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Downregulation of Akt and FAK phosphorylation reduces invasion of glioblastoma cells by impairment of MT1-MMP shuttling to lamellipodia and downregulates *MMPs* expression

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ABSTRACT

Human malignant glioblastomas are highly invasive tumors. Increased cell motility and degradation of the surrounding extracellular matrix are essential for tumor invasion. PI3K/Akt signaling pathway emerges as a common pathway regulating cellular proliferation, migration and invasion; however, its contribution to particular process and downstream cascades remain poorly defined. We have previously demonstrated that Cyclosporin A (CsA) affects glioblastoma invasion in organotypic brain slices and tumorigenicity in mice. Here we show that CsA impairs migration and invasion of human glioblastoma cells by downregulation of Akt phosphorylation. Interference with PI-3K/Akt signaling was crucial for CsA effect on invasion, because overexpression of constitutively active myr-Akt antagonized drug action. Furthermore, the drug was not effective in T98G glioblastoma cells with constitutively high level of phosphorylated Akt. CsA, comparably to pharmacological inhibitors of PI3K/Akt signaling (LY294002, A443654), reduced motility of glioblastoma cells, diminished MMP-2 gelatinolytic activity and *MMP-2* and *MT1-MMP* expression. The latter effect was mimicked by overexpression of dominant negative Akt mutants. We demonstrate that CsA and LY294002 reduced *MMP* transcription partly via modulation of IκB phosphorylation and NFκB transcriptional activity. Those effects were not mediated by inhibition of calcineurin, a classical CsA target. Additionally, CsA reduced phosphorylation and activity of focal adhesion kinase that was associated with rapid morphological alterations, rearrangement of lamellipodia and impairment of MT1-MMP translocation to membrane protrusions. Our results document novel, Akt-dependent mechanisms of interference with motility/invasion of human glioblastoma cells: through a rapid modulation of cell adhesion and MT1-MMP translocation to membrane protrusions and delayed, partly NFκB-dependent, downregulation of *MMP-2* and *MT1-MMP* expression.

1. Introduction

The invasion of neoplastic cells into brain parenchyma and fast proliferation are hallmarks of glioblastomas, the most malignant brain tumors [1,2]. In order to penetrate brain parenchyma glioma cells generate actin-rich membrane protrusions with extracellular matrix (ECM) proteolytic activity such as lamellipodia and invadopodia. These dynamic structures penetrate the microenvironment, anchor motile cells by focal adhesions and release proteinases that degrade ECM. Invasiveness and migration are complex processes which are regulated by phosphoinositide 3-kinase (PI3K), downstream Akt kinase and focal adhesion kinase (FAK) signaling pathways [3–5]. Binding of ECM proteins or growth factor receptor activation triggers focal adhesion kinase phosphorylation initiating focal adhesions turnover [6–10] and allows PI 3-kinase recruitment to the membrane and stimulation of Akt signaling [2,6]. PI3K/Akt signaling enhances actin remodeling and formation of membrane protrusions influencing Rac proteins [11], and through the activation of p70S6K modulates cell migration and invasion [12]. Akt is localized at the leading edge of moving cells in actin-rich structures and interacts with actin binding proteins [4,13]. Downregulation of Akt expression (in particular Akt2) with antisense or dominant negative constructs resulted in inhibition of glioma cell invasion *in vitro* [14] and *in vivo* [15]. The expression of matrix metalloproteinases (MMPs)-2 and -9 was inhibited in the rat tumor tissue with reduced Akt2 expression [15].

Local modification of ECM by the peptidases in gliomas involves the plasminogen activators, matrix metalloproteinases and cathepsins. Among various MMPs, the increased expression of the gelatinases MMP-2 and MMP-9 strongly correlates with glioma progression [16–18] and malignancy [19–21]. Physiological levels of MMPs are low and the amount of active enzyme is tightly regulated at several levels that involve regulation of gene expression, activation of

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zymogens and inhibition of active enzymes by specific inhibitors [20,22]. The expression of many MMPs and their inhibitors TIMPs (tissue inhibitor of metalloproteinases) is regulated by transcriptional and post-transcriptional mechanisms by a variety of growth factors, cytokines and chemokines [23–26]. Membrane bound MT-MMPs, in particular membrane-type MT1- and MT2-MMP, play a major role in activating MMP-2. Newly synthesized MMP-2 is secreted as an inactive pro-enzyme, which is cleaved on the cell surface by membrane-type MT1-MMP (MMP-14) complexed with TIMP-2 [17,19,27,28]. Development of pharmaceutical approaches that affect expression or regulation of MMPs may be beneficial in targeting invasion of glioma cells but specific inhibitors are still to be found.

We have previously demonstrated that cyclosporin A (CsA), a calcineurin inhibitor, affects growth of glioma cells [29–32] and downregulates PI3K/Akt signaling and Akt-dependent phosphorylation of downstream targets [33]. Moreover, at low micromolar concentrations CsA suppresses glioma cell invasion *in vitro*, in organotypic brain slice cultures, and reduces tumorigenicity *in vivo* [34]. We showed that CsA may directly block glioma invasion without affecting cell proliferation or viability.

In the present study we studied molecular mechanisms underlying the inhibitory effect of CsA on migration/invasion of human glioblastoma cells with different alterations of PI3K/Akt signaling pathway and contribution of PI3K/Akt signaling in the regulation of tumor cell migration and invasion. We demonstrate that CsA impairs Akt and FAK signaling that results in reduction of motility and invasion of glioblastoma cells. CsA, as well as pharmacological and genetic inhibitors of PI3K/Akt signaling, reduced invasion and MMP-2 proteolytic activity likely in two mechanisms: by rapid impairment of shuttling MT1-MMP to lamellipodia and delayed downregulation of NFκB-dependent MMP expression. Our findings show for the first time anti-invasive action of CsA and define a complex involvement of Akt signaling into the regulation of cellular motility and invasion.

