Influence of Melanoma Cells and Cancer-associated Fibroblasts on (Lymph-)angiogenesis in Conjunctival Melanoma

Abstract

Ocular melanomas represent the most common and most fatal primary malignant tumors of the eye in adults. There are two distinct melanoma entities of the eye. Intraocular uveal melanoma (UM) occurs in the choroid, ciliary body and/or iris, while extraocular conjunctival melanoma (CM) occurs at the ocular surface. Typical driver mutations of UMs occur in *GNAQ* or *GNA11* genes, while CMs often harbor *BRAF*, *NRAS*, or *NF1* mutations. In Germany, about 400 new cases of UM and 100 cases of CM are diagnosed every year. UM spreads almost exclusively via the hematogenic path with strong hepatic tropism, while CM has a propensity for lymphogenic spread into the regional lymph nodes. Despite successful treatment of the primary tumor, about half of the patients with UM and about a third of the patients with CM die of metastases in the long term. For both entities, metastatic UM and metastatic CM, there are currently no effective treatment options available. This might be because none of the current approaches takes sufficient account of the complexity of interactions within the tumor microenvironment (TME). The TME is a heterogeneous and dynamic complex that interacts with tumor cells, but has not been investigated to date in CMs. Cancer-associated fibroblasts (CAFs) represent a prominent and heterogeneous cell population of the TME that significantly promotes progression and metastatic spread in many tumors by simultaneously stimulating tumor and neighboring non-tumor cells, such as lymphatic or blood endothelial cells (LECs and BECs), and by weakening the anti-tumoral activity of immune cells.

The present study is the first to investigate CAFs derived from CMs, i.e. human conjunctival melanoma fibroblasts (HCMFs). We focused on the functionality and influence of HCMFs - and additionally of conjunctival melanoma cells (CMCs) - on tumor-associated lymph- and hemangiogenesis, tumor growth, and metastasis. Characterization of HCMFs using immunocytochemistry and flow cytometry identified no distinct phenotype, which is likely due to the known heterogeneity of CAFs, consisting of different subtypes. Similar inconsistent data regarding CAF markers has been published for other tumor entities. Stable isotope labeling by amino acids in cell culture- (SILAC) based quantitative proteomics of HCMFs and CMCs compared to corresponding healthy controls revealed significantly regulated proteins in the proteome and secretome. Furthermore, we identified subsets of significantly regulated proteins in HCMFs as well as in CMCs that were associated with angiogenesis, lymphangiogenesis as well as proliferation and migration. Of particular note, HCMFs exhibited highly upregulated sFLT1 (soluble VEGFR-1) secretion. While sFLT1 showed no stimulatory effect on LECs, BECs, and CMC proliferation in functional assays, it increased the activity of the MAPK

signaling pathway in CMCs. Stimulation with HCMF conditioned media significantly increased LEC and CMC proliferation. It promoted LEC and BEC migration, and had slight effects on vascularization properties. Not only LEC and BEC but also CMC proliferation increased significantly when stimulated with CMC conditioned media, suggesting an autocrine effect of CMCs. A phosphoproteomics screen of CMCs identified a high number of activated kinases in all CMCs that, using kinase substrate enrichment analysis (KSEA), were found to be involved in MAPK and PI3K/AKT/mTOR signaling pathways. CMCs revealed upregulated phosphorylation in typical activation sites of the MAPK signaling pathway downstream effector ERK and of the AKT downstream effector TSC2, resulting in inhibition of the tumor suppressor complex of TSC1 and TSC2 and activation of mTOR. The activating phosphorylation site of PI3K/AKT/mTOR signaling pathway downstream effector RPS6 was increased in CMCs with *BRAF* mutation pointing to a possible connection between PI3K/AKT/mTOR signaling pathway and *BRAF* mutation. These data demonstrate that MAPK and PI3K/AKT/mTOR signaling pathways are activated independent of their mutation status (*BRAF* or *NRAS*).

Taken together, the current data demonstrates for the first time a notable impact of HCMFs and CMCs on lymphangiogenesis, metastasis, and tumor growth. Independent of the mutational status, inhibiting MAPK and PI3K/AKT/mTOR pathways in CMCs as well as tumor-promoting properties of CAFs as one of the predominant TME components, should be considered as promising and potentially combinable treatment options, which will need further *in vitro* and *in vivo* evaluation in the future.