Role of RIP3 in PDT-induced glioblastoma Cell Death
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Introduction
Glioblastoma are the deadliest type of brain cancer. They are associated with poor survival and a high degree of recurrence despite removal by surgical resection and treatment with chemo and radiotherapy. 5-aminolevulinic acid (5-ALA)-based photodynamic therapy (PDT) was recently shown to sensitize human glioblastoma cells (LN18) to programmed necrosis, also called necroptosis. RIP3 (Receptor Interacting Protein 3) kinase, a key factor of the necrotic signaling pathway, is clearly implicated in PDT-induced glioblastoma cell death. It was shown to associate with RIP1 kinase in a protein complex called necrosome, where it autophosphorylates and allows the downstream necroptotic events to take place. Intriguingly, the other factors commonly present in the necrosome, namely Caspase-8 and FADD were not encountered in PDT-induced pro-necrotic complex. In order to characterise the member of the PDT-induced complex, we transduce LN18 cells with tagged-RIP3 construction and analyses RIP3 immunoprecipitate by mass spectrometry. This approach gave us a list of proteins potentially associated with RIP3 after PDT. Among those, we confirmed the interaction of 14-3-3 with RIP3. Further experiments allowed us to show the presence of 14-3-3 and TSC2 in the complex both in cells overexpressing RIP3 and in an endogenous model.

1. Glioblastoma cells die by an atypical RIP3-dependent necrosis after 5-ALA-PDT

A.

Fig. 1.A. Depletion of RIP3 by siRNA leads to a decreased necrosis induction in response to PDT in LN18 cells (as shown by Lactated Dehydrogenase release assay). **p < 0.01.

B.

Fig. 1.B. Analysis of the necrosome complex components shows that usually present proteins as FADD and Caspase-8 do not take part in PDT-induced pro-necrotic complex. U937 treated with smac mimetic, Staurosporine and JAVAD-fmk (STFv), are used as a positive control for “canonical” necrosome formation. *p < 0.05.

C.

Fig. 1.C. Immunofluorescence showing the clustering of 3XFLAG-RIP3-eGFP after 5-ALA-PDT and TNF-α/Smac-mimetic (STzVd-fmk) treatment (NI = non-irradiated, *p < 0.05).

2. Investigation of RIP3 partners by a Proteomic approach

A.

Fig. 2.A. LN18 human glioblastoma cells were transduced with 3XFLAG-RIP3-eGFP and 60 μg of total lysate were pre-cleared for 4h at 4°C and then incubated with anti-FLAG coupled-beads overnight at 4°C. Beads were washed with 50 μl of a 400mM NaCl buffer and then eluated with 2mL of 200 μg/mL 3X FLAG peptide. Proteins were concentrated and loaded on a 4-12% gel. Proteins were stained with Sypro ruby, bands cut and analyzed by Mass spectrometry.

B.

Fig. 2.B. Immunofluorescence showing the clustering of 3XFLAG-RIP3-eGFP after 5-ALA-PDT and TNF-α/Smac-mimetic (STzVd-fmk) treatment (NI = non-irradiated, *p < 0.05).

C.

Fig. 2.C. Validation of the 3XFLAG-RIP3-eGFP construct by FLAG immunoprecipitation under TNF-α/Smac-mimetic (STzVd-fmk) treatment (NI) showing a complex containing FLAG-RIP3-eGFP, the Caspase-8 and RIP1 (NT = non-irradiated, *p < 0.05).

D.

Fig. 2.D. FLAG-RIP3-gFP and RIP1 forms a complex after PDT treatment here showed by an immunoprecipitation of RIP3. (NT = non-irradiated, 4h 4h post-irradiation).

3. Formation of a Flag-RIP3-eGFP/TSC2/14-3-3 complex after PDT treatment

A.

Fig. 3.A. Immunoprecipitation of YWHAZ shows its interaction with FLAG-RIP3-eGFP 4 hours after 5-ALA-PDT treatment. **p < 0.01.

B.

Fig. 3.B. FLAG immunoprecipitation reveals interaction between TSC2, YWHAZ and FLAG-RIP3-eGFP 4 hours after 5-ALA-PDT treatment. (NT = non-irradiated, 4h 4h post-irradiation, CTR = LN18 transduced with eGFP).

4. RIP3 KD or RHIM domain mutations do not affect the TSC2/RIP3/14-3-3 complex formation

A.

Fig. 4.A. Immunoprecipitation of TSC2 shows its interaction with FLAG-RIP3-eGFP and YM142 4 hours after 5-ALA-PDT treatment independent of RIP3 Kinase Dead or RHIM domain mutations. (NT = non-irradiated, 4h 4h post-irradiation, CTR = LN18 transduced with eGFP, KD = Kinase Dead, RHIM = Rip Homology Interaction Motif)

B.

Fig. 4.B. Immunoprecipitation of YWHAZ shows its interaction with FLAG-RIP3-eGFP and YM142 4 hours after 5-ALA-PDT treatment independent of RIP3 Kinase Dead or RHIM domain mutations. (NT = non-irradiated, 4h 4h post-irradiation, CTR = LN18 transduced with eGFP).

C.

Fig. 4.C. RIP3 interacts with TSC2, YM142 and RIP1 4 hours after 5-ALA-PDT treatment as showed by the immunoprecipitation of endogenous RIP3. (CTR = control).

5. Conclusions

- Propidium Iodide positive F98 cell population analyzed by flow cytometry increases 4 hours after 5-ALA-PDT treatment, which is reinforced in the presence of JAVAD-fmk (as shown by Lactated Dehydrogenase release assay). (NI = non-irradiated, *p < 0.05, **p < 0.01).

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