Monitoring the glioma tropism of bone marrow–derived progenitor cells by 2-photon laser scanning microscopy and positron emission tomography

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Intracerebral experimental gliomas attract intravenously injected murine or human bone marrow–derived hematopoietic progenitor and stem cells (HPC) in vitro, ex vivo, and in vivo, indicating that these progenitor cells might be suitable vehicles for a cell-based delivery of therapeutic molecules to malignant gliomas. With regard to therapeutic application, it is important to investigate cell fates in vivo (i.e., the time-dependent intratumoral and systemic distribution after intravenously injection). Conventional histological analysis has limitations in this regard because longitudinal monitoring is precluded. Here, we used 2-photon laser scanning microscopy (2PLSM), positron emission tomography (PET), and MRI to study the fate of intravenously injected HPC carrying fluorescence, bioluminescence, and PET reporter genes in glioma-bearing mice. Our 2PLSM-based monitoring studies revealed that HPC homing to intracerebral experimental gliomas occurred already within the first 6 h and was most efficient within the first 24 h after intravenous injection. The highest PET signals were detected in intracerebral gliomas, whereas the tracer uptake in other organs, notably spleen, lung, liver, and muscle, remained at background levels. The results have important implications for designing schedules for therapeutic cell-based anti-glioma approaches. Moreover, the PET reporter-based imaging technique will allow noninvasive monitoring of cell fate in future cell-based therapeutic antglioma approaches.

Keywords: adult stem cells, cell-based therapy, cellular trafficking, Glioma, positron emission tomography, 2-photon laser scanning microscopy.

Glioblastomas are the most common and most malignant primary brain tumors. The median survival among patients with glioblastoma has not exceeded 15 months in selected clinical trial populations, despite surgical resection, radiotherapy, and recent advances in pharmacotherapy and is <5 months in population-based analyses. Typical features of glioblastoma cells include an invasive phenotype leading to deep infiltration of the adjacent normal brain parenchyma. Accordingly, there is an urgent need for novel tumor-selective treatments that specifically target migratory glioma cells. Cellular vehicles that are attracted by glioma cells would be ideal for the delivery of therapeutic molecules to tumor cells, because (1) cellular carriers could migrate and also target tumor satellite cells that have already deeply
infiltrated the normal brain parenchyma, and (2) cellular carriers could continuously produce and release the therapeutic molecule in the tumor microenvironment. Ideally, these cellular carriers have a low proliferation rate, are available from autologous sources, and are not tumorigenic per se. In this regard, adult stem cells have recently gained attention.

The seminal study in this field was conducted by Aboody et al. (2000), who showed that intravenously (IV) injected C17.2 cells, an immortalized neural stem cell (NSC) line, are attracted by intracerebral U87MG gliomas. Moreover, these cells mainly infiltrated the tumor borders, indicating that they might serve as carriers for delivering therapeutic molecules to infiltrating glioma cells. Characterization of spatial NSC distribution by confocal microscopy and 3-dimensional modeling in a U251-glioma model revealed that NSC accumulated in the tumor mass, indicating that a NSC-based therapy would potentially reach both the primary tumor mass and the infiltration zone. Indeed, intratumoral injection of interleukin (IL) 12–secreting NSC led to enhanced T cell infiltration and prolonged survival in a syngeneic GL261-C57Bl/6 glioma model. One obstacle is, however, that NSC-based anti-glioma therapies preclude the possibility to obtain the cellular carrier from autologous sources in the patient (i.e., from the brain). Therefore, only immortalized cell lines or primary embryonic or fetal NSC can be applied. This might require immunosuppressive comedication to avoid rejection of the cellular carrier. Currently, a clinical phase I study is recruiting patients with recurrent malignant gliomas to investigate the safety and feasibility of immortalized fetal NSC (HBLF3.CD) that are transduced with cytosine deaminase. These cellular carriers will be injected at 10 sites in the wall of the surgical cavity after removal of the tumor bulk. Patients will then receive 5-fluorocytosine, which will be converted to 5-fluorouracil by cytosine deaminase (ClinicalTrials.gov Identifier: NCT 01172964).

