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Differentiation and apoptosis induction by lovastatin and γ-tocotrienol in HL-60 cells via Ras/ERK/NF-κB and Ras/Akt/NF-κB signaling dependent down-regulation of glyoxalase 1 and HMG-CoA reductase

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ABSTRACT

Glyoxalase 1 (GLO1) and HMG-CoA reductase (HMGCR) are highly expressed in most tumor cells and little in normal cells. In this study, treatment of HL-60 cells with lovastatin induced characteristic apoptosis in a dose-dependent manner. We demonstrated that lovastatin treatment inhibited Ras and Raf protein translocation to cell membrane and eliminated the phosphorylation of the downstream effectors Akt and ERK, and the subsequent NF-κB translocation into nucleus. Specific inhibitors and γ-tocotrienol confirmed the Ras/Raf/ERK/NF-κB/GLO1 and Ras/Akt/NF-κB/GLO1 pathways. Data revealed that lovastatin induced HL-60 cell death was attenuated by mevalonate treatment. We demonstrated also that γ-tocotrienol showed its apoptotic effect on the HL-60 cell through the same pathway. γ-Tocotrienol enhanced the apoptotic effect of lovastatin through the down-regulation of GLO1 and HMGCR resulting in an increase of methylglyoxal and a decrease of cholesterol and led to the apoptosis of HL-60 cells.
Data also revealed that both lovastatin and gamma-tocotrienol induced significant HL-60 cell differentiation. These results suggest that both lovastatin and gamma-tocotrienol could induce differentiation and followed by apoptosis.

1. Introduction

The biological activities of tocotrienol and lovastatin have been individually investigated. Recently the combined effect of tocotrienol and lovastatin has emerged as a subject of broad interest. The combination of a tocotrienol-rich fraction of rice bran and lovastatin plus the American Heart Association Step-1 diet significantly reduces serum total cholesterol (20%) and LDL-cholesterol (25%) in 28 hypercholesterolemic subjects [1]. The combined treatment with δ-tocotrienol plus lovastatin showed additive effects on increasing bone formation and reducing bone resorption in postmenopausal women patients with both osteoporosis and hypercholesterolemia [2]. Dudakovic et al. proposed that inhibition of the mevalonate pathway by statins and tocotrienols suppresses the prenylation of GTPase binding proteins, reduces the activity of osteoclasts and induces their apoptosis [3]. The combined treatment with lovastatin and tocotrienol using a targeted drug delivery system to be administered directly to the fracture site significantly promotes fracture healing of bone in postmenopausal osteoporosis rats through the induction of osteoclast apoptosis [4].

Cholesterol homeostasis is abnormal in malignant cells. Tumor cells consume a larger amount of glucose and use a less efficient glycolysis pathway (2 ATP) over mitochondrial oxidative phosphorylation (36 ATP) for tumor formation and growth [5]. It is well established that malignant cells increase glucose uptake and utilization to compensate for the shortage in ATP supply due to the transformation from mitochondrial oxidative phosphorylation to cytosol glycolysis for adaptation to intermittent micro-environmental hypoxia [6]. The suppression of mitochondrial respiration results in the shortage of acetyl-CoA for cholesterol biosynthesis [7]. Cholesterol and sphingolipid are the major components of lipid rafts in cell membranes affecting cell survival mechanisms through the regulation of downstream phosphorylation cascades in vitro and in vivo. Administration of cholesterol increases phosphorylation of Akt, and reduces apoptosis in prostate cancer (PCa) and the LNCaP/sHB xenograft tumors in SCID mice [8].

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is the key enzyme in the mevalonate pathway for cholesterol biosynthesis. It is clear now that HMGCR activity is up-regulated in some malignant

Abbreviations: GLO1, glyoxalase 1; HMGCR, HMG-CoA reductase; ERK, extracellular signal regulated kinases; Akt, protein kinase B; NF-κB, nuclear factor kappa-B; MG, methylglyoxal.

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cells compared with their normal counterparts [9]. High endogenous cholesterol synthesis is observed in acute myeloid leukemia (AML) cells [10,11]. Proliferating leukemic cells require high cellular cholesterol for rapid membrane turnover and maintaining cell survival. Freshly isolated AML and chronic myelogenous leukemia cells display much higher specific HMGCR activity than leukocytes from healthy subjects [12]. To meet the increased cholesterol demand for membrane synthesis, rapidly proliferating leukemic cells elevate HMGCR gene expression and increase cholesterol synthesis. In addition to leukemic cells, elevated expression of the HMGCR is observed in breast, ovarian and colorectal cancer [9].

Ras gene mutation is the most frequently mutated oncogene in human cancers. The frequencies of mutations of N-Ras and K-Ras are 9.7% and 2.9% in Chinese patients with acute myeloid leukemia (AML) [13]. H-Ras is localized predominantly in disordered plasma membrane and cholesterol-rich lipid rafts [14]. H-Ras requires cholesterol-rich lipid raft domains for efficient activation of Raf [15]. The Ras small GTPase family is responsible for many signaling processes. Mutations in the gene level convert these genes into active Ras oncogenes. Mutated Ras genes encode activated Ras proteins, which stimulate tumorigenesis and cancer cell growth [16]. Activated Ras recruits serine/threonine kinases of the Raf family to the plasma membrane and constitutively triggers the extracellular-signal-regulated kinase (ERK) pathway [17] and phosphatidylinositol 3-kinase (PI3K) pathway [18]. Constantly active RAS/Raf/MEK/ERK and PI3K/Akt signaling cascades have been observed in a variety of leukemia samples [19]. Farnesyltransferase (FTase) catalyzed the addition of a farnesyl isoprenoid moiety to the Ras protein. Farnesyltransferase inhibitors that inhibit the prenylation necessary for Ras activation prevent Ras from maturing into its biologically active form [20]. It is clear now that FTase is the key enzyme that connects the mevalonate pathway to RAS/Raf/MEK/ERK and PI3K/Akt signaling cascades.

