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Original Contribution

ROS-dependent activation of autophagy is a critical mechanism for the induction of anti-glioma effect of sanguinarine

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ABSTRACT

Malignant gliomas are notoriously resistant to therapies that induce apoptosis, but are less resistant to therapies that induce autophagy. Therefore, drugs targeting autophagy are promising candidates in the treatment of malignant gliomas. In this study, we investigated the anti-glioma potential of sanguinarine (SNG) *in vitro*, and further examined the molecular mechanisms of SNG-induced cell death. In human malignant glioma cells SNG activated autophagic cell death pathway characterized by increased acidic vesicular organelles formation, GFP-LC3 punctate formation, LC3-II conversion, and expression of autophagy related proteins, such as Atg5 and Beclin-1. The autophagy inhibitor bafilomycin A1 or knockdown of Atg5 markedly inhibited the SNG-induced autophagic cell death. Apart from inducing autophagic cell death, SNG has also been shown to induce apoptotic cell death in these cell lines. Importantly, the study also identified the crucial role of reactive oxygen species (ROS)-dependent activation of the extracellular signal-regulated kinase1/2 (ERK1/2) in the facilitation of SNG-induced autophagic cell death. Antioxidants, such as glutathione and N-acetyl cysteine, significantly abrogated ROS production, ERK1/2 activation, and in turn, prevented SNG-induced autophagic cell death. Moreover, scavengers of H2O2 (sodium pyruvate and catalase) significantly attenuated the activity of SNG. Down-regulation of ERK1/2 activity, by using selective ERK1/2 inhibitor (U0126) or siERK1/2, led to an inhibition of SNG-induced autophagic cell death. Furthermore, tumor cells transfected with constitutively active ERK2-MEK1-LA fusion protein accentuated SNG-induced autophagic cell death. Overall, our findings unveil a novel anti-tumor mechanism of action of SNG in human malignant glioma cells, opening up the possibility of using SNG based pro-autophagic drugs for the treatment of malignant glioma.

1. Introduction

Glioma is the most aggressive primary brain tumor in humans. Despite the availability of many therapeutic approaches, the median survival duration of patients with malignant glioma remains to be 12 months for the past decade [1,2]. Thus, new therapeutic agents and strategies are needed for the management of this disease. In recent years, the use of natural products as a realistic option for the treatment of different malignancies has broadly been accepted. Novel anti-tumor compounds from herbal components represent an attractive alternative for drug development. Sanguinarine (SNG), a benzophenanthridine alkaloid isolated from *Sanguinaria Canadensis,* exhibits an ample array of pharmacological activities such as anti-bacterial, anti-fungal, and anti-inflammatory properties [3–8]. It has also been reported that SNG exerts significant inhibitory effects on diverse cellular events associated with initiation, promotion and progression of different malignancies [9,10]. Interestingly, whether SNG suppresses the growth of human malignant glioma has never been investigated. To date, research in to the anti-cancer effect of SNG was predominantly focused on its ability to induce apoptotic cell death [11,12]. Yet, whether SNG mediates its anti-cancer effect *via* activating autophagic cell death signaling pathway has not been elucidated.

Abbreviations: AO, acridine orange; AVOs, acidic vesicular organelles; Baf A1, bafilomycin A1; Cat, Catalase; DCFH-DA, 2', 7'-dichlorodihydrofluorescin diacetate; DMEM, Dulbecco's modified essential medium; DMSO, dimethyl sulfoxide; ERK1/2, extracellular regulated kinase1/2; GFP-LC3, green fluorescent protein tagged microtubule-associated protein light chain 3; GSH, glutathione; GSSG, glutathione disulfide; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein light chain 3; MAPK, mitogen activated protein kinases; MTT, 3-[4, 5–dimethylthiazol–2–yl]-2,5-diphenyl tetrazolium bromide; MitoPY1, Mito Peroxy Yellow 1; Na Py, Sodium pyruvate; NAC, N-acetylcysteine; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; SNG, sanguinarine; SOD, superoxide dismutase; z-VAD-fmk, N-benzoyloxycarbonyl-Val-Ala-Asp fluoromethylketone

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There are two major morphologically distinctive forms of programmed cell death, apoptosis (Type-1 programmed cell death) and autophagy (Type-II programmed cell death). The mechanism of apoptotic cell death is best-described in the literature, and it is initiated and executed by a family of cysteine-dependent aspartate-directed proteases called caspases. Apoptosis plays a crucial role in chemotherapy against a variety of cancers [13]. In contrast to apoptosis, autophagy is a caspase-independent mode of cell death, in which different cellular components and proteins are engulfed by double-membrane vesicles, which are then directed to lysosomes for massive degradation [14]. Recently, attention in autophagy has been renewed amongst cancer biologists. This is due to the fact that depending on the cellular context, autophagy could serve both as a cell survival and cell death mechanism. Autophagy may also function in collaboration with apoptosis to promote cell death, or serves as a back-up mechanism when the former is defective [15]. The molecular regulators for the initiation and execution of apoptotic and autophagic cell death pathways are inter-connected, and may be triggered by common upstream signaling molecule(s) [15]. Therefore, it is suggested that there is a significant and complex cross-talk between apoptosis and autophagy [15].

