Differentiation Therapy Exerts Antitumor Effects on Stem-like Glioma Cells

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Abstract

**Purpose:** Stem-like tumor cells comprise a highly tumorigenic and therapy-resistant tumor subpopulation, which is believed to substantially influence tumor initiation and therapy resistance in glioma. Currently, therapeutic, drug-induced differentiation is considered as a promising approach to eradicate this tumor-driving cell population; retinoic acid is well known as a potent modulator of differentiation and proliferation in normal stem cells. In glioma, knowledge about the efficacy of retinoic acid–induced differentiation to target the stem-like tumor cell pool could have therapeutic implications.

**Experimental Design:** Stem-like glioma cells (SLGC) were differentiated with all-trans retinoic acid–containing medium to study the effect of differentiation on angiogenesis, invasive growth, as well as radioresistance and chemoresistance of SLGCs. *In vivo* effects were studied using live microscopy in a cranial window model.

**Results:** Our data suggest that *in vitro* differentiation of SLGCs induces therapy-sensitizing effects, impairing the secretion of angiogenic cytokines, and disrupts SLGCs motility. Further, *ex vivo* differentiation reduces tumorigenicity of SLGCs. Finally, we show that all-trans retinoic acid treatment alone can induce antitumor effects *in vivo*.

**Conclusions:** Altogether, these results highlight the potential of differentiation treatment to target the stem-like cell population in glioblastoma. *Clin Cancer Res*; 16(10); 2715–28. ©2010 AACR.

The current tumor stem cell hypothesis is helping to understand the nature of malignancies such as glioblastoma, an incurable cancer with median patient survival times of 12 to 15 months (1). According to the tumor stem cell concept, resistance to conventional therapy may reside in a small subset of glioblastoma cells. These so-called stem-like glioma cells (SLGC) comprise a subpopulation of tumor cells, capable of differentiating into the actively expanding tumor bulk (2–4). SLGCs were originally defined by expression of the cell surface antigen AC133, a glycosylation-dependent epitope of the transmembrane protein CD133 (4). Although recent observations have shown that tumorigenicity is not entirely restricted to the AC133/CD133-positive tumor compartment (5, 6), there is a large, growing set of data linking the expression of AC133/CD133 with poor patient prognosis in several extracranial and intracranial malignancies throughout the body (7, 8). In glioma, AC133/CD133 proved to be an independent prognostic marker for adverse progression-free and overall survival, thus strengthening its role in tumor growth (7, 9). Moreover, a recently published report by Bao and coworkers (10) described higher DNA damage repair efficiency in the AC133/CD133-positive cell population after irradiation. Similarly, Liu and colleagues (11) have shown a positive selection for AC133/CD133-positive tumor cells in glioblastoma patients after chemotherapy most probably due to a resistance of AC133/CD133-positive cells to different cytotoxic drugs. Taken together, these data indicate that tumorigenicity, as well as radioresistance and chemoresistance, may be general attributes of AC133/CD133-expressing SLGCs.

If eradication of SLGCs is the critical determinant in achieving cure (12), it must be reasoned that depletion of the AC133/CD133-positive cell pool through controlled, drug-induced differentiation could have profound therapeutic implications. Although many therapeutic agents have been studied, only few anticancer drugs affect
Translational Relevance

Stem-like tumor cells are held responsible for tumor initiation and therapy resistance in a variety of cancers. Drug-induced differentiation is considered as a promising approach to eradicate this tumor-driving cell population. In the present study, differentiation of stem-like glioma cells induced antimitogenic, antangiogenic, and therapy-sensitizing effects in vitro and impaired the tumor-initiating capacity of these cells in vivo. Our results strengthen the therapeutic value of a drug-induced differentiation and give insight into how differentiation affects the stem-like tumor compartment in glioma.

cancer cell differentiation, e.g., retinoic acid, bone morphogenetic proteins (BMP), and drugs that target tumor epigenetics such as histone deacetylase inhibitors and hypomethylating agents (3, 13–16). Although the role of differentiation-inducing drugs as promising therapeutics agents is receiving increasing attention, some of these substances are being tested in clinical trials or are already in clinical use (16). Among these, retinoic acid, which is known as potent modulator of cellular differentiation and proliferation, proved to be a powerful antitumor agent in the treatment of certain cancers: for instance, through application of all-trans-retinoic acid (ATRA) in addition to chemotherapy, acute promyelocytic leukemia has become a curable disease (13, 17).

Although it has been shown that ATRA induces morphologic differentiation of SLGCs in vitro (18), it remains unknown whether and how tumor growth–relevant features of these cells are affected, especially because SLGCs are suspected to contribute substantially to malignancy-associated properties of glioblastoma (4, 10, 11). In this context, potential effects on the hallmark features of malignant gliomas, namely angiogenesis, infiltrative growth, tumorigenicity, as well as radioresistance and chemoresistance, constitute the benchmark criteria to determine the therapeutic value of differentiation treatment.

In the present study, we report that ATRA-based differentiation targets the AC133/CD133-positive SLGC population, abrogating major malignancy-related properties of these cells and inducing long-term antiangiogenic, antimigratory, antitumorigenic, proapoptotic, and therapy-sensitizing effects. These results should encourage future research on retinoids as potential anticancer agents in glioma, particularly in combination with other differentiation-inducing agents.

