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ERK1/2 acts as a switch between necrotic and apoptotic cell death in ether phospholipid edelfosine-treated glioblastoma cells

Sara Melo-Lima[a,b], Maria C. Lopes[b,c], Faustino Mollinedo[a,d,*]

[a] Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain
[b] Centre for Neuroscience and Cell Biology, University of Coimbra, 3000 Coimbra, Portugal
[c] Faculty of Pharmacy, University of Coimbra, 3000 Coimbra, Portugal
[d] Instituto de Investigación Biomédica de Salamanca (IBSAL), Hospital Universitario de Salamanca, E-37007 Salamanca, Spain

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ABSTRACT

Glioblastoma is characterized by constitutive apoptosis resistance and survival signaling expression, but paradoxically is a necrosis-prone neoplasm. Incubation of human U118 glioblastoma cells with the anti-tumor alkylphospholipid analog edelfosine induced a potent necrotic cell death, whereas apoptosis was scarce. Preincubation of U118 cells with the selective MEK1/2 inhibitor U0126, which inhibits MEK1/2-mediated activation of ERK1/2, led to a switch from necrosis to caspase-dependent apoptosis following edelfosine treatment. Combined treatment of U0126 and edelfosine totally inhibited ERK1/2 phosphorylation, and led to RIPK1 and RelA/NF-κB degradation, together with a strong activation of caspase-3 and -8. This apoptotic response was accompanied by the activation of the intrinsic apoptotic pathway with mitochondrial transmembrane potential loss, Bcl-x_L degradation and caspase-9 activation. Inhibition of ERK phosphorylation also led to a dramatic increase in edelfosine-induced apoptosis when the alkylphospholipid analog was used at a low micromolar range, suggesting that ERK phosphorylation acts as a potent regulator of apoptotic cell death in edelfosine-treated U118 cells. These data show that inhibition of MEK1/2-ERK1/2 signaling pathway highly potentiates edelfosine-induced apoptosis in glioblastoma U118 cells and switches the type of edelfosine-induced cell death from necrosis to apoptosis.

1. Introduction

Glioblastoma (formerly known as glioblastoma multiforme, GBM) is the highest grade of glioma (grade IV astrocytoma) and represents the most aggressive malignant and most common primary brain tumor, accounting for about 50% of gliomas. The survival rate for glioblastoma is extremely low with a 5-year survival of just 3.4% [1], and a median survival of about 12–15 months [2]. Current therapies include a combination of surgery, radiotherapy and chemotherapy, and even combined treatments including the oral alkylating drug temozolomide, considered the gold standard for the therapy of glioblastoma, are faulty and have had only limited success, showing disease relapse in most patients [3]. Therefore search for new chemotherapeutic agents is urgently needed to improve clinical outcome. One of the reasons for the chemotherapy's lack of effectiveness in the treatment of glioblastoma lies in the constitutive activation of survival signaling pathways and intrinsic apoptosis resistance of glioblastoma cells [4,5]. Interestingly, a major hallmark and diagnostic feature of glioblastoma is the presence of necrosis [6,7], being present in over 85% of cases and a predictor of poor patient diagnosis [8,9]. The presence of tumor hypoxia together with antiapoptotic mechanisms in glioblastoma cells could lead to the selection of tumor cells that are more aggressive and resistant to apoptosis-inducing therapies as well as might promote necrosis as the final mode of cell death [10]. This might explain the inverse correlation that exists between tumor necrosis and the survival of patients with glioblastoma [10]. Constitutive activity of survival signaling molecules, including the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and extracellular signal-regulated kinase (ERK)-mediated routes, occurring in up to 90% of glioblastomas [11–13], contributes to chemoresistance and to avoid apoptotic cell death

Abbreviations: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK/ERK kinase 1/2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI, propidium iodide; RIPK1, receptor-interacting serine/threonine-protein kinase 1; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene.

* Corresponding author at: Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain. Tel.: +34 923 294806; fax: +34 923 294795.
E-mail address: fmollin@usal.es (F. Mollinedo).

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[14–16]. Glioblastoma remains an incurable tumor, and despite aggressive treatment including surgery, adjuvant temozolomide-based chemotherapy and radiotherapy, glioblastoma still has a dismal prognosis [17–19]. Therefore, there is a desperate need for more effective therapies and novel approaches in order to improve therapeutic responses in this devastating disease.

Among the distinct types of cell death, apoptosis and necrosis stand out as the major processes for cell demise. Apoptosis is mainly characterized by internucleosomal DNA fragmentation and in most cases by activation of initiator caspases, such as caspase-8, or executioner/effector caspases, such as caspase-3, with a main involvement of mitochondria that leads to caspase-9 activation, which in turn cleaves and activates downstream effector caspase-3, leading to cell death [20]. Unlike apoptosis, necrosis is characterized by an early plasma membrane permeabilization and is caspase-independent [20,21]. Necrosis has been widely considered as an accidental cell death caused by overwhelming physical or chemical trauma, but recent data have shown that at least a type of necrotic cell death named necroptosis can be regulated, involving the formation of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3 complexes, and being specifically inhibited by the RIPK1 inhibitor necrostatin-1 [22,23]. Differences in cell types and stimuli seem to be critical factors in the onset of either apoptosis or necrosis. In addition, increasing the intensity of the insult could predispose to change the cell death type from apoptotic to necrotic cell death [24]. The relative expression and activation of major players of each type of cell death, such as caspase-8, RIPK1 or RIPK3, can tilt the balance toward either apoptotic or necrotic cell death [22,23].

