In vitro Apoptotic Effect of Terfenadine in Human Oral Cancer Cells
Via the Regulation of Caspase 8 and Bid

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Terfenadine (TFN) was a second generation histamine receptor antagonist. Although several studies have reported the regulatory effect of H1-histamine receptor antagonists in human cancer cell lines, its effect in oral cancer remains unclear. In this study, we focused on addressing the anti-cancer activity of TFN in human oral cancer cell lines. The anti-cancer activities of TFN were performed by tryphan blue exclusion assay, 4′-6-diamidino-2-phenylindole (DAPI) staining, live/dead assay and Western blot analysis. TFN induced a significant reduction of the growth in three different human oral cancer cell lines (MC3, HSC4 and Ca9.22). TFN markedly induced apoptosis through DNA damage and increase in cytotoxicity. It also accumulated cleaved PARP and caspase 3. This process was due to cleavage of caspase 8 and Bid protein. The results from this study strongly demonstrated that the cleavages of caspase 8 and Bid are required for the apoptotic activity of TFN in human oral cancer cells. Taken together, these findings suggest TFN as a potent anticancer drug candidate for the treatment of oral cancer.

Key words: Terfenadine, Apoptosis, Oral Cancer, Caspase 8, Truncated Bid

I. INTRODUCTION

Histamine has been reported to be associated with cell proliferation, tumor growth and embryonic development. It favors the proliferation of normal and malignant cells, so several anti-histamine drugs including astemizole have gained enormous interest by inhibiting tumor cell proliferation. Terfenadine (TFN) is a prototype non-sedating histamine H1 receptor antagonist. Initially, TFN was used for the treatment of allergic diseases by its antagonistic reaction against histamine receptor. Numerous studies also have reported that TFN may have anti-cancer activity in many cancer cell lines such as leukemia and myeloma. However, the anti-cancer effects of TFN as well as H1 histamine receptor antagonists in a human oral cancer cells have not yet elucidated.

In the present study, the effects of TFN, one of H1-antihistamines on anti-proliferation and apoptosis were investigated in three different human oral cancer cell lines,
The critical molecules involved in TFN-mediated anti-cancer activity were also evaluated.

II. MATERIALS AND METHODS

1. Chemicals and antibodies

TFN (Figure 1A) and DAPI were supplied by Sigma-Aldrich (Louis, MO, USA). LIVE/DEAD Viability/cytotoxicity kit was obtained from Invitrogen (Carlsbad, CA, USA). The antibodies for cleaved Bid and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Cleaved caspase 3, cleaved caspase 8 and cleaved poly-ADP ribose polymerase (PARP) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2. Cell culture and chemical treatment

MC3 cells were kindly provided by Professor Wu Junzheng (Forth Military Medical University, Xi’an, China). HSC4 and Ca9.22 cells were provided by Hokkaido University (Hokkaido, Japan). Cells were cultured in DMEM supplemented with 10% FBS and 100 U/mL of penicillin and streptomycin at 37°C in an atmosphere containing 5% CO2. When cells reached to 50% confluence, they were treated with 0.1% DMSO (vehicle control) or TFN.

3. Trypan blue exclusion assay

MC3, HSC4 and Ca9.22 cells were exposed to designated concentration of TFN (12.5, 8 and 12 μM is for MC3, HSC4 and Ca9.22, respectively) for 24 hr and the number of viable cells was counted with trypan blue (0.4%) using a hemocytometer. Each experiment was carried out in triplicate and the results were expressed as means ± standard deviation (SD).

4. DAPI staining

The apoptotic effect of TFN in three cell lines was measured using a fluorescent nuclear dye, DAPI. Cells were harvested by trypsinization and resuspended in phosphate buffered saline (PBS). The cells were fixed in 100% methanol at room temperature (RT) for 10 min, deposited on slides, and then stained with DAPI solution (2μg/ml). DAPI-stained cell morphology was observed under a fluorescence microscope.

Figure 1. Effect of Terfenadine (TFN) on cell viability in MC3, HSC4 and Ca9.22 cells
(A) Structure of TFN. (B) MC3, HSC4 and Ca9.22 cells were treated with DMSO or designated concentrations of TFN for each cell line for 24 hr. Cell viability was determined using a trypan blue exclusion assay. The graphs are expressed as means ± SD of triplicate experiments, *, P < 0.05, significance compared with the DMSO treatment group.
5. Viability/cytotoxicity assay

Cell viability was investigated with the LIVE/DEAD viability/cytotoxicity kit according to the manufacturer’s instructions. This kit provides a two-color fluorescence cell viability assay, based on the simultaneous determination of the live and dead cells with two probes that measure intracellular esterase activity (live cells) and plasma membrane integrity (dead cells).

6. Western blot analysis

Whole-cell lysates were extracted with lysis buffer and protein concentrations were measured using a DC Protein Assay Kit (Bio-Rad Laboratories, Madison, WI, USA). Equal amounts of protein were separated by SDS-PAGE and then transferred to Immuno-Blot PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with 5% skim milk in Tris-Buffered Saline with Tween 20 (TBST) at RT for 2 hr and incubated overnight at 4°C with designated primary antibodies. The membranes were washed with TBST, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at RT for 2 hr. Antibody-bound proteins were detected using an ECL western blotting luminol reagent.

7. Statistical analysis

Student’s t-test was used to determine the significance of differences between the control and the treatment groups; values of P less than 0.05 were considered significant.

