Selective enrichment of hypericin in malignant glioma: Pioneering in vivo results

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Abstract. Malignant gliomas are diffuse infiltrative growing tumors with a poor prognosis despite treatment with a combination of surgery, radiotherapy and chemotherapy. It has been shown recently that complete tumor resection improves the survival time significantly. Hypericin, a component of St. Johns Wort, is one of the most powerful photosensitizers in nature. The aim of the present study was to investigate accumulation of hypericin in intracerebral implanted malignant glioma in vivo. Rats underwent stereotactic implantation of C6 glioma cells. After intravenous administration of hypericin (5 mg per kg body weight), accumulation of the compound was studied in tumor, the infiltration zone surrounding the tumor and healthy brain (contralateral hemisphere) by fluorescence microscopy between 0 and 48 h after injection. Results were compared by one-way analysis of variance. For post hoc pair-wise comparison the Tukey-Kramer HSD test was used. Accumulation of hypericin was significantly higher in C6 glioma as compared to normal tissue. Maximum hypericin uptake was achieved at 24 h after injection. Ratios of fluorescence intensity between tumor and normal tissue as well as infiltration zone and normal tissue of about 6.1:1 and 1.4:1 were found. Considering tissue auto-fluorescence, fluorescence ratios of about 19.8:1 and 2.5:1 were calculated, respectively. Therefore, hypericin seems to be quite an effective fluorescence marker for the detection of glioma in vivo. To the best of our knowledge, the present study demonstrates for the first time that hypericin accumulates selectively in intracerebral implanted C6 glioma in vivo after systemic (intravenous) administration.

Introduction

Patients who are suffering from glioblastoma represent the main proportion of patients with malignant brain tumors and show simultaneously the shortest survival time (1). Surgical therapy aims for a total resection of the tumor while preserving the surrounding normal brain tissue and thus the functional integrity of patients. Glioblastoma is an ill-defined, diffuse infiltrative growing tumor. Discrimination between tumor and normal brain tissue during surgery is elementary. A method to visualize residual tumor tissue will be a considerable advantage leading to safer and more complete tumor resection, because tumors relapse immediately at their margins of resection despite adjuvant therapy in approximately 90% of patients (2-10). Improvement of radicality in tumor resection by intraoperative visualization was demonstrated by Stummer et al. They used oral administration of 5-aminolevulinic acid (5-ALA), which is metabolized in the tumor cells to fluorescent protoporphyrin IX (PpIX). With this approach complete tumor resection was achieved in 65% of patients as compared to 36%, when tumor resection is performed under conventional white light conditions. This resulted in an extension of median survival time from 11.8 to 16.7 months in the fluorescence-guided resection group (11,12). Additionally, new therapeutic options, i.e., chemotherapy with the alkylating agent temozolomide and antiangiogenic drugs (e.g., bevacizumab), ensured further progress in glioma therapy (13-15). Despite enormous progress in the surgical and chemotherapeutical treatment of malignant brain tumors during the last decade, the prognosis of glioblastoma patients is still unfavourable. Photodynamic therapy (PDT) as an additional local treatment tool might be of interest in glioblastoma therapy in the future (16-19). PDT is an interesting modality of cancer treatment, which is based on the selective accumulation of a photosensitizer (PS) in tumor tissue. Illumination with light of appropriate wavelength produces reactive oxygen species (ROS), which react with biomolecules inducing mechanisms of apoptosis or necrosis and leading finally to cell death (20).

Hypericin, a constituent of St. Johns Wort (SJW; Hypericum perforatum), is one of the most powerful natural photosensitizers (21). In vitro and in vivo photodynamic effects inducing apoptosis and/or necrosis of various malignant cells, such as human glioblastoma cell lines (22), human HL-60 promyelocytic leukaemia cells and CD4+ T-cells (23), rat bladder transitional cell carcinoma (24), human colon carcinoma and hepatoma cell lines (25) as well as human pancreatic cancer (26), were demonstrated. In vitro hypericin accumulates in glioma cells significantly higher than in neurones (27,28). However, hypericin could not be detected in healthy rat brain in vivo after oral administration of either alcoholic H. perforatum extracts or
the pure compound (29-31). These findings encouraged us to investigate accumulation of hypericin after systemic (intravenous) administration in C6 rat glioma, which is characterized by a disrupted blood-brain barrier (BBB). Particular interest was given to the relation between application time and hypericin enrichment in the tumor. Additionally, hypericin uptake in the tumor was compared to that in the infiltration zone around the tumor as well as in healthy brain of the contralateral hemisphere.