Adult stem cells from autologous sources (i.e., from the bone marrow (BM) or from peripheral blood) have been investigated (e.g., mesenchymal stem cells [MSCs] and hematopoietic progenitor cells [HPCs]). MSC exclusively accumulated in the tumor region after injection into the carotid artery of LN-229-, U87MG-, or U251 glioma-bearing mice. Moreover, intra-arterial injection of interferon-β–releasing MSC led to prolonged survival. We previously focused on the characterization of HPC (i.e., murine BM–derived lineage-depleted (lin−) BM stem cell antigen-1 [Sca-1]+ Kit+ cells or human peripherally mobilized CD34+ cells) as suitable glioma-selective cellular vehicles in preclinical glioma models. Particularly important (1) to allow the design of schedules for therapeutic cellular infusions and (2) to estimate potential adverse effects resulting from potential cellular accumulation in other organs.

Material and Methods

Cells

The murine SMA-560 and human LNT-229 glioma cells have been characterized extensively in our laboratory. In addition to established cell lines, the primary patient-derived glioma stem-like culture T269 that had been established in our laboratory was used. Human adult CD34+ cells were isolated by anti-CD34 immunomagnetic microbeads. After informed consent, peripheral blood mononuclear cells were obtained from granulocyte colony-stimulating factor–induced stem cell mobilization in healthy donors or patients undergoing autologous hematopoietic stem cell transplantation for conditions unrelated to brain tumors, according to the guidelines of the ethics committee of the University of Tübingen. Mononuclear cells were separated by Ficoll density gradient centrifugation. CD34+ cells were isolated using immunomagnetic microbeads (MACS system; Miltenyi Biotec). The purity of CD34+ cells isolated by positive selection was 95.1%–98%. The human CD34 antigen is a reliable marker for enrichment of a small fraction of human BM and peripheral blood mononuclear cells containing uncommitted and pluripotent HPCs and hematopoietic stem cells.

For isolation of murine BM cells, femurs and tibiae of VM/Dk mice were flushed with phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS). BM cells (106/mL) were incubated in PBS containing 2% FCS with the lineage marker antibodies from BD Biosciences Pharmingen, rat antimouse CD4, CD8a, CD45R/B220, Gr-1, CD11b, and TER119 at 4°C for 60 min and were washed 3 times with PBS containing 2% FCS. Prewashed sheep antirat IgG magnetic beads (Dynabeads; Dynal Biotech ASA) were added at a 4:1 ratio of beads/cells in PBS containing 2% FCS and incubated on a rotating platform for 45 min at 4°C. Beads and attached cells were magnetically removed, and lineage-depleted BM cells (lin− BM) were harvested. For simplification, we use the term murine or human HPC for murine lin− BM or peripherally mobilized human CD34+ cells, although these cell populations are not a pure hematopoietic stem cell fraction.

Labeling of Murine or Human HPC

Staining of HPC with PKH26 was performed according to manufacturer’s protocols (Sigma-Aldrich). Some researchers had experienced microenvironmental contamination when using PKH26-labeled cells after long-term in vivo studies. Because the duration of our in vivo PKH26 monitoring did not extend beyond...
14 days, the risk of microenvironmental contamination by PKH26-labeled cells remained low. Nevertheless, we performed the experiments not only with PKH26-stained cells but also with ex vivo lentivirally transduced GFP-positive or RFP-positive cells. Using this multimodal labeling approach, we avoided quantification errors resulting from potential contaminations toward other cells or the environment by in vitro labeling.

**Lentivirus Production and Transduction**

The transfer vector FRT encoded an ubiquitin C promoter–driven triple fusion cassette consisting of firefly luciferase, monomeric red fluorescent protein (RFP), and Herpes simplex virus type-1 thymidine kinase (HSV-1 TK) PET reporter gene. Lentivirus was generated by cotransfecting 293T cells with the vector and the packaging constructs. Supernatant containing lentivirus was collected 48 h and 72 h after transfection and concentrated by ultracentrifugation. Target cells were always transduced with freshly produced virus.

