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Journal of Cancer Immunology Commentary

Susceptibility of Malignant Brain Tumors to 5-aminolaevulinic Acid Mediated Photodynamic Therapy: Direct Phototoxicity and Immunological Effects

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Commentary

Recently we published the article 'Accumulation of protoporphyrin IX in medulloblastoma cell lines and sensitivity to subsequent photodynamic treatment' [1]. In this commentary, we review protoporphyrin IX accumulation after application of 5-aminolaevulinic acid and the resulting sensitivity of medulloblastoma cells to photodynamic therapy. We compare the results to glioblastoma cells, including glioblastoma stem-like cells, and address the contribution of the transporter adenosine triphosphate binding cassette subfamily G member 2 (ABCG2) as well as the enzyme ferrochelatase to the process. We discuss possible strategies to improve efficiency of photodynamic therapy, particularly in the clinical setting and highlight the contribution of the anti-tumoral immune response to the efficacy of this novel treatment modality for brain tumors.

Novel Therapeutic Options for Malignant Brain Tumors

Primary brain tumors are a heterogeneous group of tumors arising within the central nervous system. They can be benign or malignant and develop in children as well as in adults. The most frequent malignant brain tumor in adults is the glioblastoma (GBM), a grade IV glioma of astrocytic lineage with a yearly incidence of 3 – 4 per 100,000 [2]. Despite multimodal therapy, consisting of maximal safe resection combined with radiotherapy and alkylating chemotherapy with temozolomide, prognosis of patients is poor: median overall survival (mOS) is 15 months, and after three years, virtually all patients have relapsed with a mOS after recurrence of 6 months [3,4].

In children, GBM is rare (1 – 2 per million; [5]). Here, medulloblastoma (MB), a neuroepithelial tumor located in the cerebellum and the fourth ventricle is the most common malignant brain tumor, accounting for 20% of all pediatric brain tumors, with incidences ranging from 0.6 – 9 per million, depending on age [6,7]. The current treatment combines surgical resection with craniospinal radiotherapy and chemotherapy. However, due to therapy-associated sequelae, radiation sparing protocols are used in infants and reduced-dose protocols in patients at lower risk, e.g. when gross-total resection of the tumor (< 1.5 cm^2 residual tumor) has been achieved. Five-year survival ranges from 60 to 95%, depending on subtype and risk group [6,8,9].

In both tumors, GBM and MB, the extent of resection is a strong prognostic factor. However, total or even gross-total resection is frequently a neurosurgical challenge. The infiltrative growth of the tumor into adjacent functional areas, the frequently eloquent locations of the tumor and the difficulties to clearly identify the tumor intraoperatively increase the risk for resection-associated morbidity. Moreover, current prognosis for GBM patients and particularly high-risk MB patients even after gross-total resection and despite the multimodal therapies is poor. Thus, new therapeutic modalities are needed.

Photodiagnosis (PD) and photodynamic therapy (PDT) are novel therapeutic approaches in brain tumors. Both rely on the preferential enrichment of a photosensitizer in the tumor tissue. In photodiagnosis, the photosensitizer

is used to visualize the tumor intraoperatively, whereas in photodynamic therapy, the photosensitizer is excited with light of a defined wavelength, which initiates a photochemical reaction that results in the generation of reactive oxygen species, which kill the tumor cells [10].

Protoporphyrin IX (PPIX) is a well-established photosensitizer. It is an intermediate of the heme biosynthesis pathway, and is normally converted by the enzyme ferrochelatase to heme by addition of a ferrous ion. 5-aminolaevulinic acid (5-ALA) is generated from glycin and succinyl-CoA by ALA-synthase in the initial rate limiting step of heme biosynthesis. When it is added to cells exogenously, it is transported into the cytosol by the peptide transporters PEPT1 and PEPT2 and metabolized to coproporphyrinogen III, which is transported into the mitochondria by the transporter ABCB6 and further metabolized via PPIX to heme [11]. In numerous tumor cells, however, conversion of PPIX to heme is impaired due to different mechanisms, including a lack or down-regulation of ferrochelatase [12]. Hence, non-metabolized PPIX accumulates in the mitochondria and the cytosol of the tumor cells.

