Abstract

Melanoma is a highly aggressive skin cancer of melanocytic origin. Late-stage disease, when distant metastases develop, is practically incurable, despite the introduction of new treatments (e.g. targeted $BRAF^{V600E/V600K}$ therapies or immune checkpoint inhibitors). Resistance to therapy is largely due to exceptionally high heterogeneity of melanoma, attributed, among others, to the existence of the cancer stem cells (CSC). It is supposed that CSC can initiate tumor growth from a single cell and then re-establish tumor heterogeneity. They give rise to highly proliferating tumor cells, but at the same time remain quiescent, and resistant to conventional therapies. Melanoma initiating cells (MIC) are thought to be responsible for tumorigenicity and aggressiveness of this cancer, but it is still not clear which factors can regulate their behavior.

One of the enzymes important in cancer biology is heme oxygenase-1 (HO-1), that degrades heme to carbon monoxide (CO), biliverdin, and ferrous ions (Fe^{2+}). Upon activation in response to oxidative stress (e.g. caused by chemo- and radiotherapies or UV radiation) HO-1 acts as a cytoprotective agent, affecting also angiogenesis and immune response. The final outcome of the HO-1 activation in cancer is cell type-specific. In melanoma, overexpression of HO-1 increases aggressiveness and therapy resistance in growing tumors. However, nothing is known about the possible effects of HO-1 in MIC.

We addressed this issue and investigated the role of HO-1 in regulation of MIC activity. We used the B16-F10 murine melanoma cell line and found that it contains cells expressing MIC surface markers (CD20, CD133, CD24, ABCB1, ABCB5, Sca-1) and cells with functional CSC features (high activity of ALDH and PKH26 label retention). Expression levels of HO-1 in the MIC^{+} and bulk cells were comparable. However, HO-1 activity facilitated non-adherent growth of melanoma cells, a typical feature of CSC.

We found that it was HO-1 overexpression, not the MIC markers expression, that predominantly affected melanoma clonogenicity. Interestingly, overexpression of HO-1 led to decreased clonogenicity of single melanoma cells *in vitro* and was unfavorable for clonal growth. Consistently, *in vivo* serial transplantation of a small fraction of B16-F10 cell line (10 or 100 cells) into syngeneic C57BL/6 recipients revealed that HO-1 overexpression decreased tumorigenicity in secondary and tertiary recipients, most probably through reduced cell self-renewal.

Our data show that phenotypic or functional MIC markers in B16-F10 murine melanoma ($CD20^{+}$, $ALDH^{high}$, $PKH26^{+}$ slowly cycling cells) do not select CSC-like cells. We observed

similar clonogenicity *in vitro* and tumorigenicity *in vivo* between MIC^- and MIC^+ subsets. Moreover, MIC^- and MIC^+ cells had similar differentiation status reflected by expression of melanoma-associated antigens (MAAs; *Tyr, Gp100, Mart-1*) and similar expression of CSC-associated genes (e.g. *Notch1, Myc*). Finally, the progeny of both MIC^- and MIC^+ cells regain heterogeneity of the bulk subpopulation.

We also demonstrated that silencing of HO-1 with shRNAs led to the de-pigmentation of B16-F10 melanoma cells despite the fact that it did not change the expression of genes involved in melanogenesis. Instead, HO-1 overexpression was associated with decreased activity of tyrosinase, a rate-limiting enzyme in melanin synthesis. To check whether HO-1 is necessary for development of pigmentation, we performed experiments in the induced pluripotent stem cells (iPSC), generated from the tail-tips fibroblasts of *Hmox-1*$^{-/-}$ and *Hmox-1*$^{+/+}$ mice and differentiated towards pigmented melanocytes. We detected pigmentation and upregulation of melanocytic markers in both groups, meaning that HO-1 is not required for induction of melanocytic differentiation. Moreover, experiments performed in Melan A cell line showed that HO-1 is dispensable for pigmentation of melanocytes.

To sum up, our data points at the dual role of HO-1 in melanoma. Although overexpression of HO-1 promoted the progression of growing B16-F10-derived tumors, on the other hand it seems to decrease a risk of melanoma initiation. We demonstrated that overexpression of HO-1 during clonal growth induction *in vitro* and *in vivo* can play an anti-tumorigenic role. Thus, our results suggest that pharmacological inhibitors of HO-1 or drugs that indirectly induce HO-1 in melanoma treatment, might have a different effect on tumor growth than on tumor initiation.