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Induction of apoptosis by streptochlorin isolated from *Streptomyces* sp. in human leukemic U937 cells

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ABSTRACT

Streptochlorin is a small molecule that produced by marine *Streptomyces* sp. that is known to have anti-angiogenic and anti-cancer properties. However, the mechanism by which streptochlorin functions is not well understood. In this study, we investigated the pro-apoptotic effect of streptochlorin in human leukemic U937 cells. Streptochlorin treatment resulted in concentration- and time-dependent growth inhibition by inducing apoptosis. The increase in apoptosis that was induced by streptochlorin was correlated with down-regulation of anti-apoptotic Bcl-2 expression, up-regulation of pro-apoptotic Bax and FasL, a decrease in the mitochondrial membrane potential (MMP), activation of caspases and degradation of poly-(ADP-ribose)polymerase and phospholipase C-γ1 protein. In addition, the cytotoxic effects and apoptotic characteristics induced by streptochlorin were significantly inhibited by z-DEVD-fmk, a caspase-3 inhibitor, which demonstrates the important role that caspase-3 played in the process. Furthermore, Bcl-2 overexpression significantly reversed the streptochlorin-induced growth inhibitory effects via inhibition of the MMP collapse and caspases activation and effectively attenuated the apoptotic response to streptochlorin. However, the elevated levels of FasL expression induced by streptochlorin were not reduced by Bcl-2 overexpression. Taken together, these findings demonstrate that the pro-apoptotic effect of streptochlorin is mediated through activation of caspases and mitochondria in U937 cells.

1. Introduction

Apoptosis is the active process of programmed cell death that occurs during many important physiological conditions, such as embryonic development and tissue remodeling. However, most cancer cells block apoptosis, which allows them to survive despite undergoing genetic and morphologic transformations. Apoptotic cells are characterized by several unique features, including cell shrinkage, chromatin condensation, DNA fragmentation, cell surface expression of phosphatidylserine, and membrane blebbing (Okada and Mak, 2004; Jin and el-Deiry, 2005; Han et al., 2008). A central component of the apoptotic machinery is a family of cystein-containing aspartate-specific proteases, termed caspase. Caspases are present in cells as inactive proenzymes, with the active tetramer being formed as a result of removal of the prodomain and cleavage between the large and small subunits (Hengartner, 2000; Suda et al., 1993). Caspase activity is either directly or indirectly responsible for the cleavage of several intracellular proteins that are characteristically proteolysed during apoptosis. It has been reported that two major pathways, the Fas/tumor necrosis factor (TNF) death receptor (extrinsic) pathway and the mitochondria-dependent (intrinsic) pathway, lead to activation of caspases and

Abbreviations: DAPI, 4,6-diamidino-2-phenylindile; DEVD, Asp-Glu-Val-Asp; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EtBr, ethidium bromide; FasL, Fas ligand; FBS, fetal bovine serum; IETD, Ile-Glu-Thr-Asp; JC-1, 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; LEHD, Leu-Glu-His-Asp; MMP, mitochondrial membrane potential; PARP, poly-(ADP-ribose)polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; PLC, phospholipase C; pNA, *p*-nitroaniline; TNF, tumor necrosis factor.

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consequent apoptosis in mammalian cells (Fulda and Debatin, 2006; Wajant et al., 2005). The Fas/TNF death receptor pathway is initiated by binding of a ligand to the Fas/TNF death receptor on the cell surface, which then activates caspase-8 and apoptotic cell death. However, changes in the mitochondrial integrity in response to a broad range of physical and chemical stimuli can trigger the intrinsic pathway of apoptosis (Chowdhury et al., 2006; Fulda and Debatin, 2006). It is believed that the release of cytochrome *c* from the mitochondria into the cytosol is a key event in the intrinsic pathway (Mow et al., 2001; Mohamad et al., 2005). Once in the cytosol, cytochrome *c* can activate caspase-9, which in turn cleaves and activates the executioner, caspase-3. After activation, several specific substrates for caspase-3 including poly-(ADP-ribose)polymerase (PARP) and phospholipase C (PLC)-γ1 are cleaved, which eventually leads to apoptosis (Lazebnik et al., 1994; Bae et al., 2000). Consistent with the role that the mitochondria plays in the control of cell death, survival or apoptotic factors such as the Bcl-2 family act on the organelle to prevent or facilitate the release of apoptogenic factors (Yin, 2000; Zinkel et al., 2006; Jeong and Seol, 2008). Many recent studies have shown that many chemopreventive and/or chemotherapeutic agents can cause tumor cell death via the induction of apoptosis, which is the preferred method of managing cancer. Therefore, the induction of apoptotic cell death is an important mechanism in the anti-cancer properties of many drugs.

