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RIP3 antagonizes a TSC2-mediated pro-survival pathway in glioblastoma cell death

Gregory Fettweis [a], Emmanuel Di Valentin [b], Laurent L'homme [a], Cédric Lassence [a], Franck Dequiedt [c], Marianne Fillet [d], Isabelle Coupienne [a], Jacques Piette [a,*]

[a] Laboratory of Virology and Immunology, GIGA-I³, University of Liège, Liège, Belgium
[b] GIGA-Viral Vector Platform, University of Liège, Liège, Belgium
[c] Laboratory of Protein Signaling and Interactions, GIGA-Molecular Biology of Diseases, University of Liège, Liège, Belgium
[d] Laboratory for the Analysis of Medicines (LAM), Department of Pharmaceutical Sciences, CIRM, University of Liège, Liège, Belgium

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ABSTRACT

Glioblastomas are the deadliest type of brain cancer and are frequently associated with poor prognosis and a high degree of recurrence despite removal by surgical resection and treatment by chemo- and radio-therapy. Photodynamic therapy (PDT) is a treatment well known to induce mainly necrotic and apoptotic cell death in solid tumors. 5-Aminolevulinic acid (5-ALA)-based PDT was recently shown to sensitize human glioblastoma cells (LN-18) to a RIP3 (Receptor Interacting Protein 3)-dependent cell death which is counter-acted by activation of autophagy. These promising results led us to investigate the pathways involved in cell death and survival mechanisms occurring in glioblastoma following PDT. In the present study, we describe a new TSC2 (Tuberous Sclerosis 2)-dependent survival pathway implicating MK2 (MAPKAPK2) kinase and 14-3-3 proteins which conducts to the activation of a pro-survival autophagy. Moreover, we characterized a new RIP3/TSC2 complex where RIP3 is suggested to promote cell death by targeting TSC2-dependent survival pathway. These results highlight (i) a new role of TSC2 to protect glioblastoma against PDT-induced cell death and (ii) TSC2 and 14-3-3 as new RIP3 partners.

1. Introduction

Glioblastomas are among the most frequent and deadliest human brain tumors [1]. The current therapeutic strategy involves surgical resection combined with chemo- and radio-therapy [2–4]. Despite extensive research, efficient long-term treatments remain elusive and glioblastomas are associated with a high degree of recurrence and a median of survival of 14 months. Several genetic alterations have been associated with glioblastoma, which culminate in deregulation of major signaling pathways such as those including EGFR, p53 and overactivation of the NF-κB and AKT/mTOR axis [5,6].

Photodynamic therapy (PDT) is a promising treatment against solid tumors, especially glioblastoma for which this treatment is considered now as a treatment of choice among a few neurosurgical therapeutic opportunities [7]. This therapy relies on a photosensitizer, which upon illumination by visible light of specific wavelength, produces a burst of ROS (Reactive Oxygen Species) that can lead to cell death. In this study we used the 5-aminolevulinic acid (5-ALA) prodrug approach which is known to lead to the accumulation of protoporphyrin IX (PPIX) in mitochondria [8]. Indeed, in the heme cycle, 5-ALA is metabolized in photosensitive PPIX which selectively accumulates in tumor cells due to their low ferrochelatase levels [9]. Interestingly, this high selectivity for tumor cells is frequently used for fluorescence guided resection by neuro-surgeons, and notably for glioblastomas [10]. In addition, it was shown that PDT can successfully expand the survival of patients suffering from non-resectable glioblastoma tumors [11–13].

Previously, we showed that two antagonistic signaling pathways are involved in PDT-induced glioblastoma cell death. First, 5-ALA-PDT induces in glioblastoma a strong pro-survival autophagy *via* a still incompletely described mechanism [14]. This autophagy process is directly regulated by TSC2 (Tuberous Sclerosis 2), a protein known to be regulated by phosphorylation [15], ubiquitination [16] and acetylation [17], which associates with TSC1. The TSC2-TSC1 complex down-regulates the mTOR complex activity, which represses the autophagy pathway and promotes survival and proliferation [18]. As we have previously shown, activation of mTOR downstream effectors was reduced after PDT, suggesting a down-regulation of the mTOR complex activity and a subsequent possible rise of the autophagic flux [14]. On the other hand, PDT is known to induce both necrosis and apoptosis in

Abbreviations: 5-ALA, 5-aminolevulinic acid; MK2, MAP Kinase-Activated Protein kinase 2; p38, Mitogen-Activated Protein kinase p38; PDT, Photodynamic Therapy; RIP, Receptor Interacting Protein; TSC, Tuberous Sclerosis; YWHA, Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, 14-3-3 proteins.