2. Materials and methods

2.1. Cell cultures

Human glioblastoma cell lines LN229, T98G and U373 (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, MD, USA) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37 °C (Heraeus, Hanau, Germany).

2.2. Reagents

Antibodies recognizing phosphorylated forms of Akt (Thr308/Ser473), IκB (Ser32), FAK (Thr925/Thr397), GSK3β (Ser-9), as well as corresponding anti-total Akt, IκB, FAK, GSK3β, antibodies and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly MA, USA). For MMPs Western blot, antibodies anti-MT1-MMP from Chemicon Intern. and anti-MMP-2 from Santa Cruz Biotechnology were used. Immunocomplexes were detected using enhanced chemiluminescence detection system (ECL). Cyclosporin A was from Novartis (Novartis Pharma GmbH Nurnberg, Germany), LY294002 was from Cell Signaling Technology (Beverly MA, USA), FK506 (Prograf) from Fujisawa GmbH (Monachium, Germany) and BAY11-7082 was purchased from Sigma Aldrich Inc. (St. Louis, MO). A-443654–Akt inhibitor was a kind gift from Dr. V. Giranda, Abbott Laboratories.

2.3. Western blot analysis

Whole-cell lysates were prepared as previously described [35]. Cells were lysed in a buffer containing 20 mM Tris, pH 6.8, 137 mM NaCl, 2 mM EDTA, 25 mM glycerophosphate, 2 mM NaPPi, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 5 μg/ml leupeptin (Sigma), 5 μg/ml aprotinin (Sigma), 2 mM benzamidine and 0.5 mM DTT (Roche Applied Science). The cell lysates were centrifuged and the protein concentration was evaluated with BCA protein assay (Pierce). Total protein extracts (50 μg of proteins) were mixed with 5×Laemmli buffer (50 mM Tris–HCl pH 6.8, 100 mM DTT, 10% glicerol), denaturated by boiling for 10 min and equivalent amounts of protein were loaded onto 4–10% Tris glycine/SDS-polyacrylamide gels and electrotransferred to ECL nitrocellulose membranes. The specific antibodies as indicated were used for immunoblots. Primary antibodies were detected with either anti-mouse IgG or anti-rabbit IgG antibody linked to horseradish peroxidase (Sigma). Immunocomplexes were visualized by ECL (Amersham Pharmacia Biotech). Bands intensity was determined by densitometry with BioRad Molecular Imager FX and Quantity One software.

2.4. Measurements of cell viability and proliferation

LN229, T98G and U373 cells (8×10^4 cells/well) were seeded in 24-well plates, incubated overnight and treated with CsA for next 12 or 24 h, respectively. Then MTT metabolism test was performed as previously described [36]. Cell proliferation was determined with BrdU assay. Cells were seeded in 96-well plates (6×10^3 cells/well), transfected with specific plasmids and after overnight incubation at 37 °C. Cells were treated for 2 h with BrdU labeling solution (Cell Proliferation ELISA BrdU assay, Roche Diagnostics GmbH Mannheim, Germany), fixed and incubated with a mouse monoclonal anti-BrdU antibody conjugated with peroxidase. Adding 1 M H_2SO_4 enables photometric detection by Thermo labsystem Multiscan EX at 450 nm.

2.5. Plasmids, transfection and gene reporter assay

LN229 cells (8×10^4 cells/well) were seeded on 24-well plates 24 h before transfection. Cells were transfected using Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA) with the plasmid carrying a firefly luciferase gene under promoter consisting multiple binding sites for NFκB (NF-κB *cis*-Reporting System, Stratagene-Agilent Technologies Company, CA, USA). After cell lysis the luciferase activity was measured using Luciferase Reporter Assay System (Promega Corporation Madison, WI, USA) according to manufacturer's protocol.

The plasmid coding for wtAkt was a kind gift from Dr. T. Franke and was previously described [33,37,38]. Plasmid encoding constitutively active myrAkt was kind gift from Dr. M. Weber [33,39]. LN229 cells were transfected using Cell Line Nucleofector™ Kit V according to manufacturer's protocol. Twenty-four hours after transfection cells were detached with 0.25% trypsin/0.02% EDTA (Sigma), counted and used in the matrigel invasion or BrdU assays.

The plasmids coding for dominant negative Akt mutants: ΔPHAkt and K179MAkt were kind gifts from Dr. T. Franke and have been previously described [37,38]. The K179MAkt construct contains a point mutation within the ATP-binding pocket rendering the kinase inactive and the ΔPHAkt construct has the plecstrin homology domain of Akt deleted activation. The LN229 cells were transfected using Cell Line Nucleofector™ Kit V following manufacturer's protocol. Forty-eight hours later total RNA was isolated and used as a template in RT-PCR.

2.6. Invasion and migration assays

The invasion assay was performed as described [34,40]. Briefly, 24-well tissue culture inserts (12 μm pore size Transwell, Corning, NY, USA) coated with the Growth Factor Reduced Matrigel™ Matrix (BD Biosciences, San Diego. CA, USA) were used. One hundred microliters of the Matrigel™ Matrix (1 mg/ml) diluted in distilled water, was dried under sterile conditions (37 °C) for 5–6 h and reconstituted for 30 min