Time dependent of epigenetic effect of disulfiram on tumor suppressor gene of RASSF1A in Hela cancer cell line

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Abstract

Introduction: Cervical cancer is the third most common tumor among women. Surgery, radiotherapy, and chemotherapy are common treatments, however high stage tumors have frequently poor prognosis. Nowadays, the epigenetic reversion introduced as an efficient strategy of treatment of cervical cancer. In the process, inhibitors of DNA methyltransferase (DNMT) induce re-expression of tumor suppressor genes. Among these inhibitors, disulfiram (DSF) has been suggested as non-nucleoside analogous. In this research, we evaluated the epigenetic effect of DSF on demethylation of the tumor suppressor gene, RASSF1A, in Hela cell line.

Materials and methods: Hela cells were culturated and treated with different doses from 2.5 to 37.5µM during 24, 48 and 72 hours. MTT assay was carried out to find half maximal inhibitory concentration (IC₅₀). The methylation specific PCR (MSP) assay was applied to evaluate methylation pattern.

Results: The IC₅₀ of DSF was determined at the 2.5, 12.5, and 15µM after 72 hours. The MSP results showed partial demethylation at mentioned concentrations after 72h but unmethylated band was not observed after 24h.

Conclusion: Our findings indicated that, IC₅₀ of DSF exerted a biphasic effect in Hela cell line and at least 72 hours treatment is needed for the epigenetic reversion of DSF on RASSF1A in Hela cell line.

Keywords: Disulfiram, RASSF1A, Epigenetic reversion, Methylation, Cervical cancer

Introduction

Cervical cancer is the third most common cancer among women; around 70% of the patients are infected by HPV (16/18) virus(1-2). Unfortunately, this neoplasia is one of the most common life-threatening diseases in the world and the patients usually have less than 5 years survival time after diagnosis. Although combined treatment of chemotherapy, surgery and radiotherapy are recommended for this cancer, still it has poor prognosis(3-4).
Recent studies have demonstrated the role of epigenetic and genetic factors in tumorogenesis. Epigenetic events show phenotype alterations without genetic alterations(5). DNA hypermethylation and histone modification are two important epigenetic events (6). DNA hypermethylation in CPG Islands of promoter region in tumor suppressor genes induces gene silencing without alteration in DNA sequences which are inherited from one generation to the next (7-8).

RASSF1A is one of the critical tumor suppressor genes that induce apoptotic signaling, microtubule stability and cell cycle arrest (9). In one hand, the inactivation of tumor suppressor genes usually occurs by mutation in one allele and hypermethylation in the others. RASSF1A inactivation was reported in different kind of cancers such as lung, breast, bladder, prostate, kidney and cervix (10). On the other hand, RASSF1A is routinely expressed in normal tissue and regulates normal cell cycle, apoptosis, DNA repair and inhibits tumorigenesis (9). Fortunately, the epigenetic events are reversible, for instance using DNA methyl-transferase inhibitor (DNMTi) drugs are recommended to revise abnormal methylation in promoter region of tumor suppressor genes(11). The DNMTi inhibitors drugs are characterized in two groups, which called the cytosine analogous and non-cytosine analogous(12). The cytosine analogous DNMTi such as 5-Aza-CdR which was approved by FDA and used as combined cancer therapy in lymphoma and hematopoietic cancer is toxic (13). As a result, cytosine-like structure in this group can replace cytosine during DNA duplication that induces mutation. Thus, taking steps to find a new approach of cancer therapy of non-nucleoside analogous of DNMTi inhibitors seems to be obligatory (14).

For more than 50 years, DSF has been advised for alcohol abuse, but, recent studies have demonstrated the anti-neoplastic effect of this drug on different tumors and cancer cell lines such as Melanoma, glioma, lung carcinoma, and leukemia(15). Because of strong reactive functional thiol groups in chemical structure of DSF, DSF is suggested as a non-nucleoside analogous of DNMT inhibitors that can be able to attack the cytosine sites on DNA to remove methylated group (16). The exact anti-neoplastic effect of DSF is not clear, so in this study we investigated the epigenetic effect of DSF on re-expression of RASFF1A on cervical cancer cell line.

**Material and methods**

**Cell line and cell culture:** Hela was prepared from the cellular and molecular lab of Babol University of Medical Sciences, and MRC-5 was obtained from National Cell Bank of Pasteur Institute of Iran. Cells were cultured in RPMI (Biowest, France), 10% FBS, 1% penicillin streptomycin (Sigma, USA) at 37°C and 5% CO₂. When they reach 80% confluence, 2×10⁴ cells were transferred to 24 well cell cultures.

**DSF treatment:** The stock solution of DSF (50mM) was dissolved in DMSO (Sigma, USA) and stored at 20°C. Serial dilutions were prepared in different concentrations based on drug reference. Cells were treated with 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, 32.5, 35 µM one day after seeding for 24, 48 and 72h. The IC₅₀ of DSF was obtained at 2.5, 12.5, 15 µM for 72h.

**MTT assay:** The cytotoxicity of DSF was determined in Hela cell line using MTT assay. 2×10⁴ cells were cultured in each well of a 24 well plate overnight, after that and treatment, the MTT assay was performed as described previously (17).