GBM cells show reduced expression of ferrochelatase and accumulate PPIX when subjected to exogenous 5-ALA *in vitro* [13]. Oral intake of 5-ALA prior to surgery allows intraoperative identification of the tumor when exposed to light of 400 nm wavelength during fluorescence-guided surgery [14]. Thereby, the extent of tumor resection can safely be increased, which provides a survival benefit for the patients [15,16]. Thus, in GBM application of 5-ALA is a powerful photodiagnostic tool.

PPIX accumulation has also been reported for MB cells *in vitro* [1,17-20], however, *in vivo* results have been contradictory. In several studies on pediatric brain tumors, PPIX fluorescence has been detected during fluorescence-guided surgery in only 4 out of 11 MB patients [21-25], and in only 2 of the patients intraoperative identification of the tumor was possible [26]. Moreover, a patchy rather than homogenous fluorescence of the tumors has been described [22,27], which may indicate the presence of distinct subsets of MB cells differing in PPIX accumulation within the same tumor. Indeed, when we compared PPIX accumulation in MB and GBM cell lines, accumulation was more efficient in the GBM cells and not all of the MB cells became PPIX positive [1], supporting this conclusion.

There was no difference in ferrochelatase expression and activity between GBM and MB cells, which would account for the differences [1]. However, we observed expression of the transporter ABCG2 (CD338) on the surface of MB, but not GBM cells [1]. Thus, MB cells express a protein that transports PPIX out of the cells and, therefore, could counteract PPIX accumulation [28]. Whether ABCG2 expression contributes to the differences and identifies different subsets of MB cells is currently under investigation. However, it is tempting to speculate that inhibition of ABCG2, e.g. by imatinib, verapamil or genestein [11,29-31] may increase the efficiency of PPIX accumulation in MB.

Besides the presence of ABCG2, the efficiency of 5-ALA uptake and its metabolization towards PPIX, the availability of ferrous iron or even the formation of non-fluorescent aggregates can affect PPIX accumulation. *In vivo* there may also be differences in pharmacokinetics of 5-ALA and, particularly, the access to the MB tissue could be limited, e.g. when the blood-brain-barrier is undisturbed or capillary permeability and blood flow are low [32,33].

Interestingly, in GBM high expression levels of ABCG2 have been described in glioma stem-like cells (GSC) [34], thus, a cellular subset within the tumor, which has been proposed to initiate and maintain tumor growth [35,36] and to represent the driving force for disease recurrence [35,37]. In GSC, ABCG2 contributes to multidrug resistance, including resistance to temozolomide [38], but probably also to self-renewal and maintenance of stemness [39]. We reported recently that there are differences between GBM cells and primary GSC lines in PPIX accumulation [40] and could show that blocking ABCG2 in GSC increases PPIX accumulation (Ebbert et al., manuscript in preparation).

Little is known about MB stem-like cells and their accumulation of PPIX. Lee et al. isolated $CD133^+$ neuronal stem cells from the cerebellum, which may represent the cells of origin of MB [41], and della Puppa et al. identified $CD133^+$ MB cells, which were located in the faintly fluorescent external areas of the tumor, whereas the inner core consisted of more fluorescent tissue [27]. However, from these data no conclusion can be drawn on PPIX accumulation in MB cells versus MB stem-like cells and why in MB not all cells appear to accumulate PPIX.

Exposure of GBM or MB cells, which had been subjected to 5-ALA, to laser light of 635 nm wavelength (PDT) initiates a photochemical reaction and results in the generation of reactive oxygen species, including singlet-oxygen, which ultimately causes death of the tumor cells. Consistent with the reduced efficiency of PPIX accumulation in MB, GBM cells are more sensitive to the phototoxic effect of 5-ALA-mediated PDT [1], and a substantial fraction of MB cells survives the treatment. Indeed, photosensitivity of MB cells appears to be directly dependent on PPIX accumulation, since PPIX accumulation and survival after 5-ALA/PDT treatment show an almost linear relationship. Thus, other factors such as oxygen concentration seem to have a minor influence, and to enhance efficacy of 5-ALA/PDT in MB,

it will be important to improve PPIX accumulation as discussed before or other photosensitizers have to be used, such as hypericin [19] and 5-ALA-hexylester [20].