III. RESULTS

1. Terfenadine (TFN) inhibits cell viability in three different human oral cancer cell lines.

Previously, several groups found that TFN, an H1 histamine receptor antagonist, acts as a potent growth suppressor in various cancer cell lines. Thus, in order to demonstrate the growth-inhibitory effect of TFN in MC3, HSC4 and Ca9.22 oral cancer cell lines, we treated those cell lines with DMSO or designated concentrations of TFN. We found that the number of cells was significantly reduced and cells were detached in a concentration-dependent manner (Figure 1B).

2. TFN accelerates cytotoxicity and induces apoptosis in human oral cancer cell lines by regulating caspase 8 and Bid.

To demonstrate whether TFN-mediated growth inhibition was related to cell death, we performed a currently developed cytotoxicity assay that utilizes two-color fluorescence dyes to discriminate between live and dead cells. Dead cells are recognized by a red fluorescence and viable cells are confirmed by a green (fluorescein) stain. The results clearly showed that TNF increased red fluorescence-positive cells implying that it has a cytotoxicity activity (Figures 2A and 2B). Then, we performed DAPI staining to investigate whether TFN-induced cytotoxicity is related to apoptotic cell death. We found that TFN significantly increased the number of cells with nuclear condensation and fragmentation which were characteristic features in cells undergoing apoptosis (Figures 3A and 3B). To confirm its apoptotic activity, Western blot analysis with antibodies against cleaved PARP and caspase 3 was carried out. The results clearly showed that TFN caused great increase in the cleavages of PARP and caspase 3 (Figure 4A). Next, to clarify how TFN can induce apoptosis, we performed Western blot analysis to detect the expression levels of caspase 8 and Bid. We found that TFN resulted in a considerable induction of cleaved caspase 8 and truncated Bid protein (Figure 4B).
Figure 2. Effect of TFN on cytotoxicity in MC3, HSC4 and Ca9.22 cells
(A) Cytotoxicity was measured using a live/dead assay and live and dead cells were observed under a fluorescence microscopy with the appropriate excitation and emission filters. (B) The graph represents three independent experiments. *, P < 0.05, significance compared with the control group.

Figure 3. Effect of TFN on apoptosis in MC3, HSC4 and Ca9.22 cells
(A) Nuclear fragmentation and condensation were detected using DAPI staining by fluorescence microscopy (Magnification, X400). (B) The graph represents three independent experiments. *, P < 0.05, significance compared with the control group.

Figure 4. Effect of TFN on PARP, caspase 3, caspase 8 and Bid in MC3, HSC4 and Ca9.22 cells
(A) Cells treated with TNF or DMSO were harvested and prepared for Western blot analysis as described under Materials and Methods, PARP and cleaved caspase 3 in whole cell lysates were detected and actin was used to normalize the protein loading from each treatment. (B) Cleaved caspase 8 and truncated Bid protein expression were evaluated.
These results suggest that up-regulation of cleaved caspase 8 and truncated Bid may be associated with TFN-induced apoptosis in human oral cancer cells.

IV. DISCUSSION

H1 histamine receptor antagonists serve to remove histamine-associated allergic reactions and were mostly used in clinical practice for the treatment of allergic diseases\(^8\). The first-generation anti-histamines such as diphenhydramine have sedative and anticholinergic adverse effects\(^6\). In order to minimize the above side effects, second generation anti-histamines have been developed, TFN, one of the second-generation H1 antagonists, has been shown to block voltage-dependent ion channels and to reverse drug resistance in a variety of cell types via its interaction with P-glycoprotein\(^9\).

Recently, it has been reported that TFN exhibits strong activity against human cancer in vitro and in vivo implying that it may be anticancer activity. In the present study, we have also found that TFN clearly decreases cell viability and induces cell death. These results are in accordance with previous reports\(^6,10\).

Programmed cell death (apoptosis) is a physiological type of cell death that play a critical role during tissue homeostasis as well as therapy of malignant diseases\(^11,12\). It is widely considered to be one of the important mechanisms by which anticancer agents kill tumor cells. To clarify whether the growth-inhibitory and cytotoxic activities of TFN were due to apoptosis, we performed DAPI staining and Western blot analysis. The results showed that TFN resulted in nuclear condensation and fragmentation as well as PARP cleavage. Other groups have also demonstrated similar apoptotic effects of TFN in several types of human cancer cell lines by supporting our present results\(^13,14\). These suggest that TFN can be a potent apoptotic inducer in human oral cancer cell lines, TFN was effective against melanoma, prostate and hepatocellular cancer cells through various apoptotic proteins mechanisms\(^7,10,35\). For examples, TFN-induced apoptosis in human melanoma cells was mediated by Ca\(^{2+}\) homeostasis modulation and tyrosine kinase activity, independently of H1 histamine receptor\(^10\) and also induced apoptosis and autophagy in human melanoma cells through ROS-dependent and independent mechanisms\(^9\).

Recently, TFN induced anti-proliferative and apoptotic activities in human prostate cancer cells through histamine receptor-independent Mcl-1 cleavage and Bak up-regulation\(^6\) suggesting that TFN can affect Bcl-2 family members to induce apoptosis, Bid, a pro-apoptotic BH3-only protein of Bcl-2 family members, is truncated via the cleavage of caspase 8 during apoptotic cell death. In the present study, we found that TFN activated caspase 8, so we evaluated Bid protein in human oral cancer cell lines. The results showed that TFN truncated Bid protein implying Bid may be related to TFN-induced apoptosis.

In summary, we conclude that H1 receptor antagonist, TFN induces apoptotic cell death in human oral cancer cells, Caspase 8 and truncated Bid is related with TFN-mediated programmed cell death, These findings suggest that TFN may have a potential therapeutic intervention for the treatment of oral cancer.

V. REFERENCES

3. Woodward JKMunro NL: Terfenadine, the first non-sedating antihistamine, Arzneimittelforschung 1982; 32:1154-1156.