RESEARCH ARTICLE

Combination of Nimbolide and TNF-α-Increases Human Colon Adenocarcinoma Cell Death through JNK-mediated DR5 Up-regulation

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Abstract

Tumor necrosis factor (TNF-α), an inflammatory cytokine that plays an important role in the control of cell proliferation, differentiation, and apoptosis, has previously been used in anti-cancer therapy. However, the therapeutic applications of TNF-α are largely limited due to its general toxicity and anti-apoptotic influence. To overcome this problem, the present study focused on the effect of active constituents isolated from a medicinal plant on TNF-α-induced apoptosis in human colon adenocarcinoma (HT-29) cells. Nimbolide from *Azadirachta indica* was evaluated for cytotoxicity by methyl tetrazolium 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay and phase contrast microscopy. Effects on apoptotic signaling proteins were investigated using Western blot analysis. Nimbolide showed cytotoxicity against HT-29 cells that was significantly different from the control group ($p<0.01$), a concentration of 10 μM significantly inducing cell death ($p<0.01$). In combination with TNF-α, nimbolide significantly enhanced-induced cell death. In apoptotic pathway, nimbolide activated c-Jun N-terminal kinase (JNK) phosphorylation, BH3 interacting-domain death agonist (Bid) and up-regulated the death receptor 5 (DR5) level. In the combination group, nimbolide markedly sensitized TNF-α-induced JNK, Bid, caspase-3 activation and the up-regulation of DR5. Our findings overall indicate that nimbolide may enhance TNF-α-mediated cellular proliferation inhibition through increasing cell apoptosis of HT-29 cells by up-reglation of DR5 expression via the JNK pathway.

Keywords: TNF-α - apoptosis - HT-29 human colon adenocarcinoma cells - nimbolide - DR5 - JNK pathway

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Introduction

Tumor necrosis factor alpha (TNF-α) is one of cytokines produced from monocytes, macrophage, lymphocytes, keratinocytes and fibroblasts play a crucial role in cell proliferation, differentiation and apoptosis (Brenner et al., 2015). As a death ligand, the TNF-α plays a significant role in cancer cell apoptosis induction through death receptor or extrinsic pathway (Jang et al., 2014). The extrinsic apoptosis can be initiated by the activation of death receptors including, DR4, DR5, TNFR1, TNFR2, and Fas by their specific respective ligands, which in turn results in activation of caspase cascades.

Death receptor 5 (DR5) or TRAIL receptor 2 is an apoptosis-inducing receptor for TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) by selection cancer cells but not in normal cells (Debatin and Krammer, 2004; Walczak, 2013). In TNF-α pathway, the TNF-α ligand binds to TNFR-1 (TNF-receptor-associated factor 1) to activated JNK signaling cascades and caspases cascade subsequent apoptosis (Deng et al., 2003). Many clinical trials have evaluated the efficacy of TNF-α in cancer therapy for patients. However, the therapeutic doses of TNF-α as an anticancer agent is limited by the severe side effects including hypotension and systemic side effects (Walsh et al., 1991; Muc and Baranowski 1995; Clark, 2007). The combination treatment may represent a good choice for treating cancer cells with TNF-α.

Several chemical substances and natural products have been combined with TNF-α to sensitized TNF-α-induced cancer cell death including, gomisin N, genistein, honokiol, doxorubicin and curcumin (Paul et al., 2006; Waiwut et al., 2011). Among natural products that showed anticancer property, some medicinal plants such as

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Clausena harmandiana and *Azadirachta indica* have been showed cytotoxicity on cancer cells. (Sritanaudomchai et al., 2005; Boonyarat et al., 2014; Patel et al., 2016)

Nimbolide (Figure 1A) is a terpenoid lactone derived from *Azadirachta indica* (Neem tree) that features a variety of biological activities including antioxidant, anti-inflamatory, anti-malarial and anticancer activity (Rochanakij et al., 1985; Gupta et al., 2013). However, the anticancer effect in a combination of TNF-α on human adenocarcinoma cells (HT-29) cells through DR5 has remain not well understood. In this study, we investigated the effect of nimbolide on TNF-α-induces apoptosis in HT-29 cells via DR5 induction.

Materials and Methods

Cell culture

HT-29 cells (ATCC) are grow in DMEM (Dulbecco's modified Eagle's medium (high glucose) (Gibco Life Technologies, USA) supplemented with 10 % FBS (fetal bovine serum), 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO_2.

Cell cytotoxicity assay

Cells at 6 x 10^3 cells/well were plated in 96-well microplate, and then incubated for 24 h. The different concentrations of nimbolide and doxorubicin (standard compound) with or without80 ng/ml TNF-α were added to HT-29 cells in 96 wells plate, incubated for 24 h. The cell viability is quntified by using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay, measured the absorbance at 570 nm and calculated percentage of cell viability using the formula as following;

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells x 100}}{\text{Absorbace of control (untreated cells)}}$$

The cell morphological change was observed by is by Phase contrast microscope.

