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Luteolin sensitizes human 786-O renal cell carcinoma cells to TRAIL-induced apoptosis

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ABSTRACT

Aims: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been considered to be one of the most promising candidates in research on treatments for cancer, including renal cell carcinoma (RCC). However, many cells are resistant to TRAIL-induced apoptosis which limits the potential application of TRAIL in cancer therapy. Luteolin, a naturally occurring flavonoid, has been identified as a potential therapeutic and preventive agent for cancer because of its potent cancer cell-killing activity. In this study, we investigated whether luteolin treatment could modulate TRAIL-induced apoptosis in RCC.
Main methods: The effect of luteolin on TRAIL sensitivity was assessed in human RCC 786-O, ACHN, and A498 cells. The underlying regulatory cascades were approached by biochemical and pharmacological strategies.
Key findings: We found that nontoxic concentration of luteolin alone had no effect on the level of apoptosis, but a combination treatment of TRAIL and luteolin caused significant extrinsic and intrinsic apoptosis. The sensitization was accompanied by Bid cleavage, Mcl-1 and FLIP down-regulation, DR4/DR5 protein expression and cell surface presentation, and Akt and signal transducer and activator of transcription-3 (STAT3) inactivation. Among these phenomena, changes in FLIP, Akt, and, STAT3 are more prone to the effects of luteolin treatment. Studies have further demonstrated that inactivation of Akt or STAT3 alone was sufficient to down-regulate FLIP expression and sensitized 786-O cells to TRAIL-induced apoptosis.
Significance: Data from this study thus provide in vitro evidence supporting the notion that luteolin is a potential sensitizer of TRAIL in anticancer therapy against human RCC involving Akt and STAT3 inactivation.

Introduction

Renal cell carcinoma (RCC) is the most lethal malignant tumor of the kidney and exhibits highly vascularized and metastatic characteristics. Therapeutic options for unresectable and/or metastatic RCC are limited, as RCC is typically refractory to traditional chemotherapy, hormonal therapy, and radiation therapy. Although immunotherapy, including interleukin-2 and interferon-α, is an alternative choice of treatment, it provides only limited benefit (Coppin et al., 2005; Rini et al., 2009). Recently, molecular targeted therapy has been developed or actively investigated (Rini, 2009). These targeted therapies have improved the outlook of advanced RCC, but cure remains rare and novel forms of treatment strategies are being intensively investigated.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been considered to be one of the most promising anticancer agents in cancer research because it preferentially causes apoptosis in cancer cells rather than normal cells. TRAIL induces apoptosis through its interaction with death receptor-4 (DR4) or DR5, leading to the formation of the death-inducing signal complex (DISC) with binding of caspase-8 and induction of apoptosis (Tan et al., 2009; Wu, 2009). However, studies indicate that only a minority of cancer cells undergo apoptosis in response to TRAIL. Since TRAIL is recognized as a potential therapeutic

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agent against RCC, efforts are being undertaken to develop an effective regimen which can overcome TRAIL resistance (Brooks et al., 2010; Clark et al., 2010; Jang et al., 2010; Kim et al., 2012; Lee et al., 2011; Macher-Goeppinger et al., 2009; Norian et al., 2012; Woo et al., 2012).

There is growing evidence of and interest in the health benefits of foods of plant origin due to their diversity of biological activities. Luteolin (3′,4′,5′,7′-tetrahydroxyflavone), belonging to the flavone subclass of flavonoids, possesses pro-apoptotic potential. Evidence suggests that signaling molecules such as Akt, signal transducer and activator of transcription-3 (STAT3), as well as the heat shock protein-90 (HSP90) and Bcl-2 family proteins are crucial targets of action by luteolin (Cheng et al., 2006; Fu et al., 2012; Horinaka et al., 2005a,b; Ou et al., 2013; Rao et al., 2012; Selvendiran et al., 2006). Several studies have reported that TRAIL resistance is intimately associated with deficiency of death receptors, overexpression of cellular FLICE inhibiting protein (FLIP) and antiapoptotic Bcl-2 family proteins, and the constitutive activation of Akt and STAT3 (Aggarwal et al., 2009; Brooks et al., 2010; Jang et al., 2010; Panner et al., 2007; Wang et al., 2008). The aforementioned findings imply that luteolin might be a potential candidate for adjuvant therapy to overcome TRAIL resistance (Horinaka et al., 2005a,b; Shi et al., 2005; Yan et al., 2012).

Currently, the outcome of a combined treatment of RCC with TRAIL and luteolin is unknown. Recently, we published a report showing that luteolin caused apoptotic cell death in human 786-O RCC cells involving Akt and HSP90 inactivation (Ou et al., 2013). Given the combination of adjuvant compounds with anticancer agents in RCC appears a rational option, this study explored the possibility that a combination treatment with luteolin could potentiate the cytotoxicity of TRAIL on human RCC cells.

