Angiogenesis inhibition in high grade glioma
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Chapter 2.

Tumor microvasculature supports proliferation and expansion of glioma-propagating cells
Tumor microvasculature supports proliferation and expansion of glioma-propagating cells

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Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor. The identification of ‘cancer stem cells’ (CSC) has shed new light on the potential mechanism of therapy resistance of these tumors. Because these cells appear to be more resistant to conventional treatments, they are thought to drive tumor regrowth after therapy. Therefore, novel therapeutic approaches that target these cells are needed. Tumor cells interact with their microenvironment. It has been reported that close contact between CSCs and tumor microvascular endothelium in GBM is important for CSCs to preserve their undifferentiated state and self-renewal ability. However, our understanding of this interaction is still rudimentary. This is in part due to a lack of suitable in vitro models that accurately represent the in vivo situation. Therefore, we set up a co-culture system consisting of primary brain tumor microvascular endothelial cells (tMVECs) and glioma propagating cells (GPCs) derived from biopsies of GBM patients. We found that tMVECs support the growth of GPCs resulting in higher proliferation rates comparing to GPCs cultured alone. This effect was dependent on direct contact between the 2 cell types. In contrast to GPCs, the FCS-cultured cell line U87 was stimulated by culturing on tMVEC-derived ECM alone, suggesting that both cell types interact different with their microenvironment. Together, these results demonstrate the feasibility and utility of our system to model the interaction of GPCs with their microenvironment. Identification of molecules that mediate this interaction could provide novel targets for directed therapy for GBM. © 2009 UICC

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Material and methods

**Co-purification of tumor microvascular endothelial cells and glioblastoma-propagating cells**

Glioblastoma-derived tumor microvascular endothelial cells (tMVECs) were purified essentially as described with some modifications. Initially, the glioblastoma specimens were digested in 1 mg/ml Liberase-1 (Roche) for 10 min at room-temperature. The cell suspension was passed through a cell strainer with 70-μm pore size (BD-Biosciences). The flow-through containing the glioblastoma cells was washed 3 times in GPC-medium and subsequently cultured as described below. The endothelial cells that were contained in the filter residue were further digested in 0.05 U/ml Collagenase/Dispase (Roche) for 60 min at 37°C under constant agitation. The resulting suspension was filtered through a cell-strainer with 40-μm pore-size (BD-Biosciences) to remove undigested tissue components. The flow-through containing the endothelial cells was washed 3 times in IMDM containing 10% FCS and subsequently cultured as described below. Occasionally, excess lipids were removed by centrifugation of the cell suspension through a layer of 20% Dextran in PBS. Patient specimens were obtained according to established and approved protocols.

**Cell culture**

Purified GPC spheroids were cultured in “GPC-medium.” GPC medium consists of advanced DMEM/F12 medium (Invitrogen, 12634) supplemented with N2 supplement (Invitrogen, 17502-048), 2 mM glutamine, 0.3% glucose, 100 μM β-Mercaptoethanol, Trace-Elements B and C (VWR, 99-175-CL, 99-176-CL), 5 mM HEPES, 2 μg/ml heparin, lipid mixture (Sigma, L0288), 25 μg/ml insulin, 50 ng/ml h-bFGF and 20 ng/ml h-EGF (Peprotech, 500-P18, 100-15) in ultra low attachment flasks (Corning). Growth factors were supplemented twice weekly and spheroids were dissociated and resuspended in PBS in 3 ml aliquots containing 2×10⁶ cells. These aliquots were injected stereotactically into the frontal cortex of 5- to 8-week-old NOD-SCID mice (tMVECs) or 12- to 20-week-old NOD-SCID mice (Promocell, C-12200) were cultured in Endothelial Cell Medium MV 2 (Promocell, C-22221). GPCs were used at passages 10–20 (GPC 006 and 011) or 5–10 (Roche). tMVECs and HUVECs (Promocell, C-12200) were cultured as described below. The endothelial cells that were contained in the filter residue were further digested in 0.05 U/ml Collagenase/Dispase (Roche) for 60 min at 37°C under constant agitation. The resulting suspension was filtered through a cell-strainer with 40-μm pore-size (BD-Biosciences) to remove undigested tissue components. The flow-through containing the endothelial cells was washed 3 times in IMDM containing 10% FCS and subsequently cultured as described below. Occasionally, excess lipids were removed by centrifugation of the cell suspension through a layer of 20% Dextran in PBS. Patient specimens were obtained according to established and approved protocols.