Preparation of cell extracts

In order to investigate the mechanism of the nimbolide on TNF-α-induced apoptotic pathway in HT-29 cells, the cells are treated with the nimbolide at different concentrations for 4 h in the presence of absence of 80 ng/ml TNF-α. Whole cell lysates are prepared by adding cell extract buffer (Gibco Life Technologies, USA), 1 mM dithiothreitol (DTT), 10 µg/ml aprotinin, and 10 µg/ml leupeptin), collected the cell lysate from supernatant fraction after centrifugation at 14,000 rpm for 10 min. The sample protein concentration was determined by using Bradford's assay.

Immunoblotting

Cell lysate is separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred the proteins to Polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The membrane is treated with Blocking solution and probed with primary antibodies (anti-caspase-3, DR5, phosphor-JNK, JNK, BID, and anti-actin antibodies). The antibodies are detected by the use of horseradish peroxidase-conjugated anti-rabbit, and anti-goat IgG (DAKO, Glostrup, Denmark), and visualized by the enhanced chemiluminescence system (Bio-Rad Laboratories, Hercules, CA).

Results

Nimbolide sensitizes TNF-α induced morphology changing of HT-29 cancer cells

At first, we examined the effects of nimbolide on TNF-α-induced cytotoxicity of HT-29 cell. The result in Figure. 1B suggested that nimbolide at 10 µM alone showed cytotoxicity in a MTT assay; however, combination with TNF-α markedly increased the sensitivity to the

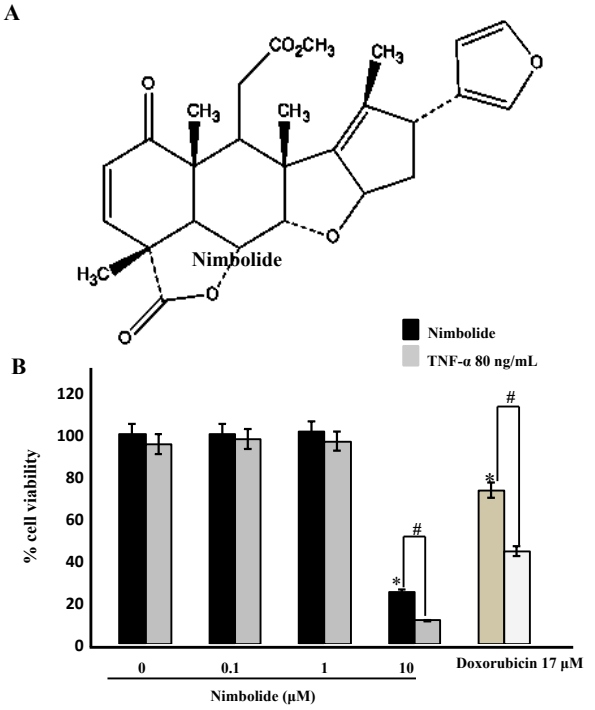

Figure 1. Effects of Nimbolide and TNF-α on Viability of HT-29 Cells. A) Chemical structure of nimbolde. B. HT-29 cells were treated with various concentrations of mimbolide and 17 µM doxorubicin (reference compound) in the presence of absence of TNF-α for 24 h. Cell proliferation was determined by MTT assay. #,*$p < 0.001$

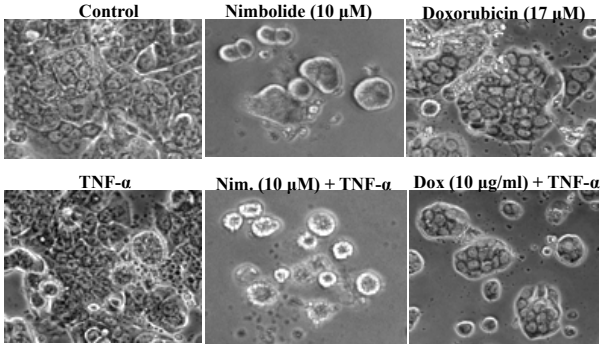

Figure 2. Effects of Nimbolide on TNF-α-induced HT-29 Cell Apoptosis. Cells were treated with 10 µM nimbolide and 17µM doxorubicin (reference compound) for with or without TNF-α for 24 h. Cell morphology was investigated by phase contrast microscopy

