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Experimental radiotherapy

Ibulocydine sensitizes human cancers to radiotherapy by induction of mitochondria-mediated apoptosis

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Background and purpose: Ibulocydine (IB), a novel prodrug of CDK inhibitor, has been reported to have anti-cancer effect in human hepatoma cells. In order to address its feasibility as a radiosensitizer to improve radiotherapeutic efficacy for human cancers, this study was designed.
Material and methods: Human cancer cells of lung and colon were treated with IB and/or radiotherapy (RT). The cellular effects were assessed by CCK-8, clonogenic, flow cytometric, and western blotting assays. *In vivo* radiotherapeutic efficacy was evaluated using the xenograft mouse model.
Results: Combined treatment of IB and RT significantly reduced viability and survival fraction of the cells. Apoptotic cell death accompanied with activation of caspases, decrease in Bcl-2/Bax expression, loss of mitochondrial membrane potential (MMP) leading to release of cytochrome *c* into cytosol was observed. Recovery of Bcl-2 expression level by introducing Bcl-2 expressing plasmid DNA compromised the loss of MMP and apoptosis induced by IB and RT. *In vivo* therapeutic efficacy of combined treatment was verified in the xenograft mouse model, in which tumor growth was markedly delayed by RT with IB.
Conclusions: IB demonstrated the property of sensitizing human cancer cells to RT by induction of mitochondria-mediated apoptosis, suggesting that IB deserves to be applied for chemoradiotherapy.
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Radiotherapy (RT) plays a crucial role in the local treatment of solid tumors by inducing DNA damage, triggering cell cycle arrest and apoptosis [1,2]. However, the therapeutic efficacy of RT as a major modality for human cancer treatment is often limited due to RT-resistance in many cancers [1,3]. RT-resistance in cancer cells appears to occur through various molecular mechanisms, including differential high activity of Akt, overexpression of Bcl-2 and Bcl-xL, and defects in the release of mitochondrial proteins [4,5]. High expression of inhibitors of apoptosis proteins (IAPs) may also contribute to RT-resistance, since IAPs such as survivin and XIAP block apoptosis at the effector phase [6]. Thus, scientists are currently seeking to identify RT-sensitizers capable of overcoming RT-resistance in cancer cells. Among these, several cyclin-dependent kinase (Cdk) inhibitors including flavopiridol, roscovitine, SNS-032, and AZD5438 were shown to exhibit anticancer activity and to increase the cellular sensitivity to both radiation and chemotherapeutic drugs in a variety of human cancer cells.

The Cdks were recognized as key regulators of cell cycle progression through their association with cyclins [7]. Deregulation of Cdk activation or overexpression of cyclin was frequently found in human cancers. Because uncontrolled cell growth is the hallmark of neoplasic cells, Cdk inhibition appeared to be a potent target to cancer treatment. Ibulocydine (IB) is an isobutyrate ester prodrug of a novel synthetic Cdk inhibitor and has activity against Cdk7 and Cdk9. IB displayed antiproliferative and antitumoral effects in human hepatocellular carcinoma cells as in our previous report [8]. IB caused rapid downregulation of anti-apoptotic protein, thus inducing apoptosis.

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In the present study, we investigated the effect of IB in combination with RT in human cancers to assess a possibility for clinical application of IB. We demonstrated that RT in combination with low doses of IB significantly induced apoptotic cell death through activation of caspases led from loss of MMP and release of cytochrome *c* by down-regulation of Bcl-2/Bax, suggesting that IB can be clinically applied as a radiosensitizer for chemoradiotherapy of human cancer.

Materials and methods

Reagents

The antibodies against PARP and cytochrome *c* (BD biosciences Pharmingen); caspase-9 (Upstate); caspase-8 (Millipore); Bcl-2 (Invitrogen); Mcl-1 (Calbiochem); Flag and β-actin (Sigma); VDAC and survivin (Santa Cruz Biotechnology); caspase-3, cleaved caspase-3, Bax, Bcl-xL, cIAP1, Bad, Bid, XIAP and cIAP2 (Cell Signaling); p-RNA pol II (S2), p-RNA pol II (S5), NOXA and RNA pol II (Abcam); rabbit IgG horseradish peroxidase, mouse IgG, and goat IgG (Zymed Laboratories, Inc.) were used.

Culture of cancer cells

Human lung cancer A549 cells were cultured in F-12 K containing 10% fetal bovine serum and antibiotics (Life Technologies). Human colon cancer RKO cells and human hepatocellular carcinoma cancer Hep3B cells were cultured in MEM containing 10% fetal bovine serum and antibiotics (Life Technologies). Human breast cancer MCF-7 cells were cultured in RPMI containing 10% fetal bovine serum and antibiotics (Life Technologies).