Reactive Oxygen species (ROS) are oxygen containing highly reactive molecules that play key roles in deciding the fate of cells, depending on the strength and duration of their exposure. Under normal physiological conditions a low level of ROS mediates growth adaptation and survival [16,17]. In contrast, excessive ROS production in response to anti-cancer agents causes cellular damage and induces different modes of cell death [16–18]. Considering the fact that cancer cells generally produce high levels of ROS when compared to normal cells, they are more vulnerable to damage by further ROS insult induced by anti-cancer drugs [17]. It is well known that ROS participate in the activation of both apoptotic and autophagic cell death pathways, through the activation of several transcription factors that are involved in either or both apoptosis and autophagy [18–20].

The extracellular signal-regulated kinase 1/2 (ERK; members of the mitogen activated protein kinase superfamily) signaling pathway plays a crucial role in many cellular events including apoptosis and autophagy [21,22]. Accumulating evidence indicate that ROS can induce sustained activation of ERK by direct oxidation of its up-stream regulators or through inhibition of ERK-specific phosphatases [19,21]. Depending on the cell type, the stimulus, and the duration of activation, ERK is associated with a variety of biological responses such as proliferation, migration, differentiation, apoptosis, and autophagy amongst other [22–25].

In the present study and for the first time, we elucidated the inhibitory effect of SNG on human malignant glioma cells *in vitro*. We demonstrate that SNG induces both apoptosis and autophagy in our cell model, which is regulated by the ERK1/2 activation *via* a ROS-dependent mechanism. Our study provides a rationale for the identification of SNG as a potent chemotherapeutic agent for the treatment of malignant gliomas.

2. Materials and methods

2.1. Chemicals and antibodies

Sanguinarine, glutathione (GSH), glutathione disulfide (GSSG), N-acetyl cysteine (NAC), 3-[4, 5–dimethylthiazol–2–yl]-2,5-diphenyl tetrazolium bromide (MTT), acridine orange (AO), bafilomycin A1 (Baf A1), sodium pyruvate (Na Py), catalase (Cat), superoxide dismutase (SOD), crystal violet, Hoechst 33342, dimethyl sulfoxide (DMSO), Mito Peroxy Yellow 1 (MitoPY1), 2-Vinylpyridine, triethanolamine, anti-rabbit IgG, and anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oxidation sensitive 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Dulbecco's modified essential medium (DMEM), Opti MEM medium, phosphate buffered saline (PBS), trypsin-EDTA, and fetal bovine serum (FBS) were bought from GIBCO BRL (Grand Island, NY, USA). U0126, z-VAD-fmk, Ac-DEVD-CHO, z-LETD-fmk, and z-LEHD-fmk were from Enzo Life Sciences (San Diego, CA, USA). Anti-actin, anti-MAP LC3β, anti-phospho ERK1/2, anti-ERK1/2, anti-phospho JNK1/2, anti-JNK1/2, anti-phospho p38, anti-p38, anti-p53, anti-p21, anti-XIAP, anti-cyclin E, anti-Bcl-xL, and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-PARP, anti-caspase-9, anti-caspase-3, anti-caspase-8, and anti-LC3B antibodies were from Cell Signaling Technology (Beverly, MA, USA). MegaTran 1.0 transfection reagent was from OriGene (Rockville, MD, USA). GSH assay kit was from Cayman chemical (MI, USA)

2.2. Glioma cell lines, cell culture conditions and drug treatment

The human malignant glioma cell lines, U87MG and U118MG (ATCC, Rockville, MD, USA) were grown in DMEM supplemented with 10% heat inactivated FBS. All cell lines were grown without antibiotics in an incubator containing humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Sanguinarine was dissolved in DMSO at a concentration of 10 mM and was stored in a dark colored bottle at −20 °C. The stock was diluted to required concentration with DMSO when needed. Prior to the SNG treatment, cells were grown to about 70% confluence, and then exposed to SNG at different concentrations (0–10 μM) and for different period of time (0–24 h). Cells grown in a medium containing an equivalent amount of DMSO without SNG served as control.

2.3. Cell viability

Cell viability assay was carried out as described previously [26]. Briefly, cells were grown in 96 well microtiter plates (5000 cells/well) and then incubated with different doses of SNG, or for different time periods. After the treatment, 25 μl of MTT (5 mg/ml) was added to each well and the assay was performed as described previously [26].

Cell viability following SNG treatment was also determined by trypan blue dye exclusion test. After the treatment with SNG, floating cells in the medium of each plate were transferred to centrifuge tubes. Adherent cells were detached with trypsin and then added to the corresponding floating cells before centrifugation. The pellets were re-suspended, the cells were stained with 0.04% trypan blue, and number of trypan blue positive and negative cells was counted on a haemocytometer under light microscopy.

2.4. Clonogenic assay

Cells (1500 cell/well) were seeded in 6-well dishes with complete medium and allowed to grow for 24 h. The cells were then incubated in the presence or absence of different concentrations of SNG for up to 24 h. The SNG-containing medium was then removed, the cells were washed in PBS, and incubated for an additional 14 days in complete medium. The cells were supplied with fresh complete medium in every alternative day. Each treatment was carried out in triplicate. The colonies obtained were stained in clonogenic reagent (50% methanol and 0.25% crystal violet) for 30 min at room temperature followed by washing twice with PBS. The colonies were counted and compared with those formed by untreated cells.