Stably FRT-transduced target cells were viable, as assessed by trypan blue exclusion. Moreover, the migration capacity of FRT-transduced HPC did not change compared to nontransduced controls in vitro (data not shown). This is in line with our previous experiments comparing viability and migration capacity of GFP lentivirus-transduced HPC and controls by trans-well migration assays in vitro and by characterization of their glioma tropism in vivo. The functionality of the RFP cassette was confirmed by fluorescence microscopy or 2PLSM. The luciferase cassette was assessed by luciferase activity after incubation of transduced target cells with Luciferin (Supplementary material, Fig. S1). The HSV-1 TK cassette served as a reporter gene for PET studies using the tracer (9-(4-[18F]Fluoro-3-hydroxymethylbutyl) – guanine) for HSV-1 TK.

**Immunohistochemistry**

Histological analysis was performed using hematoxylin and eosin (H&E) staining. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Axxora GmbH) for fluorescent imaging. Immunohistochemistry was performed on frozen sections to determine CD45 expression.

**Chronic Cranial Glass Window Preparation**

All animal procedures were performed according to the guidelines of the local authorities. Mice were anaesthetized, placed on a warming pad, and fixed in a modified stereotactic frame. After skin disinfection with 70% ethanol, a 3-cm long longitudinal incision was cut in the middle of the skull. The skin was folded and fixed on the side by 4 bulldog clamps. The skull was thoroughly cleaned. Dental glue (GlumaBond; Heraeus Kulzer) was applied. A round circle (5 mm diameter) was carefully drilled (Fig. 1B). The round piece of skull was removed, and 5 × 10^5 GFP-positive SMA-560 glioma or 10^5 LNT-229 cells in a volume of 2 μL were implanted (Fig. 1B) in 500 μm depth. The brain surface was then covered by a custom-cut glass coverslip (thickness, 130–170 μm). The coverslip was fixed by light hardening dental cement (Flowline; Heraeus Kulze).

**2PLSM Experimental Designs**

In the first set of experiments, we focused on a long-term analysis from days 5 through 14 after SMA-560 glioma cell implantation in VM/Dk mice: day 0, chronic cranial glass window preparation and implantation of glioma...
cells; day 5, baseline 2PLSM analysis and IV injection of $2 \times 10^6$ murine HPC. The time of homing and cellular distribution in the experimental gliomas were daily analyzed by 2PLSM for up to 9 consecutive days ($n = 6$).

In a second set of experiments, we focused on a short-term analysis within the first 24 h after IV injection of murine or human HPC cells in glioma-bearing mice; on day 0, preparation of chronic cranial glass window and implantation of glioma cells, then baseline 2PLSM and IV injection of $2 \times 10^6$ murine HPC in VM/Dk mice (day 5) or human HPC in nude mice (day 8) was performed. First, we performed the 2PLSM scans every hour after IV injection. The earliest time point of reproducible HPC detection was 6 h. Thus, we designed and performed the scanning algorithm as outlined in Fig. 2A and monitored homing 6 h, 12 h, and 24 h after injection. We quantified the data of 8 mice (i.e., 3 VM/Dk and 5 nude mice).

In a third set of experiments, we analyzed the glioma tropism of murine HPC after multiple IV injections. Chronic cranial glass window preparation was performed on day 0, baseline 2PLSM analysis on day 5, followed by IV injection of murine HPC on 3 consecutive days (i.e., up to 96 h after the first injection and up to 48 h after the third injection). We quantified glioma-tropism of murine HPC in 3 VM/Dk mice.

In a fourth set of experiments, we analyzed the glioma tropism of lentivirally FRT-transduced murine or human HPC after IV injection. Chronic cranial glass window preparation was completed on day 0. The baseline 2PLSM analysis was done on day 5. Next, we IV injected murine HPC. The glioma-mediated attraction of these cells was analyzed as outlined in Fig. 2A. These experiments were performed with 3 VM/Dk mice.