Materials and Methods

Cell culture conditions. Glioblastoma samples were obtained from patients undergoing surgical resection according to the research proposals approved by the Institutional Review Board at the Medical Faculty Heidelberg. Tissues were enzymatically dissociated and cultivated as floating neurospheres in DMEM/F-12 medium containing 20% BIT serum-free supplement, basic fibroblast growth factor (bFGF), and epidermal growth factor at 20 ng/mL each (all Provitro). Primary human umbilical vein endothelial cells (HUVEC) were cultured as described (19). To induce differentiation, neurospheres were grown in DMEM containing 10% FCS and 10 nmol/L ATRA. Cell lines were used for further experiments between passages 20 and 50, whereas briefly cultured AC133/CD133-positive glioma cells and HUVECs were only used up to passage 5. After dissociation, cell numbers were determined by an automated cell counter (Casy Counter, Schäfer Reutlingen), which allows for the discrimination between living and dead cells by cell size. Thus, all cell numbers mentioned in later experiments correspond to the number of vital cells.

Immunohistochemistry. Staining of mouse tissue sections and neurospheres was carried out as described (19). Primary antibodies used were mouse antihuman CD133 (clone AC133, 4.1 μg/mL, Miltenyi), mouse antihuman nestin (1 μg/mL, R&D), mouse antihuman glial fibrillary acidic protein (GFAP; ready-to-use, Progen), mouse antihuman Ki-67 (10 μg/mL, BD Pharmingen), rat antimouse CD31 (2 μg/mL, BD Pharmingen), mouse antihuman βIII-tubulin (ascites, 1:100, Chemicon), mouse antihuman myelin basic protein myelin basic protein (culture supernatant, 1:100, Chemicon), and mouse antihuman NOTCH1 (8 μg/mL, Abcam). Mouse IgG2a or IgG2b (both Acris) were used as isotype controls in equal concentrations as primary antibodies. Histochemical detection of apoptosis was done based on the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction using the In situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer’s instructions. TUNEL staining evaluation was done on representative sections of four SLGC-derived tumors and four tumors derived from differentiated SLGCs. Necrotic areas were excluded from analysis to avoid false-positive staining results. TUNEL-positive cells were counted per visual field on four hotspots at a ×20 magnification.

Flow cytometry. Cells were trypsinized and pretreated with FcR-blocking reagent (Miltenyi). Staining was done using antihuman CD133/1-PE antibody (clone AC133, 4.1 μg/mL, Miltenyi). For intracellular staining, permeabilization with the Inside Stain kit (Miltenyi) was done before incubation with antihuman GFAP antibody (0.1 μg/mL, Progen) or antihuman retinoic acid receptor (RAR) α antibody (4 μg/mL, NEB) or antihuman NOTCH1 antibody (4 μg/mL, Abcam). Appropriate isotype controls and secondary antibodies were used according to the manufacturer’s instructions. Cells were subjected to flow cytometry analysis using a FACSCalibur cytometer and the CellQuest Software (Becton Dickinson). Only cells with staining intensities above the maximal level of isotype controls were defined as positive cells.

Fluorescence in situ hybridization analysis. Two-color interphase fluorescence in situ hybridization (FISH) was
done using FITC-labeled locus-specific BAC probes RP11-231C18 (PDGFRA), RP11-450G15 (MDM2), and RP11-155I23 (Cdk4), and rhodamine-labeled centromere probes for chromosomes 4 (PDGFRA) and 12 (MDM2, Cdk4). Pretreatment of slides, hybridization, posthybridization processing, and signal detection were done as previously described (20). Samples showing sufficient FISH efficiency (>90% nuclei with signals) were evaluated by two independent investigators. Metaphase FISH for verifying clone mapping position was done using peripheral blood cell cultures of healthy donors as outlined previously (20).

Cell proliferation. Proliferation assays were done using the BrdU Labeling and Detection Kit III (Roche Diagnostics) as described. Cells were seeded in eight replicas on 96-well plates at a cell density of 3 × 10⁴ cells. Absorbance was determined and means of control samples were defined as 100% proliferation rate.

Quantitative PCR. For quantitative real-time PCR, 1 μg of total RNA was subjected to reverse transcription with the SuperScript II first-strand synthesis kit (Invitrogen). Each cDNA sample was analyzed in triplicate using ABI PRISM 7700 (Applied Biosystems) with the Absolute SYBR Green ROX Mix (ABgene). Two endogenous housekeeping genes (ARF1 and DCTN2) were used for internal normalization. All primers were tested to exclude amplification from genomic DNA. Quantification of the transcript of interest relative to the housekeeping genes was calculated according to a previously published algorithm (21) using sense and antisense oligonucleotide primers shown in Supplementary Table S1.

In vivo tumorigenicity. Six- to 8-week-old female nonobese diabetic/severe combined immunodeficient mice were obtained from Charles River and were housed under specific pathogen-free conditions. All animal experiments were done according to the German animal protection law. Institutional guidelines for animal welfare and experimental conduct were followed. Cells were implanted stereotactically into the right hemispheres of nonobese diabetic/severe combined immunodeficient mice. To avoid reflux of cells along the needle tract, small carrier volumes (5 μL) were injected 4 mm parasagittal along the coronal suture at an adequate depth of 3.5 mm. After pausing for 10 minutes to allow the diffusion of the carrier fluid into the parenchyma, the injection needle was slowly extracted. After the injection, no macroscopic reflux was observed in any of the animals and the needle tract was sealed with biodegradable bone wax. Survival and general performance of mice were monitored daily. In the SLGC group, all animals had to be sacrificed before day 104 due to cachexia, gasping, and anergy. Mice brains were removed and snap frozen. For tumor volume assessment, each tumor-containing brain tissue was cut in corony sections over the entire tumor length and stained with hematoxylin. Tumor was measured on every 20th section (approximately every 140 μm) over the whole tumor extension with the Cell Imaging Software (Olympus) and total tumor volume was approximated by overlaying all tumor-bearing sections.