**DNA extraction, bisulfate treatment, and methylation specific PCR:** Genomic DNAs were extracted from treated and control groups of Hela and MRC-5 cells (as unmethylated control) using Prime Prep TM Genomic DNA Isolation Kit (Genet bio, Korea) according to manufacturer's instructions. The amount of DNA extraction was measured by nano drop. The
bisulfate modification of genomic DNA was performed on 2-µl of genomic DNA using Epitect Bisulfite kit (Qiagen, Germany) according to the protocol. The PCR reaction was performed in thermal cycler (Thermocycler, Germany) for methylated primers as: 95°C for 5min followed by 35 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 30s and final extension 72°C for 4min. For unmethylated primers the cycling program was as: 95°C for 5 min followed by 35 cycles of 94°C for 30s, 55.3°C for 30s, 72°C for 30s, the final extension 72°C for 4min. The PCR products were transferred to electrophoresis on 2% agarose gel and visualized with ethidium bromide (17).

Result

The IC_{50} of DSF was determined 2.5, 12.5, 15 µM for 72h by MTT assay and 50%, 45% and 43% of Hela cells were alive at mentioned concentrations, respectively. The IC_{50} and the percentage of apoptotic cells were not remarkable after 24 and 48h of treatment. The results demonstrated a biphasic effect of DSF doses on Hela cells.

Methylation specific PCR (MSP) result:
The methylation specific PCR has not demonstrated any unmethylated band after being treated with 2.5, 12.5, 15 µM after 24h treatment (Figure 1). Partial demethylation was observed after treatment in the mentioned concentrations. After 72h, both methylated and unmethylated bands were demonstrated (Figure 2).

![MSP assay](image1)

**Figure 1.** MSP assay was carried out to determine promoter RASSF1A methylation and unmethylation in Hela cancer cell line after DSF treatment for 24h. There was not observed any unmethylated band after treatment. (C: control, 2.5, 12.5, 15µM DSF treatment, F: fibroblast)

![MSP assay](image2)

**Figure 2.** MSP assay was carried out to determine promoter RASSF1A methylation and unmethylation in Hela cancer cell line after DSF treatment for 72h. Both unmethylated and methylated bands were obtained after treatment with 2.5, 12.5 and 15µM concentrations. (C: control, 2.5, 12.5, 15µM DSF treatment, F: fibroblast)
Discussion

DNA methylation is one of the significant epigenetic events in tumorogenesis(16). The important reason of alteration in DNA methylation pattern is the increase of DNMT enzyme activity in malignant tumors (5). The methylation of CpGs island promoter region of RASSF1A tumor suppressor genes are reported in different type of cancers and cancer cell lines (18). Recent studies have demonstrated DNMTs inhibitors as a new strategy in cancer therapy as combined treatment (19).

In this study, we evaluated the apoptotic effect of DSF in Hela cancer cell line. The result showed no apoptotic effect of DSF in Hela cell line after 24h of treatment. Moreover, the 48th treatment of DSF in Hela cell line declared no significant decrease of cell viability as well. Nevertheless, the IC$_{50}$ of DSF was obtained after 72h treatment. The IC$_{50}$ indicated biphasic effect at 2.5, 5, 12.5 µM concentrations in Hela cell line.

Our results were in accordance with the studies of Wiggins, Zhang, and Jones that demonstrated the biphasic effect of DSF in MCF-7, HePG2, and ovarian cancer cell line(OVCAR-3) (20-22), while Wickstorms and Nikbakht stated the monophasic effect of DSF in ACH, H69AR, PRE and PANC-1, respectively(17, 23). This controversy can be explained by different responses of various cancer cell lines and tumor cells during the exposure of DSF. Furthermore, we investigated the epigenetic effect of DSF on demethylation of RASSF1Ain Hela cancer cell line. The result exhibited no epigenetic effect and no demethylation of promoter RASSF1A after 24h treatment while partial demethylation was observed through 72h treatment. Our results are similar with the study of Sherma that explained DSF as DNMTs inhibitors, induced demethylation and re-expressed ER-B in prostate cancer cell line (24). On the other hand, our results are in contrast with Nikbakht that demonstrated no epigenetic effect but apoptotic effect of DSF on PANC-1(17). This controversy can be explained as time dependent epigenetic reversion of disulfiram, to be exact 24h is not a sufficient time for epigenetic reversion. Stresemann stated at least 72h treatment is essential for epigenetic reversion which was in accordance with our results (25). However, Christoforou explained the decrease of DNMT enzyme activity on DU145 and prostate cancer cell line(PC-3) after 48h treatment with DSF(26). This controversy can be related to different doses, time dependent and different response mechanisms of tumor cells against DSF exposure.

Conclusion

DSF showed partial demethylation after 72h treatment while no epigenetic reversion was found after 24h. In conclusion, we can found that DSF has time dependent epigenetic effect on CpG island promoter region of RASSF1A. As DSF is a safe drug and induces tumor specific toxicity, it is worth using it as a combined cancer therapy drug with chemotherapy and radiotherapy.

In summary, our results showed that DSF has biphasic effect in different cell lines and tumor cells. Partial demethylation occurred in 2.5, 12.5 and 15 µM concentrations.

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