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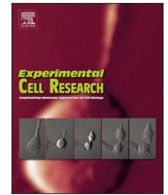

Indomethacin induced glioma apoptosis involving ceramide signals

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A B S T R A C T

Nonsteroidal anti-inflammatory drugs (NSAIDs) are increasingly implicated in the prevention and treatment of cancers apart from their known inhibitory effects on eicosanoid production. One of the NSAIDs, indomethacin, in particular shows promising antineoplastic outcome against glioma. To extend such finding, we here studied in human H4 and U87 glioma cells the possible involvement of the ceramide/protein phosphatase 2 A (PP2A)/Akt axis in the indomethacin-induced apoptosis. We found that the induced apoptosis was accompanied by a series of biochemical events, including intracellular ceramide generation, PP2A activation, Akt dephosphorylation, Mcl-1 and FLICE inhibiting protein (FLIP) transcriptional downregulation, Bax mitochondrial distribution, and caspase 3 activation. Such events were also duplicated with a cell-permeable C2-ceramide and Akt inhibitor LY294002. Pharmacological inhibition of ceramide synthase by fumonisin B1 and PP2A by okadaic acid moderately attenuated indomethacin-induced Akt dephosphorylation along with the apoptosis. Results suggested that the ceramide/PP2A/Akt axis is involved in the apoptosis and a possible cyclooxygenase-independent target for indomethacin. Furthermore, apoptosis regulatory proteins such as Mcl-1 and FLIP are potential downstream effectors of this axis and their downregulation could turn on the apoptotic program.

1. Introduction

Glioma is the leading malignancy of astrocyte origin in the brain. The most aggressive, invasive, and destructive glioma is called glioblastoma multiforme. Their highly invasive and diffuse nature precludes curative surgical therapy due to the resistance to radiotherapy, chemotherapy, immunotherapy, and others [12,19,42]. Besides, the blood-brain barrier further blocks the passage of most antineoplastic drugs into the brain, restricting their therapeutic effects. Despite the advances in clinical care, current treatment options for glioma are still limited and the prognosis with malignant glioma remains poor. To meet clinical demands, a deeper understanding of antineoplastic actions and novel therapeutic strategies are crucial to improve the treatment outcome of glioma patients.

The molecular events in the development of malignancies are multifactorial. Increasing evidence has pointed to the importance of cyclooxygenase-2 (COX-2). For example, the level of COX-2 expression is well correlated with tumor malignancy and aggressiveness. A number of epidemiological, clinical, and experimental studies suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) have promising antineoplastic effects on cancers including glioma [12,15,21,28,43,46]. Among the NSAIDs, non-selective COX inhibitor indomethacin has a profound antineoplastic effect on a variety of malignancy. Specifically, the induction of apoptosis via an off-target action could underlie its antineoplastic mechanism through mitogen-activated protein kinase (MAPK), Akt, β-catenin, CHOP, AMP-activated protein kinase (AMPK), or Aurora B kinase [11,32,33,35,45,48].

Indomethacin suppresses glioma through the inhibition of cell growth and differentiation, and in particular the induction of apoptosis [3,4,8,27,33,38]. The antineoplastic effects against glioma can be further improved by structural modification and formulation, both in vivo and in vitro models [5–7]. The proapoptotic nature of indomethacin in