For in vivo therapy, spheroids (diameter, 700-1,400 μm) of green fluorescent protein (GFP)–transfected NCH421k SLGCs (kindly provided by V. Goidts, DKFZ Heidelberg, Germany) were placed on the cerebral mouse cortex (one per hemisphere) and covered by a long-term cranial window (Irola). Microsurgical techniques and monitoring of spheroid growth by intravital multilabel fluorescence microscopy were done as described earlier (22) using a modified Axioptech Vario 100 fluorescence microscope. SLGC-derived spheroids and tumor microvasculature were visualized using an AxioCam high-speed color camera (all Zeiss). Glia microvasculature was assessed after contrast enhancement with 100 μL (2%) rhodamine-conjugated dextran administered i.v. through the tail vein (Sigma-Aldrich). To study the effects of ATRA on tumor growth as well as migratory activity and angiogenesis, mice were treated daily with 0.2 mg ATRA dissolved in 75 μL DMSO, administered i.p. from day 1 to 9 (n = 3). Control mice received the vehicle alone (n = 4). Intravital multilabel fluorescence microscopy was done on days 1, 3, 6, and 9 after cell implantation. Quantitative analysis of tumor growth and migratory activity was done with the aid of the AxioVision Rel 4.6. image analysis software (Zeiss). Tumor growth was assessed by measuring of the tissue area (mm²) covered by the fluorescent solid tumor mass. Glia cell migration was determined by measuring the distance between the spheroid and the population of cells most distant from the spheroid edge (migration distance; mm). Migration distance per spheroid was evaluated radially in eight distinct directions in a standardized fashion, i.e., stepwise by 45°. Glia cell migration was analyzed on days 3 and 6 after implantation.

Conditioned media. Twenty-four hours after seeding and cultivating 3.3 × 10⁶ SLGCs in stem cell medium as described above (see cell culture conditions), cells were shifted to DMEM/F-12 medium without additional growth factors or supplements. Twenty-four or 48 hours later, conditioned medium (CM) was harvested, centrifuged for 10 minutes at 10,000 g, and stored at −80°C. Viable cells were counted.

Tube formation assay. One hundred fifty microliters of growth factor–reduced Matrigel per well (Becton Dickinson) were filled in precooled 24-well plates and solidified at 37°C for 30 minutes. HUVECs (6 × 10⁵ cells/mL) plated on this matrix started to migrate and differentiate into tubules within 6 hours. After 16 hours, tubule formation was visualized by fixing the cell preparation in 70% methanol at 4°C for 30 seconds, rinsing in PBS, and staining with 0.1% methylene-blue for 1 minute. Tube formation was documented on the entire area of the well at ×4 magnification and subsequently quantified by automated measuring of the total area covered by stained tubules in three replicas. HUVECs grown under serum-free conditions served as negative control and reflected the spontaneous capacity to form tubes in the absence of external angiogenic promoters. In contrast, HUVECs exposed to endothelium-optimized medium containing angiogenic growth factors (Provitro) were used as positive control to mirror an effective
growth factor–induced stimulation of endothelial cells to form tubes. Computerized analysis was done using a Cell Imaging Software (Olympus).

Scrape assay. Cells were seeded on 24-well plates at a density of $2 \times 10^5$ cells per well. At postconfluent state, wounds of 1.5-mm width were created by scraping cell monolayers with a sterile pipette tip. Migration was documented at a ×4 magnification. Cell migration was quantified by measuring the area between the wound edges directly after injury and 24 hours later.

Collagen invasion assay. Twenty to 25 drops containing $2 \times 10^4$ cells/25 μL medium were plated on the lid of a 100-mm² Petri dish. Within 24 to 48 hours, cells started to form spheroids that could be harvested to place them into collagen gels. Cold Vitrogen (Nutacon BV) was mixed with cold 10-fold concentrated MEM and cold 0.1 mol/L NaOH reaching a final concentration of 2.4 mg/mL collagen. Collagen solution was distributed into 24-well plates. Spheroids placed into each well and the plates into the wells were kept at 37°C for about 30 to 60 minutes. After solidification, the gels were overlaid with 0.5 mL of medium as described above (see cell culture conditions). Cell migration out of the spheroids was monitored daily over a period of 12 days.

Cell viability assessment. Tumor cells ($6 \times 10^5$) per sample were incubated with trypsin for 3 minutes at 37°C and stained with propidium iodide (20 μg/mL in Nicoletti buffer). Cells were subjected to flow cytometry analysis of DNA content using a Becton Dickinson FACSCalibur cytometer and the CellQuest Software.

