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Shikonin induces programmed necrosis-like cell death through the formation of receptor interacting protein 1 and 3 complex

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ABSTRACT

An alternative cell demise programmed necrosis has also been proposed when apoptotic machinery is impaired or blocked during tumor necrosis factor alpha (TNFα) stimulation. Shikonin (SKN), an herbal extract from the Chinese plant, has been reported to induce either apoptosis or necrosis depending on cell types or its concentrations. In this presentation, SKN caused cell death of NIH3T3 in a dose-dependent manner. Intriguingly, SKN-mediated cell death was in part protected by necrostatin-1 (Nec-1), a specific inhibitor of programmed necrosis, but not zVAD a pan-caspase inhibitor. SKN directly mediated cell death via receptor interacting protein1 and 3 (RIP1-RIP3) complex formation, which is required for TNFα-mediated programmed necrosis. Additionally, SKN-caused cell death was reversed by a reactive oxygen species (ROS) scavenger *N*-acetylcysteine (NAC) whereas TNFα-mediated necrosis was successfully protected by butylated hydroxyanisole (BHA), implying that ROS may be differentially derived from death inducing agents. Concurrently with the protective effect of the ROS scavenger or Nec-1 on TNFα or SKN, the RIP1–RIP3 complex was significantly affected in the presence of those agents. Here, it is highlighted that SKN as well as TNFα can directly mediate cell death via a pronecrotic complex, but ROS were generated via different routes depending on cell death-inducing agents.

1. Introduction

Programmed cell death is executed in a well-orchestrated manner through a cascade of specific intracellular biochemical pathways. Therefore, apoptosis and autophagy are included in programmed cell death whereas necrosis has been regarded as unwanted cell death. However, under the specific condition that caspase is impaired or hampered by ischemic circumstances and pathogenic infection, cells switch cell death mode from apoptosis to necrosis (Graziani and Szabo, 2005). There is a growing body of evidence that specific proteins are involved in driving necrosis via a signaling pathway downstream from the death signal, such as tumor necrosis factor alpha (TNFα) (de Murcia et al., 1994; Gobeil et al., 2001; Holler et al., 2000; Lautier et al., 1993; Liu and Han, 2001). Such an alternative cell death is coined programmed necrosis or necroptosis to discriminate from classical necrosis (Cho et al., 2010; Lamkanfi et al., 2007; Rangamani and Sirovich, 2007). Unlike apoptosis, programmed necrosis is thought to exhibit distinctive characteristics of morphology, membrane integrity, and biochemical traits, similarly to necrosis. Specialized cell death by other putative necrosis-inducing agents including TNFα was corroborated by the protection of dying cells with the treatment of necrostatin-1 (Nec-1) (Thon et al., 2006). Besides TNFα, inorganic heavy metals and organic chemicals are also proposed to mediate programmed necrosis-like cell death but not apoptosis (Krumschnabel et al., 2010; Scholz et al., 2005; Xu et al., 2006).

Shikonin (SKN) is a purple naphthoquinone dye isolated from the root of a medicinal plant, *Lithospermum erythrorhizon* Sieb. et Zucc (Brigham et al., 1999). It has been widely used as a traditional herbal medicine for a variety of inflammatory and infectious diseases including macular eruption, measles, sore throat, and carbuncles, as well as burns and wounds healing (Lee et al., 2010). On the other hand, SKN itself exerts two death modes of apoptosis and necrosis in HL-60 cells depending on its concentrations (Han et al., 2009). Moreover, the cell death of MCF-7 induced by SKN is distinct from apoptosis and is characterized by a loss of plasma membrane integrity and morphology, indications of necrotic cell death (Han et al., 2007). In those articles, cells exposed to SKN at high concentrations were committed to necrotic cell death and were effectively protected with treatment of Nec-1, a specific inhibitor of receptor interacting protein-1 (RIP-1) required for

Abbreviations: RIP1, receptor interacting protein 1; RIP3, receptor interacting protein3; SKN, shikonin; TNFα, tumor necrosis factor alpha; ROS, reactive oxygen species; Nec-1, necrostatin-1; zVAD, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone.