Even in GBM, which responds to 5-ALA/PDT, clinical efficacy may depend on the treatment targeting GSC, because they represent the driving force for disease recurrence [35,37]. When GSC were subjected to 5-ALA/PDT, a 5-ALA-dose-dependent reduction in viability was observed [40]. However, different GSC preparations differed in sensitivity, which followed a negative exponential regression model, suggesting that it mainly depends on the level of PPIX accumulation, i.e. 5-ALA metabolization to PPIX and the efflux of PPIX. As discussed before, GSC express ABCG2 [34], which transports PPIX out of the cells [28], and indeed, blocking ABCG2 in GSC not only results in an increase of PPIX accumulation, but also in increased sensitivity to 5-ALA/PDT (Ebbert et al., manuscript in preparation). Thus, although early clinical reports on the therapeutic use of 5-ALA/PDT are promising [42-45], efficacy in GBM may depend on combining it with at least an inhibitor of the transporter ABCG2. In addition, more effective illumination by novel devices, which allow a more homogenous and controlled delivery of the laser light to the surgical cavity [46], may result in an increased sensitivity of the tumor cells at a given PPIX accumulation, and could thereby contribute to increase efficacy of the treatment.

Irrespective of the device, however, the phototoxic damage caused by 5-ALA/PDT can extend only up to a depth of about 4 mm in cerebral tissue [47]. Therefore, it will not reach the more distant tumor cells. However, 5-ALA/PDT not only directly kills the tumor cells, but it is well established that it generates pro-inflammatory conditions and contributes to the induction of anti-tumoral T-cell responses, with $CD8^+$ T cells as main effectors and a supportive role of $CD4^+$ T cells [10]. For glioblastoma cells we could demonstrate that 5-ALA/PDT initiates the afferent phase of adaptive immune responses leading to attraction, antigen-uptake and activation of dendritic cells mediated by the release of the heat-shock protein 70 by the dying cells [48], pre-requisites for the initiation of T cell immunity. Yet, it needs to be elucidated whether this results in cross-presentation of tumor antigens and induction of cytotoxic T-cell-mediated anti-tumoral immunity *in vivo*. The importance of such anti-tumoral immune responses to target particularly GSC has been documented by Pellegatta et al., who showed that using neurosphere-derived GSC as a source of tumor antigens improves efficacy of dendritic cell-based immunotherapy in a GL261 glioma mouse model [49]. Since GSC are sensitive to 5-ALA/PDT, the T-cell responses may also be directed towards the GSC compartment in the tumor, further highlighting the possible therapeutic potential of photodynamic therapy.

In conclusion, the selective accumulation of PPIX after the application of 5-ALA in brain tumors such as glioblastoma and medulloblastoma has the potential to improve the outcome of cytoreductive procedures by allowing to identify the tumor intraoperatively during fluorescence-guided surgery. Furthermore, when PPIX is excited with laser light of 635 nm wavelength, a photochemical reaction is initiated, generating reactive oxygen species, which kill the tumor cells. At least in glioblastoma, the respective cancer stem cells appear to be sensitive to 5-ALA/PDT as well, potentially reducing the risk of disease recurrence. Moreover, 5-ALA/PDT not only kills the tumor cells directly due to the phototoxic effect, but also results in the initiation of an anti-tumoral immune response, by attracting dendritic cells, promoting uptake of dying tumor cells and inducing dendritic cell maturation. This immunological effect is likely to contribute to the clinical responses to 5-ALA/PDT. Thus, 5-ALA and PPIX hold photodiagnostic as well therapeutic potential. Whether this approach can be extended to other brain tumors or enhanced, e.g. by inhibiting the enzyme ferrochelatase or the ABCG2 transporter to increase PPIX accumulation, requires further investigations.

Conflict of Interest

The authors declare no conflict of interest

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