In a fifth set of experiments, we analyzed the glioma tropism of lentivirally GFP-transduced murine HPC after IV injection. The transfer vector encoded a spleen focus-forming virus (SFFV) promoter-driven green fluorescent protein (GFP). Chronic cranial glass window preparation was on day 0, and baseline 2PLSM analysis was on day 5. GFP-transduced murine HPC were injected into the tail vein after the baseline scan. The attraction of these cells was analyzed as outlined in Fig. 2A. These experiments were performed with 2 VM/Dk mice.

2PLSM Data Recording and Analysis

On the first day of 2PLSM imaging, a titanium ring was fixed on the cranial window to avoid breathing artefacts. Prior to each imaging session, the animals were anesthetized with isoflurane (constant anesthesia at 1.5%). The window was cleaned, and the mouse was fixed under the microscope and held stable within a custom-build titanium plate, which embedded the titanium ring (outer diameter: 14 mm, inner diameter: 7 mm, 2 mm height) fixed on the mouse head.

The titanium ring was fixed on the glass window by dental cement (Flowline; Heraeus Kulze) and superglue. The titanium ring was then connected to the microscope by a custom-built bridge to a motorized x–y stage (Luigs & Neumann). Using a 10× objective under ambient illumination, the growth of GFP-positive experimental SMA-560, LNT-229, or T269 gliomas was monitored. All scans were performed with a 40× water immersion lens (0.8 numerical aperture, U-V-I O/D; Leica Microsystems). A Leica DMLF5 microscope attached to a Spectra Physics Mai-Tai laser (tunable 770–990 nm) was used to provide multiphoton excitation at 910 nm; the emitted fluorescence light was detected via 2 non-descanned detectors (R6357 P.M.T.; Hamamatsu) located close to the objective lens. The 2 channels were acquired simultaneously and split by an FITC/TRITC filter (reflection short pass, 560; bandpass, 525/50; bandpass, 610/75). Five different

![Fig. 2. Experimental setup for 2PLSM studies. (A) Experimental flowchart for 2PLSM LT (long-term) and ST (short-term) studies. (B) 2PLSM image of SMA-560 experimental glioma (green) and attracted murine HPC (red signal dots; arrows). (C) Combined volume view of a 2PLSM Z-stack (50 μm depth). HPC are indicated as a representative example by two arrows. (D) Combined volume view of a 2PLSM Z-stack under data analysis. Defined HPC cells can be recognized as white dots and are marked here by arrows. Background signals are red. (E) 3D reconstruction after complete data analysis. Attracted HPC are white, GFP-positive glioma cells are green.](https://academic.oup.com/neuro-oncology/article-abstract/14/4/471/1052114)
representative $xyz$ locations (starting depth: 20–100 $\mu$m) were permanently imaged across all sessions for each mouse. Fifty single images were recorded to 1 z-stack for each location (size: $300 \mu m \times 300 \mu m \times 50 \mu m$), 1 image per $1 \mu m$ depth.

Baseline scans were performed on day 5 after glioma implantation at 2 different locations in the tumor. After the baseline scans, murine or human HPCs were injected as indicated above (see section 2PLSM experimental design).

For the quantification of attracted HPC (Supplementary material, Fig. S1), 1 z-stack was recorded for each location (size: $300 \mu m \times 300 \mu m \times 50 \mu m$). The images were combined and volumes were generated. Then, the spot detection function from Bitplane Imaris was used to count murine or human HPCs. Different filters were used to exclude false-positive signals. Of note, the number of attracted HPCs to the tumor is the summation of 5 representative $xyz$ areas as indicated above. It is not the total number of all attracted cells in the whole glioma volume.

Small Animal PET and MRI

The PET studies were performed with the syngeneic SMA-560/VM/Dk glioma model.

On day 0, $5 \times 10^3$ SMA-560 cells were stereotactically implanted into the right striatum of VM/Dk mice. On day 14, FRT-transduced murine HPC were injected either IV into the tail vein or intratumoral (i.t.) into the glioma. All experimental groups are outlined below and summarized in Table 1.

