EXPERIMENTAL STUDY

Effects of ceranib-2 on cell survival and TNF-alpha in colon cancer cell line

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ABSTRACT

OBJECTIVE: The aim of this study was to investigate the effects of a novel anti-cancer drug, ceranib-2, which targets the acid ceramidase, in human colon cancer cell line.
MATERIALS AND METHODS: The cell lines were treated with 50 μM of ceranib-2. Relative mRNA expression of TNF-alpha, TNF-R1 and ASAH were assessed by quantitative RT-PCR. RESULTS: Ceranib-2 reduced cell viability in a dose-dependent manner and the apoptotic values of cells following treatment with the dose of 50 μM were reduced significantly both at 24 h and 48 h compared to the control cells ($p < 0.001$). TNF-alpha receptor 1 (TNF-R1) mRNA levels were reduced significantly in the cell lines treated with both 25 μM and 50 μM of ceranib-2 for 24 h compared to the control cells ($p < 0.05$), whereas the difference between the treatment and the control cell lines diminished at 48 h. The human acid ceramidase gene (ASAH) mRNA levels were significantly higher in the cell lines treated with 50 μM of ceranib-2 for 48 h than in the other cell lines ($p < 0.001$).
CONCLUSION: The study shows that ceranib-2 increased apoptosis by inducing ASAH expression and reduced TNF-R1 expression in human colon cancer cell lines in a dose and time-dependent manner *(Fig. 3, Ref. 17)*. Text in PDF *www.elis.sk.*
KEY WORDS: ceranib-2, ceramide, ceramidase, colon cancer.

Introduction

Ceramides, a type of the sphingolipids, in the cell membranes may be produced either by the breakdown of sphingomyelin or glycosphingolipids, by the condensation of serine and palmitoyl-CoA or by the dephosphorylation of ceramide-1-phosphate (S1P) (1). Hydrolysis of ceramide by ceramidase yields in the formation of sphingosine. Ceramides potentiate cellular proliferation and differentiation and pro-apoptotic activity in cancer cells (2). Therefore, defects in ceramide synthesis and signaling have been implicated in cancer pathophysiology (3, 4). It has been suggested that over-expression of ceramidases is associated with an increased resistance to apoptosis, which results in poor prognosis in several types of cancer including melanoma, prostate cancer, head and neck cancers (5, 6). Entirely, these findings indicate that ceramidases are potential targets in cancer therapy. Despite the known actions of sphingolipid-mediated signaling in cancer cells, the effects of ceramidase inhibition are poorly understood.

It has been reported that colorectal cancer (CRC) cells showed a > 50 % decrease in ceramide levels, when compared with normal cells in colon mucosa (7). Actually, ceramide has an important role in the response to chemotherapy in CRC (8). Inhibition of ceramidase activity by ceranib-2 may induced apoptosis in CRC cells by the activation of ceramide levels. Despite that ceranib-2 appears promising as a chemotherapeutic in some cancer cells (9), little is known about the role of the sphingolipid rheostat on its actions, and particularly in CRC.

The present study was designed to investigate the effects of ceranib-2 on the viability of human CRC cells as well as TNF-α, TNF-R1 and ASAH (acid ceramidase) expressions. It reveals for the first time the sphingolipid pathway as a target of ceranib-2 in CRC.

Materials and methods

The human colon cancer cell line Caco-2 (the American Type Culture Collection - ATTC; Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma; St. Louis, MO) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Ceranib-2 (Cayman Chemical, MI) solution was dissolved in dimethyl sulfoxide (DMSO, Sigma) and DMEM was applied for optimal dilution.

Test groups were assigned as followed: control (untreated medium), DMSO (0.01%, control for solvent) and six different doses (1, 2, 5, 10, 25, 50 μM) of ceranib-2.

Cell survival

Following detachment with 0.25% trypsin+EDTA (Sigma), cultured cells were centrifuged at 1200 rpm and 4 °C for 5 minutes and counted with a CEDEX (Roche; Mannheim, Germany) cell counter, seeded overnight in 96 well plates (approximately 10^4 cells per 0.25 mL). The medium was then removed and replaced with either 250 µL of untreated medium (the control) or with ceranib-2 (1 to 50 µM) for 24 or 48 h. The effects of ceranib-2 on colon cancer cell survival were determined by 3-3-(4,5-D-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue (MTT) test (Mossmann 1983). The optical density read at 550 nm with a microplate reader (BioTek; Winooski, VT) from the treated wells was converted to a percentage of living cells against the control using the following formula:

Absorbance of treated cells in each well x 100 / the mean absorbance of control cells.

RNA extraction and determination of TNF-alpha, TNF-R1 and ASAH mRNA levels by q-PCR

The expression of TNF-alpha, TNFR1 and ASAH mRNA was examined in control and ceranib-2 treated Caco-2 cells. The mRNA levels of TNF-alpha, TNFR1 and ASAH in relation to the housekeeping gene were determined by qT-PCR with TaqMan probes. RT-PCR data were collected using the Roche lightcycler nano system. Gene expression levels were reported using the median as a point estimator and the range of values.