The degree of necrosis and inflammatory markers has been correlated with a worse prognosis in glioblastoma patients [10,25,26]. Thus, the presence of necrosis in glioblastoma may raise some concerns about undesirable inflammatory side effects. On these grounds, it would be advisable to find out effective drugs able to promote apoptosis rather than necrosis in glioblastoma cells and to elucidate the mechanisms regulating the switch between apoptosis and necrosis to modulate the onset of different cell death types.

The ether phospholipid edelfosine is the prototypic molecule of a series of structurally related compounds collectively known as alkylphospholipid analogs (APLs) [27–29], and induces apoptosis in a series of tumor cells [30–32] by the activation of apoptotic extrinsic and intrinsic signaling pathways, involving membrane-mediated processes at the cell surface, mitochondria and endoplasmic reticulum [33–42]. Edelfosine is also able to promote distinct types of cell death in tumor cells, including apoptosis and necrosis/necroptosis, depending on the cell's ability to set off specific deadly signaling pathways [43], and recent experiments have shown the triggering of a necroptotic cell death in edelfosine-treated human glioblastoma U118 cells [44].

The compound U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) was originally identified as an inhibitor of AP-1 transactivation in a cell-based reporter assay, which turned out to be due to direct and specific inhibition of the mitogen-activated protein kinase kinase (MAPKK) family members mitogen-activated protein kinase (MAPK)/ERK kinase 1 and 2 (MEK1/2) [45]. Because ERK1/2 are isoforms of the MAPK family that are activated by MEK1/2, the specific MEK1/2 inhibitor U0126 serves as a powerful tool for *in vitro* and cellular investigations of ERK1/2-mediated signal transduction [45–49].

Here we report that edelfosine induces necrosis in the U118 glioblastoma cell line, but inhibition of ERK phosphorylation by the specific MEK1/2 inhibitor U0126 switches edelfosine-induced necrotic response to a potent apoptosis. In addition, we report here that edelfosine is also able to promote apoptotic signaling in U118 cells at concentrations unable to trigger necrosis, but the apoptosis response is mostly blocked unless ERK1/2 phosphorylation is prevented.

2. Materials and methods

2.1. Reagents

Edelfosine was obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland). A stock solution was prepared at 2 mM in culture medium containing 10% (v/v) fetal bovine serum (FBS) by heating at 50 °C for 45 min, as previously described [31]. Versene, FBS, L-glutamine, penicillin, and streptomycin were from Gibco, Life Technologies Corporation (Carlsbad, CA). U0126 was from Calbiochem (Merck Millipore, Darmstadt, Germany) and 10 mM stock solutions were prepared in DMSO, in order to use the solvent at ≤0.1% final concentration that did not affect any of the parameters analyzed in this study. All other chemicals and reagents were from Sigma (St. Louis, MO) unless otherwise indicated.

2.2. Cell culture

The human glioblastoma U118 (U-118 MG) and T98G cell lines were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies Corporation) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2.

2.3. Measurement of apoptosis by flow cytometry and fluorescence microscopy

Following drug treatment, both cells in suspension and adherent cells, which were detached with Versene, were collected and brought together, centrifuged at 1200 rpm for 5 min, and fixed overnight in 70% ethanol at 4 °C. Cells were washed three times with PBS, incubated for 1 h with 100 μg/ml RNase A and 20 μg/ml propidium iodide (PI) at room temperature and then analyzed with a Becton Dickinson (San Jose, CA, USA) FACSCalibur flow cytometer. Quantitation of apoptotic cells was calculated as the percentage of cells in the sub-G1 region (hypodiploidy) following cell-cycle analysis as previously described [33].

Apoptosis was also measured by observing morphological changes in the nuclear chromatin of cells detected by staining with 100 nM SYTOX-Green Nucleic Acid Stain (Invitrogen, Eugene, OR) for 15 min at 37 °C, followed by examination on a Nikon Eclipse Ti-S inverted fluorescence microscope (Tokyo, Japan). Photographs were acquired with ProgRes Capture Pro 2.6.

2.4. PI exclusion assay

Attached and detached cells from each experimental point were collected, brought together, centrifuged and washed once with PBS. Cells were then resuspended in PBS containing 10 μg/ml PI. After incubation in the dark for 15 min at room temperature, cells were analyzed by flow cytometry at 590 nm. The proportion of cells with increased permeability to PI (PI^+ cells) was calculated as the percentage of cells with increased red fluorescence (strong shift in FL-2 values, log scale) with respect to the basal red fluorescence observed in untreated control cells using FCS Express 4 Plus software.

2.5. Western blot analysis

Cells ($4–5 \times 10^6$) were detached using Versene, washed twice with PBS, and lysed with 60 μl of lysis buffer containing HEPES