The roles of antiapoptotic sphingosine kinase-1 and glucosylceramide genes in drug induced cell death of MCF-7 breast cancer cells

G. Gucluler[1], O. Piskin[2], Y. Baran[1]

[1]*Izmir Institute of Technology, Department of Molecular Biology and Genetics, Urla, Izmir;* [2]*Dokuz Eylul University, School of Medicine, Department of Internal Medicine, Narlidere, Izmir, Turkey*

Summary

Purpose: Sphingolipids are important signaling molecules mediating cell survival, proliferation, cell cycle regulation and apoptosis. Ceramide is the most vital sphingolipid which induces growth arrest, senescence, and apoptosis. In this study, we aimed to determine the roles of sphingosine kinase-1 (SK-1) and glucosylceramide synthase (GCS) genes in paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel induced apoptosis in human MCF-7 breast cancer cells.

Methods: IC50 values (drug concentration inhibiting cell growth by 50%) of the anticancer agents were calculated using XTT cell proliferation assay. Changes in mitochondrial membrane potential (MMP) were determined using JC-1 assay kit. Changes in the mRNA levels of SK-1 and GCS genes were measured by using RT-PCR technique.

Results: The results demonstrated significant decrease in cellular proliferation and increase in loss of MMP in a dose-dependent manner. Paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel application downregulated SK-1 expression while paclitaxel, tamoxifen, cyclophosphamide and docetaxel but not doxorubicin downregulated GCS comparing to untreated control cells.

Conclusion: These results show for the first time that these agents induce apoptosis in MCF-7 cells by downregulating the antiapoptotic SK-1 and GCS genes that may result in accumulation of apoptotic ceramides.

Key words: bioactive sphingolipids, breast cancer, glucosylceramide synthase, MCF-7, sphingosine kinase-1

Introduction

Sphingolipids are important structural lipids mediating the fluidity and sub-domain structure of the lipid bilayers [1]. All membrane sphingolipids are composed of a long-chain sphingoid backbone and a fatty acid with an amide bond linkage [2]. There are different bioactive sphingolipids such as ceramide, glucosylceramide, sphingosine, and sphingosine-1-phosphate (S1P). In addition to structural control of sphingolipids, these bioactive sphingolipids act as bioeffector molecules and control cellular processes such as cell survival, cell proliferation, cell growth and cell death [3].

Under stress conditions such as UV irradiation or chemotherapeutic agents, cell signaling through the sphingolipids starts via *de novo* pathway by ceramide synthase or salvage pathway by sphingomyelinases. Both pathways provide intracellular accumulation of ceramides. Ceramide is the central molecule within sphingolipids mediating antiproliferative responses such as senescence, growth arrest, and apoptosis. Many apoptosis-inducing factors promote ceramide generation before triggering apoptotic cascades [3-6]. Also, inhibition of ceramide metabolism enzymes increases the intracellular ceramide level resulting in apoptosis. Once ceramide is generated, ceramidases convert ceramide to sphingosine which is also an anti-proliferative bioactive sphingolipid. Phosphorylation of apoptotic sphingosine by SK-1 generates an antiapoptotic metabolite, S1P. Unlike ceramide and sphingosine, sphingosine-1-phosphate is a pro-survival molecule inducing cell proliferation, cell growth and inhibiting apoptosis [6-8]. Glucosylceramide is another pro-survival molecule, similar to S1P, converted from ceramide by GCS. Transfer of glucose to ceramide generates glucosylceramide and glucosylceramide is known

to be associated with drug resistance in different types of cancers [9].

Breast cancer is the most common cancer type among women. Breast cancer can be treated surgically, with systemic therapies (chemotherapy and endocrine therapy) and radiotherapy. However, limitations in chemotherapy are a formidable obstacle in breast cancer treatment [10]. Cancer cells have inherited or acquired resistance in order to reduce the intracellular levels of chemotherapeutic agents. This multidrug resistance (MDR) phenomenon is very common and known to be linked to several mechanisms such as overexpression of MDR genes and bcl-2 [11]. Recent studies showed that the antiapoptotic sphingolipid glucosylceramide and S1P have important effects on chemotherapeutic resistance in ovarian and breast cancer [12-14].

