The combined effects of all-trans-retinoic acid and docosahexaenoic acid on the induction of apoptosis in human breast cancer MCF-7 cells

ABSTRACT

Introduction: Breast cancer is one of the most women's cancers in the worldwide. In vivo and in vitro studies showed that all-trans-retinoic acid (ATRA) and docosahexaenoic acid (DHA) can modulate differentiation and apoptosis in both cancer and immune cells. Nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) activation in the presence of their ligands, plays a critical role in the proliferation, differentiation, and apoptosis of normal cells.

Aim of Study: We hypothesized that ATRA and DHA, as ligands of RARs and RXRs respectively, may have synergistic effects on the induction of apoptosis in MCF-7 human mammary carcinoma cell lines.

Materials and Methods: MCF-7 cells were seeded in a 24-well plate at 3 x 10⁵ cells per well. The cells were treated with 5 µM ATRA, 30 µM DHA, and various combinations of them over a 3-day trial. Apoptosis was measured by Annexin V-FITC kit and flow cytometry.

Results: Our results showed that the combination treatment of ATRA and DHA (5 µM and 30 µM and half dose at 2.5 µM and 15 µM, respectively) in a dose-dependent manner induced apoptosis rate in MCF-7 cells significantly more than single treatment of ATRA or DHA, as compared to control group (P < 0.05).

Conclusion: We conclude that the combination of ATRA and DHA at the well-balanced proportion may be effective in cancer cell apoptosis. Further studies provide details about the potential synergistically effects of combination treatment of ATRA and DHA in growth inhibition and differentiation of human mammary cancer cells.

KEY WORDS: All-trans-retinoic acid, apoptosis, breast cancer, docosahexaenoic acid, MCF-7 cell

INTRODUCTION

Breast cancer is one of the most women's cancers in the worldwide and leading cause of death from cancer is fifth leading cause of death from cancer.[1] Breast cancer incidence rate and its mortality are increased rapidly in developing countries over the last 20 years.[2] Some breast cancer risk factors are nonmodifiable including family history, obesity, lactation, menstrual and reproductive history.[3] The critical role of nutrition and diet as a modifiable risk factor in cancer etiology derived from antioxidant benefits and anti-cancer properties of selected nutrients.[4] In cancer, balance between cell division and death is disrupted.[5] Apoptosis or programmed cell death is an important control mechanism following DNA damage.[6] Therefore, reduced apoptosis or its resistance can modulate cancer cell growth.[7] Current strategies for cancer therapy is focused on apoptosis as a desirable target of cell control in cancer.[8] Early studies have shown the protective effects of omega-3 fatty acids and retinoids in breast cancer.[9,10] Possible mechanisms include cell abnormal growth suppression, neoplastic suppression, induction of differentiation, and apoptosis.[11,12] These components in contrast to their effects in cancer cells do not induce apoptosis significantly in the normal and noncancerous cells including breast and colon cancer cells.[13-15]

Epidemiological studies have shown fish oil may reduce breast cancer. Fish oil is a rich source of long chain n-3 fatty acids, especially docosahexaenoic acid (DHA).[16] Enhanced apoptotic effects of DHA were shown in MCF-7 human breast carcinoma cells in vitro and in vivo in animal models.[17,18] DHA inhibits breast tumors incidence, progression, metastasis, and reactive oxygen species production lead to cell death.[19] Numerous studies have indicated that all-trans-retinoic acid (ATRA), the active metabolite of Vitamin A, has anti-cancer...
effects and considered a potent inhibitor of malignant transformation.[20]

All-trans-retinoic acid regulates cell cycle progression[21] and enhances sensitivity of cells to the anti-cancer agents in some type of cancers.[22] ATRA can reduce MCF-7 breast carcinoma cell proliferation and induce apoptosis in vitro.[23] An even greater reduction of cancer cell progression is observed when retinoids were treated in combination with omega-3 fatty acids.[24] Heterodimerization of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) and binding to their ligands, DHA and ATRA, is responsible for synergistic effects of retinoids and fatty acids on carcinogenesis. The RXR-RAR heterodimers bind to DNA and activate gene expression, cell proliferation, and cell differentiation.[25] According to previous studies, there is a potential for combined synergistic effects of ATRA and DHA on induction of apoptosis in breast cancer cells. The purpose of this study was to investigate the effects of a combination treatment of ATRA and DHA on the induction of apoptosis in MCF-7 human mammary carcinoma cell lines.

