Project 9: **The regulation of Ripoptosome-associated cell death pathways in keratinocyte skin cancer**

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Short Summary

For progression and metastasis of SCC, the crosstalk of transformed keratinocytes with tumor stroma and immune cells is of importance. Cell death resistance is a prerequisite for progression and metastasis. We recently showed that the Ripoptosome – an intracellular signalling platform containing RIP1, Caspase 8, FADD, and cFLIP - controls apoptosis and necroptosis in SCC. The Ripoptosome is necessary for signaling initiated by membrane-bound receptors (death receptors, TLR 3) and thus shapes the quality not only of cell death but also of the immune response potentially activated by necroptosis. The project will investigate the structure, function, and assembly of the Ripoptosome in different progression stages of primary and transformed keratinocytes and SCC *in vitro* and *in vivo*. We will use in vitro model systems (HaCaT tumor progression model) and representative tumor cell lines *in vitro*. Functional studies will make use of lentiviral knockdown or overexpression of constituents of the Ripoptosome depending on their expression. Our project promotes understanding of the function of different components of the Ripoptosome for tumor progression and metastasis of SCC.

3 State of the Art

3.1 State of knowledge in the field

The major problem of SCC is its multiplicity, whereas metastasis only occurs in locally progressed stages. The reason for this relative resistance to metastasis is currently unclear but may involve immune surveillance by the host activated by inflammatory signals. Cellular death and inflammatory pathways in SCC are regulated by membrane bound receptors that can activate caspases in a pro-apoptotic or inflammatory manner. Activation of cell death via TLR7 by its ligand imiquimod is sufficient for elimination of a high proportion of in situ carcinoma by TRAIL-dependent cell death. Cell death activation can occur via the so-called Death Inducing Signaling Complex (DISC) for death receptors (DR). Alternatively cell death is initiated by recruitment of adaptor molecules such as TRIF or MyD88 to TLR3 or other TLRs. Most of these signaling platforms mediate apoptosis via FADD and caspase 8, whereas more recently the role of the RIP1-RIP3 module (necrosome) for receptor-induced necroptosis has been demonstrated. The decision for the outcome of receptor activation is dependent on the regulation of the components and activity within the Ripoptosome, a recently described novel intracellular signaling platform. The inhibitor of apoptosis proteins (IAPs) such as cIAP1/2 and XIAP were shown to suppress the formation of some of these signaling platforms, in particular the Ripoptosome. The importance of the Ripoptosome for receptor-induced cell death responses in different progression stages of SCC is unknown to date and will be investigated in this project.

3.2 Preliminary work by the participants

In our preliminary work we have studied the formation of the molecular complex that we named the Ripoptosome, which consists of FADD, RIP1, caspase 8 and cFLIP. Our studies were performed in HaCaT and in advanced tumor progression forms derived from HaCaT. By studying these cellular models we could demonstrate the decisive role of RIP1 for the formation of the Ripoptosome, and a crucial role of the RIP1 kinase activity for necroptosis execution induced by death receptors or TLR3 ligands. Furthermore, we could identify the critical role of cFLIP in promoting cell death resistance of A5RT3 (the metastatic cell line of the HaCaT tumor progression model) to cell death mediated by the Ripoptosome. We will therefore aim to further study assembly, activation, and execution of cell death by the Ripoptosome, a complex known to mediate both apoptosis and

necroptosis. Ultimately we will aim to investigate if modulation of this complex can be exploited as a novel target for tumor therapy of early or late SCC of the skin.

4 Project Plan

4.1 Specific Aims

1. Compare expression of the key Ripoptosome components in SCC tumor progression models in vitro, primary keratinocytes and SCC cell lines and primary tumor samples to identify differential expression.
2. Investigate the qualitative and quantitative cell death responses in SCC tumor progression models and compare it to primary human keratinocytes.
3. Compare Ripoptosome formation and activity in selected cell lines and investigate the functional relevance of its components by knockdown or overexpression studies.

4.2 Experimental program

1. First we will analyze the differences in expression of key complex components (FADD, RIP1, RIP3, initiator caspases 8 and 10, *Mixed lineage kinase domain-like* (MLKL)) in various cell lines at the mRNA (qPCR) and protein levels (Western blotting). To this end we will use a number of model cell lines (HaCaT and MET SCC progression models) as well as primary keratinocytes and SCC cells and compare results with data derived from *ex vivo* analysis of human tumors (immunohistochemistry, qPCR, Western blotting). These studies will help to define the main differences between primary keratinocytes and SCC cells.
2. The next step will be the functional analysis of cell death in the above-mentioned primary cells and cell lines. We will analyze the caspase- and RIP1 kinase-dependency of cell death in response to a number of stimuli (TLR or death ligands) by using inhibitors (zVAD-fmk, Necrostatin-1, necrosulfonamide). The morphology of cell death will be determined with combinations of Hoechst and SYTOX Green staining in a kinetic manner using fluorescent microscopy. Caspase-dependent cell death will be monitored by initiator/effector caspase cleavage. Using reverse transfection, transient siRNA transfection against RIP3, MLKL, or caspase-8 will be utilized to dissect which signalling pathways are required for cell death execution.
3. We will then study Ripoptosome formation and activity in selected cell lines (primary and lines derived from SCC progression models) using caspase-8 coimmunoprecipitation. Finally we want to analyze SCC cell lines that show primary resistance to cell death stimuli. We will investigate which Ripoptosome components are critical for cell death resistance, and if proteins such as RIP1, RIP3, MLKL, or others can reconstitute or block the ability of cells to undergo Ripoptosome-induced cell death using retro/lentiviral knockdown of these key components. For selected questions we will extend these studies to organotypic model systems to investigate if identified genes shape a differential cell death response in a three-dimensional environment.

4.3 Collaborations with other Projects in the RTG

Our project will utilize nude mouse model systems (Project 2) and skin carcinogenesis models (Project 5) to extend the *in vitro* findings *in vivo.* Genetic manipulation to eliminate Ripoptosome-associated genes in SCC cell lines via CRISPR/Cas9 will be done in collaboration with project 3 and 4.

5. References

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