Materials and methods

Cell cultures

Human RCC cells, 786-O (ATCC CRL1932), ACHN (ATCC CRL1611), and A498 (ATCC HTB44), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). When experimenting, cells were switched to DMEM containing 2% FBS.

Cell viability

Cell viability was assessed by the measurement of formazan production after the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS, Promega, Madison, WI). The number of surviving cells after treatment was determined by measurement of the A_{490} nm of the dissolved formazan product after the addition of MTS for 1 h according to the manufacturer's instructions.

Caspase activity assay

Caspase activity assay was carried out using a fluorometric protease assay kit following the instructions provided by the manufacturer (BioVision, Mountain View, CA). Enzymatic release of free fluorogenic moiety was measured by a fluorometer (E_x 380 nm and E_m 460 nm). The arbitrary unit was expressed as the fluorescent change per amount of protein.

Western blot

Obtained cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with the indicated antibodies against PARP-1, Bax, Bad, Bid, Mcl-1, FLIP, Akt, phospho-Akt (Ser-473), STAT3, and phospho-STAT3 (Tyr-705) (Santa Cruz Biotechnology, Santa Cruz, CA), DR4 and DR5 (Genetex, Irvine, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (R&D Systems, Minneapolis, MN). Then the membranes were incubated with horseradish peroxidase-labeled IgG. The blots were developed using enhanced chemiluminescence Western blotting reagents. The intensity of each signal was determined by a computer image analysis system (IS1000; Alpha Innotech Corporation). The relative protein content was depicted under panels and the intensity of the first lane was defined as 100.

Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and the cDNA was prepared using M-MLV reverse transcriptase (Epicentre Biotechnologies, Madison, WI) according to the manufacturers' instructions. The PCR reaction was performed with a DNA thermal cycler under the following conditions: one cycle at 94 °C for 3 min, 28 cycles at 94 °C for 50 s, 58 °C for 40 s, and 72 °C for 45 s, and then 72 °C for 5 min. Relative mRNA levels were expressed as the intensity ratio of each gene and internal control (β-actin) after determination by a computer image analysis system (Alpha Innotech Corporation, IS1000). Oligonucleotides used in this study were as follows: 5′-GTCTGCTCTGATCACCCAAC and 5′-CTGCAACTGTGACTCCTATG for DR5; 5′-TGGCACACAGCAATGGGAACATAG and 5′-GAAACACACCCTGTCCATGCACTT for DR4; 5′-CGGACTATAGAGTGCTGATGG and 5′-GATTATCAGGCAGATTCCTAG for FLIP; and 5′-GGCATCGTCACCAACTGGGAC and 5′-CGATTTCCCGCTCGGCCGTGG for β-actin.

Flow cytometry assay

For the detection of DR4- and DR5-positive cells, the detached cells were washed in phosphate-buffered saline (PBS) and stained with phycoerythrin (PE)-conjugated antibodies against DR4 and DR5, respectively (eBioscience, San Diego, CA). Antibody-labeled cells were washed and fixed in PBS with 0.37% formaldehyde. Characterization of antibody-labeled cells was performed on a BD FACSCalibur flow cytometer. For the analysis of cell cycle distribution, the detached cells were fixed in 80% ethanol, washed in PBS, incubated with 100 μg/ml RNase at 37 °C for 30 min, and stained with propidium iodide (50 μg/ml). The resultant cells were analyzed on a BD FACSCanto II flow cytometer.

Statistical analysis

The data are expressed as mean values ± standard deviation. Statistical analysis was carried out using one-way analysis of variance, followed by Dunnett's test to assess the statistical significance between treated and untreated groups. A level of $p < 0.05$ was considered statistically significant.

Results

Luteolin sensitizes 786-O cells to TRAIL-induced apoptosis

As previously described (Clark et al., 2010), 786-O cells were relatively resistant to TRAIL at doses up to 500 ng/ml by measuring cell viability (Fig. 1A) and caspase activities (Fig. 1B). In contrast, MTS reduction assay revealed that luteolin reduced cell viability (Fig. 1C). In parallel with viability loss, the elevation of caspase-3, caspase-8, and caspase-9 activities was observed in luteolin-treated cells (Fig. 1D). In an attempt to search for novel strategies to overcome TRAIL resistance in 786-O cells, we investigated the effect of the combined treatment with TRAIL and luteolin. Co-treatment of 786-O cells with TRAIL and luteolin resulted in a marked reduction of cell viability, compared with that of cells treated with TRAIL or luteolin alone (Fig. 2A). To understand the mechanism by which a combination of TRAIL and luteolin caused