TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand) mediated Apoptosis of human breast cancer cells sensitized by dietary flavonoid Kaempferol

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Abstract-Kaempferol is one of the most prevailing flavonoid distributed in edible plants while TRAIL(tumor necrosis factor-related apoptosis-inducing ligand)is a promising anti-cancer agent. Resistance to TRAIL, limits its potential as a drug for cancer therapy. Cytotoxicity induced by Kaempferol and TRAIL alone and in combination was measured by MTT assay. Apoptosis was detected by DNA fragmentation assay and measured by real time PCR and western blotting techniques. The study clearly showed the dose dependent cytotoxic effect of Kaempferol in combination with TRAIL in all the breast cancer cells. The dying cells showed characteristics of apoptosis such as DNA fragmentation in combined treatment with Kaempferol and TRAIL in human breast cancer cells appreciably, compared to single treatments. Furthermore, it has also been found that treatment with Kaempferol enhances TRAIL-induced apoptosis by increasing expression of apoptosis-related proteins including caspase-3, 8, 9 and Bax and by decreasing the expression of Bcl-2. The present work for the first time shows that the combined treatment with Kaempferol and TRAIL drastically induced apoptosis in human breast cancer cells as compared to single treatment. Thus, the data clearly demonstrates that Kaempferol sensitizes human breast cancer cells to TRAIL-mediated apoptosis, which could have potential therapeutic significance in treating breast cancer.

Keywords: Apoptosis, Kaempferol; TRAIL; Breast cancer; Cytotoxicity

I. INTRODUCTION

In the past few decades, medicinal plants and their biologically active derivatives has increased, in relation to the possible development of novel potential drugs for several pathologies of relevant social impact [1,2]. Recently, the possible applications of medicinal plants as an anti-tumor agent have been widely described [3-5]. Natural products, from the extracts of medicinal plants, are used in the treatment of several health disorders such as skin, respiratory, neuromuscular, mental, obstetrics and gynecology illness [6-8]. Epidemiologic studies in humans have shown that regular consumption of fruits and vegetables is associated with reduced risk of cancer [9]. It is believed that many fruits and vegetables contain flavonoids, which could be one of the reasons to exert potential antitumorigenic activities [10]. Flavonoids possess pharmacological and biochemical effects which are capable of modulating the activity of enzymes and affect the behaviour of many cell systems in the body [11,12]. Kaempferol is a well known flavonoid in nature, which is widely distributed in plant kingdom and heavily consumed in large amounts in our daily life. Kaempferol is known to possess cancer-preventive properties [13-15]. Studies have shown that Kaempferol sensitizes the TRAIL induced apoptosis in glioma cells [16]. TRAIL also induces apoptosis in various types of malignant tumor cells, through its interaction with the death domain-containing receptor, death receptor 5 (DR5), which is also called TRAIL-R2 [17-18]. In vitro and in vivo data indicates that TRAIL is not cytotoxic to the normal cells [19-20].

Therefore, TRAIL is one of the most promising candidates for cancer therapeutics. However, some tumor cells are resistant to TRAIL-induced apoptosis[21]. It is, therefore, important to overcome this resistance for the clinical use of TRAIL in cancer.
therapy. Based on this hypothesis and reported data, the present study is aimed to evaluate the nature of Kaempferol–TRAIL induced apoptosis in breast cancer cells. We hypothesized that treatment with Kaempferol enhances TRAIL-induced apoptosis by increasing expression of apoptosis-related proteins including Caspase-3, 8, 9 and Bax whereas by decreasing the expression of Bcl-2.

II. MATERIALS AND METHODS

2.1 Cell Culture and Treatment: To broadly cover thein vitrophenotypes exhibited by breast cancer cells, MCF-7, T47D, and ZR-75 were selected and procured from NCCS, Pune. All cell lines were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS and 50 IU/ml penicillin, 50μg/ml streptomycin and 2 mM L-glutamine. Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in air. Further, cells were treated with Kaempferol (25-200 μM) alone and in different combinations with TRAIL.

2.2 Cytotoxicity assay: The cytotoxic effect was assessed in human breast cancer cells exposed to different concentrations of Kaempferol alone and in different combinations with TRAIL for 48 hours by the MTT assay as described previously (22,23). Percent inhibition of cytotoxicity were calculated as a fraction of control verses the cytotoxicity of Kaempferol, alone and in different combinations with TRAIL and expressed as IC₅₀.

