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Original article

Temozolomide and sorafenib as programmed cell death inducers of human glioma cells

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ABSTRACT

Background: Gliomas are aggressive brain tumors with very high resistance to chemotherapy. Therefore, the aim of the present study was to investigate the effectiveness of sorafenib and Temozolomide in elimination of human glioma cells through apoptosis and autophagy.
Methods: MOGGCCM (anaplastic astrocytoma) and T98G (glioblastoma multiforme) cell lines incubated with sorafenib and/or Temozolomide were used in the experiments. Cell morphology (ER stress, apoptosis, autophagy, and necrosis) was analyzed microscopically while apoptosis and mitochondrial membrane potential were assessed with flow cytometry. Beclin1, LC3, p62, Hsp27, and Hsp72 levels were analyzed by immunoblotting. The activity of caspase 3, 8, and 9 was evaluated fluorometrically. Expression of Hsps was blocked by transfection with specific siRNA.
Results: In MOGGCCM cells, Temozolomide most frequently induced autophagy, which was accompanied by decreased p62 and increased beclin1 and LC3II levels. Sorafenib initiated mainly apoptosis. Additional incubation with Temozolomide, synergistically potentiated the pro-apoptotic properties of sorafenib, but it was mediated in a caspase-independent way. In T98G cells, the effect of the analyzed drugs on programmed cell death induction was different from that in MOGGCCM cells. Sorafenib induced autophagy, while Temozolomide initiated mainly apoptosis. After simultaneous drug application, apoptosis dominated, suggesting synergistic action of both drugs. Inhibition of Hsp27 and Hsp72 expression increased the sensitivity of both cell lines to ER stress and, to a lesser extent, to induction of apoptosis, but not autophagy.
Conclusions: Sorafenib and Temozolomide applied in combination are potent apoptosis inducers in T98G and MOGGCCM cells. ER stress precedes the elimination. Blocking of Hsp expression has a greater impact on ER stress rather than apoptosis induction.
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Introduction

Anaplastic astrocytoma (AA, WHO grade III) and glioblastoma multiforme (GBM, WHO grade IV) are highly malignant, aggressive, and invasive primary brain tumors characterized by increased proliferation and migration rates. Prognosis for patients with standard treatment including surgical removal of the tumor followed by radio- and chemotherapy is poor. Therefore, continuous investigation of the complex biology of gliomas is needed to improve the effectiveness of new treatments [1].

It is known that, at the molecular level, brain tumors have developed many mechanisms enabling cell survival during chemotherapy. One of these is the activation of a mitogenic Ras-Raf-MEK-ERK pathway that takes part in promotion of cellular proliferation. Therefore, inhibition of this signaling cascade, which is altered in gliomas, may be a valuable target in developing new treatments [2]. Sorafenib, a novel bi-aryl urea compound, is a multikinase inhibitor specifically diminishing the activity of Raf

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kinase. *In vitro*, it is engaged in reduction of cell proliferation, inhibition of angiogenesis, and initiation of autophagy or apoptosis in glioma cells. The drug possesses the ability to effectively cross the blood brain barrier and is well tolerated after systemic administration [3,4].

Temozolomide is an oral alkylating chemotherapeutic agent, which prolongs survival when administered alone as well as during and after radiotherapy. Due to its lipophilicity, the drug crosses the BBB readily and is bioactive in the central nervous system. Temozolomide possesses anti-proliferative and cytotoxic activity, due to guanine methylation in the O^6 position. It leads to base pair mismatch and strand breaks, thereby preventing mitotic division and cell death by autophagy or apoptosis [5,6]. It is known that combined therapy is more effective that drugs in the separate application. Therefore, the aim of this study was to estimate the effect of sorafenib and Temozolomide on programmed cell death-based elimination of human glioma cells. The expression of anti-apoptotic heat shock proteins 27 (Hsp27) and 72 (Hsp72), Raf, pro-autophagic LC3, and beclin1, autophagy flux marker p62, as well as the pro-apoptotic caspase 3, 8, and 9 activity were also studied. Heat shock proteins are well-known molecular chaperones, the overexpression of which in cancer cells is responsible for increased cell survival. Therefore, the next goal of our study was to examine the sensitivity of the MOGGCCM and T98G lines to cell death after blocking Hsp27 and Hsp72 expression by specific siRNA.