Western blot. Cells were rinsed with ice-cold PBS and resuspended with cold lysis buffer [120 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, and 0.5% NP40] containing phenylmethylsulfonylfluoride (1mmol/L), proteinase inhibitors (Complete, Roche Diagnostics), and phosphatase inhibitors (10 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L Na4PPi, 2 mmol/L phenylmethylsulfonyl fluoride). After 15 minutes on ice, lysates were centrifuged at 16,100 $g$ for 20 minutes. Total protein concentration of lysates was measured using the Bradford-Assay (Bio-Rad Laboratories). Soluble protein (75 μg/lane) was separated on 15% polyacrylamide gels and blotted onto nitrocellulose by standard procedures. Primary antibodies used were mouse antihuman caspase-3 (1:1,000, Imgenex) and mouse antihuman actin (1:10,000, Chemicon). Membranes were washed, incubated with the primary antibody, washed again, and incubated with the secondary antibody (1:3,000, horseradish peroxidase–conjugated, Bio-Rad Laboratories). Bound antibodies were visualized using an enhanced chemiluminescence detection system (GE-Healthcare).

Irradiation. Irradiation was done at room temperature as single exposure to doses of photon irradiation of 10 Gy delivered by a linear accelerator (Siemens). An initial set of experiments was done to assess the optimal dose of irradiation for subsequent experiments. Proliferation of
Table 1. Clinical data related to the different tumor tissue donors

<table>
<thead>
<tr>
<th>Histology</th>
<th>Sex</th>
<th>Age (y)</th>
<th>PFS (wk)</th>
<th>OS (wk)</th>
</tr>
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<tbody>
<tr>
<td>NCH421k</td>
<td>GBM</td>
<td>66</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td>NCH440</td>
<td>GBM</td>
<td>43</td>
<td>19</td>
<td>37</td>
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<tr>
<td>NCH441</td>
<td>GBM</td>
<td>75</td>
<td>22</td>
<td>31</td>
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NOTE: PFS, progression-free survival; OS, overall survival.

Table 2. Quantification of AC133/CD133 expression in corresponding SLGC lines as assessed by flow cytometry

<table>
<thead>
<tr>
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<th>% CD133+ in culture</th>
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<tbody>
<tr>
<td>NCH421k</td>
<td>93 ± 5.2</td>
</tr>
<tr>
<td>NCH440</td>
<td>68 ± 6.5</td>
</tr>
<tr>
<td>NCH441</td>
<td>93 ± 2.3</td>
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NOTE: Data indicate mean ± SD and are representative of at least 2 independent experiments.

Results

Efficient differentiation of SLGCs in vitro. To study the differentiation of SLGCs, we established SLGC lines from primary glioblastomas. Like normal neural stem cells (NSC), SLGCs grew as floating, dense cell aggregates, so-called neurospheres. All three cell lines (NCH421k, NCH440, and NCH441) expressed high levels of the NSC markers nestin and CD133 with contents of AC133/CD133-positive cells ranging from 68% to 93% (Fig. 1A-D, Tables 1 and 2). SLGCs could be clonally expanded and were able to grow large, infiltrating tumors in nude mice with 100% penetrance compared with AC133/CD133-positive glioma cells isolated from glioblastoma tissue donors (Fig. 1A, left column), a phenomenon well-known from differentiating NSCs (24). To assess the degree of differentiation, AC133/CD133 levels were measured by flow cytometry. AC133/CD133 expression decreased in SLGC lines under the influence of ATRA-containing medium in a dose-dependant manner (Supplementary Fig. S6), whereas serum-containing medium alone resulted only in a minor decrease of AC133/CD133 (Supplementary Table S2; Fig. 2A, right column). Confirming ATRA effects in the absence of serum, exposure to ATRA alone applied under stem cell conditions markedly decreased the expression of AC133/CD133 in SLGC lines (Supplementary Fig. S11A). It is worth mentioning that repeated administration and withdrawal of ATRA induced minor oscillations of AC133/CD133 expression. Although AC133/CD133 expression after ATRA treatment never reached initial expression levels, these experiments suggest a certain plasticity of the SLGC phenotype after differentiation (Supplementary Fig. S11C). Finally, it has been reported that SLGCs cultured under stem cell conditions show a high degree of similarity to the original tumor (18); however, it cannot be excluded that differentiation effects on AC133/CD133 are caused or at least enhanced under artificial stem cell conditions that not necessarily mirror actual tumor conditions. Interestingly, AC133/CD133-positive glioma cells isolated from glioblastoma tissue that had only been allowed to expand briefly (less than five passages) were even more sensitive than our SLGC lines and responded with marked downregulation of AC133/CD133 and pronounced morphologic differentiation upon exposure to ATRA-containing medium (Fig. 2B).

Finally, we assessed the effects of differentiation on βIII-tubulin, GFAP, and myelin basic protein, which are surrogate markers of the neuronal, glial, and oligodendroglial lineage, respectively, and are expected to be upregulated upon differentiation (24). Although all lineage markers were expressed in differentiated SLGCs, the predominant phenotype consisted in an augmented glial staining pattern as quantified by flow-cytometric analysis (Supplementary Fig. S17).
Fig. 2. Efficient differentiation of SLGCs. A, phase-contrast image of SLGC line NCH421k before and after differentiation with serum alone or with serum and ATRA, and corresponding CD133 content as determined by flow cytometry. B, phase contrast image of briefly cultured SLGC cells before and after differentiation with FCS alone or with ATRA and corresponding CD133 content. C, immunofluorescence staining of lineage markers βIII-tubulin, myelin basic protein (MBP), and GFAP before and after differentiation with serum and ATRA depicted for the NCH421k SLGC line and quantification of GFAP expression as assessed by flow cytometry and shown here exemplarily for NCH421k (columns, mean of representative of at least two independent experiments; bars, SD). D, quantitative PCR analysis of NOTCH and BMP pathway-related gene transcripts. Bars, x-fold upregulation or downregulation after differentiation compared with undifferentiated SLGCs (columns, mean value of all three cell lines and are representative of at least two independent experiments; bars, SD); P, parenchyme; T, tumor.
Figs. S6 and S11B-C; Fig. 2C). Taken together, we have evidenced efficient, ATRA-induced differentiation of SLGCs based on the differentiation criteria applied to NSCs.