Materials and methods

Cell culture. Rat glioma C6 cells (ATCC Number CCL-107; American Type Culture Collection, Rockville, MD, USA) were grown in 75 cm² flasks (Corning, NY, USA) with RPMI medium containing 10% fetal bovine serum and penicillin (10,000 U/ml) as well as streptomycin (10,000 µg/ml) (Invitrogen, Karlsruhe, Germany). Cells were cultured for 3 days until confluence was achieved. After washing with phosphate buffered saline (PBS, Invitrogen) cells were detached, centrifuged and resuspended in PBS for implantation in rat brain.

In vivo implantation of cultivated C6 cells. The animals were treated in accordance to the guidelines of the University of Tübingen animal ethics committee and the German Animal Welfare Act. A total of 16 male Wistar rats purchased from Charles River (Sulzfeld, Germany) weighting 200-250 g underwent stereotactic implantation of glioma C6 cells (10⁵) in a volume of 2 µl PBS. Rats were anaesthetized using a combination of fentanyl (0.005 mg/kg), midazolam (2.0 mg/kg) and medetomidine (0.15 mg/kg) body weight by intraperitoneal injection. A frontal, 10 mm sagittal scalp incision in the midline was performed. A burr hole was made 2 mm lateral to the sagittal suture and 2 mm in front of the coronal suture using a microsurgical drill. Rats were fixed in the stereotactic frame and the cell suspension was slowly inoculated intracerebrally at a depth of 2 mm. The burr hole was closed using bone wax and the scalp was sewed.

On day ten after tumor implantation the animals were anaesthetized as mentioned before. Hypericin [obtained from Phytochem, Ichenhausen, Germany, with a purity higher than 99% and dissolved in DMSO, PEG and aque injectabile as described by van de Putte et al (32)] was administered at a dose of 5 mg/kg body weight via the lateral tail vein. Animals were sacrificed at 2, 6, 12, 24, 36 and 48 h after injection and the brain was withdrawn, covered in Tissue Tek (Invitrogen) and immediately frozen in liquid nitrogen. Rats bearing C6 glioma without photosensitization as well as healthy rats after hypericin administration served as controls.

Fluorescence microscopy. Coronal slices with a thickness of 7 µm were prepared with a cryomicrotome (Microm HM 560, Microm International GmbH, Walldorf, Germany). Slices for fluorescence microscopy were stained with DAPI Vectashield Mounting medium at a concentration of 1.5 µg/ml (Vector Laboratories, Burlingame, USA) and covered with antifade medium (Dako, Hamburg, Germany) on cover slips (Super Frost Plus, Langenbrinck Labor und Medizintechnik, Emmendingen, Germany). Corresponding serial slices were stained by hematoxylin and eosin (H&E) for histologic assessment.

Fluorescence microscopy was carried out using an Olympus BX61 microscope (Olympus-Europa GmbH, Hamburg, Germany) equipped with a UPlanFl 40x/0.75 objective and filter sets for the detection of red fluorescence (hypericin, U MWG2; excitation: 510-550 nm, emission: >590 nm) as well as blue fluorescence (DAPI, U MWU; Excitation 330-385 nm, emission: >420 nm). Fluorescence images were obtained using an F-View II charge-coupled device camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Each slice used for fluorescence microscopy was divided into three zones: tumor, infiltration zone and normal tissue, i.e., corresponding contralateral area usually 50-100 µm distant to the tumor border. In every zone 30 regions of interest (ROIs) were defined and fluorescence intensity was measured under identical conditions. Values of fluorescence intensity and autofluorescence, defined as the fluorescence measured with the U MWG2 filter in slices from healthy brain without hypericin administration, were given in arbitrary units. For each animal at least 12 slices were investigated. All procedures, i.e., preparation and storage of slices and fluorescence measurements, were performed under subdued light or in the dark.

Results

In order to determine the tumor selectivity of hypericin uptake coronar cryosections of rat brains were investigated. To identify tumor tissue histologically, H&E staining was performed for every second serial section. The subsequent serial sections were stained by DAPI and investigated by fluorescence microscopy using the appropriate filter settings to detect DAPI and hypericin fluorescence. As demonstrated in Fig. 1, hypericin accumulates selectively in C6 glioma, but not in normal rat brain tissue, after intravenous administration at a dose of 5 mg/kg body weight. Note the intensive red fluorescence of the C6 glioma due to hypericin accumulation. Additionally, the infiltration zone around the solid tumor shows also some hypericin uptake. This finding is shown in more detail in Fig. 2. Solid C6 glioma is characterized by a high density of cell nuclei (right side after H&E and DAPI staining) and the intense red fluorescence of hypericin. In contrast, no hypericin fluorescence is detected in the contralateral hemisphere without tumor (left side). Furthermore, hypericin fluorescence correlates well with the decreasing density of tumor cells in the infiltration zone of C6 glioma (middle).