2.3 Detection of apoptotic DNA fragments: DNA fragmentation was detected by agarose gel electrophoresis. 1x10⁶ breast cancer cells were plated in 30 mm culture plate. When the cells reached approximately 70% confluency, Kaempferol was added alone and in different combinations with TRAIL and the cells were incubated for 48 h. After 48 hours, cells were harvested and pelleted by centrifugation (Eppendorf 5804R, Germany). Cellular DNA was extracted by SDS/proteinase K treatment, phenol–chloroform extraction, and ethanol precipitation as described previously[22] and then dissolved and stored in TE buffer and the DNA samples obtained were analyzed by 2% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide, and visualized as a DNA ladder with UV.

2.4 Apoptosis measurements

2.4.1 Quantitative real-time PCR: Harvested cells were lysed in RLT buffer and total RNA was extracted using RNaseasy Plus Mini Kit (Qiagen, USA). RNA was quantified and 1 μg RNA was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). 20 ng of synthesized cDNA were used for quantitative real time according to the manufacturer’s protocol using 2x SYBR Green qPCR Master Mix (Biotool, USA) on BioRad CFX 96 well machine.

2.4.2 Western Blotting: Harvested cells were lysed and cleared by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was stored at −70°C until use. The protein concentration was quantified by BCA Assay method. Next, total proteins (15–20 μg) were electrophoresed using 12% reducing SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 0.1% Tween-20 in PBS (PBST) containing 1% skim milk and 1% BSA for 1 h, the membranes were incubated overnight at 4°C with the indicated primary antibodies. After washing in 1X PBST for 15 min (3 times x 5 min), the membranes were incubated with diluted enzyme-linked secondary antibodies. After washing in 1X PBST for 1 h (4 times x 15 min), the protein bands were detected using the EZ-western chemiluminescent detection kit and visualized by exposing the membranes to X-ray films. All protein bands were normalized against β-actin protein.

III. RESULTS

3.1 Kaempferol sensitizes MCF-7, T47D and ZR-75 cells for TRAIL induced cytotoxicity

Effect of Kaempferol on breast cancer cells were shown in Figure 1. Kaempferol alone is not inducing cytotoxicity in all the breast cancer cells even at higher concentration i.e.,200μM. Figure 2 showed that Kaempferol (200 μM) alone did not inhibit cell viability in all the breast cancer cells in a dose-dependent manner within 24h. Similarly, TRAIL (25-200 ng/mL) did not have a significant effect on cell viability. Kaempferol enhanced the inhibitory effects of TRAIL on cell viability in all the breast cancer cells within 24 h (Figure 2). Compared with control cells, addition of Kaempferol in combination with TRAIL decreased viability by more than 50% in all the breast cancer cells.

3.2 Validation of apoptosis measurement by DNA laddering

In the given results, the cells were treated with Kaempferol alone and in different combinations with TRAIL, and the DNA was directly extracted and run on agarose gel. DNA hyperfragmentation, if presented, was seen as a stepwise ladder of DNA fragments. Figure 3 shows that DNA laddering is pronounced in the samples where cells were treated with Kaempferol and TRAIL together in all the breast cancer cell lines. These results confirm that Kaempferol promotes TRAIL induced apoptosis in breast cancer cells.

3.3 Kaempferol Augments TRAIL-Induced Apoptosis through Caspase Activation

Quantitative real time PCR was performed to analyze the extrinsic and intrinsic apoptotic pathways in TRAIL-induced and TRAIL-Kaempferol − induced apoptosis. By analyzing caspase-3,8 and 9 it has been found that all the three caspases are upregulated in TRAIL-Kaempferol−
induced apoptosis in all the three breast cancer cells (Figure 4).