* Corresponding author at: Laboratory of Virology and Immunology, GIGA-R B34, University of Liege, 1 Avenue de l'Hôpital, Liège 4000, Belgium.
E-mail address: jpiette@ulg.ac.be (J. Piette).

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glioblastoma [19,20]. Recently, Receptor Interacting Protein kinase 3 (RIP3), a key protein of the programmed necrosis pathway (necroptosis) was demonstrated to be involved in PDT-induced glioblastoma cell death induced by PDT [21–23] and other cell death inducers [24–26]. The current model of necroptosis relies on the assembly of the "necrosome" complex, in which Receptor Interacting Protein kinase 3 (RIP3) interacts with RIP1, Fas-Associated protein with Death Domain (FADD) and Caspase-8 upon a combined treatment of TNF-α, Smac-mimetic and pan-caspases inhibitors [27]. Unexpectedly, our latest data strongly suggested that the classical necrosome partners of RIP3 were not present in RIP3 containing complex in LN-18 glioma cells after 5-ALA-PDT treatment [21].

In the present study, we highlight the interference between a pro-necrotic RIP3 complex and a pro-survival TSC2-dependent pathway in glioblastoma treated by PDT.

2. Materials and methods

2.1. Cell culture

Human glioblastoma LN18, U87-MG and U373 cell lines were a kind gift of Pierre Robe (Human Genetics, GIGA-R, University of Liege, Belgium). F98 cells were kindly provided by Emmanuel Garçion (INSERM, Angers, France). T98G cell line was obtained from the ATCC. Cells were grown in DMEM complemented with 10% fetal bovine serum and 100 units mL^{-1} penicillin/streptomycin (Gibco) and maintained at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Plasmids single site directed mutagenesis and transfections

pcDNA3.0 3XHA-TSC2 (rat) WT (24939), and pcDNA3.0 FLAG-TSC2 (human) WT (14129) [28] plasmids were purchased *via* the Addgene they were deposited respectively by the Pr Kun-Liang Guan (University of California, San Diego) and Brendan D. Manning (Harvard T.H. School of Public Health). Control empty vector (EV) pcDNA3.0 3XHA was generated by digestion of the plasmid 24939, with *Not*I, *Xba*I and *Kpn*I restriction enzymes. pcDNA3.0 3XHA TSC2 (human) was constructed by cloning human TSC2 from the plasmid 14129 with the In-Fusion cloning system (Clontech). The Q5 High Fidelity DNA-Polymerase (NEB) was then used according the manufacturer to perform site directed mutagenesis to generate 3XHA-TSC2 (rat) S1254A and P1256A; and 3XHA-TSC2 (human) S1254A and P1256A mutants. For the establishment of stable cell lines, target cells were cultured until 80–90% of confluence in 6-well plates and transfected with 1 μg of the desired plasmid using the Lipofectamine LTX with Plus reagent (ThermoFisher Scientific) according to the manufacturer's instructions. 24 h later, cells were selected by a treatment with G-418 (1 $mg \cdot mL^{-1}$) (Roche).

2.3. Generation of TSC2 KO cells via the CRISPR system

Lentiviral plasmid targeting human TSC2 gene *via* CRISPR system was purchased from Sigma-Aldrich (pLenti U6gRNA TSC2-Cas9: Sigma-Aldrich HS0000020001 and HS0000019924) as well as the non-target guide (pLenti CRISPR-NT CONTROL: Sigma-Aldrich, CRISPR13-1EA). Guides will be referred lately as TSC2 #1, #2 and N.Targ, respectively. Briefly, Lenti-X 293T cells (Clontech) were co-transfected together with pLenti U6gRNA TSC2-Cas9 or pLenti CRISPR-NT CONTROL and pSPAX2-D64A (Addgene plasmid 12260 modified in order to mutate HIV-1 integrase catalytic site, non-integrative vector) or pSPAX2 (integrative vector) and a VSV-G encoding plasmids [29]. Lentiviral supernatants were collected 48 h, 72 h and 96 h post-transfection, filtrated and concentrated 100× by ultracentrifugation. The lentiviral vectors were then titrated with qPCR Lentivirus Titration (Titer) Kit (ABM, LV900) and used for cells transduction. After 72 h of transduction, cells expressing GFP were isolated by FACS. Each clone was allowed to grow and later tested by western blotting analysis and clones that were shown depleted for TSC2 were selected. For non-integrative clones a second FACS analysis was done after 2 weeks of culture in order to remove GFP expressing cells (rare event of integration of CAS9 DNA sequence). For integrative clones, cells were subjected to a regular puromycin treatment (5 $\mu g \cdot mL^{-1}$) (Invivogen).

