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Antitumor activity of methyl gallate by inhibition of focal adhesion formation and Akt phosphorylation in glioma cells

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ABSTRACT

Background: Methyl gallate (MG) possesses a wide range of biological properties that include anti-oxidant, anti-inflammatory, and anti-microbial activities. However, its anti-tumor activity has not been extensively examined in cancer cells. Thus, we examined the effect of MG in both glutamate-induced rat C6 and human U373 glioma cell proliferation and migration.
Methods: MG was isolated from the stem bark of *Acer barbinerve*. Cell viability and migration were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and scratch wound-healing assay, respectively. Focal adhesion formation was detected with immunofluorescence.
Results: Treatment of C6 and U373 glioma cells with MG significantly reduced cell viability, migration, and Akt phosphorylation level. Glutamate stimulation markedly increased the level of ERK1/2 phosphorylation. However, cells treated with MG displayed decreased ERK1/2 phosphorylation. Inhibition of ERK1/2 by MG or MEK1/2 inhibitor significantly inhibited paxillin phosphorylation at Ser^{83} and focal adhesion turn-over produced inefficient glioma cell migration. In addition, activation of Akt and ERK1/2 upon glutamate stimulation was independently regulated by Ca^{2+} and protein kinase C activity, respectively, via the α-amino-3-hydroxy-5-methy-4-isoxazolepropionate acid glutamate receptor and metabotropic glutamate receptor.
General significance: Our results clearly indicate that MG has a strong anti-tumor effect through the down-regulation of the Akt and ERK1/2 signaling pathways. Thus, methyl gallate is a potent anti-tumor and novel therapeutic agent for glioma.

1. Introduction

Glioma is the most common and highly malignant type of primary brain tumor in children and adults [1]. Even though glioma incidence is far lower than other cancers, its mortality is extremely high. In particular, glioblastoma (GBM) is infamous for its highly infiltrative and invasive behavior into brain parenchyma, which produces the worst prognosis after conventional therapies, including surgery, radiotherapy, and chemotherapy [2,3]. In addition, the proliferation and invasion of glioblastoma progress very quickly after surgery, making the likelihood of tumor recurrence very high. Thus, establishment of an effective therapy for glioma will require better understanding of the molecular mechanisms underlying the proliferation and migration of the tumor cells.

Malignant gliomas release excessive glutamate to promote tumor growth and excitotoxic neuronal cell death caused by the increased ratio of invasive cells to brain parenchyma. The extracellular glutamate can activate ionotropic and metabotropic glutamate receptors (mGluRs) on glioma cells in paracrine and autocrine manners [4–6]. The activation of the α-amino-3-hydroxy-5-methy-4-isoxazolepropionate acid glutamate (AMPA) receptor by glutamate has an important role in the growth and invasion of glioma, involving control of the intracellular Ca^{2+} influx. Ca^{2+} influx by glutamate or AMPA activates Akt, which controls glioma cell growth [7–9]. Pharmacological blockade of mGluR 2/3 significantly reduces the activation of ERK1/2 induced by epidermal growth factor (EGF) as a result of inhibition of the glioma cell growth [10]. Furthermore, inhibition of Akt and ERK1/2 activation by gallic acid significantly suppresses glioma cell proliferation and invasion [11]. These reports indicated that the activations of Akt and ERK1/2 are important in cell proliferation and migration. However, molecular details of signaling events leading to Akt and ERK1/2 activation upon glutamate stimulation

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and regulatory roles of PI3K/Akt and ERK1/2 in glioma migration remain poorly understood.

Paxillin is a major component of the focal complex (FX) and focal adhesion (FA) [12,13], as well as being a key scaffolding protein bringing together signaling molecules, structural components, and the regulatory proteins that play important roles in the regulation of FAs and the actin cytoskeleton [14]. Many other cell types are exposed to a chemoattractant gradient, PI3K and F-actin polymerization overlap at the front of migrating neutrophils and fibroblasts [15,16]. Inhibition of the PI3K activity by pharmacological blockade reduces membrane ruffle and F-actin formation, which leads to inefficient cell migration [17,18]. Previous reports have shown that ERK1/2 directly binds to paxillin and also induces the phosphorylation of paxillin in the epithelial cells and in vitro assay [19,20]. Furthermore, activation of ERK1/2 leads to an increase in the phosphorylation of paxillin at Ser^{83} that is required for FA disassembly [21].

Methyl gallate (MG) is a derivative of gallic acid. MG possesses a wide range of biological properties that include anti-oxidant, anti-inflammatory, and anti-microbial activities [22–25]. MG also has a significant anti-tumor effect by inhibiting tumor infiltration of $CD4^+CD25^+$ regulatory T cells [26]. However, its biological activity has not been extensively examined in glioma and other cancer cells. In the present study, we examined the effect of MG isolated from the stem bark of *Acer barbinerve* in both glutamate-induced rat C6 and human U373 glioma cell proliferation and migration. The results demonstrate that MG has a significant inhibitory effect on glioma cell proliferation and migration via inhibition of Akt, ERK1/2, and paxillin phosphorylation at serine 83 (Ser^{83}). Furthermore, the activation of Akt and ERK1/2 is mediated by Ca^{2+} and protein kinase C (PKC) activity, respectively. The results indicate that activation of Akt is involved in cell proliferation and migration, while ERK1/2 activity is involved only in the migration of glioma cells.