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programmed necrosis, suggesting that SKN may induce programmed necrosis-like phenotype. More intriguingly, two different research groups pronounced that a RIP1–RIP3 pronecrotic complex was a prerequisite for TNFα-induced programmed necrosis (Cho et al., 2009; Zhang et al., 2009). Therefore, RIP3-directed necrotic cell death contributes to not only the development of diseases but also innate immune response to microbial infection. We raised questions as to how cells would die by other chemical insults aside from TNFα. As a result, we showed that SKN mediated necrotic cell death via a RIP1–RIP3 complex similar to TNFα-directed necrotic cell death, and this pronecrotic complex was blocked by a reactive oxygen species (ROS) scavenger or Nec-1 concomitantly with protection against cell death. To our knowledge, this is the first report that a chemical itself directly facilitates the formation of a pronecrotic complex without activation of a death receptor, consequentially leading to necrotic death. It is suggested that a variety of chemicals or inorganic agents in addition to TNFα might induce programmed necrosis but not apoptosis.

2. Materials and methods

2.1. Chemicals

For cell culture, Dulbecco's modified Eagles medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (South Logan, UT, USA). Antibiotics/antimycotics and trypsin EDTA were purchased from Gibco (Grand Island, NY, USA), and SKN and TNFα were purchased from Cayman Chemical (Ann Arbor, MI, USA). Phosphatase and protease inhibitors were obtained from Thermo Scientific (Rockford, IL, USA). We used antibodies against RIP3 from Prosci (Poway, CA, USA) and RIP1 from BD Pharmingen (San Jose, CA, USA) for detection and validation of the RIP1–RIP3 complex. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was purchased from Promega (Madison, WI, USA). Precast 4–12% NuPAGE gel and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) were purchased from Invitrogen (Carlsbad, CA, USA). Butylated hydroxyanisole (BHA), zVAD (benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone), an extended duration developing solution, and propidium iodide (PI) were obtained from BD Pharmingen (Franklin Lakes, NJ, USA). All other analytical chemicals were purchased from Sigma (Steinheim, Germany).

2.2. Cell culture

Mouse fibroblast cell line NIH3T3 (CRL-1658) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in high glucose–DMEM, 10% FBS, and 1% antibiotics/antimycotics, and allowed to incubate at 37 °C in a 5% CO_2 incubator.

2.3. Cell viability assay

The viability of the cells was determined by a non-radioactive proliferation assay, MTS. The trypsinized cells were replated in 96-well plates at a density of 1×10^4 cells/each well. The next day, cells were subjected to treatment with SKN or TNFα for the induction of apoptosis or necrosis. For apoptosis, cells were stimulated with TNFα in the presence of CHX (10 mg/ml) for 16 h. To shift cell death mode from apoptosis to necrosis, zVAD (10 μM) was added to cells under the apoptosis conditions described above. Besides TNFα, a chemical cell death inducer SKN was tested in NIH3T3 by varying the concentrations of SKN or combining SKN with either Nec-1 or zVAD. Unless otherwise stated, DMSO was used as a solvent of the chemical at a concentration of less than 0.5% so as not to disturb cell viability in the pilot study. Following the indicated times of drug treatment, MTS was directly added to the culture media. The plates were further incubated for development for 1 h at 37 °C and the color development was read immediately at a wavelength of 490 nm in a plate reader.

2.4. Detection of intracellular ROS generated by SKN

NIH3T3 cells were exposed to SKN at the indicated concentrations for 3 h. For the detection of ROS, DCFDA (1 μM) was added to cells, which were further kept in an incubator for 30 min so as to emit fluorescence derived from oxidized DCFDA. ROS production in cells treated with SKN was observed under a fluorescence microscope. Additionally, to examine membrane integrity of the cells, an indicator of necrotic cell death, cells were stained with PI, which permeates damaged cells, and were microscopically imaged.