3.4 Regulation of Bcl-2 Family Members by Kaempferol
Bcl-2 family members regulate apoptosis induced by stress stimuli primarily at the level of mitochondria [23]. Therefore, the effects of Kaempferol in combination with TRAIL on the expression of Bcl-2 family proteins have been examined here. Real time PCR and western blot analysis showed that on treatment with Kaempferol in combination with TRAIL, the expression of the anti-apoptotic proteins Bcl-2 was decreased significantly in all the three breast cancer cells whereas Bax expression is increased (Figure 5). Furthermore, we also performed western blot analysis to confirm our findings at protein level. Treatment of Kaempferol significantly increases the expression of caspase-3, caspase-9, caspase-8 and Bax whereas Bcl-2 expression is decreased in TRAIL induced apoptosis in all the three breast cancer cells (Figure 6). Results suggested that the combination of Kaempferol and TRAIL could be an effective approach for breast cancer therapy and sensitization of TRAIL-induced cytotoxicity in cancer cells by a combination of TRAIL and Kaempferol may be particularly relevant in retaining the cancer-killing activity and circumventing the cancer-promoting potential of TRAIL. In vivo experiments with animal models are needed to verify the efficacy of the TRAIL and Kaempferol combination for breast cancer therapy.

IV. DISCUSSION

We have been made to enhance the survival of breast cancer patients by targeting resistance to apoptosis. TRAIL induces apoptosis in cancer cells with almost minimal toxicity[24]. However, breast cancer cells develop resistant to TRAIL therapy [25]. Therefore, in the present investigation it has been aimed to sensitize the TRAIL induced apoptosis in breast cancer cells by using Kaempferol, an essentialflavonoid. Kaempferol is known to have anticancer efficacy against a broad range of cancers in cell culture studies. Our findings suggests that Kaempferol may be useful as an anticancer drug or as an adjunct in combination therapy to enhance TRAIL induced apoptosis in breast cancer cells. Cellular proliferation depends on the rates of cell division and death and thus, many anticancer drugs have been used to prevent cancer cell division in order to inhibit cancer cell proliferation. In vitro cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals [26,27]. In this study, it has been shown for the first time that Kaempferol in combination with TRAIL reduced the viability of breast cancer cell in culture. However, separate administration of Kaempferol and TRAIL did not show significant reduction in cell growth in all the three breast cancer cells. Combined application of TRAIL and Kaempferol strongly reduced the cell viability upto ~80%. Apoptosis is a physiological process of cell death. DNA fragmentation is one of the hallmarks of cell apoptosis. The results revealed that the apoptosis proportion of cells was increased by the administration of Kaempferol and TRAIL in combination in all the breast cancer cells. Studies also have shown that Kaempferol modulates expression of genes involved in apoptosis [14,15]. The present findings detected the suppression of Bcl-2 and significantly increased expression of caspase-3 caspase-9 and caspase-8 and Bax in all the three breast cancer cells.

V. CONCLUSION

Previous findings have not shown many reports on TRAIL mediated apoptosis of human breast cancer cells sensitized by dietary flavonoid Kaempferol till date. In the present study, kaempferol sensitizes the TRAIN induced apoptosis in breast cancer cells. However, the future studies are needed to confirm role of kaempferol in TRAIN mediated apoptosis in breast cancer cells as well as other cancer types.

VI. CONFLICT OF INTEREST

None

VII. ACKNOWLEDGEMENT

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REFERENCES

**Figure I:** Kaempferol effect on breast cancer cells

**Figure II:** Kaempferol sensitized TRAIL induced cytotoxicity in breast cancer cells. (NOTE: NK: No Kaempferol, 50T: 50ng/ml TRAIL, 100T: 100 ng/ml TRAIL, 100K: 100μM Kaempferol, NT: No TRAIL, 200K: 200μM Kaempferol).

**Figure III:** DNA fragmentation analysis in TRAIL-kaempferol – induced apoptosis in human breast cancer cells.(Note: M=Marker; 1= Control; 2= NK, 50T; 3= NK, 100T; 4= 100K, NT; 5= 200K, NT; 6= 100K, 50T; 7= 100K, 100T).
Figure IV: Quantitative Real Time PCR showing the effect of kaempferol on TRAIL induced caspase-3, caspase-9, caspase-8 expression in human breast cancer cells.
Figure V: Quantitative Real Time PCR showing the effect of kaempferol on TRAIL induced Bcl-2 and Bax expression in human breast cancer cells.

Figure VI: Immunoblotting showing the effect of kaempferol on TRAIL induced caspase-3, caspase-9, caspase-8, Bax and Bcl-2 expression in human breast cancer cells.