As shown in Fig. 3, maximum of hypericin accumulation in C6 glioma is reached 24 h after intravenous administration. For longer time intervals, i.e., 36 and 48 h after injection, fluorescence intensity decreases rapidly. Mean values of hypericin fluorescence within tumor, infiltration zone and healthy brain tissue are summarized in Table I. There is significant higher fluorescence in tumor tissue as compared to normal tissue after administration of hypericin regardless to the time interval between injection.
and brain removal. Additionally, the infiltration zone exhibits about 1.5-fold higher hypericin fluorescence as the contralateral hemisphere at 24 h after administration. Control experiments were performed in rats after stereotactic injection of PBS. Fluorescence intensity after PBS injection (16.2 a.u., area 5, Fig. 3) was quite similar to that of normal brain (16.0 a.u., area 4, Fig. 3). Ratios of fluorescence intensity between tumor and normal tissue as well as infiltration zone and normal tissue of about 6.1:1 and 1.4:1 were found at 24 h after hypericin administration; considering tissue autofluorescence (Table I at 0 h) the ratios of about 19.8:1 and 2.5:1 were calculated, respectively. It should be mentioned that the autofluorescence values of C6 glioma (19 a.u.), infiltration zone (20.25 a.u.) and normal brain tissue (19.6 a.u.) are quite similar.
Table I. Fluorescence intensities of different zones in C6 glioma bearing rat brains after i.v. administration of hypericin (5 mg/kg b.w.).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Tumor (T) (95% confidence interval)</th>
<th>Infiltration zone (IZ)</th>
<th>Healthy brain (HB) (95% confidence interval)</th>
<th>Tukey-Kramer HSD test (significance level: p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19.0 (18.7-19.3)</td>
<td>20.25 (19.9-20.6)</td>
<td>19.60 (19.3-19.9)</td>
<td>T vs HB 0.0180 &lt;10^-4 0.0096</td>
</tr>
<tr>
<td>2</td>
<td>38.34 (37.3-39.4)</td>
<td>28.93 (28.3-29.6)</td>
<td>26.30 (25.8-26.8)</td>
<td>T vs HB &lt;10^-5 &lt;10^-5 &lt;10^-4</td>
</tr>
<tr>
<td>6</td>
<td>30.55 (29.8-31.3)</td>
<td>23.85 (23.4-24.3)</td>
<td>22.67 (22.2-23.2)</td>
<td>T vs HB &lt;10^-4 &lt;10^-4 0.0168</td>
</tr>
<tr>
<td>12</td>
<td>35.85 (34.9-36.8)</td>
<td>23.08 (22.7-23.5)</td>
<td>23.05 (22.6-23.5)</td>
<td>T vs HB &lt;10^-4 &lt;10^-4 0.9973</td>
</tr>
<tr>
<td>24</td>
<td>163.27 (159.4-167.2)</td>
<td>38.58 (37.0-40.1)</td>
<td>26.89 (26.2-27.6)</td>
<td>T vs HB &lt;10^-5 &lt;10^-5 &lt;10^-5</td>
</tr>
<tr>
<td>36</td>
<td>91.05 (88.2-93.9)</td>
<td>20.86 (20.5-21.2)</td>
<td>19.86 (19.5-20.2)</td>
<td>T vs HB &lt;10^-5 &lt;10^-5 0.7734</td>
</tr>
<tr>
<td>48</td>
<td>67.65 (64.1-71.2)</td>
<td>18.37 (18.0-18.7)</td>
<td>16.12 (15.9-16.3)</td>
<td>T vs HB &lt;10^-5 &lt;10^-5 0.2820</td>
</tr>
</tbody>
</table>

Mean fluorescence intensity and the 95% confidence interval (CI) in parentheses are given. Tukey-Kramer HSD test was applied to test the significance of fluorescence intensity according to localization. Significance level was p<0.05. Non-significant p-values are given in italic numbers.

Discussion

Hypericin is a component of SJW, also known as H. perforatum. SJW, a herbal medication, used over 2,000 years for various complaints, such as bronchitis, inflammation of the throat or gastrointestinal tract disorders. Extracts of SJW contain a complex mixture of more than 150 components, including phloroglucinols such as hyperforin, naphtodianthrones such as hypericin and pseudohypericin, flavonoids such as quercetin, quercitrin and rutin, as well as other constituents (33).

