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Proapoptotic effects of ceranib-2 in combination with radiation therapy on human breast cancer cells

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Abstract. – OBJECTIVE: Strategies for cancer therapy involve radiation therapy (RT), which accounts for about 40% of all cancer treatment types. As to current chemotherapeutics, cancer cells also develop resistance that remains a clinical problem, such as disease recurrence. Recent studies focused on understanding the molecular mechanisms of radiation-induced cell death. Conventional RT aims at treatment with a single fraction per day of 8-30 Gy per fraction. Radiotherapy increases intracellular ceramide levels that trigger cell death. Additionally, increasing intracellular ceramide by radiation may restore therapeutic sensitivity to cancer treatments. Drugs that inhibit ceramide-metabolizing enzymes like ceramidases are expected to be radiotherapy sensitizers.

MATERIALS AND METHODS: In this research, we investigated the proapoptotic effects of SRS alone and in combination with ceranib-2, a ceramidase inhibitor in human breast adenocarcinoma cells. The molecular mechanism of action of RT and ceranib-2 was investigated on MCF-7 cells exposed to 13 μM ceranib-2 for 24 hours following 20 Gy radiation using MTT, radiotherapy, and annexin-V analyses.

RESULTS: Results indicated that the dose of 20 Gy radiation induces apoptosis on human breast cancer cells with and without co-treatment with ceranib-2 by causing cytotoxicity in the cells. Based on the results of ceranib-2 exposure, it can be concluded that the mechanism of action may rely on an increase of intracellular ceramides, also called apoptotic lipids.

CONCLUSIONS: The study results suggest that co-treatment of human breast adenocarcinoma cells with a ceramidase inhibitor, ceranib-2, and a high dose of radiation of 20 Gy exerted cytotoxicity and apoptosis and might be a solid, potent alternative to current therapy strategies.

Key Words:
Breast cancer, Radiotherapy, Ceranib-2, Apoptosis, Cytotoxicity.

Introduction

Breast cancer (BC) is an essential leading health problem among women due to its high mortality and morbidity rates. Today, several treatments are available to treat BC, including surgery, radiotherapy, chemotherapy, hormone therapy, and immunotherapy. Despite the availability of current medicines, their incidence and mortality remain high[1]. Overall, it is the second leading cause of cancer-related deaths among women after lung cancer. Therefore, there is a need to develop new treatment strategies and agents[2].

Radiation therapy (RT) is an essential method for cancer treatment and accounts for at least 40% of all cancer treatments. However, resistance to treatment remains a clinical problem[3]. Significant progress has recently been made in understanding the molecular mechanisms of radiation-induced cell death. The type of cell death after radiation depends on several factors, including cell type, radiation dose and quality, oxygen tension, TP53 status, DNA repair capacity, cell cycle stage at the time of radiation exposure, and microenvironment. Traditionally, there are four mechanisms of radiation-induced cell death: mitotic cell death/mitotic catastrophe, apoptosis, necrosis, and autophagy. The trials of new radio-sensitizers that target key cell-death pathways and the stromal and immune microenvironment have drawn significant attention, especially in combination with stereotactic ablative body radiotherapy (SABR)[4].

Conventional fractionation aims at treatment with a single fraction per day, from Monday to Friday, at a dose of 1.8-2 Gy per fraction[5]. Stereotactic radiosurgery (SRS) and stereotactic body radiation therapy (SBRT), known as SABR, contain the delivery of single or several large dose fractions of 8 to 30 Gy per fraction[6].

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DNA damage represents the primary tumoricidal process in conventional fractionated RT. Growing evidence in the literature reports that high doses of irradiation, commonly used in SBRT and SRS, lead to indirect cell death as a result of vascular damage and also directly kill tumor cells through DNA damage. In addition, high-dose irradiation per fraction has been shown to augment an antitumor immune response. Such secondary cell death due to vascular damage and immune response seems to play a crucial role in response to treatment with SBRT and SRS[7].