- **Group 1**, naive wild-type VM/Dk mice without orthotopically implanted glioma cells and without injected lin- BM ($n = 8$ mice).
- **Group 2**, wild-type VM/Dk mice with $5 \times 10^3$ orthotopically implanted SMA560 glioma cells and without injected lin- BM ($n = 7$ mice).
- **Group 3**, wild-type VM/Dk mice with $5 \times 10^3$ orthotopically implanted SMA560 glioma cells and with ipsilateral i.t. injected HSV1-TK-negative (non-transduced) lin- BM cells 24 h before PET analysis ($n = 9$ mice).
- **Group 4**, wild-type VM/Dk mice with $5 \times 10^3$ orthotopically implanted SMA560 glioma cells and with IV injected HSV1-TK-negative (non-transduced) lin- BM cells 24 h before PET analysis ($n = 8$ mice).

- **Group 5**, wild-type VM/Dk mice with $5 \times 10^3$ orthotopically implanted SMA560 glioma cells and with ipsilateral i.t. injected HSV1-TK-positive lin- BM cells 24 h before PET analysis ($n = 11$ mice).
- **Group 6**, wild-type VM/Dk mice with $5 \times 10^3$ orthotopically implanted SMA560 glioma cells and with IV injected HSV1-TK-positive lin- BM cells 24 h before PET analysis ($n = 12$ mice).
- **Group 7**, wild-type VM/Dk mice with $5 \times 10^3$ orthotopically implanted SMA560 glioma cells and with IV injected HSV1-TK-positive lin- BM cells 7 days before PET analysis ($n = 10$ mice).
- **Group 8**, wild-type VM/Dk mice with $5 \times 10^3$ orthotopically implanted SMA560 glioma cells and with IV injected HSV1-TK-positive lin- BM cells 7 days before PET analysis ($n = 10$ mice).

Animals were imaged using a small animal PET scanner (Inveon; Siemens Preclinical Solutions) yielding a spatial resolution of $\sim 1.3$ mm in the reconstructed images. All animals were shortly anaesthetized with isoflurane and IV injected with 11–15 MBq of $[18F]$FHBG. During PET and MRI, the animals were anaesthetized with 1.5% isoflurane mixed with 100% oxygen. Static (10 min) PET scans were acquired 60 min after tracer injection. Dynamic 90 min PET data were acquired in list mode, starting with tracer injection and graphed in 30 frames and reconstructed using an iterative ordered-subset expectation maximization (OSEM) algorithm. MRI was performed on a dedicated 7 T small animal MR tomograph (Bruker Biopin MRI). A T2-weighted 3D space sequence (TE/TR 205/3000 ms, matrix of 161 $\times$ 256, slice thickness 0.22 mm) was used for the detection of gliomas in the mouse brain and to obtain morphological information. Fused PET and MRIs were normalized to each other and analyzed using Inveon Research Workplace software (Siemens Preclinical Solutions). We used fiducial markers with $^{18}$F activity for image fusion. The $[18F]$FHBG uptake as a quantitative measure of TK expression was reported by percent injected dose per cubic centimeter (%ID/cc) yielded by drawn regions of interests in various organs such as experimental glioma tissue, brain parenchyma liver, lung, and muscle tissue. $[18F]$FHBG uptake ratios were calculated between experimental glioma tissue and brain parenchyma in groups 2, 4, 6, and 8. After imaging, animals were euthanized, and the brains were dissected; autoradiography, revealing radioactivity

**Table 1. Experimental groups for PET and MRI**

<table>
<thead>
<tr>
<th>Group</th>
<th>SMA-560 glioma</th>
<th>Injection site of HPC</th>
<th>Murine HPC</th>
<th>Time point of PET analysis after HPC injection</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Control group</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>Control group</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Intratumoral</td>
<td>Control</td>
<td>24 h</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Intravenous</td>
<td>Control</td>
<td>24 h</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>Intratumoral</td>
<td>HSV-TK+</td>
<td>24 h</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>Intravenous</td>
<td>HSV-TK+</td>
<td>24 h</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>Intratumoral</td>
<td>HSV-TK+</td>
<td>7 d</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>Intravenous</td>
<td>HSV-TK+</td>
<td>7 d</td>
<td>10</td>
</tr>
</tbody>
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distribution in ex vivo tissue slices, and histology were performed after cryosectioning (Leica CM1850) the brain.