**Mouse brain fixation and histopathology**

Mice were killed and their brains were immediately removed and fixed in 4% freshly depolymerized formaldehyde in PBS, embedded in paraffin or frozen in liquid nitrogen and stored at −80°C. Brains were sectioned at 6-μm thickness and stained with Haematoxylin and Eosin stain.

**Generation of GFP⁺-GPCs**

We used the lentiviral transfer vector pWPT-GFP (Addgene plasmid 12255) to generate lentiviral particles coding for GFP. Lentiviral particles were produced by using the packaging vector psPAX2 (Addgene plasmid 12260) and the envelope vector pMD2.G (Addgene plasmid 12259). The viral supernatants were concentrated and resuspended in PBS containing 2% BSA. For transduction, GPC006 were dissociated by trypsinization and lentiviral particles were added in the presence of 10 μg/ml polybrene for 12 hr.

**Co-cultures, transwell experiments and ECM preparation**

TMVECs and HUVECs were grown in 12-well culture dishes until confluence. Before plating GPCs, endothelial cells were kept in GPC medium without FGF and EGF for 2 days. GPCs were mechanically dissociated into a single cell suspension and washed 3 times in PBS/1% BSA. Subsequently, 5,000 GPCs were added to each well containing fresh growth-factor free GPC medium. After 7 days the cells were incubated with BrdU or EdU and processed further.

For transwell experiments, endothelial cells were seeded in transwell inserts (0.4-μm pore size, Corning 3470). After the endothelial cells reached confluency the inserts were moved to fresh wells containing GPCs in the bottom compartment.

To prepare cell-free ECM, endothelial cells were grown to confluence and then removed by incubation with 10 mM EDTA in PBS for 20 min. The cells were then gently scraped off and the remaining matrix-deposits were washed extensively with PBS before seeding of GPCs.

**Immunocytochemistry**

Cells were fixed with 4% freshly depolymerized formaldehyde in PBS, permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS), blocked with 1% BSA (Sigma, A3744) in PBS and immunostaining was performed with rabbit anti-GFAP (Sigma, G9269) and mouse anti-β-3-Tubulin (Clone Tuj1, R&D systems, MAB1195). After incubation with primary antibodies for 2 hr at 37°C, the cells were washed 3 times with PBS and incubated with secondary antibodies for 30 min. Secondary antibodies were anti-rabbit Alexa-546 (Invitrogen, A10001) and goat anti-mouse Alexa-488 (Invitrogen, A11029). Cells were washed 3 times with PBS and incubated with 5 μg/ml DAPI in PBS before analysis by fluorescence microscopy.

**Scoring of proliferation and absolute cell numbers**

To detect proliferating cells, 5-bromo-2-deoxyuridine (BrdU) (or in some experiments EdU) incorporation was used. Cells were incubated for 90-120 min with 10 μM of the respective nucleotide-analogs at 37°C. Staining was performed according to the manufacturers’ recommendation with anti-BrdU Alexa Fluor 488 conjugate (Invitrogen, A21303). EdU staining was performed with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, C35002) according to the manufacturers’ instructions. Fluorescent images of random fields containing 1,000 cells in total were scored by 2 independent observers. Absolute cell numbers were determined by harvesting the cells by trypsinization. The wells were washed with PBS and the wash-fractions pooled with the first harvest. The cells were washed and resuspended in a 500 μl of FACS-buffer. Subsequently, the whole cell suspension was added to a Trucount tube (BD Biosciences #340334) and the cell numbers were then determined by FACS. The co-cultures were previously stained with the anti-CD105 antibody to separate glioma cells from endothelial cells.