Cell viability assay and clonogenic assay

Cytotoxicity was assayed using Cell Counting Kit-8 (Dojindo) according to the manufacturer's protocol. For clonogenic assay, cells were seeded in a 6-well plate and exposed to different doses of RT after pretreatment with IB at 0.01 or 0.1 μM for 2 h. The cells were incubated for 9 days to allow colony formation, and stained with 0.5% crystal violet solution in 10% methanol. The colonies composing >50 cells were counted, and the survival fraction for each treatment group was normalized to the surviving fraction from the untreated controls.

Establishment of the stable cell lines overexpressing Bcl-2

A549 and RKO cells were transfected with Flag-tagged backbone vector (3261) or *bcl-2* cDNA (kindly provided by Dr. A. Strasser, The Walter and Eliza Hall Institute of Medical Research) and selected in fresh media containing 4 μg/ml puromycin. Overexpression of Bcl-2 in these stable cells was verified by western blotting using Flag-antibody and Bcl-2-antibody.

Annexin V-Fluorescein and PI staining

Cells were digested with trypsin and washed twice with PBS. The experiment was performed using the Annexin-V-FLUOS Staining Kit (Roche) according to the manual. Cells were stained with 5 μl Annexin-V-FLUOS and 1 μl propidium iodide (PI) staining solution in the dark at room temperature for 15 min. The cell samples were analyzed by flow cytometry (Becton–Dickinson).

Cell cycle analysis

Trypsinized and floating cells were pooled, washed with PBS–EDTA, and fixed in 70% (v/v) ethanol. DNA contents were assessed by staining cells with propidium iodide and monitoring by flow cytometry. Cell distribution was determined with a ModFit LT program (Verity Software House).

In vivo tumor growth delay

A549 cells were used to produce a xenograft tumor model. A suspension of 1×10^6 cells in a 50 μl volume was subcutaneously injected into the right hind limb of male Balb/c nude mice (5-week old; SLC). Tumors were grown until average tumor volume reached 70–80 mm^3. Mice were then divided into groups ($n = 4$/group). At 2 h after intratumoral injection of IB (100 μg/mouse), tumors were irradiated to 2 Gy using a 6-MV photon beam linear accelerator (CL/1800, Varian Medical System Inc.). The treatment of IB and RT was repeated three times in three-day interval. The tumor volume was calculated using the formula [$V = (L \times W^2) \times 0.5$, where V = volume, L = length, and W = width] [9].

Statistical analysis and determination of synergy

All data are presented as mean ± SE from at least three separate experiments. The statistical analyses were performed using SPSS v12.0.1 software (SPSS). Mean values were calculated using *T*-tests and analysis of variance with Bonferroni correction. All *P*-values <0.05 were considered to represent significant differences. The possible synergistic effect of IB and RT was evaluated using the isobologram method. In brief, the cells were treated with different concentrations of IB and RT alone or in combination. After 24 h, relative survival was assessed and the concentration-effect curves were used to determine the IC_{50} (the half-maximal inhibitory concentration) values for each agent alone and in combination with a fixed concentration of the second agent.

Supplementary information

Details regarding the other methods including measurement of mitochondrial membrane potential (MMP), isolation of cytosolic fractions, analysis of mitochondrial protein release, Quantitative real-time PCR analysis and Western blot analysis are provided in the Supplementary Information.

Results

To evaluate the cytotoxic effect of IB on human lung cancer A549 and human colon cancer RKO cells, cells were treated with varying doses of IB for 24 h. The viability of cells was reduced by IB at doses of 1 μM for A549 cells and above 0.8 μM for RKO cells (Supplementary Fig. 1A). Although RT alone up to 10 Gy induced a limited cell death (<10%) over 24 h, RT in the presence of IB significantly decreased cell viability in both cells (Supplementary Fig. 1B). In addition, other human cancer cells, Hep3B and MCF-7 cells, induced cell death by IB plus RT (Supplementary Fig. 1B). Furthermore, an isobologram analysis demonstrated that there were synergistic interactions between IB and RT in A549, RKO, Hep3B and MCF-7 cells (Supplementary Fig. 1C). The effect of IB on long-term survival of the cells subjected to RT was determined by clonogenic assay. The survival fraction was markedly decreased by the combination of IB with RT in both cells (Supplementary Fig. 1D). These results demonstrated that a sub-lethal dose of IB efficiently sensitized human cancer cells to RT.

We addressed the characterization of cell death associated with radiosensitization by IB. The change of DNA content was examined upon treatment with IB and/or RT. Co-treatment of IB and RT for 24 h increased the accumulation of sub-G1 phase cells, whereas IB or RT alone did not (Fig. 1A). The annexin V/PI double-staining detecting phosphatidylserine externalization showed that cells

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