor Mevastatin and HDACs Inhibitor TSA on Cell Death

To examine the pro-apoptotic effects of the two classes of inhibitors, we treated HeLa cells with TSA (1 μ g/ml), or mevastatin (40 μ M), or both for 36 h. The dead cells demonstrated morphological shrinkage and eventually floating (Fig 1), which were consistent with apoptotic features. Cell viabilities were further examined by trypan blue exclusion assay; and approximately $6.3 \pm 4.2\%$ cells cultured in the normal control media were stained positive for the same period as the cells receiving treatments. Combined treatment with mevastatin and TSA induced $92.8 \pm 1.6\%$ cell death; whereas TSA alone or mevastatin alone only induced $25.4 \pm 3.1\%$ and $32.2 \pm 2.9\%$ cell death, respectively (Fig. 1B). However, GGPP (10 μ M) reduced the apoptotic effect induced by mevastatin alone and also with the combination of mevastatin and TSA (Fig. 1).

Effects of Mevastatin and TSA on RhoA Expression and Membrane Location

RhoA is normally cycling between membrane-bound and soluble forms in a tightly regulated manner. When RhoA is geranylgeranylated, it translocates to membrane for loading GTP and to be activated [30,31]. RhoA is believed to play an important role in statin-induced apoptosis [7–10,32,33]. Therefore, we first examined whether RhoA was transcriptionally influenced by the treatments with mevastatin (20 and 40 μ M), or TSA (1 μ g/ml), or both in HeLa cells for 24 h. As shown in Figure 2A, RhoA mRNA was dose-dependently induced by the mevastatin treatment but not by TSA. Interestingly, combined treatment with mevastatin and TSA synergistically induced RhoA mRNA expression from 3.2 ± 5.8 fold (mevastatin alone) to 8.0 ± 13.0 fold (mevastatin and TSA) of the control (Fig. 2A). We further quantified cytosolic (non-geranylgeranylated) RhoA and membrane-bound (geranylgeranylated) RhoA after the treatments with mevastatin (40 μ M) or TSA (1 μ g/ml) or both in HeLa cells. After 24 h treatment, in accordance with the induction of RhoA mRNA, cytosolic RhoA (in aqueous phase of Triton X-114 extraction) was dramatically increased by the mevastatin treatment, but not by TSA alone. The synergistic induction was clearly evident by the combined mevastatin and TSA treatments (Fig. 2B). In contrast, treatment with mevastatin alone significantly decreased membrane-bound RhoA (in detergent phase of Triton X-114 extraction), but not by TSA alone. Surprisingly, membrane-bound RhoA was further decreased by the combinational treatment with mevastatin and TSA (Fig. 2B). Taken together, it appears that in addition to the upregulation on RhoA mRNA expression, mevastatin has blocked the translocation of RhoA from cytosol to membrane. This blockage was accentuated by the additional treatment with TSA although TSA alone has no effect on either RhoA expression or membrane translocation.

Modulation of GGTase-I β and GGPS1 mRNA by the Mevastatin and TSA Treatments

To explore the possible mechanisms involved in mevastatin-mediated, and indeed the combined treatment-mediated decrease in geranylgeranylated RhoA, we examined expressions of the GGTase-I β subunit and GGPS1 in HeLa cells. GGTase-I consists of two subunits, α and β , whereas α subunit is also a component of protein farnesyltransferase (FTase) [34]. However, the expression of β subunit determines the GGTase-I level. GGTase-I is responsible for the geranylgeranylation of proteins including RhoA. Therefore, we examined the expression of the GGTase-I β subunit in the HeLa cells after the cells were treated with mevastatin (20, 40 μ M), or TSA (1 μ g/ml), or both for 24 h. As shown in Figure 3A, expression of GGTase-I β mRNA was dose-dependently increased by the mevastatin treatment. In contrast, expression of GGTase-I β mRNA was decreased to 36.6% of the control by the TSA treatment. The TSA treatment also diminished the mevastatin's upregulating effect with the expression level at 50% of the control (Fig. 3A). We further examined GGPS1 expression in the HeLa cells after they were treated with mevastatin (20, 40 μ M), or TSA (1 μ g/ml), or both for 24 h. GGPS1 is responsible for the production of GGPP. As shown in Figure 3B, expression of GGPS1 mRNA was dose-dependently increased by mevastatin. The expression was also increased by TSA and more significantly by the combined treatment.

DISCUSSION

In the present study, we reported that combined treatment with HMG-CoA inhibitor mevastatin and HDACs inhibitor TSA synergistically induced apoptosis in HeLa cells. Although there are extensive studies about HMG-CoA inhibitors or HDACs inhibitors in apoptosis induction, the pro-apoptotic effects of the two inhibitors in combination have not been explored. Given that HDACs inhibitors and HMG-CoA inhibitors could be developed to the next generation anti-tumor drugs [1,13,19,35,36], our findings of the synergistic effects of the two classes of inhibitors on apoptosis may have significant clinical implications.

Studies have also demonstrated that inhibitors of histone deacetylases could down-regulate expression of endothelial nitric oxide synthase (eNOS) and compromise endothelial cell functions, implying that administration of HDACs inhibitors may have increased cardiovascular risk [25][26]. Basic and clinical studies have shown that statins can significantly improve endothelial functions [37][38]; mevastatin could rescue TSA-induced down-regulation of eNOS[26]. Therefore, it is possible that clinical co-administration of HDACs inhibitors and HMG-CoA inhibitor as an anti-tumor therapy may have advantages that not only enhance cancer cell apoptosis but also reduce possible cardiovascular side-effect of HDACs inhibitors.