A good number of widely used anticancer agents for the treatment of breast cancer is available. The microtubule stabilizing agents paclitaxel and docetaxel have been used since 1990s for breast cancer treatment, being members of the taxane family. They function at the microtubule dynamics resulting in cell cycle arrest and induction of apoptosis [15]. Tamoxifen is a widely used anticancer agent used in hormone-responsive breast cancer types. Inhibition of estrogen receptor by tamoxifen induces cell cycle arrest resulting in apoptosis [16]. Doxorubicin is one of the most effective anthracyclines that intercalates DNA bases, DNA unwinding, reactive oxygen generation, and changes in membrane structure. Thus, doxorubicin results in several important processes of a cell, such as cellular growth and differentiation, and programmed cell death [17]. Cyclophosphamide is also an important alkylating agent for the treatment of breast cancer [15].

In this study, we examined the involvement of SK-1 and GCS genes in paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel induced apoptosis in human MCF-7 breast cancer cells.

Methods

Cell lines and culture conditions

Human MCF-7 breast cancer cells were kindly provided by Dr. Ali Ugur Ural from Gulhane Military Medical Academy. Anticancer agents used in this study (paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel) were kindly provided by Dr. Guray Saydam from Ege University Hematology Department. These cells were maintained in RPMI-1640 growth medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) at 37° C in 5% CO_2.

Measurement of cell growth by XTT

The IC50 values of paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel were determined by XTT cell proliferation assay as previously described [18,19]. Briefly, 1×10^4 cells were seeded into 96-well plates containing 200 μl growth medium and were incubated at 37° C in 5% CO_2 for 24 h. Then, they were exposed to increasing concentrations of the agents separately and incubated at 37° C in 5% CO_2 for 72 h. After that, the cells were treated with the 40 μl XTT for 4 h and then the plates were read under 490 nm wavelength by Elisa Reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Cell proliferation plots obtained from the measurement were used to calculate the IC50 values.

Measurement of mitochondrial membrane potential (MMP)

MMP in MCF-7 cells was determined by using APO LOGIX JC-1 Assay Kit (Cell Technology, CA) as previously described [20]. Briefly, the cells that had been induced to apoptosis with exposure to paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel for 72 h were centrifuged at 1000 rpm for 10 min. The collected pellets were treated with 500 μl JC-1 dye and incubated at 37° C in 5% CO_2 for 15 min. After incubation, they were centrifuged at 1000 rpm for 5 min. Supernatants were removed and the pellets were resuspended with 2 ml of assay buffer. Then, they were centrifuged at 1000 rpm for 5 min. Pellets were resuspended again by adding 500 μl of assay buffer. Then, 150 μl from each sample were added into 96-well plate, and the plates were read under 585/590 nm and 510/527 nm wavelengths for the aggregate red form and the green monomeric form, respectively, by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland).

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

mRNA levels of SK-1 and GCS genes were determined in docetaxel, doxorubicin, paclitaxel, cyclophosphamide, and tamoxifen applied to MCF-7 cells by RT-PCR. To this aim, total RNA was isolated from the cells that had been induced to apoptosis through treatment with paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel for 72 h by using Nucleospin RNA isolation kit (Macherey-Nagel) according to the manufacturer instructions. Then, 1 μg of RNA was reverse-transcribed by using reverse transcriptase (Fermentas, USA). First, the reaction tubes were incubated at room temperature for 10 min. Then, they were

incubated at 42° C for 50 min. After incubation at 95° C for 5 min and on ice for 5 min, the cDNAs were used for the mRNA level detection of β-actin, SK-1 and GCS genes by PCR reaction. The primer sequences and the PCR conditions were as follows:
β-actin forward 5'CAGAGCAAGAGAGGCATCCT3',
β-actin reverse 5'TTGAAGGTCTCAAACATGAT3',
SK-1 forward 5'CCGACGAGGACTTTGTGCTAAT3',
SK-1 reverse 5'GCCTGTCCCCCCAAAGCATAAC3',
GCS forward 5'ATGACAGAAAAAGTAGGCT3',
GCS reverse 5'GGACACCCCTGAGTGGAA3'.

2 µl cDNA were used for the amplification of β-actin, SK-1, and GCS [20]. PCR reactions were performed for 35 cycles at 94° C for 5 min, 94° C for 1 min, 53° C for 1 min, 72° C for 1 min and 72° C for 5 min. Finally, RT-PCR products were run on 2% agarose gel for 1 h at 90 V.