Luciferase Assay

A total of $5 \times 10^6$ triple-fusion lentivirally transduced or nontransduced SMA-560 glioma cells were seeded in 12-well plates and incubated for 10 min with 200 μg D-Luciferin (potassium salt; Promega) before imaging. In vitro bioluminescence was imaged using a cooled ORCA-II-ER-coupled charged device (CCD) camera (Hamamatsu) in photon counting mode.

Statistical Analysis

Statistical analysis was performed using analysis of variance, with post-hoc Tukey test performed with Origin 8 software (OriginLab). Data were considered to be statistically significant for $P < .01$ and $P < .001$. All quantitative results are shown either as box plots showing the minimum, the 25th and 75th percentile (box), whiskers depicting the fifth and 95th percentile, the median (line), the mean (small box), and the maximum or as bar charts, error bars representing 1 standard derivation.

Results

Monitoring the Interval of HPC Homing to Intracerebral Gliomas by 2PLSM

For characterizing the cell fate of IV injected murine or human HPC in glioma-bearing mice (i.e., interval of homing to experimental gliomas), tracking in situ, and accumulation in noncerebral organs, we used a multimodal imaging approach using 2PLSM and PET and MRI allowing longitudinal monitoring.

Real-time monitoring of cellular migration in the brain is possible by 2PLSM after insertion of a chronic cranial glass window in the skull of animals (Fig. 1A). We prepared a chronic cranial glass window and injected GFP-positive SMA-560 or LNT-229 glioma cells in the cortices of mice (Fig. 1B). We monitored the growth of experimental SMA-560, LNT-229, or T269 gliomas. Monitoring glioma growth by 2PLSM was feasible for up to 14 days after implantation, limited only by expansive growth of the glioma cells outside the area of the cranial window, as shown representatively for SMA-560 (Fig. 1C). Consequently, we scheduled our next experiments for real-time monitoring glioma-mediated attration of IV HPC until day 14 (Fig. 2A). We injected PKH26-labeled or ex vivo lentivirally transduced HPC on day 5 after glioma cell implantation. Then, we investigated their glioma tropism by 2PLSM (Fig. 2A). In the first set of experiments, we focused on a long-term analysis from days 5 through 14 after SMA-560 glioma cell implantation in VM/Dk mice. The interval of homing and cellular distribution in the experimental gliomas were daily analyzed by 2PLSM for up to 9 consecutive days. We detected attracted HPC already after 24 h in experimental gliomas. Therefore, we continued with a short-term analysis by scanning glioma-mediated attraction of murine or human HPC hourly within the first 24 h after injection into the tail vein of glioma-bearing mice. We started with times of 1–6 h after HPC injection. The first time of reliable and reproducible homing of IV HPC to intracerebral gliomas was 6 h after injection. For the following experiments, we focused on short-term scanning for quantifying glioma tropism of IV injected HPC at 6 h, 12 h, and 24 h after IV injection (Fig. 2A).

In Situ Tracking of HPC Fate in Glioma-Bearing Mice by PET and MRI

To noninvasively monitor HPC trafficking in glioma-bearing mice, we used PET and MRI (Fig. 4, Table 1). HSV-1 TK-positive murine HPCs were injected into the tail vein (IV) or into the tumor of glioma-bearing mice (Table 1). The tracer $[^{18}F]FHBG$ was used to detect TK-expressing HPC. The HSV-1 TK enzyme monophosphorylates $[^{18}F]FHBG$, and cells expressing the HSV-1 TK (PET-) reporter gene can be imaged in living animals by PET (Fig. 4). PET and MRI were scheduled either 24 h or 7 days after the injection of HSV-1 TK-positive HPC in glioma-bearing mice (Table 1).

