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Phosphodiesterase inhibitors control A172 human glioblastoma cell death through cAMP-mediated activation of protein kinase A and Epac1/Rap1 pathways

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ABSTRACT

Aims: We investigated whether cAMP-mediated protein kinase A(PKA) and Epac1/Rap1 pathways differentially affect brain tumor cell death using 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone(rolipram), specific phosphodiesterase type IV(PDE IV) inhibitor.
Main methods: A172 and U87MG human glioblastoma cells were used. Percentage of cell survival was determined by MTT assay. PKA and Epac1/Rap1 activation was determined by western blotting and pull-down assay, respectively. Cell cycle and hypodiploid cell formation were assessed by flow cytometry analysis.
Key findings: Non-specific PDE inhibitors, isobutylmethylxanthine(IBMX) and theophylline reduce survival percentage of A172 and U87MG cells. The expression of PDE4A and PDE4B was detected in A172 and U87MG cells. Rolipram-treated A172 or U87MG cell survival was lower in the presence of forskolin, adenylate cyclase activator, than that in its absence. Co-treatment with rolipram and forskolin also enhanced CREB phosphorylation on serine 133 that was inhibited by H-89, PKA inhibitor and cAMP-responsive guanine nucleotide exchange factor 1(Epac1), a Rap GDP exchange factor-mediated Rap1 activity in A172 cells. When A172 cells were treated with cell-permeable dibutyryl-cAMP(dbcAMP), PKA activator or 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate(CPT), Epac1 activator, basal level of cell death was increased and cell cycle was arrested at the phase of G2/M. Rolipram-induced A172 cell death was also increased by the co-treatment with dbcAMP or CPT, but it was inhibited by the pre-treatment with H-89.
Significance: These findings demonstrate that PKA and Epac1/Rap1 pathways could cooperatively play a role in rolipram-induced brain tumor cell death. It suggests that rolipram might regulate glioblastoma cell density through dual pathways of PKA- and Epac1/Rap1-mediated cell death and cell cycle arrest.

Introduction

Glioblastoma is the most frequent and devastating primary malignant brain tumor in adults. Common genetic abnormalities in glioblastoma are associated with aberrant activation or suppression of cellular signal transduction pathways and resistance to radiation and chemotherapy. Despite advances in diagnosis and standard therapies such as surgery, radiation, and chemotherapy, the prognosis remains poor (Kesari et al. 2005; Lee et al. 2011; Minniti et al. 2009). It has been reported that brain region-specific differences in cyclic AMP (cAMP) levels account for the pattern of gliomagenesis and low levels of cAMP promote glioma formation in neurofibromatosis-1 genetically engineered mouse models (Brown et al. 2010; Warrington et al. 2010).

cAMP is produced by the activation of Gs protein-coupled receptors and adenylate cyclase. cAMP degradation is regulated by a diverse set of phosphodiesterase (PDE) families (Caggiano and Kraig 1999; Lee et al. 2002; Nakamura et al. 1999). PDE4 is widely expressed in brain tumors and promotes their growth (Goldhoff et al. 2008). Pharmacologic elevation of cAMP with the PDE4-specific inhibitor rolipram overcomes tumor resistance (Goldhoff et al. 2008) and suppresses tumor cell growth *in vitro*. Rolipram also inhibits intracranial growth in xenograft models of malignant brain tumors upon oral administration (Yang et al. 2007). In addition, rolipram reduces neuronal damage induced by intrastriatal QA application (Block et al. 2001; Hatzelmann and Schudt 2001; Zhang et al. 2002).

cAMP is considered to be a ubiquitous regulator of inflammatory and immunological reactions (Caggiano and Kraig 1999; Cho et al. 2011; Moon et al. 2011; Nakamura et al. 1999). It regulates many physiological processes via the activation of protein kinase A (PKA) (Moon et al. 2011; Sands and Palmer 2008). Epac1 was characterized as another effector molecule of cAMP and it was demonstrated to exert a regulatory effect on the activity of Rap1 (Bos 2003; de Rooij

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et al. 1998). Through rational drug design, it has been developed a novel cAMP analog, 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (CPT), which activates Epac, but not PKA, both *in vitro* and *in vivo*. CPT has proven to be very useful to study an independent pathway of PKA and Epac (Enserink et al. 2002). Although PDE4 inhibitor is effective therapeutics on brain tumor, differential role of cAMP-mediated pathways in glioblastoma required for apoptosis studies have not yet become available.

Here, we have examined the hypothesis that PKA and Epac is responsible for the particular sensitivity of glioblastoma to PDE4 inhibitor-induced decrease of cell density. We confirm that glioblastoma cells express easily detectable levels of Epac and PDE4B transcript as well as PDE4A. We found that rolipram increase PKA-dependent CREB phosphorylation at serine 133 and Epac1-mediated Rap1 activity. We also found that cell cycle was arrested and cell survival was decreased by the treatment with PKA activator, dibutyryl-cAMP (dbcAMP) or the Epac agonist CPT in glioblastoma cells. In addition, while glioblastoma cell survival was decreased by the co-treatment of rolipram with dbcAMP or CPT, rolipram-induced glioblastoma cell death was inhibited by H-89, PKA inhibitor. Given the result of glioblastoma cell sensitivity to PDE4 inhibitor, it suggests that brain tumor regression by rolipram could be associated with the activation of PKA and Epac1/Rap1 pathways.