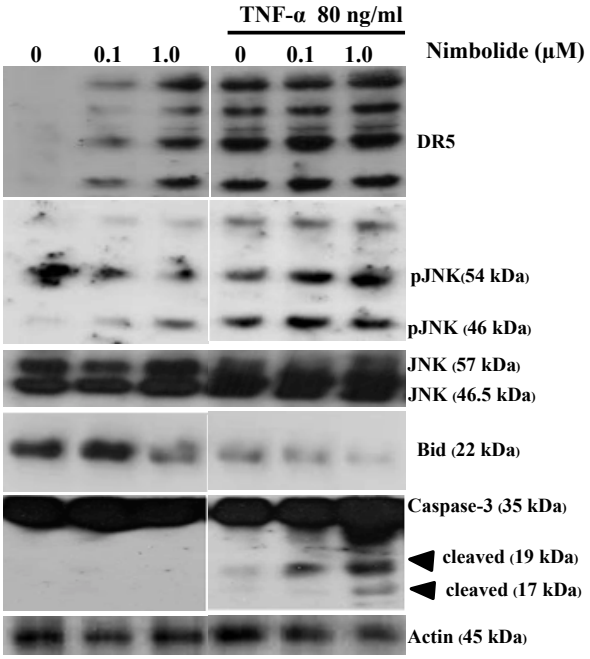

Figure 3. Effects of Nimbolide on TNF-α-induced Apoptotic Signaling Pathway. A) Cells were treated with various concentrations of nimbolide in combination with TNF-α for 4 h. Whole cell extract was prepared, and analyzed by Western blotting using anti-caspase-3, DR5, phosphor-JNK, JNK, Bid and β-actin antibodies. Arrows indicate cleaved forms of caspase-3

cytokine. The results indicated that nimbolide dramatically enhanced TNF-α -induced HT-29 cancer cell death.

Nimbolide enhances TNF-α induced morphological change of HT-29 cancer cells

To investigate the effect of nimbolide on HT-29 cancer cell morphology, the cells were treated with 10 μM of nimbolide or 17 μM doxorubicin (positive control) in the presence or absence of TNF-α for 24 h and morphological changes were observed by phase contrast microscopy. The result showed that nimbolide and doxorubicin induced morphology change of cell death consisting of rounding and shrinkage of cells and the cell treated with TNF-α slightly induced morphological change. In the combination group, when treated the cell with nimbolide in the present of TNF-α markedly, it induced cell death comparing with doxorubicin as positive control (Figure 2). The result indicated that nimbolide markedly induced morphological changes during HT-29 cell death.

Nimbolide sensitizes TNF-α induced apoptosis of HT-29 cancer cells through by increasing DR5 expression

To explain the molecular basis of nimbolide sensitized TNF-α induced apoptosis of HT-29 cancer cells, cells were pretreated with various concentrations of nimbolide for 30 min, and then stimulated with or without TNF-α for 4 h. The apoptotic effect determined by observed the cleavage of caspase-3, apoptosis-related cysteine peptidase. TNF-α alone slightly induced cleavage of caspase-3 and nimbolide enhanced the TNF-α-induced cleavage in a concentration-dependent manner (Figure 3). The combined treatment also induced up-regulation of DR5 and Phospho-JNK. In addition, mitochondrial-type Bid and subsequent cytochrome C release were increased. This result suggested that nimbolide potentiated TNF-α-induced apoptosis through DR-5 pathway.

Discussion

Cancer is diseases of abnormality of cell growth that lead to rapid divide and uncontrolled growth of cells. These problems can cause a tumor that become cancer which metastasis and invade to other tissues that spread to other parts of human body. Many cells can transform to be cancer cells, resulting in many types of cancers such as breast cancer, colon and rectal cancer, brain cancer and bladder cancer (Gerard et al., 2001; Hanahan and Weinberg, 2011). Apoptosis is one of the important terminal mechanisms for damage cells, explained by morphological changes, including membrane bleb, cell shrinkage, chromatin condensation and nuclear fragmentation (Elmore, 2007). In cancer cells, cell division and cell death are imbalance, cell division is greather than cell death, therefore many studies tried to induces apoptosis in cancer cell (Ouyang et al., 2012; Kalimuthu and Kwon, 2013; Su et al., 2014). Nimbolide was isolated from the leaves and flowers of neem (Azadirachta indica), used as medicinal plant, has been reported for containing antifungal, anti-inflammatory, antihistamine, antitubercular, antimalarial, antiarthritic, and anticancer activities (Rochanakij et al., 1985; Patel et al., 2016). Nimbolide showed potent effect to induced human adenocarcinoma cells (HT-29), suggesting that nimbolide is a strong anticancer agent to kill cancer cells. Death receptor-mediated tumor cell death, either alone or in combination with other anticancer drugs, is considered as a new strategy for anticancer therapy (Chung et al., 2014). Treatment of cancer cells with many natural compounds have been potentiated TNF-α-induced cancer cell death (Doss, 2014). In this study, nimboline was combined with TNF-α, the apoptosis inducing death receptor ligand and examined the apoptotic effects on HT-29 cancer cells. Nimbolide showed its activity to promoted TNF-α-induced cancer cell death. Death receptor 5 (or Apo2), one of the TRAIL receptors, belongs to the TNF receptor superfamily, located on the cell surface, and becomes trimerized on binding to its ligand TRAIL and recruits to Fas-associated death domain (FADD), caspase-8 drives its auto-activation through oligomerization and subsequently induces signaling complex and activates the downstream effector caspases, such as caspase-3. The caspase-8 activation can also cleave and activate the BH3 domain containing pro-apoptotic molecule Bid, which then translocates to the mitochondria triggering the pre-apoptotic mitochondrial events, resulting in caspase-3 activation and apoptosis (Lavrik, 2014; Wu, 2014). Many studies have shown that inducing the expression of DR5 contributes to certain cancer therapeutic agents induced apoptosis and enhances TRAIL-induced apoptotic pathway (Chung et al., 2014; Jang et al., 2014; Koff et al., 2015). However, the mechanism of DR5 in TNF-α pathway has not been investigated. To better understand the role of DR5 in TNF-α induced apoptosis, we treated

