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Apoptotic toxicity of destruxin B in human non-Hodgkin lymphoma cells

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ABSTRACT

Destruxins are fungal toxins used as insecticides. Recent reports demonstrated the potential anti-cancer activities of destruxin B (DB). This study is to discover the effects of DB in lymphoma. Flow cytometry and Western blotting were used to analyze apoptosis and protein expression, respectively, in Toledo human non-Hodgkin lymphoma cells in response to DB. Administration of DB, induced apoptosis via death receptor pathway activating Fas associated death domain (FADD), caspase 8 and caspase 3, and suppressed the cell growth. In addition, DB alterated mitochondrial membrane potential by increasing the expressions of tBid and Bax, but decreasing the levels of Bcl-2, resulting in the release of apoptosis-inducing factor (AIF). In conclusion, apoptosis of human non-Hodgkin lymphoma cells in response to DB is induced through the death receptor pathway and involves an alteration of the mitochondrial membrane potential. These findings may aid the development of novel treatment of non-Hodgkin lymphoma.

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1. Introduction

Lymphomas comprise a heterogeneous group of neoplasms characterized by the proliferation of lymphoid cells or their precursors and are the most common nonepithelial head and neck malignancies (Yuen and Jacobs, 1999). They often present as multiple enlarged lymph nodes in the neck and are frequently identified first by otolaryngologists. Head and neck surgeons often obtain tissue for diagnosis and follow management (Nayak and Deschler, 2003). Lymphoma is typically categorized as Hodgkin or non-Hodgkin. Non-Hodgkin lymphoma is five times more common than Hodgkin lymphoma in the head and neck region (Lu, 2005). Various treatments for late-stage patients, including radiation and chemotherapy, result in tremendous morbidity and little gains in terms of survival (The International Non-Hodgkin's Lymphoma Prognostic Factors Project, 1993; Mawardi et al., 2009). The Toledo cell line, a non-Hodgkin lymphoma cell line established from peripheral blood leukocytes of patients with diffuse B-cell lymphoma in 1990, has been used to study the effects and mechanisms of different therapeutic regimens for non-Hodgkin lymphoma (Gabay et al., 1999).

Destruxins, first reported in 1961, are secondary metabolites and biological toxins secreted by a range of fungi, including *Metarhizium anisopliae,* one of the entomopathic fungi that produces mycotoxins, and have been used as insecticides for decades (Pedras et al., 2002). According to their chemical structures, destruxins are cyclic hexadepsipeptides and may be categorized into subtypes A through F, with A, B, and E subtypes being most common (Pedras et al., 2000). Destruxins possess a wide variety of biological activity, with diverse side chains being linked as different functional groups. They were used as insect neurotoxins and biological control agents (Skrobek and Butt, 2005). Other activities, including suppression of the immune response (Pal et al., 2007; Vey et al., 2002), inhibition of bone-resorbing osteoclasts (Nakagawa et al., 2003), antivirus (Yeh et al., 1996), and phytotoxic activity (Pedras et al., 2000), have also been investigated. Among the derivatives, destruxin B (DB) is less toxic. The LC_{50} of DB at 48 h was 137 mg/L to an early nymph stage of the insect Bemisia tabaci, and higher to late stages (Hu et al., 2009). DB was reported to inhibit the activity of vacuolar-type H^+-translocating ATPase in insects

Abbreviations: AIF, apoptosis-inducing factor; Apaf, apoptotic protease-activating factor; DB, destruxin B; FACS, fluorescence-activated cell sorting; FADD, Fas associated death domain; FITC, fluorescein isothiocyanate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; PI, propidium iodide.

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(Bandani et al., 2001). It was also shown to suppress the expression of hepatitis B surface antigen in human hepatoma Hep3B cells (Chen et al., 1997). Recently it was evaluated as an anti-cancer agent inducing apoptosis and suppressing proliferation in colorectal cancer cells (Yeh et al., 2012). DB administered subcutaneously up to 15 mg/kg daily was proven to be safe and effective in a murine xenograft model of human colon cancer (Lee et al., 2012).

Interference with the mechanism directing cells to undergo apoptosis results in tumorigenesis. Therefore, this mechanism is a logical target for potential therapeutic intervention. Apoptosis occurs principally via two separate yet interlinked signaling mechanisms: the death receptor pathway (extrinsic pathway), activated by proapoptotic receptor signals at the cell surface, and the mitochondrial pathway (intrinsic pathway), activated by mitochondrial signals from within the cell. These pathways converge through "effector" caspases, which orchestrate the apoptotic program (Ashkenazi and Herbst, 2008; Danial and Korsmeyer, 2004). Extrinsic apoptosis signals are initiated by the activation of specialized proapoptotic membrane receptors, so-called death receptors, by ligands such as the Fas ligand (CD95 ligand) and Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) (Guicciardi and Gores, 2009). Recruitment of the adapter molecule Fas-associated death domain (FADD) promotes downstream caspase activation (Jin and El-Deiry, 2005; Ward et al., 2008). The intrinsic pathway is controlled by interactions between pro- and antiapoptotic members of the Bcl-2 protein family, causing permeabilization of the mitochondrial outer membrane, membrane potential alteration, opening of the mitochondrial permeability transition pore (Kang and Reynolds, 2009), and the release of soluble molecules such as cytochrome *c*, apoptosis-inducing factor (AIF), and Second Mitochondrial Activator of Caspases/Direct IAP Binding protein with Low pI (Smac/DIABLO) into the cytoplasm (Armstrong, 2006; Mayer and Oberbauer, 2003). Many researchers have tried to regulate the expression of apoptosis-related proteins and/or their corresponding inhibitory proteins to counteract the proliferation of cancer cells. An antitumor effect of DB has been demonstrated in some tumor cell lines, but its effect on lymphoma cells has not been investigated. The purpose of this study was to evaluate the effects of DB on the Toledo human non-Hodgkin lymphoma cells, and to elucidate the underlying apoptotic mechanisms.