Thus far, there are only few studies analyzing changes in molecular pathways after differentiation of SLGCs (3). In this regard, self-renewal pathways such as the NOTCH and the BMP signaling cascade could be of interest because they are involved in the maintenance of stem cell properties and preservation of the SLGC pool. Accordingly, we analyzed several signaling molecules relevant to these pathways in SLGCs (3, 25). Interestingly, expression of NOTCH1 was reduced on mRNA and protein level after differentiation (Supplementary Fig. S7; Fig. 2D). In addition, NOTCH signaling components 6-like 3 (DLL3) and the downstream effectors hairy and enhancer of split (HES) 5 and 6 were significantly downregulated after differentiation treatment (Fig. 2D), whereas expression of transcripts belonging to the BMP pathway such as BMP receptor (BMPR) 1A, 1B, and 2 as well as small mothers against decapentaplegic homologue 4 (SMAD4) was not altered by differentiation.

Angiogenic activity of SLGCs is reduced after differentiation. In gliomas, angiogenesis is linked to the aggressive phenotype of high-grade tumors. Recruitment of blood
vessels depends on angiogenic cytokines that are secreted by tumor cells and act in a paracrine manner on proliferation, migration, and tube formation of endothelial cells (26). It has been reported that SLGCs are a crucial source of key angiogenic factors such as vascular endothelial growth factor (VEGF) and bFGF, two potent proangiogenic cytokines in glioblastoma (27, 28). Therefore, we investigated possible effects of differentiation on the angiogenic behavior of SLGCs. First, we measured tumor cell–secreted amounts of VEGF and bFGF in CM from differentiated and undifferentiated SLGCs. Upon differentiation, levels of VEGF and bFGF decreased up to 82% and 99%, respectively, in CM of all SLGC lines (Supplementary Fig. S8A; Fig. 3A and B). Interestingly, differentiation exerted similar effects on VEGF and bFGF secretion of AC133/CD133-negative glioma cells cultivated under identical stem cell conditions (NCH465), although initial expression levels of these cytokines were much lower compared with SLGCs (Supplementary Fig. S9A and B).

To evaluate the biological significance of our findings, we analyzed the paracrine response of HUVECs to CM containing tumor-secreted factors. When applying CM of differentiated NCH421k and NCH440 cells, HUVECs showed a significant reduction of migration as well as of invasion. Additionally, levels of MMP2 protein measured in CM of SLGCs before and after differentiation (*, P < 0.01). D, assessment of cell viability using propidium iodide incorporation of undifferentiated and differentiated SLGCs 72 h after irradiation or (E) treatment with 100 μmol/L BCNU (*, P < 0.005). Columns, mean of at least two representative independent experiments; bars, SD.

Fig. 4. Differentiation affects the invasiveness of SLGCs and induces therapy-sensitizing effects. A, collagen invasion assay of NCH441 SLGC line at 0, 5, and 12 d. B, the average migration distance in mm assessed at day 12 (*, P < 0.001). C, levels of MMP2 protein measured in CM of SLGCs before and after differentiation (*, P < 0.01). D, assessment of cell viability using propidium iodide incorporation of undifferentiated and differentiated SLGCs 72 h after irradiation or (E) treatment with 100 μmol/L BCNU (*, P < 0.005). Columns, mean of at least two representative independent experiments; bars, SD.
proliferation by up to 52% and 29%, respectively, compared with HUVECs incubated with CM of undifferentiated SLGCs (Fig. 3C and D). Interestingly, CM of differentiated NCH441 cells did not elicit significant antiproliferative or antimigratory effects, which might be attributed to the remaining postdifferentiation levels of VEGF (Fig. 3A). In contrast, tube formation, a further prerequisite for endothelial growth, which mirrors the capacity of endothelial cells

Fig. 5. Differentiation elicits long-lasting antitumorigenic, anti-invasive, antiangiogenic, and proapoptotic effects of SLGCs in vivo. (A and B, top) H&E-stainings of mouse brains bearing tumors derived from SLGCs and differentiated SLGCs, respectively. Tumor tissues were stained with antibodies against GFAP, CD133, NOTCH1, Ki-67, and CD31. For SLGCs, see correspondingly labeled pictures in A, and for differentiated SLGCs, see correspondingly labeled pictures in B. A and B, TUNEL, TUNEL-based staining of tumors derived from SLGCs and differentiated SLGCs. C, Kaplan-Meier blot showing survival of mice bearing differentiated SLGCs (n = 4; dotted line) compared with animals with undifferentiated SLGC xenografts (n = 7; continuous line, P = 0.016). D, tumor volume of SLGC-derived tumors in cubic millimeters compared with xenografts of differentiated cells (*, P < 0.005). E, number of TUNEL-positive (TUNEL+) cells per visual field in tumors derived from undifferentiated and differentiated SLGCs (*, P < 0.0005). Columns, mean of at least two representative independent experiments; bars, SD; P, parenchyme; T, tumor.
to form endothelial networks, was significantly lowered by up to 55% in the presence of CM from all differentiated SLGC lines (Supplementary Fig. S8B; Fig. 3E). In summary, we showed the downregulation of angiogenic surrogate markers in SLGC following differentiation.