Apoptosis is a highly regulated form of cell death with characteristic morphological and molecular features. It occurs in 3 different ways: the intrinsic (mitochondrial) pathway, the extrinsic (tumor necrosis factor) pathway, and the ceramide pathway. Sphingolipids regulate biological processes such as growth, proliferation, migration, invasion, and metastasis by controlling signaling functions within the cancer cell signaling network. Sphingolipid metabolites such as ceramide, sphingosine, and sphingosine-1-phosphate (S1P) are involved in the control of cell proliferation and apoptosis. Ceramide and sphingosine, the production of which is induced by chemotherapy, radiation, or oxidative stress, mediate cell death, aging, and cell-cycle arrest[8]. S1P is a suppressive agent of the ceramide-dependent apoptosis mechanism. Therefore, the balance of ceramide and S1P levels in cells is closely related to the fate of cells to survive or die. Thus, regulation of the ceramidase enzyme and control of intracellular ceramide, sphingosine, and S1P rates play a vital role in the mechanism of apoptosis[9]. The intracellular level of the acid ceramidase (AC) enzyme determines the ceramide/S1P ratio, which determines cell fate. The expression of AC, which is upregulated during the cancer process, acts as a "double-edged sword" by converting pro-apoptotic ceramide into anti-apoptotic S1P, leading to a decrease in the anti-apoptotic ceramide levels and an increase in the S1P levels and aggravating the progression of cancer[10]. The types of sphingolipids believed to be most relevant to BC development, disease progression, and response to treatment are ceramide and S1P[11].

Ceranib-2, a new type of AC inhibitor, is known to significantly induce apoptosis in different cells such as human ovarian adenocarcinoma cells[12], rat fibroblast cancer cells[13], and breast adenocarcinoma MCF7 cells[14]. The literature has reported that these enzyme inhibitors have excellent potential in developing new anticancer drugs[12].

As a radiation-induced pathway, the ceramide pathway is triggered by radiation-induced activation of acid sphingomyelinase in the plasma membrane, producing ceramide *via* hydrolysis of sphingomyelin. Radiation-induced DNA damage can activate mitochondrial ceramide synthase for *de novo* ceramide synthesis. Ceramide acts as a second messenger in the initiation of the apoptosis[15]. The ceramide apoptotic pathway in tumor vascular endothelium, activated by ablative radiation doses, is particularly important[16].

Ceramide-based cell death induced by ionizing radiation has been reported to be both dose- and time-dependent, and this has been confirmed in various *in vitro*[17] and *in vivo* studies[18]. The mechanism of radiation-induced cell death varies depending on low or high doses of ionizing radiation. It is known[19,20] that cell death induced after single high-dose radiation (>8-10 Gy) is governed by the ceramide pathway.

Radiotherapy increases intracellular ceramide levels, possibly restoring therapeutic sensitivity to these treatments[21-24]. Therefore, drugs that inhibit ceramide-metabolizing enzymes are expected to be radiotherapy sensitizers. In this research, we investigated the effects of SRS alone or in combination with ceranib-2 on apoptosis in human breast adenocarcinoma cells.

Materials and Methods

Human breast adenocarcinoma MCF-7 (ATCC® HTB-22™) cells were purchased from the American Type Culture Collection (ATCC, VA, USA). Ceranib-2, Fetal bovine serum (FBS), Penicillin/Streptomycin, Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT), Trypsin/ETDA and Roswell Park Memorial Institute (RPMI-1640) were obtained from Sigma-Aldrich (St. Louis, USA). Annexin-V Kit was purchased from Merck (Millipore, USA).

Cell Culture

MCF-7 (ATCC® HTB-22™) cells were cultured in fresh RPMI-1640 medium supplemented with fetal bovine serum (10%) and penicillin/streptomycin (1%) in a humidified CO_2 (5%) incubator. Cells were passed each third day. Flasks with a confluency of 80% were used in the tests. The passage number of the used MCF-7 cells was 15.

Radiation Therapy

Human breast adenocarcinoma cells MCF-7 were plated in sterilized Petri dishes and kept

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in an adequate cell culture condition since they became confluent. Three replicates with MCF-7 cells were irradiated with 20 Gy per fraction using an Elekta Versa HD linear accelerator device (Elektra Solutions AB, Stockholm, Sweden) in Eskisehir City Hospital, having 6 MV FFF energy in a 10x10 cm^2 area with a source-skin distance (SSD) of 100 cm, giving the total dose of 2,300 MU at a maximum dose rate of 1,400 MU/minute. To release the maximum effective dose of 6 MV FFF energy to the culture in the plate, the periphery of the plate was covered with a bolus (skin equivalent) of 2 cm thickness and 1 cm on the top. These groups of cells were used as positive controls. Another three plates with MCF-7 cells were exposed to the IC_{50} value of ceranib-2 obtained from previous studies[25]. This group of cells was also exposed to 20 Gy radiation and used as test cells. Briefly, the molecular mechanism of action was investigated on MCF-7 cells exposed to 13 µM ceranib-2 for 24 hours following 20 Gy radiation. The other three plates with MCF-7 cells were untreated and used as negative controls. After 24 hours of exposure, each group was separately collected with trypsinization and used for further experimentation. The workflow of the study is given in Figure 1.