MATERIALS AND METHODS

Cell culture
MCF-7 human mammary carcinoma cells were obtained from National Cell Bank of Iran (NCBI Code: C135), which is affiliated to Pasteur Institute of Iran. Cells were cultured in RPMI-1640 (GIBCO, USA) containing 10% fetal bovine serum (FBS, GIBCO, USA) and 1% penicillin-streptomycin (GIBCO, USA) at 37°C with 5% CO2. The cells cultured in 1 × 75 cm2 cell culture flasks until reach 80% confluence. Then MCF-7 cells were washed with trypsin solution and centrifuged for 5 min at 1800 RPM. The cells were resuspended by gently vortexing in 1 mL fresh media after discarding the supernatant. Cells were counted using the trypan blue method on a hemocytometer to count the number of viable cells in 1 mL.

For cell counting, once 80% of confluence is reached, MCF-7 cells were washed with trypsin solution and centrifuged for 5 min at 1800 RPM. The cells were resuspended by light gently vortexing in 1 mL fresh media after discarding the supernatant. Cells were counted using the trypan blue method on a hemocytometer to count the number of viable cells in 1 mL.

Preparation all-trans-retinoic acid and docosahexaenoic acid and cell treatments
All-trans-retinoic acid and DHA were purchased from Sigma–Aldrich (St. Louis, MO, USA). They were dissolved in 100% dimethyl sulfoxide (DMSO) (MP Biomedicals, The Netherlands) under dim-light condition and were stored at −80°C until use. The compounds were diluted from the stock solutions to a final concentration of ATRA at 1, 5, and 10 µM and DHA at 15, 30, and 40 µM prior to being incubated with the cells. Negative control cells were treated with 0.1% DMSO.

For cell treatments, cells were seeded in 24-well plates with a density of 3 × 104 cells per well. The cells were incubated for 24 h at above-mentioned condition until the cells reached 80% confluence. Then media was removed and replaced with fresh FBS-free media containing ATRA and DHA alone at different concentrations of 1, 5, and 10 µM for ATRA and 15, 30, and 40 µM for DHA; Both at 24, 48, 72 and 96 h as pilot. At the next step on the based on pilot results, the cells treated with 5 µM of ATRA, 30 µM of DHA, and combination of them including ATRA 5 µM + DHA 30 µM and ATRA 2.5 µM + DHA 15 µM for an incubation period of 72 h. The last treatments run in two independent experiments each performed in triplicates.

Cell apoptosis assay
Apoptosis were analyzed using an Annexin V-FITC/PI apoptosis detection kit (BioVision, K101-25, Milpitas, CA, USA) according to the manufacture’s protocol. Briefly, MCF-7 cells incubated with different proportion of treatment solutions for different incubation times, as mentioned above. Control wells treated with (0.1% DMSO). Cells were trypsinized, and washed with 500 µl of binding buffer, and incubated with 5 µl conjugate Annexin V-FITC and 5 µl propidium iodide (PI) for 15 min in the dark and room temperature and analyzed by flow cytometry (FACS Calibur, BD Biosciences, USA) method.

Statistical analysis
Student’s paired t-test and ANOVA test were used to determine a statistically significant difference between treatment and control groups. A P < 0.05 was considered statistically significant.

RESULTS

The results of the pilot experiment have shown in Figures 1 and 2. Different concentrations of ATRA and DHA induced apoptosis in MCF-7 cells compared to the control except for the first 24 h (data not shown). As shown in figures, the highest apoptosis rate were found in ATRA at 5 µM and DHA at 30 µM concentrations that obtained statistically significant following 72 h incubation (P < 0.05).

The results of next step of experiment showed that the single treatments of ATRA at 5 µM and DHA at 30 µM and combination treatment of them (ATRA plus DHA at dosage of 2.5 µM + 15 µM or 5 µM + 30 µM, respectively) after 72 h significantly induced apoptosis in MCF-7 cells compared to the control (7.12%, 9.23%, 9.82%, and 12.49% increasing apoptosis rate respectively) (P < 0.05) [Figure 3].