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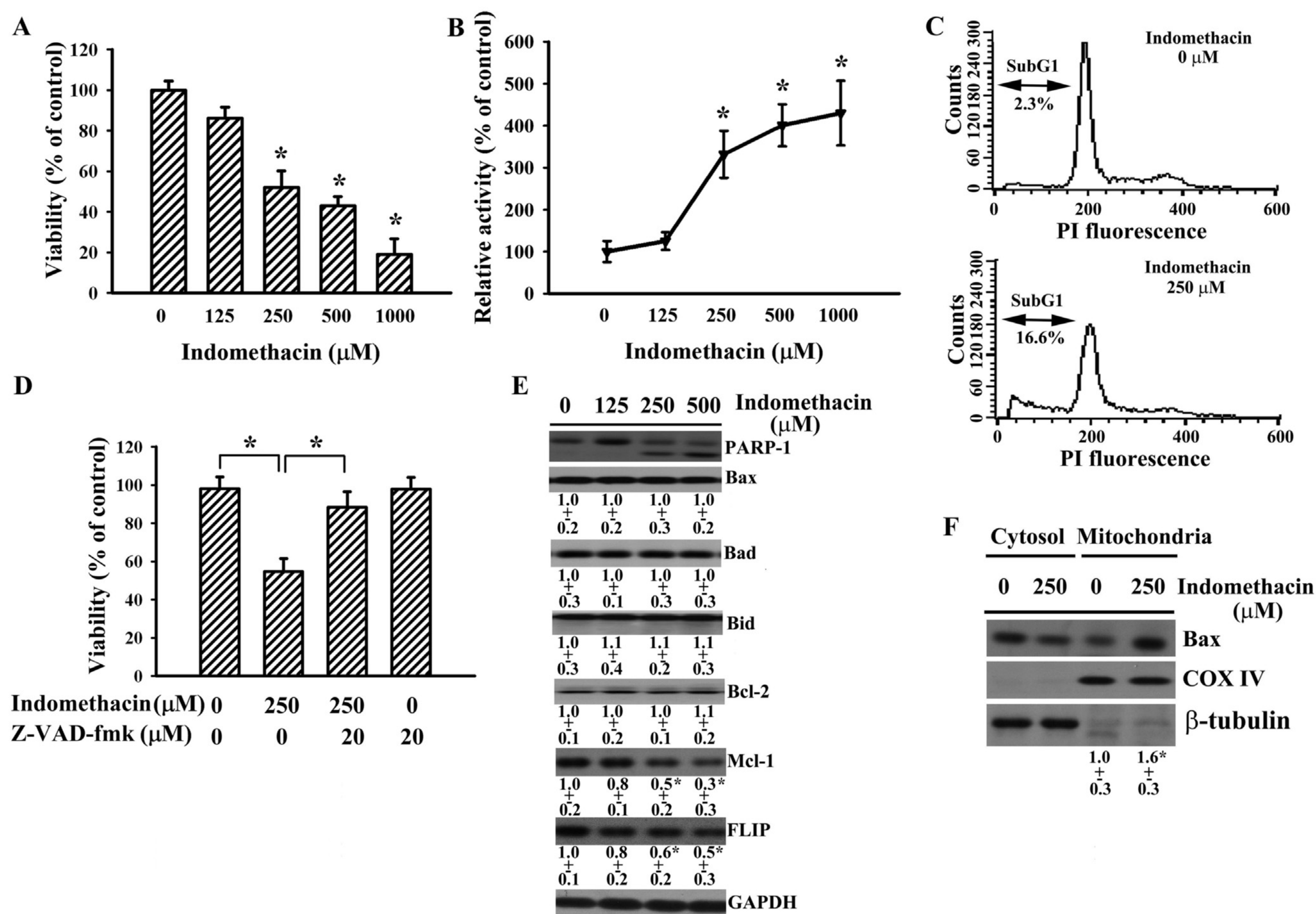

Fig. 1. Indomethacin induced cell apoptosis. (A) H4 cells were treated with various concentrations of indomethacin for 24 h. Cell viability was assessed by MTS reduction assay. (B) H4 cells were treated with various concentrations of indomethacin for 5 h. Caspase 3 activity was assessed by enzymatic assay. (C) H4 cells were treated with indomethacin (0 and 250 μM) for 24 h. The percentage of subG1 population was assessed by flow-cytometric analysis. (D) H4 cells were treated with vehicle, indomethacin, Z-VAD-fmk, or in combination for 24 h. Cell viability was assessed by MTS reduction assay. (E) H4 cells were treated with various concentrations of indomethacin for 5 h. Proteins were isolated and subjected to Western blot with indicated antibodies. (F) H4 cells were treated with indomethacin (0 and 250 μM) for 5 h. Proteins obtained from cytosolic and mitochondrial fraction were subjected to Western blot with indicated antibodies. *$p < 0.05$ vs. each control (0 μM group). Mean value was calculated through three independent experiments (A, B, and D). One of three independent experiments is shown (C, E, and F). Relative protein content was depicted under the blots.

glioma however remains unclear. Therefore, this study was conducted to determine the molecular mechanisms of indomethacin in the apoptosis it induced in glioma cells.

2. Materials and methods

2.1. Cell cultures

Human glioma cell lines U87 and H4 (American Type Culture Collection, Manasas, VA) were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) [9]. An assay kit (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit) was used to determine cell viability according to product instructions (Promega, Madison, WI).

2.2. Caspase 3 activity assay

Caspase 3 activity was measured with the Caspase Fluorometric Assay kit (BioVision, Mountain View, CA) using a fluorogenic peptide substrate. Cell lysis and enzymatic reactions were conducted with the buffers and protocols provided in the kit according to its instructions. The levels of fluorescent AMC moiety were measured using a fluorometer (E_x 380 nm and E_m 460 nm). Arbitrary units were used to mark the levels according to the fluorescence changed per amount of protein and the relative activity was expressed.

2.3. Measurement of cell cycle distribution

Cell cycle distribution was analyzed using flow cytometric assay. Prior to analysis, the detached cells were fixed, incubated with 100 μg/ml RNase, and stained with propidium iodide (50 μg/ml) according to the standard protocol [9]. Resultant cells were analyzed using a flow cytometer (BD FACSCanto II).

2.4. Subcellular fractionation

Cells were first resuspended in buffer [75 mM NaCl, 8 mM Na_2PO_4, 1 mM NaH_2PO_4 (pH 7.4), 250 mM sucrose, 1 mM EDTA, 0.4 mM PMSF,

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