Materials and methods

Reagents

Rolipram, forskolin, and IBMX were from Sigma Chemical Co. (St. Louis, MO). Theophylline was obtained from Baxter Healthcare Corporation (Deerfield, IL). Dibutyryl-cAMP was obtained from Calbiochem (San Diego, CA). 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (CPT) was purchased from Biolog Life Science Institute (Bremen, Germany). Antibodies which are reactive with CREB and phospho-CREB on serine 133 (S133) came from Cell Signaling (Beverly, MA). Rabbit anti-Rap1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies which are reactive with PDE4B were generously provided from ICOS Corporation (Bothell, WA). Except where indicated, all other materials are obtained from the Sigma chemical company (St. Louis, MO).

Cell culture

A172 (CRL-1620) and U87MG (HTB-14) human glioblastoma cell lines were obtained from American Type Cell Culture (ATCC). U373 and D54MG human glioblastoma cell lines were obtained from Korea Research Institute of Bioscience and Biotechnology (KRIBB). Cells were maintained and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Kansas City, MO), 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin.

MTT assay

We quantified paclitaxel-treated cell survival using colorimetric assay described for measuring intracellular succinate dehydrogenase content with MTT [3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Denizot and Lang 1986). Confluent cells were cultured with various concentrations of paclitaxel for 48 h. Cells were then incubated with 50 μg/ml of MTT at 37 °C for 2 h. Formazan formed were dissolved in dimethylsulfoxide (DMSO). Optical density (OD) was read at 595 nm.

Measurement of hypodiploid cell formation

For the analysis of hypodiploid cell formation, cells were fixed in 40% ethanol on ice for 30 min and then incubated with propidium iodide (50 μg/ml) and RNase (25 μg/ml) at 37 °C for 30 min. Otherwise, cells were stained with annexin V-FITC and propidium iodide (5 μg/ml). Fluorescence intensity of 10,000 cells was analyzed by CELLQuest™ software in FACScalibur™ (Becton Dickinson, San Jose, CA).

RT-PCR

RNA was isolated from A172, U87MG, U373 and D54MG cells using TRIZOL (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg of total RNA, using oligo-dT_{18} primers and superscript reverse transcriptase in a final volume of 21 μl (Bioneer, Taejeon, Korea). For standard PCR, one μl of the first strand cDNA product was then used as a template for PCR amplification with Taq DNA polymerase (Bioneer, Taejeon, Korea). PCR amplification proceeded as follows: 30–33 thermocycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, using oligonucleotides specific for human PDE4A (sense 5′-GGA GGA AGA AAT ATC AAT GGC CC-3′, antisense 5′-GAT GTG TCC TCC CCA AAT GTC) (Miki et al. 1996), human PDE4B (sense 5′ATT CTG AAG GAC CTG AGA AGG-3′, antisense 5′-CAG TGA GTT CAG TCA CTG TCG-3′) (Bolger et al. 1993) and human Epac1 (sense 5′-GCT CTC CCC TCC TGT CAT CC-3′, antisense 5′-GTT CCC GCT GGT TGT CAA TG-3′).

Western blot analysis

Cells were lysed in ice-cold lysis buffer, containing 0.5% Nonidet P-40 (vol./vol.) in 20 mM Tris–HCl, at a pH of 8.3; 150 mM NaCl; protease inhibitors (2 μg/ml aprotinin, pepstatin, and chymostatin; 1 μg/ml leupeptin and pepstatin; 1 mM phenylmethyl sulfonyl fluoride (PMSF); and 1 mM Na_4VO_3. Lysates were incubated for 30 min on ice prior to centrifugation at 14,000 rpm for 5 min at 4 °C. Proteins in the supernatant were denatured by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. Following this transfer, equal loading of protein was verified by Ponceau staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris–HCl, pH 7.6; 150 mM NaCl; 0.5% Tween 20), then incubated with the indicated antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL) (Pierce, Rockford, IL).

Rap1 GTPase activation assay

The level of activated Rap1 was determined by “pulldown” analysis. The technique for pulldown analysis has been previously described (Ren et al. 1999). Ten million cells were harvested in lysis buffer (50 mM Tris–HCl, pH 7.2, 200 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 10% glycerol, 2 μg/mL aprotinin, leupeptin and pepstatin, 1 mM PMSF). Whole cell lysate was incubated for 2 h with 6 μl of glutathione sepharose 4B beads, which had been preassociated with 6 μg GST-RalGDS. The beads were washed three times with cell lysis buffer. GTP bound GTPase was released from the beads by addition of 1X protein sample buffer, and 5 min of boiling. The released GTPases was then detected by Western blot analysis using an anti-Rap1 antibody and ECL Plus chemiluminescence (Amersham, Piscataway, NJ).

Statistical analyses

Experimental differences were tested for statistical significance using ANOVA and Student's *t*-test. P value of <0.05 or <0.01 was considered to be significant.