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Calcium entry via ORAI1 regulates glioblastoma cell proliferation and apoptosis

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ABSTRACT

Introduction: Calcium entry plays a critical role in the proliferation and survival of certain tumors. Ca^{2+} release activated Ca^{2+} (CRAC) channels constitute one of the most important pathways for calcium entry especially that of store-operated calcium entry (SOCE). ORAI1 and stromal interaction molecule1 (STIM1) are essential protein components of CRAC channels. In this study we tested the effect of inhibiting CRAC through ORAI1 and STIM1 on glioblastoma multiforme (GBM) tumor cell proliferation and survival.
Methods: Two glioblastoma cell lines, C6 (rat) and U251 (human), were used in the study. ORAI1 and STIM1 expressions were examined using Western blot and immunohistochemistry. CRAC channel activity and its components were inhibited with ion channel blockers and using siRNA knockdown. Changes in intracellular calcium concentration were recorded using Fura-2 fluorescent calcium imaging. Cell proliferation and apoptosis were examined using MTS and TUNEL assays, respectively.
Results: CRAC blockers, such as SKF-96365 (1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy]ethyl-1H-imidazole), 2-aminoethoxydiphenyl borate (2-APB) and Diethylstilbestrol (DES), inhibited cell proliferations and SOCE in GBM cells. Knockdown of ORAI1 and STIM1 proteins using siRNA significantly inhibited C6 cell proliferation and SOCE compared with those in control cells, and a more significant effect was observed in cells with ORAI1 siRNA knockdown than that of STIM1-treated cells. Both CRAC blockers and siRNA treatments increased apoptosis in C-6 cells compared with control.
Conclusion: Calcium entry via ORAI1 and CRAC channels are important for GBM proliferation and survival.
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Introduction

Glioblastoma (GBM) is the most common and deadly primary malignant brain tumor. In the United States from 2004 to 2006, GBM accounted for 53.7% of all gliomas with a median survival time of 1 year and a less than 5% five year survival rate (CBTRUS, 2010). The poor prognosis of GBM patients possibly stems from some of the unusual capabilities of GBM tumor, for example, aggressive proliferation and metastasis, evading apoptosis, and developing resistance to therapeutic agents. Recent studies have shown that GBM tumor cells attain dysregulated growth, immortality and invasiveness via several mechanisms such as hyperamplification and mutation of epidermal growth factor receptor (EGFR), deletion of tumor suppressor genes, hyperactivation of tyrosine kinases and phosphatidylinositol-3-kinase (PI3K)/AKT-signaling. Despite recent advances in our understanding of GBM pathogenesis, even the most up-to-date clinical and experimental strategies have not yielded ideal outcomes. This necessitates new therapeutic approaches based on a broader understanding of GBM pathogenesis (Grzmil and Hemmings, 2010; Krakstad and Chekenya, 2010). One potential approach for suppressing GBM cell aggression is to target the Ca^{2+} signal pathways.

A persistent elevation of intracellular Ca^{2+} concentration caused by Ca^{2+} influx has been known to be important for cellular activities such as enzyme activation, gene transcription, cell-cycle progression and apoptosis. The calcium influx can be initiated directly via receptor activation (receptor-operated calcium entry, ROCE), or through depleting internal calcium store in the endoplasmic reticulum (SOCE) (Parekh and Putney, 2005). CRAC channels are best studied SOCE pathways. CRAC channels are formed by at least two essential components: a pore forming protein ORAI1 sitting on plasma membrane and a calcium sensing protein STIM1 located in the ER membrane. STIM1 monitors endoplasmic reticulum (ER) Ca^{2+} store concentration depletion and activates ORAI1, allowing store-operated Ca^{2+} entry, or CRAC, to occur (Feske et al., 2006; Liou et al., 2005; Prakriya et al., 2006; Roos et al.,

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; CPA, cyclopiazonic acid; CRAC, Ca^{2+} release activated Ca^{2+}; DES, Diethylstilbestrol; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; GBM, glioblastoma multiforme; ROCE, receptor-operated calcium entry; SKF, 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy]ethyl-1H-imidazole; SOCE, store-operated calcium entry; STIM1, stromal interaction molecule1; TRPC1, transient receptor potential canonical channel 1.

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2005). CRAC inhibition has been shown to induce suppression of tumor cell proliferation by arresting cell cycle at G0/G1 phase and inhibiting tumor cell metastasis in tumor cells such as leukemic cells (Holmuhamedov et al., 2002), breast cancer cells (Yang et al., 2009) and prostate cancer cells (Abeele et al., 2005).

Notable studies regarding the role of Ca^{2+} entry in GBM have been performed; recent studies showed that the disruption of Transient Receptor Potential Canonical Channel 1 (TRPC1) channels inhibits proliferation in GBM cells through disrupting cytokinesis and inducing cell cycle arresting (Bomben and Sontheimer, 2008, 2010; Bomben et al., 2011). Nevertheless no research has been focused on the CRAC, possibly the most important pathway of SOCE. In the present study, we examined the effect of inhibiting CRAC on the GBM cell proliferation and apoptosis by using pharmacological intervention and siRNA knockdown of CRAC components: ORAI1 and STIM1. We show here that ORAI1 and STIM1-mediated Ca^{2+} entry are crucial to sustained GBM cell proliferation and survival.