We detected enhanced tracer uptake in experimental gliomas after IV injection of HSV-1 TK-positive murine HPC by PET (Fig. 5A) and by simultaneous PET/MRI (Fig. 5B). We validated PET signals by autoradiography and histology (Fig. 5C). Quantification of PET signals revealed 4–5-fold higher tracer signals in...
gliomas after IV injection of HSV-1 TK-positive murine HPC, compared with tracer signals in gliomas after injection of HSV-1 TK-negative HPC or with tracer signals in gliomas alone (Fig. 5D). The marginally increased tracer signal in experimental gliomas after IV injection of HSV-1 TK-negative murine HPC, compared with tracer signals in gliomas alone, is not statistically significant (Fig. 5D). This is in line with a representative qualitative image where a slightly increased tracer signal after injection of HSV-1 TK-negative HPC in glioma-bearing mice is visible (Fig. 5A). Of note, the significantly enhanced tracer uptake in gliomas after IV injection of HSV-1 TK-positive murine HPC remained at comparable levels after 24 h and after 7 days (Fig. 5D), as was suspected from the qualitative images (Fig. 5A). Of importance, tracer signals in the normal brain parenchyma were similar in all experimental groups (Fig. 5E).

These findings were further confirmed by analyzing the tracer uptake ratios (glioma/normal brain) for each mouse (Fig. 5F). The uptake ratio was significantly higher at 24 h and 7 days after IV injection of HSV-1 TK-positive murine HPC in glioma-bearing mice (Fig. 5F), compared with the uptake ratio after injection...
of HSV-1 TK-negative murine HPC (Fig. 5F) or the uptake ratio in gliomas alone (Fig. 5F). Quantitative analysis of systemic distribution of HSV-1 TK-positive HPC in glioma-bearing mice confirmed a significantly enhanced tracer signal in experimental gliomas. Of note, the uptake in muscle, liver, and lung was not significantly enhanced, compared with control or background signal (Fig. 5G).

In parallel to the in vivo PET experiments, tracer uptake was tested in vitro by gamma counting to assess the function of the HSV-TK PET reporter cassette after lentiviral transduction: As expected, tracer accumulation was highest in HSV-1 TK-positive murine HPC, compared with medium, nonneoplastic NIH3T3 fibroblasts, murine SMA-560 glioma cells, or control HPC (Fig. 5H).

**Discussion**

Cell-based antiglioma therapies using cellular carriers that specifically target invading tumor cells might be an efficient therapeutic approach to selectively target tumor cells without harming nonneoplastic resident cells. We previously studied the glioma tropism of autologous, easily accessible HPC in this regard. The estimation of potential risks and adverse effects of a therapeutic HPC-based approach requires real-time monitoring of the cellular fate in vivo. For designing therapeutic schedules (i.e., injection frequencies or application routes), it is important to define the time of homing to the tumor and potential cellular accumulation in other organs. Furthermore, a noninvasive imaging tool is required to monitor cellular carriers. Finally, it will be important to have safety strategies if misbehavior of cellular carriers occurs.

Our longitudinal monitoring data using 2PLSM through a chronic cranial glass window (Fig. 1) aimed at defining the time of earliest HPC homing to experimental gliomas. We detected murine or human HPC within the first 6 h after injection (Figs. 2 and 3). The migration capacities of MSC were recently assessed by 2PLSM. Red-labeled MSC accumulated in intracerebral gliomas after IV injection and were quantified by 2PLSM, confirming that 2PLSM is a valid tool to detect migrating cells in the brain.

To study HPC trafficking in glioma-bearing mice, we performed whole-body PET and MRI after IV and i.t. injection of HPC carrying the PET reporter gene HSV-1 TK. We included the i.t. application route because it might be more convenient for future clinical trial applications in patients to infuse tailored therapeutic HPC directly into the resection cavity (e.g., after surgical resection of glioblastoma), rather than injecting the cells via IV infusions.