**Collagen invasion of SLGCs is decreased after differentiation.** The influence of differentiation on SLGC motility was studied using a collagen invasion assay. After ATRA-induced differentiation, SLGCs displayed a significantly reduced invasion of the surrounding matrix by up to 46% (Fig. 4A and B). Because enzymes of the matrix metalloproteinase (MMP) family show matrix-degrading activity and are involved in the invasion and migration of glioma cells (29), we studied the expression of MMP 2 and 9, which are considered pivotal invasion-promoting enzymes in gliomas (29). Although MMP9 was not detectable in the CM of SLGCs, differentiation-induced downregulation of MMP2 secretion showed decrements ranging from 50% to 87% (Supplementary Fig. S8C; Fig. 4C). In line with our previous observations on SLGCs, differentiation exerted similar effects on the MMP2 secretion of AC133/CD133-negative glioma cells cultivated under stem cell conditions (NCH465), although MMP expression in nontreated cells was again much lower compared with SLGCs (Supplementary Fig. S9C). Altogether, our results indicate that the anti-migratory effects evidenced in the collagen assay might be caused at least in part by the decrease of MMP2 expression, whereas MMP9 does not seem to be involved in the invasion process of SLGCs.

**Increased cell death contributes to chemosensitizing and radiosensitizing effects after differentiation.** Previous reports described increments in the apoptotic susceptibility of glioma cells following exposure to retinoids in vitro (30). These findings encouraged us to monitor the potential effects of differentiation on the apoptotic susceptibility of our SLGC lines. In line with these observations, cell viability assessment using propidium iodide incorporation revealed a significantly increased cell death when applying radiation or the cytotoxic compound BCNU to all three differentiated SLGC lines (Fig. 4D and E), suggesting that differentiation increases the sensitivity of differentiated cells toward chemotherapeutic and radiotherapeutic influence. To corroborate our data on chemosensitivity and radiosensitivity after differentiation, we studied the expression of caspase-3, which is a key indicator of apoptosis and is activated through proteolytic cleavage (31). Western blot analysis of cleaved caspase-3 did not reveal any change in the basal apoptotic rate following our differentiation protocol (Supplementary Fig. S10A and B); however, after exposure to cytotoxic compounds such as cisplatin, BCNU, and especially after irradiation, levels of cleaved caspase-3 were found to be augmented to a higher degree in differentiated SLGC lines (Supplementary Fig. S10A and B). Notably, upon differentiation, AC133/CD133-negative cells (NCH465) also increased their sensitivity to radiotherapy and chemotherapy in agreement with the data on AC133/CD133-positive SLGCs (Supplementary Fig. S9D and E).

**Short-term differentiation of SLGCs exerts long-lasting antitumorigenic, anti-invasive, antiangiogenic, and proapoptotic effects in vivo.** Next, we asked if differentiation influences the tumorigenicity of SLGCs. Ex vivo differentiation impaired the tumorigenicity of SLGCs (Supplementary Table S3; Fig. 5A and B). Even if 1 × 10⁵ undifferentiated and 1 × 10⁵ differentiated NCH421k cells were implanted, animals inoculated with undifferentiated SLGCs quickly developed anergy, developed cachexia, and had all to be sacrificed within 15 weeks after implantation, whereas mice bearing differentiated SLGCs did not develop any of the aforementioned signs (Fig. 5C). Analyses of postmortem brains with NCH421k xenografts (Fig. 5A and B) revealed that although tumors had developed in both groups, mice xenografted with undifferentiated NCH421k cells developed more than seven times larger tumors, although implanted cell numbers were 10 times lower (Fig. 5D). Strikingly, SLGC-induced tumors showed a highly invasive growth pattern compared with the predominantly circumscribed, and thus, noninvasive tumors developed in animals grafted with differentiated SLGCs. Staining for GFAP, a typical differentiation marker found in glial tumors, revealed that in both groups, tumors reconstituted the glial phenotype of the parental tumors although xenografts derived from differentiated SLGCs showed both a higher staining intensity of GFAP and a fibrillary distribution pattern absent in the SLGC tumors. Correspondingly, CD133 expression remained high in SLGC tumors, whereas it was markedly reduced and confined to small, scattered clusters in tumors derived from differentiated SLGCs. In line with this, NOTCH1 expression was higher in xenografts from undifferentiated SLGCs.