Statistical analysis

A minimum of three independent assays were used to calculate the mean results. The *p value* < 0.05 was considered statistically significant. The data are expressed as the mean percent fraction of the control ± standard error of mean. Both one-way variance analysis and Tukey's multiple comparison tests were used to determine statistical significance. RT-PCR results were calculated by using the GraphPad software program. Transcript data were expressed relative to the control (set to 1) ± standard deviation. Following the determination of TNF-alpha, TNF-R1 and ASAH mRNA expression using beta-actin as a reference gene, the data obtained from RT-PCR were calculated using the formula $2^{-\Delta\Delta Ct}$.
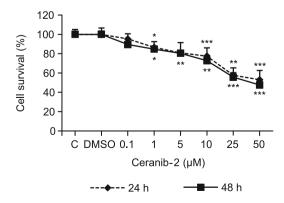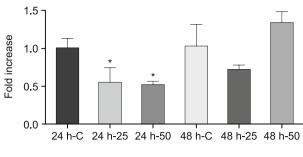

Fig. 2. TNF-alpha receptor mRNA expression.
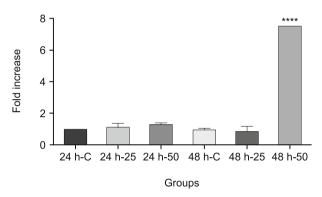

Fig. 3. ASAH mRNA expression.

Results

Cell viability assays

Viability of Caco-2 cell lines treated with 6 different concentrations of ceranib-2 for 24 and 48 h was tested. Either DMSO (solvent) or untreated medium (control) did not significantly change the cell survival rate. Following treatment with 0.1, 1, 5, 10, 25 and 50 µM ceranib-2, the percentages of living Caco-2 cells (against the control) were reduced to 95.2 % (p > 0.5), 86.5 % (* p < 0.05), 80.8 % (** p < 0.01), 77.4 %, 57.7 and 53 % (*** p < 0.001) after 24 h, and 89.4 % (p > 0.5), 84.7 % (* p < 0.05), 80.4 % (** p < 0.01), 72.7 %, 55.6 and 47.5 % (*** p < 0.001) after 48 h (Fig 1), respectively.

Gene expression

TNF-R1 mRNA levels were reduced significantly in cell lines treated with both 25 µM and 50 µM of ceranib-2 for 24 h compared to control cells (p < 0.05), whereas the difference between the treatment and control cell lines was diminished at 48 h (Fig 2). ASAH mRNA levels were significantly higher in cell lines treated with 50 µM of ceranib-2 for 48 h than in the other cell lines (p < 0.001) (Fig 3).

Discussion

Acid ceramidase catalyzes the hydrolysis of ceramide, a potent biological messenger molecule that induces apoptosis, inhibits

Fig. 1. MTT analysis results for Caco-2 cells treated with different concentrations of ceranib-2 for 24 or 48 h.

cellular proliferation and differentiation (10). Sphingosine (Sph) is only formed from ceramide by ceramidase-catalyzed hydrolysis and can be phosphorylated by sphingosine kinases (SphKs) to form S1P. Many studies in various cancer types confirmed our original observation that overexpression of SphK1 and increased S1P production promoted cell growth (11). Considering all this information, it should not be surprising that these enzymes have also been involved in cancer progression, proliferation and inflammation (12, 13) and, thus may provide novel approaches to therapy of cancer (14).

Current research indicates that ceramidase inhibition may stimulate proapoptotic signaling by limiting the conversion of ceramide to S1P, which resulted in ceramide accumulation (15). Consistent with these results, Draper et al (16) reported that ceranib-2 inhibited cell proliferation in human ovarian adenocarcinoma cell line SKOV3, with an IC50 of 28 μM. The present study suggests that ceranib-2 promotes cell death in human colon tumor cell lines at comparatively lower doses.

According to our results, TNF-alpha receptor 1 (TNFR1) mRNA levels were reduced significantly in cell lines treated with both 25 μM and 50 μM of ceranib-2 for 24 h compared to control cells, while the difference between the treatment and control cell lines diminished at 48 h. This suggests that TNFR1 expression may be reduced in time and dose dependently. Peng C et al reported that the decrease in TNFR-1 expression in ovarian cancer cells led to a decrease in cell proliferation (17). It is similar to our study, MTT levels were reduced in cell lines treated with both 25 μM and 50 μM of ceranib-2 at 24 h.

To the best of our knowledge, our study is the first one investigating the effects of ceranib-2 treatment on ASAH gene expression. We have observed that ASAH mRNA levels were significantly higher in cell lines treated with 50 μM of ceranib-2 for 48 h than in the other cell lines, which might be caused by time-dependent changes or by some other reactions, which resulted in the activation of sphingosine known as antiapoptotic lipid. It suggests that, the in vitro treatment of colon cancer cell line (Caco-2) using six different concentrations of ceranib-2, cell viability was reduced in the presence of a ceramidase inhibitor dose-dependently.

Conclusion

Our study shows that ceranib-2 has strong dose and time dependent anti-cancer effects and ceranib-2 might have some other effects on the pathways in the cancer pathogenesis. In addition, some other reactions, which resulted in formation of sphingosine by breakdown of ceramide with ceramidase might have an effect on apoptosis. In our future studies, we will focus on other possible effects of ceranib-2 on different pathways to explain our present results.

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