2. Materials and methods

2.1. Isolation of MG

MG was isolated from the stem bark of *A. barbinerve*. The air-dried stem bark of *A. barbinerve* (3.5 kg) was extracted with 70% aqueous acetone by maceration. The extract was suspended in water and then successively partitioned with *n*-hexane, CH_2Cl_2, ethyl acetate (EtOAc), and water, respectively. The EtOAc extract (48 g) was subjected to column chromatography over a Sephadex LH-20 column eluted with methanol (MeOH)–H_2O (3:1, v/v) to give four fractions (designated fractions 1–4). Fraction 2 was subjected to column chromatography on RP-18 silica gel with MeOH–H_2O (1:1, 1:3, v/v), and then purified on Sephadex LH-20 with MeOH–H_2O (1:2, 1:5, 1:7, v/v) and ethanol (EtOH)–hexane (3:1, 3:2, 5:4, v/v) to yield MG (13.7 g). MG was identified by a combination of spectroscopic methods including mass spectrometry (MS), ^1H, and ^{13}C nuclear magnetic resonance (NMR).

2.2. Materials

Five hundred micrograms of MG was dissolved in 1 ml of dimethyl sulfoxide (DMSO) as stock solution. This stock solution of MG (500 μg/ml or 2.7 mM) was further diluted to appropriate concentrations with serum-free cell culture medium immediately before use. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Duchefa (Haarlem, the Netherlands). 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX disodium salt) was obtained from Tocris Bioscience (Bristol, UK). L-glutamic acid, 1,2-bis(2-aminophenoxy) ethane-N,N,N′, N′-tetraacetic acid tetrakis (BAPTA-AM), staurosporine, sulfasalazine (SAS), a potent blocker of system Xĉ, and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Calphostin C and KT-5720 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LY294002 and U0126 were purchased from Cell Signaling Technology (Danvers, MA, USA). Diacylglycerol kinase (DAG kinase) inhibitor I was purchased from Calbiochem (Billerica, MA, USA).

2.3. Cell culture

Rat C6 glioma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco BRL, Grand Island, NY, USA). The human U373 glioma cell line was maintained in a Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL, Grand Island, NY, USA) containing 10% FBS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Cell cultures were maintained in tissue culture dishes and kept in a humidified atmosphere with 5% CO_2 at 37 °C. Primary astrocytes were prepared from the forebrain of two-day-old BALB/c mice and cultured in DMEM medium containing 10% FBS and penicillin/streptomycin as previously described [27].

2.4. Cell viability assay

Cytotoxicity of MG was assessed by a MTT assay. Cells (1×10^5 cells/ml) were seeded into 96-well plates. After overnight incubation, the culture medium was removed and cells were rinsed with phosphate buffered saline (PBS) and cells were treated with different concentrations of MG in serum-free culture medium. After 24 h of MG treatment, MTT was added to each well and incubated for 4 h to allow mitochondrial dehydrogenase to convert MTT into insoluble formazan crystals. The effect of the several kinase inhibitors, AMPA receptor antagonist, and calcium chelator on the glioma cell viability was assessed. Cells were pretreated with pharmacological inhibitors and stimulated with 150 μM glutamate for 24 h. At the end of treatment, MTT was added to the culture medium for 4 h. The medium was then aspirated, and formazan was solubilized by adding 150 μl of DMSO. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

2.5. Cell proliferation assay

Live-cell proliferation was gaged using an ELIPSE 80i microscope and NIS Elements software (Nikon, Tokyo, Japan). To investigate the effect of glutamate on glioma cell proliferation, cells were plated at a density of 10,000 cells per well in 24-well plates and allowed to settle for 4 h. The culture medium was removed, and cells were rinsed with PBS and then the cells were cultured in serum-free culture medium. For phase-contrast microscopy, images were taken with a 4× PhP objective lens. After image acquisition at 0 h, cells were treated with several concentrations of glutamate and incubated for 24 h. The images of cells after glutamate stimulation were taken using the same microscope. The number of total cells was counted and used to calculate the increase of cell number by subtracting the value at 0 h from that at 24 h.

Proliferation of glioma cells was determined using a 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay kit (Cell Signaling Technology) according to the manufacturer's instructions. Cells (5×10^4 cells/ml) were seeded into 96-well plates. After overnight incubation, cells were rinsed with PBS and starved for 4 h in the serum-free medium. They were pretreated with MG or PI3Knase inhibitor and then cells were stimulated with or without 150 μM glutamate. Finally, 10× BrdU solution was added to each well and cells were incubated for 18 h after which a BrdU assay was performed.

All experiments were performed in triplicates and results were expressed as mean ± SEM of five independent experiments.