To elucidate possible causes for tumor size differences (Fig. 5D), we analyzed tumor cell proliferation, vessel supply, and cell death in xenografts originating from undifferentiated and differentiated SLGCs. Both tumor types showed high levels of the proliferation marker Ki-67 without revealing any significant variance in their proliferative activity (Fig. 5A and B). Immunohistochemical analysis of CD31-positive endothelia, however, revealed striking differences between both tumor types. Whereas SLGC-derived xenografts displayed a homogeneous pattern of CD31-positive capillaries, tumors derived from differentiated SLGCs exhibited a predominantly avascular growth with vessels lined up at the tumor margin and only few capillary sprouts penetrating the tumor bulk (Fig. 5A and B). In addition, TUNEL-based staining of apoptotic cells revealed 5-fold higher numbers of TUNEL-positive cells in tumors derived from differentiated SLGCs (Fig. 5A and B, and E).

Finally, we assessed the effect of ex vivo differentiation on our AC133/CD133-negative glioma cell line cultivated under identical stem cell conditions as our SLGCs. Although NCH465 was not as tumorigenic as AC133/CD133-positive SLGCs (Supplementary Table S3), differentiation completely abolished tumor-initiating capacity in vivo. Altogether, ex vivo differentiation induced long-term antitumorigenic, anti-invasive, antiangiogenic, as
Fig. 6. ATRA induces antimigratory, growth-inhibiting, and antiangiogenic effects in SLGCs in vivo. A, intravital fluorescence microscopy images showing GFP-transfected SLGC spheroids treated with ATRA (bottom) or vehicle alone (top) at day 0 and 9 (dotted white lines, original spheroid sizes). B, mean increment of tumor size in percent after 9 d compared with the day of implantation in ATRA-treated and nontreated spheroids (columns, mean; bars, SD). C, change of tumor size in percent after 9 d compared with the day of implantation in individual SLGC spheres. D, mean migratory distance of SLGCs in millimeters after 3 and 6 d in ATRA-treated and nontreated animals (*, P < 0.05; columns, mean; bars, D). E, left, intravital fluorescence microscopy images showing the migratory activity of GFP-transfected SLGC spheroids treated with ATRA (bottom) or vehicle alone (top) at day 0 and 6 (red arrowheads, migrating cells; white arrow, tumor size decrement; blue arrow, tortuous vessels forming in close vicinity to the tumor; *, reference vessels). Right, intravital fluorescence microscopy images showing vessel activity in the proximity of GFP-transfected SLGC spheroids treated with ATRA (bottom) or vehicle alone (top) at day 3 and 6 with vessels counterstained with rhodamine-conjugated dextran (blue arrowheads, tortuous, newly formed tumor capillaries; *, label reference vessels).
well as proapoptotic effects in vivo, which were in good agreement with observations made in vitro.

Direct ATRA treatment induces antitumor effects in vitro and in vivo. In spite of the beneficial effects described after in vitro differentiation, it has to be emphasized that artificial ex vivo treatment with ATRA in serum-containing medium may not allow to assume a direct antitumor effect of ATRA on SLGCs. Therefore, we sought to establish if simultaneous treatment of xenografted SLGCs with ATRA could confirm in vivo antitumor effects seen after ex vivo differentiation. In a cranial window model, implantation of undifferentiated SLGC spheroids of GFP-expressing NCH421k cells was accompanied by i.p. injections of ATRA or vehicle alone. Although all SLGC-derived spheroids of control animals (n = 8) increased in size during the observation period, four of six spheroids treated with ATRA responded with growth arrest or even a decrease in tumor size (Fig. 6A-C). When analyzing the migratory activity of SLGCs at two different time points, we observed a significantly lowered migratory activity by up to 58% in ATRA-treated animals compared with that of vehicle-treated controls (Fig. 6C and D, left). Previous reports on glioma vascularization using intravital microscopy have shown that angiogenesis in glioma can be described as a two-step process: dilatation, elongation, and coiling of preexisting blood vessels constitutes an angiogenic epiphenomenon and precedes forming of new, perfused capillaries, which truly represent tumor neovascularization (32). Nontreated SLGC-derived tumors displayed elongated, dilated, and tortuous capillaries (Fig. 6E, top left) as well as the typical scenario of tumor neovascularization, in which new, tumor-specific capillaries (Fig. 6E, top right) had formed mainly in the tumor periphery as previously described (32). In contrast, ATRA-treated animals showed only moderate signs of angiogenic activity such as early budding of host vessels (Fig. 6E, bottom right) but absent neovascularization. Even in one ATRA-treated spheroid with unaffected tumor growth (Fig. 6C, last red column), vascularization as well as migration were severely impaired. In summary, the antimigratory, growth-inhibiting, and antiangiogenic effects witnessed after ATRA administration in our in vitro therapeutic setting are in good agreement with our previous data obtained after ex vivo differentiation.

Discussion

Most current cancer research is focused on the molecular and cellular analysis of the bulk tumor mass, although there is increasing evidence that curative therapies can be improved if stem-like tumor cells are considered (3, 33). In this context, differentiation-inducing drugs such as retinoids, BMFs, and histone deacetylase inhibitors, have received growing attention (3, 13, 16). Several reports have studied the effect of prodifferentiating agents on stem-like tumor cells in various human cancers (34, 35). Although retinoids have been studied extensively on established glioma cell lines of different origins (30, 35), this is the first study analyzing the effectiveness of ATRA in SLGCs. In the present study, we showed that differentiation treatment with ATRA, in combination with serum or as single substance, diminishes the AC133/CD133-positive SLGC pool and induces antitumorigenic, antiangiogenic, as well as antimigratory effects.

