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Simvastatin inhibition of mevalonate pathway induces apoptosis in human breast cancer cells via activation of JNK/CHOP/DR5 signaling pathway

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ABSTRACT

Simvastatin (SVA) was shown to up-regulate expression of death receptor-5 (DR5), CCAAT/enhancer binding protein homologous protein (CHOP) and phosphorylated c-Jun N-terminal kinase (pJNK) in human breast cancer cell lines. siRNA knockdown of DR5, CHOP or JNK significantly blocked SVA-induced apoptosis, demonstrating the importance of JNK/CHOP/DR5 signaling pathway in SVA-induced apoptosis. Exogenous addition of either mevalonate or geranylgeranyl pyrophosphate (GGPP) inhibited SVA activation of JNK/CHOP/DR5 pro-apoptotic pathway, indicating that activation of JNK/CHOP/DR5 pro-apoptotic pathway is dependent on SVA inhibition of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase and its intermediate GGPP. Data provide novel insight into better understanding the anticancer mechanisms of SVA.

1. Introduction

Breast cancer incidence and death remain major health concerns for women [1]. Systemic treatments, including cytotoxic, hormonal, and immunotherapeutic agents, are effective initially in 90% of primary breast cancers and 50% of metastases; however, after variable periods of time drug resistance and toxicity limit treatment effectiveness [2–4], highlighting the need for new treatment regimens.

Statins are widely prescribed drugs used for the reduction of cholesterol levels via inhibition of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the rate-limiting step in mevalonate synthesis. This pathway is critical for the cellular synthesis of cholesterol and its isoprenoid intermediates; such as, geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). These mevalonate-derived prenyl groups exert pleiotropic effects on many essential cellular functions including cell proliferation, differentiation, and survival [5–7]. Accumulating *in vitro* and animal studies have shown that various statins possess anti-proliferative, anti-angiogenic, anti-metastasis and pro-apoptotic properties in a variety of cancer cells including breast, colon and prostate cells [8–15], and some but not all clinical studies have shown a reverse association of statins, particularly lipophilic statins, with cancer risk [16–22]. Since the relationship between statins and cancer is promising yet complex, further basic information is needed [21].

Simvastatin (SVA) is a commonly used lipophilic statin derived from lovastatin. SVA shows *in vitro* and *in vivo* antitumor actions in a variety of cancer cells including breast, colon and prostate [9–12]. Long-term use of SVA has been reported to reduce the risk of breast cancer [20]. The ability of SVA to induce apoptosis in cancer cells has been established and several insights into the mechanisms of action have been gained [9,23–27] including activation of JNK [23], generation of reactive oxygen species (ROS) [24], activation of inducible nitric oxygen species resulting in increase of nitric oxide [25], suppression of Akt [26] and suppression of NF-kB [27], as well as up-regulation of Bax and down-regulation of Bcl-2 [9]. Despite this knowledge, a more complete understanding of SVA induced apoptosis is needed in order to improve SVA treatments by pairing SVA with agents that can act in an additive or synergistic manner.

Here, for the first time, we demonstrate that SVA induces apoptosis in human breast cancer cells via activation of a death receptor DR5 pro-apoptotic pathway in a JNK/CHOP dependent manner, which is subject to blockage by exogenously added mevalonate and geranylgeranyl pyrophosphate. Data suggest that

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mevalonate/GGPP is/are promising molecular targets for breast cancer cells and increased expressions of JNK/CHOP/DR5 are potential biomarkers for monitoring responsiveness to SVA treatment.

2. Materials and methods

2.1. Chemicals

Simvastatin sodium salt (SVA), an equivalent of simvastatin used *in vivo*, was obtained from Calbiochem (San Diego, CA). Mevalonate (Me), geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were obtained from Sigma (St. Louis, MO).

2.2. Cell culture

Human breast cancer cell lines MDA-MB-231 [American Type Culture Collection (ATCC), Manassas, VA], MCF-7 (ATCC), SUM 159 (Asterand, Detroit, MI), acquired tamoxifen resistant MCF-7 (MCF-7/TamR, gift from Dr. Linda deGraffenried, University of Texas at Austin, Austin, TX), acquired doxorubicin resistant MCF-7 (MCF-7/ADR, gift from Dr. Kapil Mehta, M.D. Anderson Cancer Center, Houston TX), and immortalized non-cancerous human epithelia breast cell line MCF-10A (ATCC) were used in this study. SUM159 cells were cultured in Ham's F12 medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum, 5 μg/ml insulin, 1 μg/ml hydrocortisone (Sigma–Aldrich, St. Louis, MO), and 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES). MCF-7/TamR, MCF-7/ADR cells and MCF-10A cells were cultured as described previously [28–30]. To mimic the low-serum exposure of tumors, studies were conducted with 2% serum. SVA was dissolved in ethanol and level of ethanol contained in the highest SVA dosage used in a given experiment was used as vehicle control (VEH).

2.3. Quantification of apoptosis

Apoptosis was quantified using an Annexin V-FITC/PI assay as described previously [31]. Annexin V-FITC/PI assays measure the amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to membranes of apoptotic cells) and amount of propidium iodide (PI), a dye that does not cross the plasma membrane of viable cells but readily enters dead cells or cells in the late stages of apoptosis and binds DNA. Fluorescence was measured using Fluorescence Activated Cell Sorter (FACS) analyses with a FACSCalibur flow cytometer and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

2.4. Western blot analyses

Preparation of whole cell protein extracts and Western blot analyses were conducted as described previously [31]. Primary antibodies used in this study were CHOP, pJNK, total JNK and poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA), DR5, Caspase-8 and Caspase-9 (Cell Signaling), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, made in house).