In addition, in combination groups significant induction of apoptosis was observed when compared to ATRA group alone (in ATRA 2.5 + DHA 15 µM group 2.7% and in ATRA 5 + DHA 30 µM group 5.37% increasing). The promotion of apoptosis between DHA 30 µM and combined treatment at half dose of single treatment (ATRA 2.5 µM + DHA 15 µM) did...
It is necessary to mention that FITC Annexin V positive cells are in early apoptosis, whereas FITC Annexin V and PI positive cells are in late apoptosis. Results are expressed as a percentage of positive cells.

**DISCUSSION**

In the present study, the combination treatment of ATRA, the most active metabolite of Vitamin A, and DHA, an omega-3 fatty acid, ligands of RAR and RXR, respectively, enhanced a synergistic and dose-time dependent effect on apoptosis in breast cancer MCF-7 cells. For the best of our knowledge, this is a novel effect that not demonstrated before.

Retinoic acid plays an important role in the gene transcription and as a necessary factor in the growth of epithelial cells. RA exerts its effects through interaction with the nuclear receptors, RARs and RXR. These receptors are ligand-dependent transcription factors. ATRA especially binds to RAR and activate it, while 9-cis RA binds to both RARs and RXRs and activate them.

Docosahexaenoic acid, a member of the omega-3 polyunsaturated fatty acids, has antineoplastic properties. It has been observed that DHA causes cancer cell death by induction of cytotoxicity in tumor cells and increasing uptake of anticancer drugs, and enhancing pro-oxidant and pro-apoptotic effects of chemotherapeutic agents. Binding of ATRA to RARs, facilitates binding of DHA to RXRs and active RAR/RXR heterodimers are formed. When ATRA binds to RAR, facilitates binding of DHA to RXR and leads to RAR-RXR heterodimers. RAR-RXR heterodimers bind to RA response elements and modulate the expression of the target genes involved in normal cell proliferation, differentiation, and apoptosis.

The results of the present study showed that DHA increased MCF-7 apoptosis which is in consistent with the literature. Previous studies demonstrated that ATRA is a ligand for RARs and may amplify the effect of RXRs ligand, that is, DHA in MCF-7 cells. It is suggested that ATRA and DHA can target enteric and extrinsic apoptosis pathways through their effects on key proteins and enzymes which are involved in apoptosis pathways. This combination can increase the expression of pro-apoptotic genes such as Bax and Bak while reduce gene expression of anti-apoptotic proteins including Bcl-2 and Bcl-XL.

Our finding is parallel with the study of Crowe and Chandraratna, which demonstrated that AGN194204, the synthetic RXR-selective ligand, and linolenic acid which binds to peroxisome proliferator-activated receptors (PPARs) can enhance the effect of apoptosis through heterodimerization of RXRs/PPARs in breast cancer cell lines. It has been...
shown PPAR-γ have anti-carcinogenesis effects on breast cancer cells as shown combination of PPAR-RXR dimers ligands enhance activity of responsive promoter in breast cancer cells.\textsuperscript{[36,37]}

In general, there are no many literatures on the field of efficacy of combination therapy trough nuclear receptors ligands on breast cancer prevention or treatment. Previous studies demonstrate that ATRA and RXR-selective retinoids in combination with omega-3 fatty acids reduce cell growth in MCF-7 cells\textsuperscript{[18,24]} but the synergistic effect of ATRA and DHA as ligands of RAR-RXR dimers on induction of apoptosis and dose-time response of them in breast cancer cells has not been demonstrated by other studies clearly. These findings indicate targeting RAR and RXR heterodimers and their ligands, presents an approach to prevent and treatment of breast cancer. Clearly, further investigations will require to measurement the gene expression of key factors involve in apoptosis such as caspase enzymes and anti-apoptotic or pro-apoptotic proteins follow combination treatment which is limited in our study. Furthermore, another experiment is needed to explore the molecular pathways involved RAR-RXRs and effects of combination therapy on mammary carcinogenesis or the potential role for synergistic aspects of combination treatment of ATRA and DHA.

REFERENCES

Abdolahi, et al.: The combined effects of ATRA and DHA on the apoptosis of breast cancer cells


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