Especially in gliomas, migration and invasiveness of tumor cells poses a major therapeutic obstacle. Invasive cells hinder in sano resection and ultimately lead to tumor recurrence. Our results suggest that collagen invasion of SLGCs in vitro may be linked to MMP2, a matrix-degrading enzyme correlated with tumor progression in gliomas (29). Although collagen may not mirror the extracellular matrix of the brain exactly, these data are in good agreement with the anti-invasive effects described in differentiated SLGC xenografts and the antimigratory effects reported after direct ATRA treatment in vivo.

However, MMPs and MMP2 in particular also play an important role in the angiogenic process (36). It has been reported that MMPs can be induced by VEGF and bFGF (36), two crucial proangiogenic and synergistically acting cytokines (27, 28, 37) that were specifically downregulated after differentiation in our experimental setting. In addition to the lowered paracrine angiogenic activity of differentiated SLGCs, three relevant surrogate markers for angiogenesis, namely tube formation, proliferation, and migration of endothelial cells, were inhibited in vitro. Although these effects are promising, it has to be argued that the HUVEC cells used in our study may not represent the optimal cell type to analyze the antioangiogenic effects of SLGCs. Further experiments using tumor-derived endothelial cells should corroborate effects witnessed in our report. Nevertheless, impaired vessel growth after ex vivo differentiation of in vivo treatment with ATRA are in good agreement with our in vitro findings.

Although there is evidence for the existence of tumorigenic AC133/CD133-independent glioblastoma cells, until now, the stem cell–related antigen AC133/CD133 still constitutes a useful marker to enrich SLGCs (4, 5, 10) and numerous findings have linked the expression of AC133/CD133 with patient prognosis in several extracranial and intracranial malignancies (7–9). Recently, we reported that AC133/CD133 content in gliomas inversely correlates with patient prognosis independent of WHO grade, patient age at diagnosis, and extent of tumor resection corroborating the importance of AC133/CD133 (9). In line with this, AC133/CD133 downregulation was associated with better survival in our mouse model, indicating that drug-induced differentiation may lead to a cellular phenotype that mirrors the less tumorigenic AC133/CD133-negative tumor population in vivo.

Finally, we present evidence that differentiation treatment elicits chemosensitizing and radiosensitizing effects in SLGCs in vitro. In response to irradiation and chemotherapeutic treatment, increments in cell death and the apoptotic susceptibility of SLGCs were observed after differentiation. These preliminary findings should encourage further in vivo experiments to assess whether differentiation can
act synergistically with treatments developed to target the tumor bulk, thus aiming at both tumor populations simultaneously.

Despite the promising antitumor effects of ATRA described in our study, previous clinical trials have reported limited efficacy of retinoids in glioma patients (38, 39). It is conceivable that ATRA effects are limited in recurrent tumors with significant tumor burden as in previous clinical studies, which only included patients with recurrent tumor disease (38, 40). It is thus tempting to speculate that patients, which underwent first-line therapy and feature low tumor burden could profit from ATRA therapy (41). In addition, it cannot be excluded that some tumor cells might be endowed with an ATRA-resistant phenotype caused by ATRA-metabolizing enzymes, which are culprits of ATRA resistance in other tumor entities (42, 43). Further, loss of RARs in a fraction of tumor cells, which occurs as a common event in several extracranial cancers (44–46), could render cells insensitive to ATRA effects and require long-term, supraphysiologic ATRA doses. In this context, one particular observation reported by Kaba and coworkers (38) could be of further interest regarding clinical ATRA application: after 4 weeks of retinoid treatment, the authors documented markedly decreased ATRA peak plasma levels measured directly after drug administration. This suggests additional extracranial metabolism, which would soon limit the effective ATRA dose and ultimately terminate ATRA effects. However, our data on reversibility of ATRA differentiation suggest that prolonged, supraphysiologic ATRA levels are required to maintain SLGCs in a differentiated state. Following this line of reasoning, three potential therapeutic approaches to enhance ATRA effects could be envisioned. This would include (a) simultaneous blocking of ATRA metabolism or (b) intracranial application of ATRA to bypass extracranial degradation and (c) the use of additional differentiation agents targeting alternative metabolic pathways, such as BMP signaling and the NOTCH pathway. In this context, it seems worth to mention differentiation-induced downregulation of NOTCH1 in SLGCs as described in our study. Deletion of NOTCH1 has been reported to deplete the NSC fraction (47), whereas NOTCH1 inhibition abrogated the tumor formation capacity of AC133/CD133-positive tumor cells in medulloblastoma and glioma (48, 49). Therefore, it would seem of interest to study potential synergisms between the pharmacologic inhibition of NOTCH and ATRA differentiation. Recently, activation of the BMP signaling cascade through BMP4 has been characterized as a further differentiation pathway in SLGCs capable of inducing antiproliferative effects in these cells (3). Acting on the assumption that ATRA-mediated antitumor effects in our study are BMP independent, a combination of both agents might also enhance retinoid effects. Such a differentiation strategy could prove to be beneficial beyond the potential synergism of the two drugs because recent findings described an impaired BMP response of SLGCs due to epigenetic-mediated silencing of BMPR1B (50). BMPR1B proved to be the determinant factor for the antiproliferative response of SLGCs in the presence of BMPs and seems to be dysfunctional in a relevant fraction of glioblastomas. Therefore, we see a further necessity for ATRA-induced differentiation to surpass these obstacles in addition to the beneficial effects described in this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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