Statistical analysis

Statistical significance was determined using two-way analysis of variance, and $p < 0.05$ was considered significant.

Results

All agents showed a dose-dependent cytotoxicity on human MCF-7 breast cancer cells.

The results revealed that increased concentrations of docetaxel (Figure 1A), doxorubicin (Figure 1B), paclitaxel (Figure 1C), cyclophosphamide (Figure 1D), and tamoxifen (Figure 1E) expressed antiproliferative effects on human MCF-7 breast cancer cells as shown by XTT cell proliferation assay. There was dose-dependent decrease in the proliferation of MCF-7 cells in response to paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel as compared with the untreated controls. IC50 values of paclitaxel, doxorubicin, tamoxifen, cyclophosphamide and docetaxel were calculated as 137 nM, 47 µM, 47 µM, 20 mM and 10 nM, respectively (Figure 1).

The MMP increased significantly with the exposure of increased concentrations of the agents in MCF-7 cells comparing to untreated control cells (Figure 2).

Significant decrease in mRNA levels of SK-1 was registered in MCF-7 cells exposed to increasing concentrations of docetaxel, doxorubicin, paclitaxel, cyclophosphamide, and tamoxifen in a dose-dependent manner as compared to untreated cells and normalized to β-actin levels (Figure 3). Similar decrease was obtained in GCS levels in response to docetaxel, paclitaxel, cyclophosphamide, and tamoxifen in a dose-dependent man-
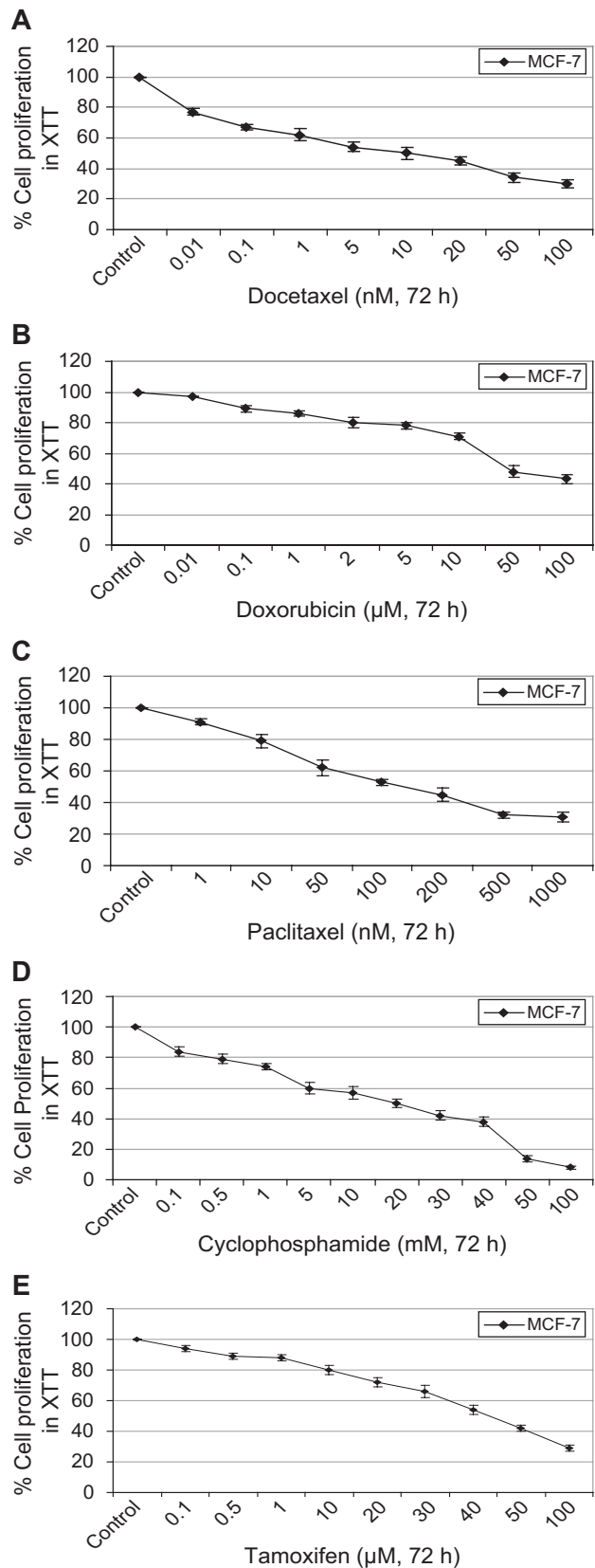
Figure 1. Antiproliferative effects of docetaxel **(A)**, doxorubicin **(B)**, paclitaxel **(C)**, cyclophosphamide **(D)**, and tamoxifen **(E)** on MCF-7 cells. XTT assay was performed using triplicate samples in at least 2 independent experiments. The error bars represent the standard deviations. Statistical significance was determined using two-way analysis of variance, and $p < 0.05$ was considered significant.
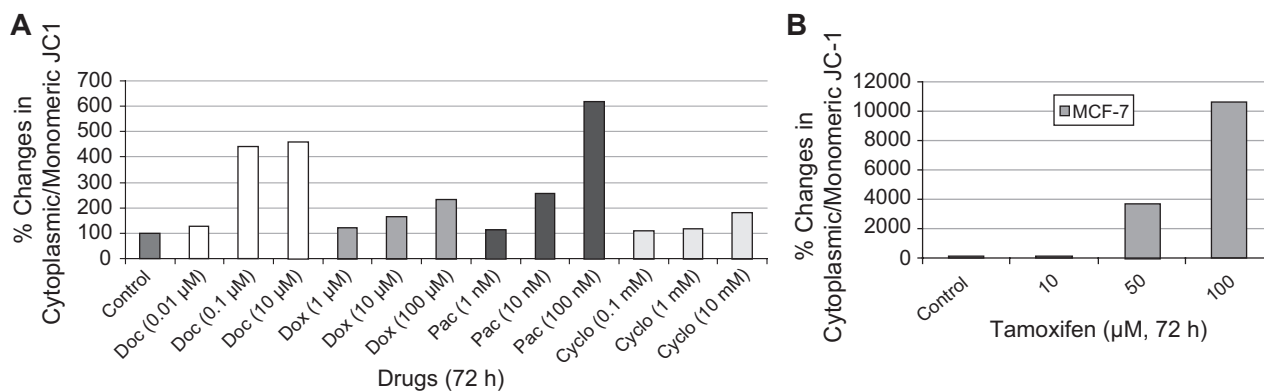
A