SJW extracts are well established in the therapy of mild to moderate depression and are of equal efficiency compared to tricyclic antidepressants, monoamine oxidase (MAO) inhibitors and selective serotonin reuptake inhibitors. The relevance of hypericin regarding the antidepressive effect is controversially discussed. However, inhibitory effects of MAO enzymes could not be confirmed. In order to gain more clarity Paulke et al. developed a new method for the detection of hypericin using a HPLC method. After oral administration of either SJW extracts or pure hypericin the authors could not find any hypericin in the brains of healthy rats (29,31). Gaccia and Gobbi confirmed this result in 2009 (30). Fox et al. investigated the pharmacokinetics of hypericin in plasma and cerebrospinal fluid in non-human primates (34). Notably, the compound was not detected in the cerebrospinal fluid after intravenous administration of pure hypericin (2 and 5 mg/kg body weight).

This findings are probably due to an intact blood-brain barrier (BBB), separating extracellular fluid in the brain from blood circulation. The BBB is formed by the endothelial cells of brain microvessels, which are connected by specialized tight junctions. These microvessels are surrounded by pericytes and astrocytic endfeets, building the so-called glio-vascular complex. Only lipophilic and small molecules with a molecular weight of less than 400 g/mol can cross the BBB via free diffusion (35). All other molecules need specific BBB transport systems, classified in a) carrier-mediated transporters (CMT), b) active efflux transporters (AET) and c) receptor-mediated transporters (RMT). The present data strongly indicate, that penetration of the highly lipophilic hypericin (molecular weight of 504 g/mol), across an intact BBB is very low. Thus, quite selective hypericin accumulation in intracerebrally implanted C6 gliomas in Wistar rats as demonstrated in Fig. 1 is likely to be due to an impairment of the BBB in the tumor.

In the present investigation, maximum hypericin accumulation in intracerebrally implanted C6 glioma was achieved at 24 h after intravenous administration. However, other in vivo investigations using different subcutaneously implanted tumor cell lines in mice found maximum hypericin accumulation at 4-6 h after intravenous injection (32,36,37). In addition, maximum hypericin accumulation in subcutaneously implanted prostate cancer cells (LNCaP) was found at 1-2 h after oral administration of 5 mg/kg hypericin (38). Tissue distribution of hypericin was studied in Fischer rats bearing subcutaneously inoculated AY-27 cells (rat bladder transitional cell carcinoma) after intravenous administration (1 and 5 mg/kg body weight). The highest concentrations of hypericin were determined in plasma as well as in liver, spleen and lung tissue. Again negligible amounts of hypericin were found in brain tissue (39). Also other fluorescence markers were investigated in in vivo rat glioma models. Kabuto et al. detected the maximum of fluorescein fluorescence at 1 h after intravenous administration in a C6 rat glioma model (40).

Madsen et al. studied 5-ALA-derived PpIX fluorescence in tumor, normal brain and by the authors the so-called brain-adjacent-to-tumor (BAT) zone after stereotactical implantation of BT4C tumor cells in BD-1X inbred rats (41). PpIX distribution was determined at 4-5 h after intraperitoneal injection of 60 mg/kg 5-ALA. In normal brain tissue fluorescence intensity was less than 1% as compared to tumor fluorescence; in the BAT zone it was up to 20% of the fluorescence intensity of tumor tissue. Angell-Petersen et al. compared the biodistribution of PpIX between tumor and contralateral brain by fluorescence measurements 4-5 h after intraperitoneal injection of 60, 125 and 250 mg/kg 5-ALA. The authors found a selectivity ratio of at least 230:1 comparing tumor to normal brain tissue (42). Stummer et al. detected maximum of 5-ALA-derived PpIX fluorescence
the C6 rat glioma model at 6 h after intravenous administration of 100 mg/kg body weight 5-ALA and a fluorescence ratio between tumor and normal tissue of 6:1 was determined (43).

In the present study maximum accumulation of hypericin in C6 glioma was found at 24 h after intravenous administration. Although the time interval between administration and maximum accumulation in tumor tissue is significantly longer in the case of hypericin as compared to 5-ALA-derived PpIX, the tissue selectivity of both photosensitizers seem to be quite similar (as deduced from the fluorescence ratios between tumor and normal tissue). Experimental studies concerning the first generation of PS, namely haematoporphyrin derivative (HDP), a mixture of different porphyrins, revealed a accumulation ratio of up to 12:1 of PS in the tumor as compared to normal brain (44,45). For meta-tetrahydroxyphenylchlorin (mTHPC), a second generation PS, a ratio of more than 80:1 has been described in vivo (implantation of C6 glioma in Spraque-Dawley rats) (46).

In conclusion, to the best of our knowledge the present study demonstrates for the first time that hypericin accumulates selectively in C6 glioma in vivo after systemic (intravenous) administration. Therefore, hypericin seems to be quite an effective fluorescence marker for the detection of glioma in vivo. Since this compound exhibits excellent photosensitizing properties, it might also be of advantage in glioma therapy. Further in vivo investigations should be initiated to prove this hypothesis.

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References