**Results and discussion**

To obtain primary tMVECs for our studies, we utilized freshly resected material from GBM patients undergoing surgery. The isolated tMVECs show the typical cobblestone-like morphology as has been reported before (Fig. 1a). To verify the purity of the isolated tMVECs we stained them for the endothelial markers CD105 and CD31. The positive control, HUVECs stained strongly for both markers while the staining on tMVECs was weaker (Fig. 3a) for both markers while the staining on tMVECs was weaker (Fig. 3a). Contamination with glioma cells and microglia was excluded by verifying the absence of β-3-Tubulin, GFAP and CD11b, respectively (Fig. 3a, lower panel and data not shown). However, later passages of the tMVECs lost the surface expression of CD31, a phenomenon that has been noted before and was dependent on the migratory state of the cells and the density of the culture. In another publication it has been suggested, that intracellular retention of the molecule could also account for a downregulation of surface-CD31. Although the cells of the passages we used
still showed the typical endothelial morphology, largely excluding overgrowth of the culture by contaminating cells, we determined the mRNA expression of a panel of markers. To that end, we subjected tMVECs of the latest passage used to RT-PCR for a panel of markers. As shown in (Fig. 1c), the tMVECs expressed the endothelial markers CD34, CD31, CD144, CD146 and CD141. Expression of desmin, a marker for pericytes, was hardly detectable. Interestingly, the tMVECs were negative for van Willebrand Factor (vWF), which is regarded as a classical endothelial marker. However, it has been reported that vWF expression differs between endothelial cells of large and small vessels with vWF expression being the strongest in endothelial cells derived from large vessels and being low or absent in microvascular endothelia.19 These results are thus in accordance with the reported phenotype of microvascular endothelial cells and largely exclude a significant contamination with other cells.