MTT Colorimetric Assay

MCF-7 cells were exposed to radiation and co-exposed to ceranib-2 with radiation. Unexposed were separately plated in flat bottom 96 well culture plates (Corning, NY, USA) in triplicates ($5x10^5$ cells per well), and 20 µL/well of MTT solution (5 mg/mL in PBS) was added to each well. Plates were incubated for 4 hours. After the incubation period, 200 µL of SDS (10% in distilled water) was added per well to dissolve the formazan crystals. All test plates were read on a plate reader (BioTek HTX Synergy, Vermont, USA) at a wavelength of 540 nm. Viability percentages were calculated as mean SD values based on the absorbances obtained from the reader.

Phosphatidylserine Translocation Assay

Annexin-V analysis was performed to detect the cell death mode of MCF-7 cells exposed to ceranib-2 and radiation of 20 Gy. This test calculated the translocation of phosphatidylserine on MCF-7 apoptotic cells. Briefly, MCF-7 cells exposed to 20 Gy, co-exposed to 20 Gy, and IC50 value of ceranib-2 and untreated cells were collected by trypsinization washed twice in PBS and resuspended (in PBS). After this period, each test group separately was transferred into tubes (Eppendorf, Hamburg, Germany) per 100 µL. Annexin-V solution (100 µL) was added to each sample tube. After gently mixing, samples are incubated for 15 minutes in the dark at room temperature. Following the incubation, cells were analyzed using Muse™ Cell Analyzer (Merck, Millipore, Hayward, CA, USA) according to the user instructions of the manufacturer of Annexin-V kit (Merck, Millipore, Hayward, CA, USA).

Statistical Analysis

One-way ANOVA analysis and Tukey post-hoc test, performed with GraphPad Prism 8 statistical package program (GraphPad Software, La Jolla,

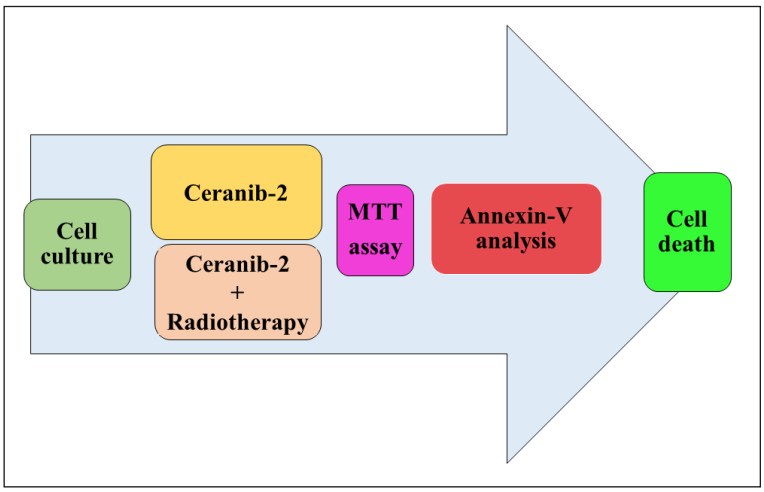

Figure 1. The workflow of the study.

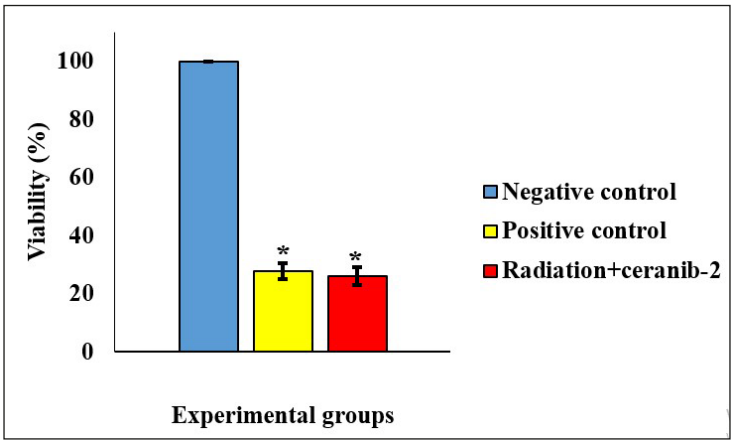

Figure 2. Growth inhibition graph of MCF-7 cells after treatments. (*p<0.5).

CA, USA), were used to evaluate the test data statistically. p<0.5 was taken as statistical significance.