Materials and methods

Cells and culture conditions

Human glioblastoma multiforme cells (T98G, European Collection of Cell Cultures) and human anaplastic astrocytoma cells (MOGGCCM, European Collection of Cell Cultures) were grown in a 3:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's nutrient mixture F-12 (Sigma) supplemented with 10% fetal bovine serum (Sigma), penicillin (100 units/ml) (Sigma), and streptomycin (100 μg/ml) (Sigma). The cultures were kept at 37 °C in humidified atmosphere of 95% air and 5% CO_2.

Drug treatment

Sorafenib (Nexavar, BAY 43-9006) (0.75 μM) and Temozolomide (Shering-Plaugh) (100 μM for MOGGCCM or 50 μM for T98G) dissolved in DMSO were studied as a continuation of our previous experiments [7–9]. The drugs doses were chosen on the basis of earlier experiments [7–9]. The cancer cells were treated with sorafenib or with Temozolomide separately or in combination (at the same time) for 48 h. As controls, T98G and MOGGCCM cells were incubated only with 0.01% of DMSO.

Microscopic detection of apoptosis, autophagy, and necrosis with fluorochromes

For identification of apoptosis and necrosis, a staining method with Hoechst 33342 (Sigma) and propidium iodide (Sigma) was chosen, as described previously [9]. In the case of autophagy, staining with acridine orange was performed. The same method facilitated observation of granules ("wholes") within the cells [8,9]. For morphological analysis of dead cells, a fluorescence microscope (Nikon E − 800) was used. At least 1000 cells in randomly selected microscopic fields were counted under the microscope. Each experiment was repeated three times with each 1000 cells.

Detection of apoptosis, necrosis, and mitochondrial membrane potential by flow cytometry

Detection of apoptosis and necrosis by flow cytometry was performed with the Annexin V-FITC apoptosis detection kit (Sigma) according to the manufacturer's protocol. For the mitochondrial membrane potential (Δy_m) (MMP) analysis, staining with fuorochrome 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was chosen according to the method described previously [8]. Both analyses were performed with the FacsCanto instrument (Becton Dickinson, San Jose, CA, USA). Each experiment was performed in triplicate.

Immunoblotting

Whole cell extracts were prepared by lysing cells in hot buffer containing 125 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, and 100 mM dithiothreitol (DTT). The protein concentration was measured with the Bradford method [10].

In total, 80 μg of proteins were separated by 10% SDS-PAGE [11] and electroblotted onto Immmobilon P membrane (Sigma). After blocking with 3% low fat milk for 1 h, the membranes were incubated overnight with primary antibodies: mouse anti-Hsp72 monoclonal antibody (SPA 810, StressGen, concentration 0.2 μg/ml), anti-Hsp27 (SPA 800, StressGen, concentration 0.1 μg/ml), rabbit anti-LC3 (Sigma, concentration 2 μg/ml), anti-beclin 1 (Sigma, concentration 3 μg/ml), anti-p62 (Sigma, concentration 1 μg/ml), and anti-Raf (Santa Cruz Biotechnology, concentration 0.5 μg/ml). After three washes with PBS enriched with 0.05% Triton X-100 (Sigma), the membranes were incubated with secondary antibodies conjugated with alkaline phosphatase (AP) for 2 h. Proteins were detected with AP substrates: 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro-blue tetrazolium (NBT) (Sigma) in *N,N*-dimethylformamide (DMF, Sigma). The results obtained were analyzed qualitatively on the basis of the band thickness, width, and color depth. The quantitative analysis of protein bands was performed using the Bio-Profil Bio-1D Windows Application V.99.03 program. The data were normalized relative to β-actin (Sigma, working dilution 1:2000). Three independent experiments were performed.

Caspase activity assay

The activity of caspases 3, 8, and 9 was analyzed with a SensoLyte® AMC Caspase Substrate Sampler Kit (AnaSpec) according to the manufacturer's protocol with a 2030 Multilabel Reader Victor™x4 (Perkin Elmer) microplate reader.

T98G and MOGGCCM transfection with siRNA

The cells at a density of 2×10^5 were incubated for 24 h at 37 °C in a CO_2 incubator to reach 60–80% of confluence. After washing with a 3:1 DMEM:Ham's F-12 mixture without serum and antibiotics, the medium was aspirated. The cells were overlaid with transfection probes containing 2 μl of specific anti-Hsp27 or anti-Hsp72 small interfering RNA (siRNA) (Santa Cruz Biotech) and 2 μl of Transfection Reagent (Santa Cruz Biotech). After 5 h of incubation at 37 °C in a CO_2 incubator, the medium was supplemented with medium containing 20% of fetal bovine serum and 200 μg/ml of antibiotics. Incubation for additional 18 h was performed. After changing the medium to the fresh normal growth one, such transfected cells were taken for further experiments.

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