2. Experimental methods

2.1. Cell culture

The human lymphoma (Toledo) cell line was purchased from American Type Culture Collection (Manassas, VA). Toledo tumor cells were cultured in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO) containing 10% fetal calf serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate in 75-cm^2 flasks in an incubator at 37 °C under 5% CO_2. After 72 h, nearly confluent cells were subcultured. When the cells again reached confluence, they were harvested and centrifuged, washed once in phosphate-buffered saline (PBS), and centrifuged again. A volume of 10 mL RPMI-1640 medium was added, the cells were dispersed evenly, and then were plated in Petri dishes for use, leaving approximately one-fifth of the cells in the original bottle for continued culture.

2.2. Drug preparation

Destruxins were prepared as described in a previous protocol (Liu et al., 2004). Briefly dextruxins were extracted from fermentation broth with the use of cyanide methane, and the four subtypes of destruxin, DA, DB, DE and desmethyl DB (DMDB), were separated and collected by semipreparative, high-performance liquid-phase chromatography. Fast atom bombardment mass spectrometry was used to test product purity, and the concentration of purified extract and peak area of chromatography were analyzed by linear regression to establish a standard curve for quantitative analysis. The extract was dissolved in acetonitrile for experiments.

2.3. Cell viability

Cell viability was assessed with the use of trypan blue (Life Technologies, Carlsbad, CA) exclusion assay and cell counting. Cells were plated at 1×10^5 cells/well in 6-well plates and cultured, and DB at a concentration of 1.26 μM (0.75 mg/L), 2.53 μM (1.5 mg/L), 5.05 μM (3 mg/L), or 10.1 μM (6 mg/L) was added. The same concentrations of acetonitrile were used as a control. After 24 h, cells were harvested every 12 h for 48 h (for a total of 3 time points), stained with trypan blue, and the number of surviving cells was quantified by cell counting.

2.4. Flow cytometric analysis of propidium iodide and annexin V double staining

During apoptosis, phosphatidylserine is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V has a strong, Ca^{2+}-dependent affinity for phosphatidylserine and therefore is used as a probe for detecting apoptosis (Vermes et al., 1995). Flow cytometry was performed with a 15-μW argon ion laser with an emission wavelength of 448 nm. Cells cultured in 10-cm^3 dishes were treated with DB (or acetonitrile as a control). After 48 h, cells were harvested at 4 °C, centrifuged at 2000 rpm for 5 min, washed with ice-cold PBS, and diluted to 2×10^5 cells/mL. The supernatant was removed from each tube, and 85 μL binding buffer (MEBCYTO Apoptosis Kit; MBL, Nagoya, Japan) was added with mixing, followed by the addition of 10 μL annexin V-fluorescein isothiocyanate (FITC) and 5 μL propidium iodide (PI; MBL) and gentle mixing at room temperature in the dark for 15 min. A volume of 400 μL binding buffer was added to each tube, and flow cytometry was performed within an hour. Each sample was tested in duplicate.

2.5. Flow cytometric analysis of mitochondrial membrane potential

The lipophilic cation-staining agent 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was used to assess changes in mitochondrial membrane potential. The JC-1 monomer is released from mitochondria into the cytoplasm when the mitochondrial membrane potential is less than 100 mV, emitting a green fluorescence signal in response to a 488-nm excitation wavelength. As the membrane potential increases, JC-1 accumulates along the inner mitochondrial membrane and emits an orange-red fluorescence signal, which appears in the upper left quadrant of the fluorescence-activated cell sorting (FACS) display (J-aggregate). When apoptosis occurs, the mitochondrial membrane potential decreases, and the JC-1 monomer emits a green signal, which appears in the upper right and lower right quadrants of the FACS display (Bedner et al., 1999; Salvioli et al., 1997). The procedure was the same as that described above for PI/annexin V double staining, with cells diluted to 1×10^6 cells/mL. A volume of 2 μL JC-1 (Invitrogen, Paisley, UK) was added to 1-mL cell suspensions, which were mixed well and incubated at room temperature for 10 min, and flow cytometry was performed within an hour.

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