Material and methods

MTS cell proliferation assay

Rat C6 and human U251 glioma cells were maintained in Eagle's minimal essential medium (EMEM) with 10% FBS and 2 mM glutamine (ATCC, Manassas VA) in 5% CO_2 at 37 °C. About 1000 C6 cells, 2000 or 5000 U251 cells were plated into each well of 96-well culture plates in 200 μl medium. Cells were allowed to attach for 1 h and the culture medium was replaced with fresh medium containing ion channel blockers. SKF-96365, 2-APB and DES were diluted in culture medium from stock solution (>1000× concentration in ddH_2O or DMSO). After 48 h of drug treatment, the culture media was decanted and replaced by 100 μl fresh media/MTS reagent (CellTiter 96® AQ one solution cell proliferation MTS assay kit, Promega, Madison MI) in the ratio of 5:1 (v/v) in each well and underwent 1 hour incubation. The OD value of light absorption at 490 nM was then recorded for plates using a spectrophotometer. Drug induced concentration inhibition of cell proliferation was analyzed in Origin software (OriginLab, Northampton, MA).

Single cell $[Ca^{2+}]_i$ measurement

Cells were detached from culture flasks by 2–3 minute incubation with 2% EDTA in PBS; detached cells were washed twice with fresh culture medium and incubated in 2 μM Fura-2 AM in medium at room temperature for 30 min. After one wash with PBS, cells were plated on a No. 1 glass cover slip mounted in a chamber on the stage of an inverted microscope (Nikon TE2000; Tokyo, Japan). A Nikon 40× UV oil-immersion objective lens was used to visualize the cells. Cells were illuminated by alternating excitation at 340 and 380 nm wavelengths every 10 s; rapid filter changes for ratiometric imaging were computer controlled via a Lambda 10–2 filter wheel and changer and MetaFluor 6.3 software (Molecular Devices, Sunnyvale, CA); fluorescence images were acquired using a CCD camera (MicroMax RTE CCD-728, Photometrics; Tucson AZ). Images were corrected for background fluorescence and shade using functions in MetaFluor and were recorded. Averages of fluorescence intensity 340/380 nM ratio in regions of interest defined in individual cells of each stored image provided an estimate of intracellular calcium concentration, were analyzed in Excel, and graphed in Origin 8.0.

siRNA knockdown of ORAI1 and STIM1

siRNA and transfection reagents were purchase from Qiagen (Valencia, CA). A reverse protocol was performed according to manufacturer's instructions. Specifically, a mixture of 0.5–1 μL siRNA stock solution (10–20 μM in RNase free water)/1.5 μL Hiperfect transfection reagent/100 μL serum free medium was applied into each well of a 96-well culture plate. The mixtures was allowed to incubate for 5 to 10 min to allow for the formation of transfection complexes, then an additional 1000 cells in 100 μL complete culture medium were added to each well for transfection. Calculated final siRNA concentrations were 50 nM. Cells were incubated in 5% CO_2 at 37 °C for 32–72 h. MTS cell proliferation assay was then performed. To measure Ca^{2+} concentration, siRNA treatment was performed in cells cultured on a 35 mm dish with a cover glass bottom (0.5 cm diameter). Apoptosis assay and immunohistochemistry studies were performed on chambered cover glass (0.7 cm^2/well culture area, Lab-Tek™ II Chambered Cover glass, Thermo scientific), with each well plated with 1000 cells (for control siRNA treatment) or 2000 cells (for ORAI1 or STIM1 SiRNA treatment). Two different sequences of ORAI1 (ORAI1a and ORAI1b) and STIM1 (STIM1a and STIM1b) siRNA were used for protein knockdown. The oligonucleotide sequences of RNA duplexes for siRNA assays were: ORAI1a, forward GGAUCAUGACUACCCGCCATT/reverse UGGCGGGUAGUCAUGAUCCGT; ORAI1b, CGGCCGUCGUCGUCGCCAATT/UUGGCGACGACGACGGCCGGT; STIM1a, GCCACGUCUUCCAAUGGUATT/UACCAUUGGAAGACGUGGCAT; STIM1b, CGGCCGUCGUCGUCGCCAATT/UUGGCGACGACGGCCGGT. Scramble sequences were used for control siRNA treatment.

Apoptosis study

TUNEL assay was performed using an in situ cell death detection kit, TMR red (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instruction. Cells grown on a cover slip were washed with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4, freshly prepared) for 20 min at room temperature. After washing, cells were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared) for 2 min on ice. After washing, cells were incubated with The TUNEL reaction mixture (100 μL Label Solution and 50 μL enzyme solution) in a humidified chamber at 37 °C for 1 h. Cells were then briefly rinsed with PBS and counterstained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 1 min for visualization of the nucleus then analyzed by fluorescence microscopy interfaced with MetaMorph (Molecular Devices) using an excitation wavelength in the range of 520–560 nm and detection in the range of 570–620 nm.

Immunocytochemistry

Cells grown on cover slips were rinsed briefly with PBS and fixed with pre-chilled acetone for 10 min at −20 °C. After washing with PBS three times for 5 min, cells were incubated for 30 min with 1% BSA in PBST and 10% serum from the species that the secondary antibody was raised from to block unspecific binding of the antibodies. Cells were then incubated with a 1:200 diluted primary antibody in 1% BSA in PBST in a humidified chamber overnight at 4 °C. After washing three times in PBS, cell samples were incubated with 1:500 secondary antibodies in 1% BSA for 1 h in the dark at room temperature, then washed and counter stained with DAPI for 1 min.The cover slips were then mounted with mounting medium and examined under fluorescence microscopy. Primary antibodies to ORAI1 (P20, goat polyclonal IgG, SC-74778) and to STIM1 (H-180, rabbit polyclonal IgG, SC-68897) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Compatible Alexa Fluor® labeled secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Western blot

Blotting was performed according to previously reported methods (Liu et al., 2005). Primary antibodies to ORAI1 (P-20) and STIM1