2.4. Establishment of stables transduced cell lines

peGFP-RIP3 WT, RIP3 KD (D161N) (Kinase Dead) and RIP3 RHIM/AAAA (here after referred to as RHIMm) which were a kind gift from FK. Chan, were subjected to PCRs where 3XFLAG tag was added at the N-terminal part. 3XFLAG-RIP3-eGFPs amplicons were then cloned *via* the Gateway Cloning system (ThermoFisher Scientific) into a pLenti PGK Blast Dest plasmid (Addgene, 19065) [30] provided by the Dr. Eric Campeau (University of Massachusetts Medical School). LN-18 cells were infected and stable pools of cells expressing 3XFLAG-RIP3-eGFP selected with a blasticidin treatment (10 $\mu g \cdot mL^{-1}$) (Invivogen).

2.5. Purification of RIP3 immuno-complex

3XFLAG-RIP3-eGFP WT LN-18 transduced cells' proteins were extracted with a lysis buffer (20 mM Tris-HCl pH 7,4; 1% Triton X-100; 10% glycerol; 150 mM NaCl; 1 mM PMSF; 3.3 mM NaF, 1 mM Na3VO4, 25 mM glycerol-phosphate, Roche Complete™ protease inhibitor (Roche Life Science)). Incubated 20 min on ice, lysates were centrifuged at 15,000*g* for 15 min. The supernatant was collected and 56 mg of proteins was pre-cleared for 4 h at 4 °C with 100 μL of Protein-G beads (Santa Cruz Biotechnology, Dallas, TX), then incubated with 120 μL of FLAG-M2 beads (Sigma-Aldrich) overnight at 4 °C. Beads were then loaded on Poly-Prep Chromatography Columns (Bio-Rad) and washed with 50 mL of wash buffer (400 mM NaCl lysis buffer) and eluted with 4 mL of elution buffer (20 mM Tris-HCl pH 7,4; 150 mM NaCl; 200 $\mu g \cdot mL^{-1}$ of 3XFLAG peptide (Sigma-Aldrich)). The eluted proteins were concentrated on Vivaspin 500 columns (Sartorius) and resolved on a 4–12% SDS-PAGE (Invitrogen). The gel was finally analyzed with Sypro ruby staining (Invitrogen).

2.6. In-gel digestion and mass spectrometry (MS) analysis

In-gel digestion was performed by addition of modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate at 37 °C overnight. The tryptic digests were air dried and then dissolved in formic acid (0.1%) for further MS-MS analysis. Each in-gel digest of an individual band was analyzed by nano-high-performance liquid chromatography (HPLC) electrospray MS-MS using an XCT ion trap mass spectrometer (Agilent, Santa Clara, CA). The HPLC separations were performed on an RP C18 Zorbax column from Agilent. The mobile phase was a 90 min gradient mixture formed as follows: mixture A, water-acetonitrile-formic acid (97/3/0.1 [v/v/v]); mixture B, acetonitrile-water-formic acid (90/10/0.1 [v/v/v]). The flow rate was fixed at 300 nL/min. The collision energy was set automatically depending on the mass of the parent ion. Each MS full scan was followed by MS-MS scans of the first four most intense peaks detected in the prior MS scan. A list of peptide masses was subsequently introduced into the database for protein identification searches using MASCOT (Matrix Sciences).

2.7. Reagents and antibodies

5-Aminolevulinic acid (5-ALA) and p38 inhibitor (S8307) was purchased from Sigma-Aldrich. MK2 inhibitor (MK2i, 475864) was bought from Merck Millipore. TNF-α was from Peprotech and zVAD-fmk was purchased from Promega. The BV6 Smac mimetic was previously described [21]. The following antibodies were used for western blot analysis: anti-FLAG-M2 (F-3165) and anti-RIP3 rat (R4277) (Sigma-Aldrich); anti-Caspase-8 (ALX-804-429) and anti-LC3 (NT-0231-100) (Enzo Life Sciences); anti-RIP1 (3493), anti-RIP3 human (13526), anti-TSC2