2.5. Immunoprecipitation (IP) assay and Western blots

Cell lysates were prepared by lysis in 150 mM NaCl, 20 mM Tri–HCl (pH 7.4), 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1× protease inhibitor, and 1× phosphatase inhibitor for 15 min on ice. After preclearing lysates with beads, cell extracts were immunoprecipitated with an antibody against RIP3 overnight at 4 °C. The resulting immune complexes were rinsed three times with a lysis buffer, resolved on 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA, USA), and finally transferred to membranes. The membranes were blocked in 5% fat-free milk for 1 h, and subsequently allowed to incubate overnight at 4 °C with either RIP1 or RIP3 primary antibody diluted in 5% fat-free milk. Probed membranes were exhaustively washed with 1× Tris-buffered saline containing Tween-20 [TBST, 10 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween 20] and were subjected to incubation for 1 h with HRP-conjugated secondary antibodies against mouse or rabbit. After rinsing the membrane four times with 1× TBST, protein bands of interest were visualized on X-ray film by an extended duration substrate system (Thermo Scientific, Rockford, IL, USA).

2.6. Statistical analysis

All results are expressed as the means ± S.E.M of data from at least three separate experiments. Statistical significance was determined with the Student's *t*-test from two points. A level of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. SKN induces necrosis in NIH3T3 cells

To delineate the cell death modes by SKN, NIH3T3 cells were exposed to SKN in combination with either zVAD or Nec-1. SKN significantly caused cell death of NIH3T3 cells in a dose dependent manner (Fig. 1A), exerting 80% cytotoxicity at 2.5 μM within 3 h. Introduction of a pan-caspase inhibitor, zVAD, did not affect cellular damage induced by SKN whereas Nec-1 effectively protected cells from SKN treatment by roughly 50% (Fig. 1B). For comparison, TNFα treatment along with CHX induces cell death, a reported typical apoptosis, but not as strongly as SKN (Fig. 1C). The addition of zVAD to cells under apoptosis-inducing condition (C/T) did not block apoptosis, but sensitized rather cells to TNFα stimulation. As can be deduced from the molecular target of Nec-1, a necroptosis inhibitor, Nec-1 significantly rescued cells from the necrotic death signals but not apoptotic cell death caused by TNFα.

3.2. SKN induces the formation of RIP1–RIP3 pronecrotic complex

In a previous report, the formation of pronecrotic complex RIP1–RIP3 is required for TNFα-mediated programmed necrosis (Cho et al., 2009; He et al., 2009). To shed light on whether RIP3 or RIP1 is involved in SKN-stimulated necrosis similarly to TNFα, the RIP1–RIP3 complex was identified by immunoprecipitation using an anti-RIP3 antibody from cells exposed to SKN (Fig. 2). As a result, the pronecrotic complex between RIP1 and RIP3 was also clearly induced by SKN treatment, but its formation was abrogated in the presence of Nec-1, reflecting that its molecular interaction is closely associated with cell death as shown in Fig. 1. As expected, TNFα caused noticeably the formation of pronecrotic complex in the presence of zVAD when compared to SKN. TNFα-driven complex formation was also ameliorated by increasing the dosage of Nec-1.

3.3. SKN-induced cell death is mediated by ROS generation

Unlike apoptosis, ROS is generally known to be generated during necrotic cell death. Therefore, we studied the involvement of ROS generation when SKN caused cell death. Similar to cells stimulated with TNFα, SKN caused considerable ROS production, as detected by a chemical probe (Fig. 3A). ROS-positive cells were increased with exposure times or doses by SKN treatment (data not shown). Based on the results that ROS were generated by SKN, well-known ROS scavengers were employed to determine