Synergistic effect of celecoxib in tumor necrosis factor-related apoptosis-inducing ligand treatment in osteosarcoma cells

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is under clinical development as a cancer therapeutic agent and in combination with other agents (18,19). Currently, CXB is being widely investigated in clinical trials for therapeutic activity against various cancer types as a single agent and in combination with other agents (18,19).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising antitumor agent since it is capable of killing tumor cells via receptor-mediated apoptosis (5,6). Preclinical studies using recombinant TRAIL (rhTRAIL) have provided evidence for exogenous TRAIL efficacy in suppressing tumor growth in vitro and in vivo (7-9). However, resistance towards TRAIL may result in failure at any step in the death signaling cascade. For example, TRAIL resistance may occur at the receptor level, due to deregulated expression, or at the death-induced signaling complex (DISC) level, mediated by proteins counteracting DISC formation (10-12).

The aim of the present study was to evaluate the potency of CXB in combination with TRAIL in inhibiting OS cancer cell growth and proliferation, and to reveal the underlying molecular mechanisms involved in TRAIL-induced apoptosis. Whether CXB, as an adjuvant agent, allows the effective dosage of TRAIL required to treat OS cancer cells to be reduced was also determined.

Materials and methods

Cell lines. The MG-63 human OS cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured immediately following unfreezing at a concentration of 5x10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA). The cells were cultured immediately following unfreezing at a concentration of 5x10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA).
USA) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco Life Technologies, Carlsbad, CA, USA). The cells were incubated in a humidified atmosphere containing 5% carbon dioxide at 37°C.

**Reagents.** CXB (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich) to create a 1 mM stock solution, stored at -20°C and diluted in fresh medium immediately prior to use. TRAIL (with Enhancer applied at a concentration of 1 µg/ml) was purchased from Alexis Biochemicals (San Diego, CA, USA). For western blot analysis, the following antibodies were used: Mouse monoclonal anti-β-actin (Sigma–Aldrich); mouse monoclonal anti-B-cell lymphoma 2 (Bcl-2) and anti-survivin, and horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Nonidet P-40 lysis buffer and MTT were obtained from Sigma–Aldrich. MTT solution was prepared by dissolving 1 mg of compound in 1 ml phosphate-buffered saline (PBS). The solution was protected from light, stored at 4°C and used within one month.

**Cell viability assay.** MG-63 cells grown in monolayers were harvested and dispensed in 96-well culture plates in 100 µl DMEM at a concentration of 5x10^3 cells per well. After 24 h, differential drug concentrations of TRAIL (0-200 ng/ml), CXB (0-250 µM) or a combination (0-100 ng/ml TRAIL plus 30 µM CXB) were added to the cells. Cell viability after 24, 48 and 72-h incubation was measured using the MTT colorimetric assay with an ELISA microplate reader (Thermo Labsystems, Helsinki, Finland) at 490 nm with minor modifications according to methods described in a previous study (20). The inhibition rate was calculated according to the following formula: Inhibition rate (%) = [1-(average absorbance of experimental group/average absorbance of blank control group)] x 100.

**Detection of apoptosis.** MG-63 cells were cultured in six-well plates in DMEM with 10% FBS medium and were treated with the respective half maximal inhibitory concentrations (IC_{50}) of TRAIL, CXB or their combination for 48 h. The cover slips were washed three times with PBS and single cell suspensions were fixed in 1% PBS. The cells were stained with 100 µg/ml acridine orange (Sigma–Aldrich) and 100 µg/ml ethidium bromide for 1 min. Subsequently, the cells were observed under a fluorescent microscope (IX73-U; Olympus, Tokyo, Japan). At least 200 cells were counted and the percentage of apoptotic cells was determined. All experiments were performed in triplicates conducted on five occasions. Caspase activation, Bax-2 and Bcl-2 expression levels were also detected by western blotting as an additional indicator of apoptosis.

**Western blot analysis.** MG-63 cells (1x10^6 cells per 100 mm plate) were treated with TRAIL, CXB or their combination at the respective IC_{50} doses for 24 h. The cells in control wells were treated with 0.1% DMSO for 1 h. All cells were activated with recombinant human epidermal growth factor (25 ng/ml; Sigma–Aldrich) for 30 min. The cells were then scraped and lysed in Nonidet P-40 lysis buffer. Cell extracts (50 µg protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were blocked in 3% bovine serum albumin (BSA) for 2 h. Subsequent to blocking, the membranes were incubated with primary antibodies β-actin (1:5,000), Bcl-2 (1:1,000) and survivin (1:3,000) overnight at 4°C and then with horseradish peroxidase-conjugated anti-mouse IgG (1:10,000) for 2 h at room temperature. The proteins were visualized by exposing the chemiluminescence substrate (Sigma–Aldrich) to X-Omat AR autoradiography film (Eastman Kodak, Rochester, NY, USA).