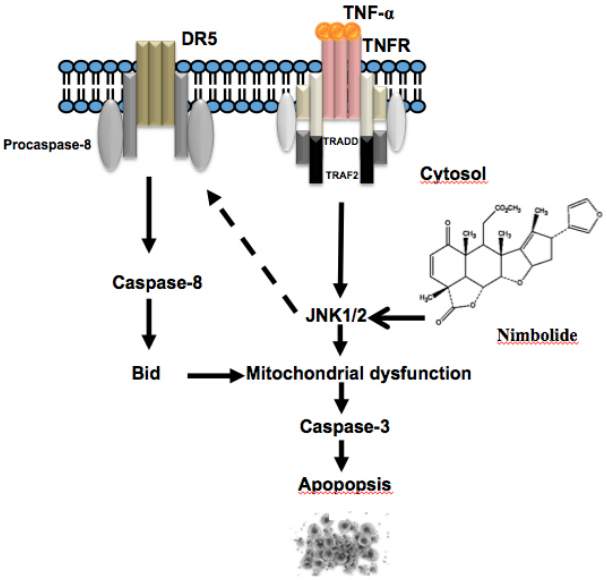

Figure 4. Nimbolide Sensitized TNF-α-Induction of HT-29 Cell Apoptosis. TNF-α-induced apoptosis pathway through phosphorylated-JNK which consequent to DR5-upregulation and bid cleavage activation that interfered mitochondrial function and resulting in caspase-3 activation

HT-29 cells with or without TNF-α and observed the DR5 protein expression. We found that TNF-α ligand induced caspase-3 activation correlating with increasing of DR5 expression, suggesting that DR5 may be the proapoptotic receptor for TNF-α induced apoptosis of the HT-29 cancer cells, and nimbolide promotes the TNF-α-mediated-DR5 induced HT-29 apoptosis. In DR5 activation signaling pathway, the phosphorylated c-Jun NH2-terminal kinase (JNK) has been reported that it involved with DR5 induction and paralleled with cancer cell apoptosis (Fassl et al., 2015; Park et al., 2016). In addition, it has been reported that TNF-α-mediated apoptosis required a sequential pathway involving JNK and Bid (Marques et al., 2013; Jin et al., 2007). To explain how nimbolide enhanced TNF-α-induced apoptosis via increasing DR5 expression, nimbolide was observed its effects on TNF-α dependent JNK and bid activation in HT-29 cancer cells.

Both nimbolide and TNF-α alone showed activity to induced of JNK phosphorylation and Bid activation compared with nimbolide in combination with TNF-α which increased JNK phosphorylation and activation of Bid. Nimbolide (5,7,4'-trihydroxy-3',5'-diprenylflavanone) structure is a tetranortriterpenoid consisting of β-unsaturated system and δ-lactonic ring (Figure 1A). The previous reports have shown that α, β unsaturated ketone structural element correlated with the anticancer activity of nimbolide (Elumalai and Arunakaran, 2014).

Nimbolide inhibited cancer progression by various mechanisms, including prevention of procarcinogen activation and oxidative DNA damage, upregulation of antioxidant and induction of apoptosis. This study revealed that nibolide showed strong anticancer activities to induce human adenocarcinoma cell death and markedly sensitized TNF-α-mediated cancer cell apopotosis. The possible mechanism is that nimboide activated TNF-α-induced JNK phosphorylation which causes DR5-upregulation, leading to Bid and caspase-3 activation, subsequence to cancer cell apoptosis (Figure 4). This report suggested that nimbolide is a promising candidate for anticancer drug development.

Acknowledgements

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