B

Figure 2. Percent changes in cytoplasmic/mitochondrial JC-1 in MCF-7 cells exposed to docetaxel **(A)**, doxorubicin **(A)**, paclitaxel **(A)**, cyclophosphamide **(A)**, and tamoxifen **(B)**.
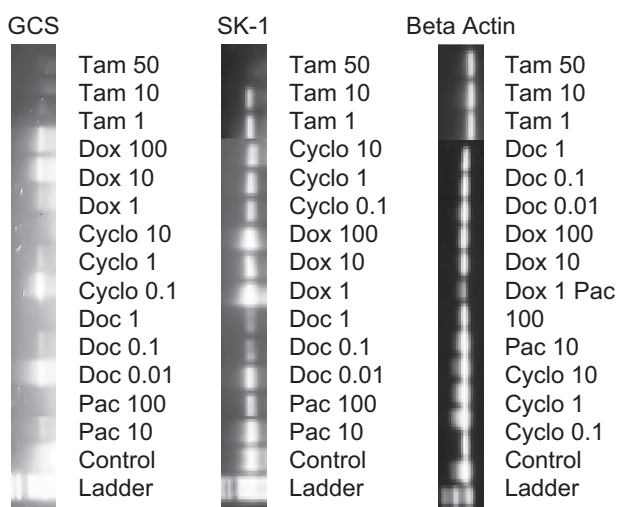

Figure 3. mRNA levels of GCS, SK-1 and beta actin in docetaxel, doxorubicin, paclitaxel, cyclophosphamide, and tamoxifen exposed MCF-7 cells. Expression levels of GCS, SK-1 and beta actin genes were determined by RT-PCR. Expression levels of beta actin was detected as an internal positive control.

ner while no changes in expression levels of GCS were detected in response to doxorubicin (Figure 3).