Cancer cells utilize glucose via glycolysis to produce ATP for energy requirement. Methylglyoxal (MG) is mainly formed as a by-product of glycolysis. Over-activated glycolysis pathway forms MG from the triose phosphate intermediates, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate in cancer cells [21,22]. MG inhibits cell proliferation by inducing cell cycle arrest and apoptosis. A physiological concentration of MG causes both ladder formation of DNA and nuclear fragmentation and leads to apoptotic cell death in monocytic leukemia U937 cells [23]. MG induces apoptosis in human leukemia HL-60 cells through the accumulation of MG-DNA adducts [24]. MG has a strong cytotoxicity and is able to cross link with protein and DNA to form adducts, resulting in an inhibition of DNA synthesis, stopping cell proliferation and inducing apoptosis [25].

The glyoxalase system is a ubiquitous detoxification pathway that transforms MG to nontoxic D-lactate and protects against cellular damage caused by MG. Recent studies have demonstrated silenced GLO1 in tumors with high rates of glycolysis resulting in marked accumulation of MG and cytotoxicity [26]. Significant high glyoxalase activity has been detected among patients with pancreatic cancer [27], hepatocellular carcinoma [28], prostate cancer [29] and differentiation of human leukemia HL-60 cells [30]. Development of a cancer cell results in the increased expression of GLO1 to eliminate the toxic MG for cell survival.

Apoptotic effects of γ-tocotrienol and lovastatin were well documented respectively. Inoue et al. reported that γ-tocotrienol induced apoptosis by regulation of the caspase cascade, Bid cleavage, and Bcl-2 gene expression in human promyelocytic HL-60 cells [31]. Previous studies demonstrated that γ-tocotrienol is able to inhibit HMGCR, NF-κB, a target of the PI3K/Akt pathway and induces cell cycle arrest or apoptosis in neoplastic mammary epithelial cells (Shah and Sylvester 2005). Wang et al. found that lovastatin induces apoptosis in HL-60 cells through the decrease of cholesterol and Ras protein isoprenylation leading to caspase-3 activation [32]. However, the mechanism underlying the effects of γ-tocotrienol and/or lovastatin on malignant hematopoietic cells has not been fully elucidated. Our previous study demonstrates that GLO1 and HMGCR are two key enzymes for leukemia cell survival and proliferation. Lovastatin attenuates GLO1 and HMGCR expression and results in a typical apoptosis in U937 cells [33]. In this contribution, we showed that similar to lovastatin, γ-tocotrienol exerted its apoptotic activity through the inhibition of Ras/Raf/ERK/NF-κB and Ras/PI3K/Akt/NF-κB pathways leading to suppression of GLO1 and HMGCR in HL-60 cells.

2. Materials and methods

2.1. Chemical reagents and antibodies

Lovastatin, 2-methylquinoxaline (2-MQ), *o*-phenylenediamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), γ-tocotrienol and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against phospho-p44/42 MAPK (Thr202/Tyr204), phospho-Akt (Ser473), ERK1/2, Akt1/PKBα and actin, were obtained from Millipore (Billerica, MA, USA). Antibodies against GLO1, HMGCR, NF-κB (p65), H-Ras, Raf-1 and lamin A were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against K-Ras and N-Ras were obtained from GeneTex (San Antonio, TX, USA). Alexa Fluor 594 goat anti-mouse IgG (H + L) was purchased from Invitrogen. U0126 (MEK1/2 inhibitor), LY294002 (PI3K inhibitor), JSH-23 (NF-κB inhibitor), IgG HRP-conjugated goat anti-mouse and IgG HRP-conjugated goat anti-rabbit were obtained from Chemicon (Temecula, CA, USA).

2.2. Cell culture

The HL-60 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI 1640 medium with 10% fetal bovine serum, 5 mM L-glutamine, and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Cytotoxicity assay

Cell viability was assessed by MTT assay. Briefly, cells at 1×10^5/mL were treated with various concentrations of compounds in 96-well plates for 48 h, followed by treatment with MTT for 4 h at 37 °C. The medium was removed, dimethyl sulfoxide (DMSO) was added to dissolve the blue formazan residue, and color intensity was measured using an ELISA reader at 570 nm.

2.4. Analysis of DNA fragmentation

After lovastatin treatment and washing with cold PBS, the cells were resuspended in 180 μL of DNA lysis buffer (0.5% lauroylsarcosine, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mg/mL proteinase K and 50 mM Tris–HCl at pH 8.0) overnight at 56 °C and treated with RNase A (0.5 μg/mL) for 1 h at 37 °C. The genomic DNA was extracted by phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) extraction and analyzed by gel electrophoresis using 1.8% agarose gel. The gel was stained with ethidium bromide. After the gel was de-stained for 5 min, the DNA was visualized under UV light (Kodak, Rochester, NY, USA).

2.5. Nuclear staining with DAPI

After treatment with lovastatin for 48 h, cells were washed with cold PBS and fixed in 4% paraformaldehyde/PBS for 30 min at room temperature. The fixed cells were washed with cold PBS and stained with 2.5 mg/mL DAPI solution for 30 min at room temperature. The cells were washed two more times with PBS and analyzed via a confocal microscope (CARV II Confocal Imager, BD Biosciences).