**Animal experiments.** The tumor response to TRAIL and CXB treatment was investigated using the MG-63 tumor-bearing female BALB mouse model (obtained from the Experimental Animal Center of Jilin University, Changchun, China). The mice were maintained in accordance with the Institute of Animal Ethics Committee guidelines approved by the University of Jilin Animal Care and Use Committee, Changchun, China. The present study was approved by the ethics committee of the First Hospital of Jilin University (Changchun, China). The mice were housed and acclimated in a pathogen-free environment at the animal facility of the First Hospital of Jilin University for one week prior to injection with tumor cells.

Exponentially growing MG-63 cells were harvested and a tumorigenic dose of 2.5x10^6 cells was injected intraperitoneally into the 6 to 7 week-old female BALB mice. Tumors were allowed to grow in the mice for seven days, then the animals were randomly assigned to one of four treatment groups (10 mice per group). The control group received 1% polysorbate resuspended in deionized water. The other three groups were treated with CXB (3.7 mg/kg body weight), TRAIL (90 mg/kg body weight) or CXB plus TRAIL (2 and 50 mg/kg body weight, respectively) intraperitoneally on alternative days for two weeks. The doses were selected as determined by previous experiments (21,22). Tumor weight and tumor volume were measured prior to administration of the treatment injections and on days 7 and 14 of treatment. On day 15, the animals were sacrificed using chloroform and spleen tissues were collected and cultured for a splenocyte surveillance study.

**Assay of splenocyte proliferation.** Single-cell spleen suspensions were generated and pooled in serum-free DMEM by filtering the suspension through a sieve mesh with the aid of a glass homogenizer, which exerted gentle pressure on the spleen fragments. The samples were washed twice in PBS with 0.1% (w/v) BSA following centrifugation at 200 xg for 10 min, the cells were seeded at 3x10^5 cells per well in 96-well flat-bottomed microplates in triplicate in DMEM supplemented with 10% FBS. The cells were incubated in a total volume of 100 µl per well. Serum-free DMEM served as a control. After 24 h, cell proliferation was measured using an MTT assay.

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism 5 software (La Jolla, CA, USA). Values are presented as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance. P<0.001 and P<0.05 were considered to indicate statistically significant differences.
Results

CXB enhances TRAIL-induced OS cancer cell viability. To determine the effect of TRAIL, CXB, and the combination treatment on the viability of OS cancer cells in vitro, MG-63 cells were treated with increasing concentrations of CXB (0-200 µM) or TRAIL (0-200 ng/ml). The IC_{50} value of TRAIL alone was determined to be 100.18±5.28 ng/ml, and the IC_{50} value of CXB alone was 100.5±0.780 µM. Combination treatment (0-100 ng/ml TRAIL in the presence of 30 µM CXB) resulted in a shift of the cell viability curve towards lower drug concentrations such that the IC_{50} value of TRAIL was reduced to 50.76±14 ng/ml. These results indicated that treatment with the two agents was more cytotoxic than either alone. According to the results, respective drug IC_{50} values were selected for further treatments throughout the study. In addition, the inhibitory rates in the combination treatment group were found to be higher than those of the single drug groups. No significant difference was detected between the TRAIL and CXB single treatment groups (Fig. 1).

CXB enhances TRAIL-induced apoptosis. The effects of TRAIL and CXB on MG-63 cell apoptosis were then analyzed. MG-63 cells (IC_{50} values: 100 µM for CXB and 100 ng/ml for TRAIL) treated with either TRAIL or CXB exhibited an increased percentage of apoptotic cells compared with untreated cells (Fig. 2). However, the low-dose combination (30 µM CXB plus 50 ng/ml TRAIL) resulted in an even greater percentage of apoptotic cells than the higher doses of either drug alone. These data are consistent with the results from the MTT assay, which indicated an additive mechanism of TRAIL and CXB in inducing cell death through apoptosis.