Results

MTT Colorimetric Assay

MTT results (Figure 2) indicated that MCF-7 cell growth was inhibited after exposure to 20 Gy radiation. Also, the viability of MCF-7 cells decreased after co-treatment with ceranib-2 and 20 Gy radiation. The inhibition of viability was detected to be statistically significant (p<0.5) in both MCF-7 cells compared with untreated MCF-7 cells used as negative control cells in the experimentations. The viability of cells exposed to 20 Gy radiation was detected to be 27.9%, which indicates a decrease of 72.1%. The cell viability of ceranib-2 and 20 Gy radiation co-treated MCF-7 cells was 26.2%, representing 73.8% growth inhibition compared to the negative control group. A statistically significant difference was not detected between positive control and ceranib-2-radiation co-treated MCF-7 cell groups. Only a 1.7% difference in viability percentages was recorded.

Phosphatidylserine Translocation Assay

The translocation of phosphatidylserine to the outer membrane of a cell is a clear sign of apoptosis. Herein, the annexin-V technique indicated the translocated phosphatidylserine, and it was detected that after exposure both to ceranib-2 and 20 Gy radiation, apoptosis was induced both in positive and co-treated MCF-7 cell groups (Figure 3). The total apoptotic cells on positive control cells (Figure 3B) had an entire apoptotic profile of 26.4% compared to the untreated cells used as controls (Figure 3A). Early apoptotic cell percentage for his group of cells was detected to be 21.9%, and 4.5% late apoptotic cells were detected. On MCF-7 ceranib-2 and 20 Gy radiation co-treated cells (Figure 3C), the total apoptotic cell percentage was 29.98%, of which 28.65% were early, and 1.35% were late apoptotic.

Discussion

Breast cancer is the most common malignancy in women worldwide, leading to 626,629 deaths worldwide in 2018. About 30% of patients develop metastases during disease, and more than 90% of deaths from BC are due to complications related to metastasis[26]. Using SBRT to treat BC-related metastases is a safe and valuable treatment[27]. Herein, it has been shown that the use of high-dose radiotherapy (20 Gy, frequently used in treating BC-related metastases) together with ceranib-2 can provide a strong synergy in inducing apoptosis compared to SBRT alone. Additionally, this combination was found to be highly cytotoxic on MCF-7 cells. The main contribution of this study to research progress in the field is that it underlines and provides a good, potent therapy strategy as an alternative to current therapy strategies after a deeper evaluation of the relationship between radiation and sphingolipid pathway members.

Studies[28] on BC consistently show that local ablative radiotherapy in BC patients with oligo-metastatic disease provides very high local control rates of up to 90% and increases disease-free survival

and overall survival, depending on condition- or patient-spe cific factors. SBRT doses used in BC treatment vary between 24 and 60 Gy per 1 to 10 fractions, depending on the location and size of the lesions[29]. Our study chose a dose of 20 Gy, frequently used in treating BC-related metastases.

Apoptosis and proliferation are in a balance in tissues under normal physiological conditions. Any impairment in this balance may lead to atrophy or cancer. Decreased apoptosis or resistance to apoptosis plays a vital role in carcinogenesis. Today, as the mechanism of apoptosis is understood, it has become an alternative target of cancer treatment. Radiotherapy can be used alone or in combination with surgery and chemotherapy in cancer treatment. Cell death due to radiotherapy occurs by different mechanisms, including apoptosis[30]. In our study, it has been shown that the use of high-dose radiotherapy in combination with ceranib-2 can provide a strong synergy in the early apoptotic effect compared to SBRT alone.

Previous studies[31,32] have reported that ceramide production in tumors and tumor vessels remains low at a dose of 2 Gy irradiation. Therefore, the interaction between the dose of X-ray irradiation and ceramide production needs to be further investigated[33]. In their study, Cheng et al[34] found that 5 Gy ionizing radiation in prostate cancer caused an increase in sphingolipid expression between 24 and 72 hours, specifically increasing the total ceramide content by 40-100%. Again, Mesicek et al[35] showed that radiation-induced apoptosis was dose-dependent in HeLa cells, with a dose-dependent elevation in total cellular ceramide levels occurring before radiation-induced apoptosis, with a maximum 2.34-fold increase after 28 hours at a dose of 15 Gy radiation compared to the dose of 5 Gy. In the study conducted by Santana et al[18] to demonstrate the role of acid sphingomyelinase in inducing apoptosis with ionizing radiation, a small apoptotic effect occurred up to the dose of 7.5 Gy, and the maximum apoptotic effect was achieved at the dose of 20 Gy. Our study found that the dose of 20 Gy radiation alone or combined with ceranib-2 caused strong apoptosis in MCF-7 breast cancer cells by increasing the amount of ceramide. Vethakanraj et al[36] showed that cancer cells with AC expression had increased cell proliferation, metastasis, chemo-resistance, and radio-resistance, stating that pharmacological inhibition of AC promoted cell death by sensitizing resistant cells to chemo/radiotherapy.