We also established several cultures from glioma cells, which we purified from freshly resected material. All lines grew in suspension as spheroids, 1 example is shown in (Fig. 2a). As our cell cultures are defined by their ability to propagate gliomas in a xenograft model, we use the term glioma-propagating cells (GPCs) to denote the cultures. We initially chose 2 GPC lines for our further experiments, GPC006 and GPC011. To verify their self-renewal capacity, we used the neurosphere assay. Cells were seeded at clonal density and after 7 days, the formation of neurospheres was assessed. The cells were then re-seeded under the same conditions for 2 additional rounds. In each case, we observed the outgrowth of spheres, indicating the presence of cells with self-renewal potential (Fig. 4c). Additionally, we stained the GPCs for CD133 and nestin, known markers for stem-cells and CSCs. Both, GPC006 and GPC011 were negative for CD133. This has been reported for a subclass of GPCs before, indicating that our GPCs belong to that subclass. Nestin expression could be detected in all GPC006 cells, although at varying intensities (Fig. 2d, upper panel), while only a subset of GPC011 was positive for this marker (Fig. 2e, upper panel). While marker expression is useful to determine stem-cell populations, it has been reported that in some cases only a subset of stem-cell marker positive cells possess true self-renewing capacity. Therefore, we used limiting dilution, a more rigorous approach to determine the frequency of stem-like cells in our cultures (Fig. 2d, and 2e lower panels). The results show the frequency of self-renewing cells to be about 1 in 510 and 1 in 430 for GPC006 and GPC011, respectively. One has to bear in mind though, that this assay provides harsh conditions for the cells and that the self-renewal capacity is likely to be higher when supporting cells are present. Finally, we assessed the tumor-initiating potential of the GPCs by orthotopic transplantation into NOD-SCID mice. One example is shown in (Figs. 2b–2d). Injection of the cells resulted in tumor formation after 20–80 days in almost all mice injected (Fig. 2b). Histological analysis of xenografts revealed that the GPCs gave rise to fast-growing and highly invasive tumors, recapitulating some of the distinctive features that
FIGURE 2 – Glioblastoma cells retain their characteristic properties under stem-cell like culture conditions. (a) Microscopic analysis of GPC spheroids. (b) Tumor-initiating potential of GPCs. Aliquots of $2 \times 10^5$ cells were injected stereotactically into the frontal cortex of 5- to 8-week-old NOD-SCID mice. Mice were killed when they showed neurological signs. In (c), the morphology of Hematoxylin and Eosin stained tumors is demonstrated. The tumors obtained from the xenografts (upper panel) show a similar gross morphology as the original tumor (lower panels). Note especially the scattered invasive tumor cells which can be identified by their dark blue staining pattern. (d) Nesting staining (upper panels) and limiting dilution assay (lower panels) to determine the frequency of self-renewing cells. Different dilutions of GPCs were seeded into 96-well plates. After 14 days, the frequency of cells without detectable sphere-growth was determined and plotted against the number of cells plated per well. The x-intercept of the linear regression indicates the frequency of self-renewing cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
FIGURE 3 – Co-culture of GPCs with tMVECs stimulates proliferation and expansion of GPCs. (a) Immunofluorescence of control cultures (upper panel) and co-cultures (lower panel) demonstrate GFAP (red) and β-3-Tubulin (green) positive cells which adhere to their substrate and undergo morphological differentiation. Note that the tMVECs in the lower panel are negative for either marker and can be further distinguished by their difference in morphology, appearing large and flat. (b) Co-culture of GPCs with tMVECs enhances the amount of EdU^+ GPCs compared to GPCs cultured alone. Proliferation of GPCs was scored by incorporation of the BrdU-analogue EdU and detected with Alexa647 in co-cultures of 2 different GPC lines. To distinguish between GPCs and tMVECs, the co-cultures were stained with CD105-FITC, which labels all tMVECs (See Fig. S2). Shown are the mean of EdU^+ cells plus SD of a representative example of 5 experiments. (c) The co-culture increases expansion of GPCs. The absolute amount of cells was determined by FACS using counting beads. To distinguish tMVECs from GPCs in the co-cultures, we previously stained for CD105 as described for (Fig. S2). The control cultures were treated in the same manner to control for cell loss during the staining procedure. The amount of GPCs in the cultures was then determined by FACS. GPCs were identified by gating on the CD105^+ population. The cell numbers are expressed as % of initially plated GPCs. Shown is one representative example of 3 independent experiments. (d) tMVECs stimulate proliferation and expansion of a GPC line with a low proliferative index. Proliferation and expansion was determined as in (b) and (c). Significance levels for (b) and (c) were determined by two-way ANOVA with Bonferroni’s post-test. For (d) the two-tailed student’s t-test was used. Stars denote the p-values (*p < 0.05, **p < 0.01, ***p < 0.001) for clarity reasons only the comparisons yielding differences with p < 0.05 are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
were found in the original patient specimen, such as invasive growth and necrosis as well as GFAP positivity (Fig. 2c compare upper and lower panel and supporting Figure S1). However, the extent of vascularization and necrosis was much less pronounced in the xenografts than in the original specimens (Fig. S1). We hypothesize that this is due to the fast growing nature of the transplants or the small absolute diameter of the xenografts, which allows the tumor to acquire nutrients and oxygen by diffusion alone.

It has been described, that GBM CSCs are located in a perivascular niche and that this niche can support the growth of self-renewing cells.6,10 We thus asked if our tMVECs, which would represent a major constituent of this perivascular niche, can influence the proliferation of GPCs. Therefore, we cultured...
the GPCs in medium devoid of bFGF and EGF either on an endothelial monolayer or on normal cell culture plastic. Both conditions promote adhesion of GPCs and expression of the glioma markers GFAP and β-3-tubulin (Fig. 3a). We next asked if the co-culture changes the amount of proliferating GPCs cells. For that reason, we used an EdU incorporation assay, shown in (Fig. 3b). To distinguish between tMVECs and GPCs in the co-culture, we stained for CD105 to label all tMVECs (Fig. S3). Interestingly, the GPCs continued to incorporate EdU even after 7 days of growth factor withdrawal (Fig. 3b). When we scored the number of EdU positive cells, we detected a significantly higher proliferation rate in the co-cultures as compared to the controls in both cell lines tested, indicating that indeed tMVECs can support the growth of GPCs in vitro (Fig. 3b).