2.5. Small interfering RNA (siRNA) assay

A scrambled RNA duplex that does not target any known genes was used as the nonspecific negative control for RNAi (referred to as control siRNA). Transfection of siRNAs to JNK2/1 (Sense:5′-AAA GAA UGU CCU ACC UUC Utt-3′), DR5 (Sense:5′-CUG AUA AAG UGG GUC AAC Att-3′), CHOP (Sense:5′-GCA CAG CUA GCU GAA GAG Att-3′) or control (Ambion, Austin, TX) was performed in 100 mm^2 cell culture dishes at a density of 2×10^6 cells/dish using Lipofectamine-2000 and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. One day after transfection, the cells were re-cultured in 100 mm^2 dishes at 5×10^5 cells/dish and incubated for 1 day followed by treatments.

2.6. Statistical analysis

The student's *t*-test was used to determine statistical differences between treatment and control values. Differences were considered statistical significant when $p < 0.05$.

3. Results

3.1. SVA induces human breast cancer cells to undergo apoptosis

Treatment of MDA-MB-231 and MCF-7 cells with 0.625–5 μM SVA for 3 days significantly induced apoptosis in both cell lines in a dose-dependent manner (Fig. 1a–c). Based on EC_{50} values, the sensitivity of breast cancer cell lines to SVA-induced apoptosis was shown to differ. MDA-MB-231 cells with an EC_{50} value of 2.72 ± 0.21 μM were more sensitive to SVA-induced apoptosis than MCF-7 or SUM 159 breast cancer cell lines with EC_{50} values of 6.00 ± 1.00 μM and 4.00 ± 0.62 μM, respectively (Table 1). Unexpectedly, data showed acquired tamoxifen and doxorubicin resistant cell lines, MCF-7/TamR and MCF-7/ADR, to be more sensitive to SVA-induced apoptosis than MCF-7 cells, with EC_{50} values of 0.80 ± 0.20 μM and 0.76 ± 0.25 μM, respectively (Table 1). These data suggest that SVA has potential to be used as a treatment for drug resistant breast cancers by itself or in combination with other agents which will lower the effective doses of SVA required for treatment. It will also be of interest to determine if the addition of SVA with tamoxifen or with doxorubicin can circumvent drug resistance. Furthermore, data show that MCF-10A, an immortalized non-cancerous human epithelial breast cell line, is more resistant to SVA with EC_{50} value >20.00 μM (Table 1), suggesting that SVA's pro-apoptotic property is selective for breast cancer cells, but not for non-cancerous human epithelial breast cells. Western blot analyses of whole cell extracts from SVA-treated MDA-MB-231 and MCF-7 cells show SVA to induce dose-dependent caspase 8 and 9 cleavage, biochemical indicators of caspase activation, as well as PARP cleavage, a marker for caspase-dependent apoptosis (Fig. 1d). These data further confirm the pro-apoptotic properties of SVA, and show that SVA induced apoptosis is associated with caspase-8 and caspase-9 activation, suggesting involvement of death receptor- and/or mitochondrial-mediated apoptotic pathways.

3.2. SVA up-regulates DR5, CHOP and pJNK2/1 during apoptosis

MDA-MB-231 and MCF-7 cells treated with different levels of SVA for 3 days showed increased levels of both the long (L) and short (S) splice variants of death receptor 5 [DR5 (L/S)] protein in comparison to vehicle control (Fig. 2a). The generality of SVA up-regulation of DR5 in human breast cancer cells was tested in MCF-7/ADR, SUM 159 and MCF-7/TamR cell lines and data showed that treatment with 0.625 μM SVA for 3 days enhanced DR5 (L/S) protein levels in all three cell lines (Fig. 2b). Notably, SVA at 5 μM did not induce changes in DR5 protein expression in MCF-10A cells (Fig. 2b). Since JNK and CHOP had been shown to act upstream in the activation of DR5 (L/S) by previous studies in our lab with other pro-apoptotic agents [31,32], we tested whether JNK/CHOP was up-regulated in SVA-induced apoptosis. SVA treatment of MDA-MB-231 and MCF-7 breast cancer cells for 3 days increased CHOP and pJNK2/1 protein expression in both cell lines in a dose-dependent manner in comparison with Vehicle control (VEH) (Fig. 2c and d).

3.3. SVA up-regulation of CHOP and DR5 is involved in SVA-induced apoptosis and mediated by JNK

To see if CHOP and DR5 were involved in SVA-induced apoptosis and mediated by JNK, we performed siRNA knockdown assays. Data show that siRNAs to CHOP and DR5, as well as JNK, significantly reduced the ability of SVA to induce apoptosis in both MCF-7 and MDA-MB 231 cell lines as measured by Annexin V/FITC-PI (Fig. 3a and b) and PARP cleavage analyses (Fig. 3c and d). Furthermore, siRNA to CHOP blocked the ability of SVA to increase DR5 (L/S) protein expression, indicating that SVA up-regulation of DR5 is CHOP dependent, and siRNA to JNK blocked the ability of SVA to increase CHOP and DR5 (L/S) protein levels (Fig. 3c and d), indicating that SVA induced up-regulation of CHOP and DR5 protein expression are downstream of JNK2/1. It was noted that the basal levels of DR5 in MDA-MB-231 cells could not be blocked by siRNAs to JNK and CHOP (Fig 3c left panel), suggesting that JNK and CHOP only regulate SVA mediated DR5 protein expression, but do not control the basal level of DR5 protein expression.