Limitations

Also, radiotherapy has some limitations, like the requirement of precise case-dependent calibration of irradiation as well as strong control of treatment conditions in order to protect the patients from the adverse effects of this treatment. Additionally, the main mechanism of ceramides to make sensitive the cancer cells to radiotherapy needs to be recovered in detail for preventing the radiotherapy-dependent degradation of the used ceramidase inhibitor-ceranib-2 and to enhance the anticancer activity in breast cancer treatment. It has been reported[33] that ceramide- and sphingosine-metabolizing enzyme inhibitors or synthetic ceramides may act as sensitizers of radiotherapy and chemotherapy for head and neck squamous cell carcinoma. Similarly, our study revealed evidence that cancer cells may become

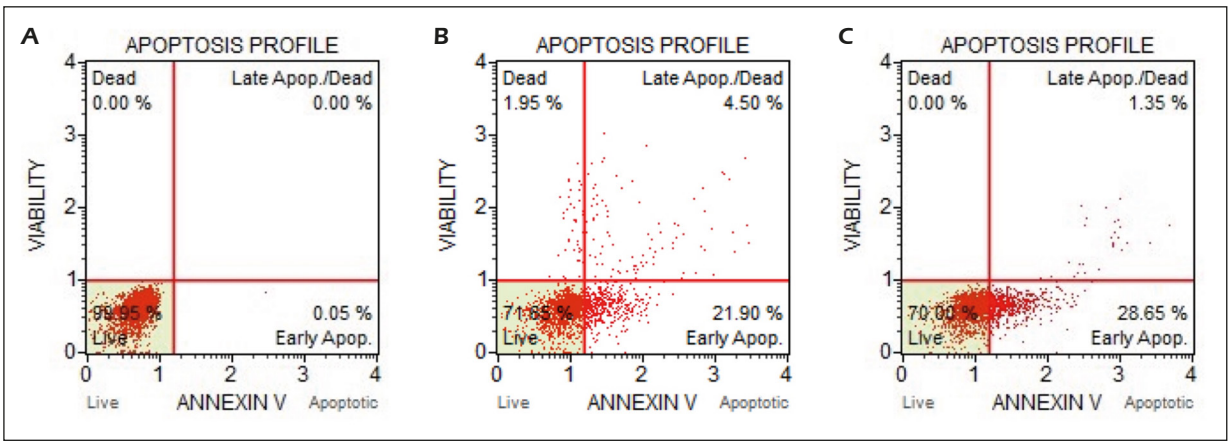

Figure 3. Cell death mode on MCF-7 cells after treatments. **A**, Untreated MCF-7 cells; (**B**) MCF-7 cells exposed to 20 Gy radiation; (**C**) MCF-7 cells co-treated with ceranib-2 and 20 Gy radiation.

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radiosensitive with the combination of SBRT and ceranib-2.

Conclusions

In conclusion, all findings of this study showed that the dose of 20 Gy radiation induces apoptosis on MCF-7 cells with and without co-treatment with a ceramidase inhibitor ceranib-2. A high dose of radiation of 20 Gy and ceranib-2 kills human breast adenocarcinoma cells by causing cytotoxicity in the cells. Based on the ceranib-2 exposure, it can be thought that the mechanism may rely on an increase of intracellular ceramides called apoptotic lipids. However, further investigations are needed to clarify the exact mechanism of action. More research regarding *in vitro* and *in vivo* anti-cancer efficacy is required for more profound conclusions. Nonetheless, it can be concluded that co-treatment of human breast adenocarcinoma cells with ceramidase inhibitor ceranib-2 and high doses of radiation might be an excellent alternative to current therapy strategies.

Conflict of Interest
The authors declare no conflict of interest in this study.

Ethics Approval
This cell-culture research is conducted using commercially available human breast cancer cells MCF-7. Thus, ethics approval was not needed for this study.

Informed Consent
This cell-culture research is conducted using commercially available human breast cancer cells, MCF-7. Therefore, informed consent was not required.

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None.

Authors' Contribution
Fuzuli Tugrul: Planning, designing, literature survey, interpretation of the results, active intellectual support.
Canan Vejselova Sezer: Planning, designing, data collection, literature survey, statistical analysis, interpretation of the study results, English editing. Hatice Mehtap Kutlu: Planning, designing, data collection, literature survey, interpretation of the results, active intellectual support.

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Availability of Data and Materials
All data for this study is presented in this paper.

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