Project 8: **Does Angiopoietin-2 protect malignant melanoma tumor cells from anoikis?**

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Short Summary

Increased levels of the Tie2 ligand Angiopoietin-2 (Ang-2) can be detected in the blood from patients suffering from metastasized malignant melanoma (MM) (AJCC III/ IV). Furthermore, different studies could show that Ang-2 is essential for primary MM tumor growth and metastasis formation in mice. Ang-2 is mainly secreted by activated endothelial cells but can also be produced by MM tumor cells themselves. Therefore, Ang-2 may act in an autocrine as well as in a paracrine manner on MM tumor cells. In the absence of its high affinity receptor Tie2, Ang-2 directly associates with and signals through integrins. Integrin activation is required during metastasis formation to protect tumor cells from anoikis, apoptosis induced by inadequate cell-matrix connection. Preliminary data by the applicants show that Tie2 negative MM tumor cells are protected from anoikis by exogenous Ang-2 stimulation. Consequently, this project aims to study if anoikis resistance mediated by Ang-2 represents an essential new pathomechanism during MM metastasis formation and if this can be used therapeutically.

3 State of the Art

3.1 State of knowledge in the field: Angiopoietin-2 (Ang-2) is essential during MM metastasis formation and increased levels of Ang-2 can be detected when metastases have been formed. Metastasis formation passes through the single steps invasion, intravasation, intravascular survival, extravasation and colony formation. Intravascular survival of tumor cells requires protection from anoikis, apoptosis induced by inadequate cell-matrix connection. Protection from anoikis is classically acquired by integrin activation [3] but may also be achieved by receptor activation. Recently, the applicants could show that integrins may also be activated by Ang-2 in the absence of Tie2 receptor. Ang-2 can be detected in some Tie2 negative malignant melanoma cell and may therefore directly bind to and activate integrins. Yet, the impact of Ang-2 stimulation of MM cells has not been studied.

3.2 Previous work by the participants: In the presence of Tie2, Ang-2 induces complex formation of Tie2-FAK-αvβ3 integrin, FAK phosphorylation at Ser910 but not at Tyrosine397, induces αvβ3 integrin internalization/ degradation and endothelial destabilization *(Thomas*, Felcht*, et al, 2010)*. In the absence of Tie2 receptor Ang-2 binds αvβ3, αvβ5 and α5β1 integrins and induces FAK phosphorylation at Tyrosine397 and RAC activation (*Felcht et al., 2012*). The binding of Ang-2 to integrins is tightly regulated and obligates absence of the high affinity receptor Tie2, an acid environment and integrin expression in their active conformation (*Felcht et al., 2012*). Functionally, Ang-2 induces in endothelial cells migration and sprouting independent of Tie2 expression (*Felcht et al., 2012*). In A375 MM tumor cells Ang-2 stimulation protects from anoikis *in vitro* (*unpublished*). A375 cells express αvβ3 integrin but not Tie2 receptor or αvβ5 (*unpublished*). Tumor specimens of metastatic MM show CD34 negative/ αvβ5 expressing and/or αvβ3 expressing cells *(unpublished).*

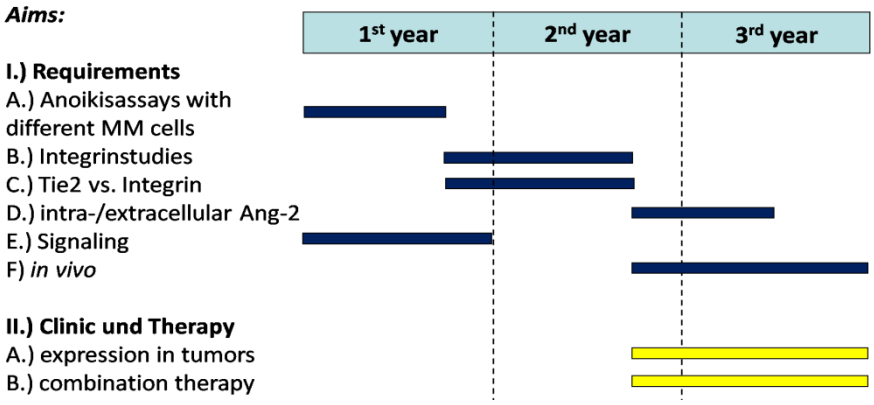
4 Project Plan

4.1 Specific Aims

I. Which requirements are needed for Ang-2 protection from anoikis?

II. Is there a therapeutic relevance of Ang-2 induced protection from anoikis?

4.2 Experimental program



(I.A.) Various MM tumor cells (C32, SK-Mel-28, RPMI 7951, HAT 144, SK-Mel2, WM9, WM35, MV3, CRL 1676, Malme 3M, MeWo, WM 115-8) will be compared for their Ang-2 induced anoikis resistance in different anoikis assays. These studies will include primary MM cells as well as Ang-2 studies in the ret transgenic melanoma mouse model. PCR/ELISA studies will compare Ang-2 levels. **(I.B.)** Integrin expression of the MM cells (see I.A.) will be compared by PCR, Western Blot, IP and FACS analyses i. Inhibition studies (antibodies, siRNA, shRNA) should unravel the relevance for anoikis resistance. **(I.C.)** Tie2 expression will be studied in the different MM cells (see I.A.) (PCR/Western blot). Inhibitory (siRNA, antibodies)/overexpression (shRNA) studies will be compared with the integrin expression/anoikis sensitivity. **(I.D.)** Intra- and extracellular Ang-2 signalling has been observed. MM cells with low, intermediate & high levels of Ang-2 (see I.A.) will be analysed in inhibition studies (siRNA, shRNA, antibody) in the anoikis assay. Exogenous (recombinant, conditional media w. adenoviral overexpression [Ad-Ang-2])) vs. endogenous (Ad-Ang-2) Ang-2 stimulation will support the analyses. **(I.E.)** AKT, ERK, mTOR, JNK, Mcl-1 and bad signalling will be analysed. Intrinsic/extrinsic apoptosis will be studied in collaboration with project 9. Pharmacological inhibitory studies (Worthmanin, UO126, Rapamycin, zVAD-fmk) will be performed in parallel. **(I.F.)** MM cells (different integrin/Ang-2/ Tie2 expression profiles) will be used for metastasis studies *in vivo*. Metastasis formation will be studied by conventional microscopy and correlated with anoikis sensitivity. Control experiments with Ang-2 overexpression and inhibition (antibodies) will support the *in vivo* study. Inhibition experiments (antibodies) within the ret transgenic melanoma mouse model will finalize the *in vivo* studies. **(II.A.)** Preliminary studies detected αvβ3 integrin in non-vascular MM areas in tumor specimens from patients. Integrin expression studies (see I.B.) will be performed in a larger cohort with co-staining against melanocytic markers. **(II.B.)** The signalling studies (see I.e.) will be followed by combination inhibitory studies *in vitro*.

4.3 Collaborations with other Projects in the RTG

Primary cutaneous MM cells will be generated in collaboration with project 2. Vascular remodelling/pruning is studied in collaboration with project 3. The studies of molecular signalling of apoptosis will be supported by project 9 and 10. The ret transgenic melanoma mouse model will be provided by project 13.

5 References

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