Discussion

Sphingolipids are known as important structural lipids. However, several studies showed that the roles of sphingolipids in most cellular processes are vital and related with cancer pathogenesis and treatment [1]. Ceramide is the most important biologically active sphingolipid acting as an antiproliferative molecule. Stress conditions increase the intracellular levels of ceramide through *de novo* synthesis or sphingomyelin hydrolysis. Conversion of ceramide to antiapoptotic glucosylceramide by GCS results in the decrease of intracellular ceramide levels and this conversion is known to be as-

sociated with the development of MDR in many cancer types [1]. On the other hand, ceramide can also be converted to antiapoptotic and prosurvival molecule, S1P, by SK-1. That's why both glucosylceramide and S1P levels have been used as resistance markers in various types of cancers. However, stress conditions including chemotherapy can induce apoptosis through downregulation of GCS and SK-1 genes.

In this study, we have shown that docetaxel, doxorubicin, paclitaxel, cyclophosphamide, and tamoxifen have significant cytotoxic effects on human MCF-7 breast cancer cells in a dose-dependent manner. In order to confirm the XTT cell proliferation data, we determined the loss of MMP in response to these agents. The results revealed that they all induce apoptosis through MMP in a dose-dependent manner. Although the mechanisms of action of all the drugs are well known, the roles of SK-1 and GCS in docetaxel-, doxorubicin-, paclitaxel-, cyclophosphamide-, and tamoxifen-induced apoptosis have not been examined before.

Our and different other groups have shown that there is significant increase in the expression levels of GCS and intracellular concentrations of glucosylceramide in drug-resistant cancer cells. Liu et al. have shown that GCS introduced in MCF-7 cells resulted in resistance to doxorubicin and ceramide comparing to wild type cells [21]. Cabot et al. have also shown that introduction of GCS sense and antisense cDNAs into doxorubicin-resistant human breast carcinoma cells affected the results of sensitivity and resistance of breast cancer cells [22]. On the other hand, it was shown that inhibition of GCS expression by the oligodeoxyribonucleotides resulted in increase of sensitivity of doxorubicin-resistant MCF-7 cells to doxorubicin [23]. There are a number of studies supporting the idea that overexpression of GCS also results in increased expression levels of a strong transporter gene, MDR1, in various types of cancers [24-26]. In this study, we examined the expres-

sion levels of GCS in response to a number of anticancer agents to determine whether GCS is involved in apoptosis triggered by these agents. Interestingly, our results showed that there was significant decrease in the expression levels of GCS in MCF-7 cells exposed to docetaxel, paclitaxel, cyclophosphamide, and tamoxifen but not to doxorubicin in a dose-dependent manner.

Increase in intracellular concentrations of the pro-survival metabolite S1P has been used as a marker for drug resistance. There are a number of studies showing increased expression levels of SK-1 and intracellular concentrations of S1P in cancer cells and in drug resistant cancer cells [1,3]. On the other hand, we and others have shown that under stress conditions generated by applying of DNA damaging agents like actinomycin D, etoposide and γ-irradiation, the expression levels of SK-1 are downregulated in different types of cancers [19,27,28]. In parallel with these results, inhibition of SK-1 gene expression by siRNA application or application of SK-1 inhibitors results in increased sensitivity of cancer cells to chemotherapeutics [19,29]. In our study, we have shown that all anticancer agents tested (docetaxel, doxorubicin, paclitaxel, cyclophosphamide, and tamoxifen) resulted in significant repression of expression levels of SK-1 gene in a dose-dependent manner in MCF-7 cells.

In conclusion, we aimed to assess the roles of antiapoptotic and prosurvival genes SK-1 and GCS in docetaxel, doxorubicin, paclitaxel, cyclophosphamide, and tamoxifen induced apoptosis in MCF-7 human breast cancer cells. We have shown for the first time that the anticancer agents docetaxel, paclitaxel, doxorubicin, cyclophosphamide and tamoxifen, induce apoptosis by downregulating the expression levels of the antiapoptotic GCS and SK-1 genes and thus increasing the intracellular concentrations of the apoptotic ceramide, besides their known effects. The results of this study may support the idea that SK-1 and GCS can be novel targets for cancer treatment.

Acknowledgement

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