For detection of cellular trafficking in vivo, MRI using iron oxide particles has been applied. Using this approach, it is feasible to assess the anatomic location, but significant drawbacks include problematic quantification of cellular signals because of dilution of labeling during cellular division and possible interference with migration capacity of cells. We used a reporter gene-based PET imaging technique to avoid these impairments of iron particle labeling strategies for MRI. This requires stable incorporation of a reporter gene regulated by a constitutive promoter into HPC before administration in vivo. The reporter probe can then be injected at any time thereafter to image the location of reporter gene-carrying HPC and to assess their survival. We chose the PET reporter gene HSV-1 TK that allows the detection of HSV-1 TK positive cells by the PET tracer [18F]FHBG (Fig. 4). Even if the [18F]FHBG tracer uptake in experimental glioma tissue seems to be low on qualitative PET images, the quantification of the tracer uptake in experimental glioma tissue, either as %ID/cc or as uptake ratios, revealed significant differences compared with the reference tissue (i.e., normal brain parenchyma) (Fig. 5D–F). Highest tracer accumulation occurred in experimental gliomas, whereas the uptake in other organs remained at background levels (Fig. 5G). After the i.t. injection, this was
expected, but even after systemic IV injection, we did not detect a significant tracer uptake in either spleen or lung. Of note, neither of the animals were myeloablative irradiated, which explains the lack of significant uptake in the bone marrow. It remains hypothetical whether a concomitant infection or other inflammatory
process at any extracerebral site in the glioma-bearing mouse would have changed this cellular distribution pattern after IV injection and would have promoted cellular accumulation at this site. For future therapeutic applications, the application of ex vivo tailored HPC carrying both a therapeutic molecule and the PET reporter gene HSV-1 TK would offer 2 major advantages.

First, therapeutic cells could be non-invasively monitored by $[^{18}F]$FHBG PET scans, as demonstrated recently in a patient with recurrent glioblastoma. The patient was enrolled in a trial of adaptive cellular immunotherapy and received cellular infusions in the tumor. Autologous cytolytic T cells were ex vivo expanded and genetically modified to express HSV-1 TK and IL-13. The therapeutic cells were infused 3 times per week by a Rickham reservoir. The first $[^{18}F]$FHBG PET scan was scheduled 3 days after completion of a 5-week cycle of cellular infusions to monitor the fate of in the tumor infused cytolytic T cells. Thus, $[^{18}F]$FHBG PET-based real-time monitoring of therapeutic cellular carriers in patients with recurrent glioblastoma is feasible.\(^2^9\) Given the successful application of FHBG tracer–based monitoring of autologous cytolytic T cells in patients with glioblastoma, we anticipate that this method can be applied to the monitoring of HPC-based therapies. Of course, it will be important to take into account that PET application for longitudinal monitoring of cellular therapy includes the issue of radiation exposure. The radiation exposure for patients during PET scans is approximately half of the radiation dose during diagnostic computed tomography examinations.\(^1^0\) For cell-based approaches, only a limited number of cells will be radiolabeled, reducing radiation exposure. Nevertheless, the scheduling of PET imaging for monitoring cellular therapies will have to be defined in explorative clinical phase I studies.

Second, the HSV-1 TK reporter gene could also be exploited as a suicide gene providing a safety mechanism if adverse effects occur or if therapeutic HPC exhibit aberrant biological activity in the glioma-bearing organism. HSV-1 TK can act as a safety gene, because cells expressing HSV-1 TK die when exposed to ganciclovir. This safety mechanism is important because transplantation of adult HPC might still carry the risk of altered function or activity over a lifetime. This could be prevented by a reliable monitoring tool using a PET reporter gene that could also serve as a suicide gene.

Taken together, we defined the time of HPC homing to experimental gliomas by 2PLSM. Furthermore, HSV-1 TK reporter gene-based PET was demonstrated to be a powerful approach to noninvasively and longitudinally monitor intra- and extracerebral cellular HPC trafficking in glioma-bearing mice. Of importance, the PET reporter probe $[^{18}F]$FHBG is a highly promising tool for monitoring HSV-1 TK-positive cells in the brain because of an excellent signal:noise ratio. These findings will be important to design injection schedules for cell-based antiglioma approaches and to noninvasively monitor trafficking of cellular carriers.

**Supplementary Material**

Supplementary material is available at Neuro-Oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

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**References**

10. Tabatabai G, Frank B, Mohle R, Wellen M, Wick W. Irradiation and hypoxia promote homing of haematopoietic progenitor cells towards...