TRAIL and CXB treatment results in significant inhibition of tumor growth. The in vivo therapeutic efficacy of TRAIL and CXB was assessed in female BALB mice bearing MG-63 cell tumors. TRAIL and CXB each induced tumor regression and inhibited tumor growth in the respective treatment groups. The mice were subsequently sacrificed on day 21 after treatment and tumor tissue was excised. The tumor weight of the animals was subsequently measured; the average tumor weight in the treatment groups was lower than that in the untreated group. Furthermore, the average tumor weight in the combination group was significantly lower than that in the single-treatment CXB or TRAIL groups (P<0.01; Fig. 3A). In addition, the tumor volume was measured on days 7, 14, and 21 of treatment. The tumor volume in the treatment groups increased at a slower rate than that in the untreated group. The tumor volume in the combination treatment group increased at a significantly slower rate than that in either CXB or TRAIL single treatment groups (P<0.01; Fig. 3B). These result demonstrated that TRAIL and CXB, and particularly their combination, induced tumor regression and inhibited tumor growth.

CXB increases TRAIL-induced splenocyte proliferation inhibition. To assess the efficacy of TRAIL and CXB in modulating splenocyte proliferation, spleen cells from treated mice with MG-63 cell tumors were isolated and cultured in DMEM supplemented with 10% FBS for 24 h and subjected to in vitro proliferation assays using the MTT method. As shown in Fig. 4, the inhibitory rate in the combination treatment group was higher than that in the single drug treatment groups, which demonstrated that combination treatment inhibited OS cell proliferation.

Preliminary clarification of the mechanism of CXB- and TRAIL-induced induction of apoptosis. To clarify the molecular mechanisms of CXB-TRAIL combination treatment on the induction of OS cell apoptosis, the effects of CXB and TRAIL on the activation of proteins Bcl-2 and survivin, which are involved in cell apoptosis, were analyzed. As shown in Fig. 5, CXB administered in combination with TRAIL significantly inhibited Bcl-2 and survivin expression levels in OS tumor cells compared with the single treatment and untreated groups. These results indicated that CXB and TRAIL combination therapy inhibited apoptosis in OS tumor cells.

Discussion

In the present study, combining TRAIL with CXB significantly induced cell apoptosis and inhibited OS tumor growth, whereas single treatment did not exert a significant effect. Although the two compounds have each been extensively investigated, to the best of our knowledge, this study is the...
first to reveal that combining clinical doses of TRAIL with CXB reduces OS tumor cell apoptosis and inhibits OS tumor growth in vivo.

Amongst the numerous methods to stimulate tumor cell apoptosis, induction of the death receptor ligand TRAIL shows particular promise. Previous preclinical studies have observed that TRAIL induces tumor cell apoptosis in vivo without causing lethal toxicity (23,24). One main issue in the clinical use of TRAIL is its limited efficacy in monotherapeutic approaches in different tumor entities. Thus, investigation into methods that enhance the capacity of TRAIL to induce apoptosis is required.

Recent preclinical studies have observed that TRAIL efficacy is increased through the use of combined chemotherapy (25,26). Furthermore, a phase I b trial was conducted with rhTRAIL administered in combination with rituximab in patients with low-grade non-Hodgkin lymphoma. Gaiser et al (27) demonstrated that CXB suppressed survivin levels by proteasomal degradation and thereby induced apoptosis and enhanced TRAIL-mediated cytotoxicity in U87, U251 and A172 glioma cells. In addition, Lu et al (28) observed that CXB and camptothecin treatment sensitized TRAIL-resistant HepG2 and Hep3B hepatocellular carcinoma (HCC) cell lines to TRAIL-induced apoptosis through downregulation of cellular Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein and cleavage of caspase-8 and caspase-3. These studies demonstrated that combination therapy with TRAIL and other anticancer drugs significantly inhibited cell viability compared with single drug treatments. The present study revealed that combining TRAIL with CXB significantly induced cell apoptosis and inhibited OS tumor growth, whereas single drug treatment did not exert statistically significant effects. These results further demonstrated that combination treatments in cancer therapy may be more effective than single drug treatments.

In combined chemotherapy, drugs with different mechanisms of action are employed simultaneously, reducing the possibility that resistant cancer cells survive and proliferate. When drugs exerting different effects are combined, each drug may be used at the optimal dose, without intolerable side effects (29). Numerous studies have demonstrated that the anticancer activity of standard chemotherapeutic agents is enhanced by the addition of CXB (30). The data from the present study revealed that CXB sensitized TRAIL-resistant MG-63 OS cell lines to TRAIL-induced apoptosis through downregulation of cellular Bcl-2 and survivin expression. These results further confirmed that the administration of CXB in combination with other anticancer drugs improves the antitumor effect.

In conclusion, the present study demonstrated that combining TRAIL with CXB significantly induced cell apop-
tosis and inhibited OS tumor growth in a nude rat model. This combination regimen requires further evaluation in clinical trials, following further preclinical studies.

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References