The Rho family of small GTPases are involved in diverse biological functions such as cytoskeleton organization, adhesion, migration, cell proliferation, apoptosis, and transcriptional regulation [13,39,40]. Depletion of geranylgeranylated RhoA (membrane-bound) by statins is believed as one of the important reasons for statins to induce cell growth arrest and apoptosis [5,7–10,32,33]. RhoA inhibitor or a dominant-negative mutant RhoA (T19N) induced apoptosis similarly to what statins did [9]. RhoA is cycling between membrane-bound and soluble forms.

The cytosolic RhoA translocates to the cell membrane only after geranylgeranylated with GGPP, and becomes activated after loading GTP (GTP-bound) [1,2]. In the presence of statins, RhoA was demonstrated to be regulated by negative feedback in endothelial cells [38]. Consistently, the present study showed that RhoA mRNA and cytosolic protein in the HeLa cells were also induced by the negative feedback. Furthermore, membrane-bound (geranylgeranylated) RhoA was expectedly decreased by mevastatin in the HeLa cells in accordance with previous studies [7–10,32,33]. Although TSA alone did not influence RhoA expression, it enhanced mevastatin-induced increase of RhoA mRNA expression and accumulation of cytosolic RhoA (Fig 2). More importantly, TSA simultaneously enhanced mevastatin-mediated decrease of the membrane-bound (geranylgeranylated) RhoA (Fig. 2B). Since RhoA is regulated by the negative feedback mechanism in the statin-mediated depletion of membrane-bound (geranylgeranylated) RhoA, the more membrane-bound RhoA decreases, the more RhoA mRNA and cytosolic RhoA is induced. Therefore, the enhancement of mevastatin-induced RhoA mRNA expression and accumulation of cytosolic RhoA by TSA (Fig. 2) is likely due to its enhancement of mevastatin-mediated depletion of membrane-bound RhoA. Considering that RhoA plays many important roles in cell survival and apoptosis [13, 39,40], our results suggest that TSA enhancement of mevastatin-mediated depletion of geranylgeranylated RhoA may be an important reason responsible for the synergistic induction of apoptosis triggered by TSA and mevastatin.

TSA down-regulated GGTase-I β expression may contribute to its enhancement on mevastatin-mediated depletion of geranylgeranylated RhoA. Given that GGTase-I is responsible for geranylgeranylation of proteins, the reduction in mevastatin-induced expression of GGTase-I could only further decrease RhoA geranylgeranylation and therefore lead to additional accumulation of RhoA in cytosol as shown in Figure 2 and 3. However, the inhibition of GGTase-I β expression by TSA alone did not influence RhoA geranylgeranylation, or only marginally (Figure 2B, 3A). The slight induction of GGPS1 expression (Fig. 3B), which is responsible for synthesis of GGPP, may be a negative feedback response to TSA-induced down-regulation of GGTase-I β expression since GGPP is the substrate of GGTase-I. Therefore, the induction of GGPS1 by mevastatin or together with TSA would be also a negative feedback response to the mevastatin-induced inhibition of mevalonate biosynthesis, and showed no effect to RhoA geranylgeranylation.

In summary, we have shown that treatment with TSA and mevastatin synergistically induced apoptosis in HeLa cells. The combined treatment also synergistically inhibited

geranylgeranylation of RhoA. Down-regulation of GGTase-I β expression by TSA could be one of the important mechanisms underlying TSA enhancement of mevastatin-induced geranylgeranylated RhoA depletion, which may be responsible for the cell death.

Acknowledgements

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Fig 1B

Fig. 1. Synergistic induction of cell death by mevastatin and TSA in HeLa cells

A: Inverted microscopy photographs of HeLa cells exposed to mevastatin (40 μ M), or TSA (1 μ g/ml), or both for 36 h. Dead cells became shrunk and floated. **B:** Cell viability assay. Dead cells were scored with trypan blue staining.

Fig. 2. Synergistic induction of RhoA and enhancement of geranylgeranylated RhoA depletion by combined treatment of mevastatin and TSA

A: Transcriptional induction of RhoA by mevastatin in negative feedback regulatory mechanisms and synergistic induction of RhoA by mevastatin and TSA. Cells were exposed to the agents as indicated for 24 h. The RhoA mRNA levels were quantified by the real-time quantitative RT-PCR. All data are presented as the mean $\pm$ SD of three separated experiments.

B: Synergistic depletion of geranylgeranylated RhoA and accumulation of cytosolic RhoA by combined mevastatin and TSA treatment. Western blot was performed for cytosolic and membrane-bound (geranylgeranylated) RhoA from the extracts of Trion X-114 partition method. HeLa cells were exposed to the agents as indicated for 24 h. Membrane-bound (geranylgeranylated) RhoA is in detergent phase while cytosolic (non-geranylgeranylated) RhoA is in aqueous phase. Actin was used as an internal control.

Fig. 3. Evaluation of GGTase-I β and GGPS1 mRNA expressions after the combined mevastatin and TSA treatment

A: Regulation of GGTase-I β mRNA expression by mevastatin and TSA. **B:** Regulation of GGPS1 mRNA expression by mevastatin and TSA. HeLa cells were exposed to the agents as indicated for 24 h. The mRNA levels were quantified by the real-time quantitative RT-PCR. All data are presented as the mean $\pm$ SD of three separated experiments. * P < 0.05 and ** P < 0.01 vs control.