Recent studies have shown that marine microorganisms are a novel and rich source of bioactive compounds due to their potential pharmacological activities. However, it is believed that molecules produced by those microorganisms have therapeutic properties that have not yet been discovered (Schweder et al., 2005; Singh and Pelaez, 2008). In addition, studies evaluating the components of marine microorganisms have shown that many are not general cytotoxic agents, but are instead targeted towards specific cellular or biochemical events and may therefore be useful as anti-microbial, anti-cancer or anti-inflammatory agents (Proksch et al., 2002; Singh and Pelaez, 2008). We previously screened marine natural products for the ability to induce apoptosis in cancer cells and found that streptochlorin isolated from *Streptomyces* sp. exhibited selective cytotoxicity against several cancer cell lines (Choi et al., 2007; Shin et al., 2007). However, the mechanism by which streptochlorin exerts its function is not yet fully understood. Therefore, we used the human leukemic U937 cell line as a model system to investigate the effects of streptochlorin-induced apoptosis. We found that streptochlorin-induced apoptosis is accompanied by modulation of the Fas/Fas ligand (FasL) system, Bcl-2 family members, mitochondrial dysfunction and activation of caspases. In addition, blocking of caspase-3 activation and Bcl-2 overexpression reduced streptochlorin-induced apoptosis in U937 cells.

2. Materials and methods

2.1. Cell culture and viability assay

U937 cells were obtained from the American type culture collection (Rockville, MD), and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Gaithersburg, MD) at 37° and 5% CO_2. The Bcl-2 overexpressing U937 (U937/Bcl-2) cells were a generous gift from Dr. T.K. Kwon (Department of Immunology, Keimyung University School of Medicine, Taegu, Korea) and maintained in a medium containing 0.7 μg/mL geneticin (G418 sulfate). Streptochlorin isolated from *Streptomyces* sp. (strain 04DH110) was prepared as described previously (Shin et al., 2007) and dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 1 mg/mL concentration, and stored in aliquots at −20 °C. Cell number and its viability were determined by tryphan blue exclusion assay and MTT assay, respectively.

Table 1
Gene-specific primers for RT-PCR

Name		Sequence of primers
TRAIL	Sence	5′-ATG GCT ATG ATG GAG TCC AG-3′
	Antisence	5′-TTG TCC TGC ATC TGC TTC AGC-3′
DR4	Sence	5′-CAG AAC GTC CTG GAG CCT GTA AC-3′
	Antisence	5′-ATG TCC ATT GCC TGA TTC TTT GTG-3′
DR5	Sence	5′-GGG AAG AAG ATT CTC CTG AGA TGT G-3′
	Antisence	5′-ACA TTG TCC TCA GCC CCA GGT CG-3′
Fas	Sence	5′-TCT AAC TTG GGG TGG CTT TGT CTT C-3′
	Antisence	5′-GTG TCA TAC GCT TTC TTT CCA T-3′
FasL	Sence	5′-GGA TTG GGC CTG GGG ATG TTT CA-3′
	Antisence	5′-AGC CCA GTT TCA TTG ATC ACA AGG-3′
Bax	Sence	5′-ATG-GAC-GGG-TCC-GGG-GAG-3′
	Antisence	5′-TGG-AAG-AAG-ATG-GGC-TGA-3′
Bcl-2	Sence	5′-CAG-CTG-CAC-CTG-ACG-3′
	Antisence	5′-GCT-GGG-TAG-GTG-CAT-3′
Bcl-xL	Sence	5′-CAG CTG CAC CTG ACG-3′
	Antisence	5′-GCT GGG TAG GTG CAT-3′
GAPDH	Sence	5′-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3′
	Antisence	5′-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3′

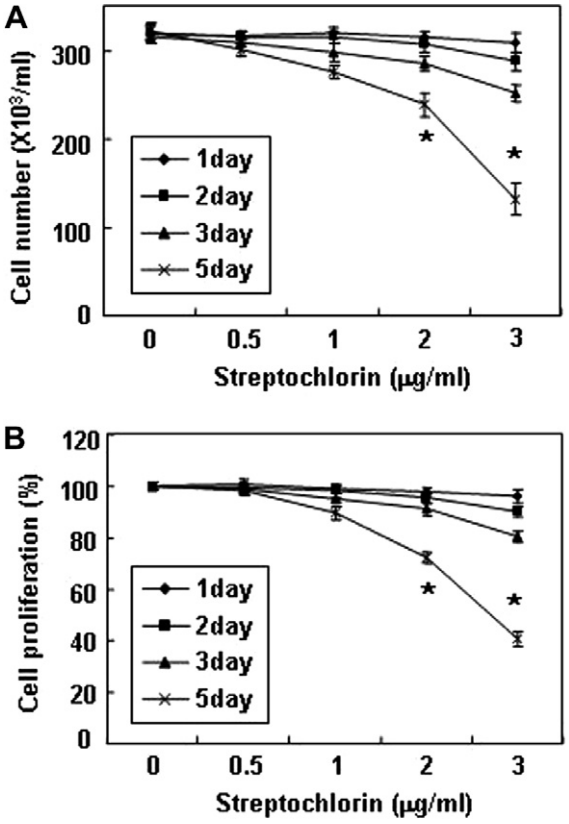

Fig. 1. Growth inhibition in response to streptochlorin treatment of U937 cells. U937 cells were plated at a concentration of 1×10^5 cells per 60-mm plate and then incubated for 24 h, after which they were treated with various concentrations of streptochlorin for the indicated times. (A) Cell number and (B) viability were determined by hemocytometer counts of tryphan blue-excluding cells and an MTT assay, respectively. Each time point represents the means ± SD of three independent experiments. The significance was determined by a Student's *t*-test (*$p < 0.05$ vs. vehicle control).