EdU-incorporation could also be the result of ongoing repair activity or DNA-replication without cytokinesis. Moreover, the balance between proliferation and cell death ultimately decides whether a tumor expands. We therefore determined the absolute numbers of GPCs in the control and in the co-cultures (Fig. 3c). Both, GPC006 (Fig. 3c, upper panel) and GPC011 (Fig. 3c, lower panel) expand in the control cultures. However, the amount of GPCs in the co-cultures was more than double (500 versus 1,100% expansion for GPC006) or 1.7-fold (180 versus 310% for GPC011) compared to the control cultures. As both GPC lines had a high basal proliferation index, we were interested if tMVECs could also support GPCs with a much lower proliferation rate. For that reason, we used a third line, GPC051, which had a 20-fold lower EdU labelling index than GPC006. Analysis of co-cultures with tMVECs revealed that proliferation and expansion of GPC051 was also stimulated in the co-cultures (Fig. 3d). Together, our results show that tMVECs can stimulate the proliferation and expansion of GPCs in vitro.

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To further confirm that the proliferation-enhancing effects we detected are not exclusive to one tMVEC preparation, we tested 2 additional independent tMVEC preparations. Both, tMVEC075 and tMVEC085 were able to significantly enhance the proliferation of GPC006, demonstrating that the stimulatory effect is of a more general nature (Fig. 4a).

It has also been described that HUVECs are able to increase the proliferation of medulloblastoma cells. To assess if the increase in proliferation we observed is specific for tMVECs, we co-incubated GPCs with HUVECs under the same conditions. In this case we did not observe a significant effect on the proliferation of GPCs, demonstrating that the stimulation is not a general endothelial cell activity (Fig. 4b). These results are in contrast to the findings by Calabrese et al. who showed that HUVECs can increase proliferation of CSCs in a transwell chamber. How could this difference be explained? It is known that microvascular endothelial cells in general and especially tumor derived microvascular endothelial cells contain a different array of secreted and cell bound factors compared to HUVECs. Additionally, Calabrese et al. used a medulloblastoma cell line in their experiments, which may differ in their requirements from GPCs and interact differentially with their microenvironment.

It has been reported that the perivascular niche in tumors is able to support the stem-like phenotype of GPCs. We thus asked, if the GPCs in our co-culture retain the potential for self-renewal even after prolonged co-culture. To that end, we co-cultured a GFP derivative of GPC006 for 10 days with tMVECs in growth-
factor free medium. Subsequently, all cells were trypsinized, plated under stem-cell like conditions and neurosphere outgrowth was assessed. The GFP marker allowed us to reliably distinguish between GPCs and tMVECs. While most of the GFP+ cells stayed as single cells, we observed a small amount of GPCs that formed sphere-like structures after 7 days, which expanded into larger spheres after 14 days (Fig. 4c). To ascertain that the expanding cells were derived from the GPCs and not the tMVECs, we subjected the cultures to FACS analysis. The results show that all cells were GFP+ and thus derived from the GPCs (Supporting Fig. S3). To determine if the co-culture with tMVECs supports the self-renewal capacity of GPCs better than the control cultures where GPCs are cultured alone, we determined the numbers of GFP+ cells at the day of replating, as well as a week and 2 weeks after. We normalized the numbers of cells to the amount of live cells present in the co-cultures, even after prolonged co-culturing (Fig. 5). These results indicate that a significantly higher percentage of cells with self-renewing properties was present in the co-cultures, even after prolonged co-culturing without the addition of exogenous growth factors.

The interaction between normal neural stem cells with their niche is mediated by both, soluble factors such as VEGF, PDGF and PEDGF, and a contact-dependent interaction of the stem cell with their niche.20,21 It is thus tempting to speculate that the same mechanisms in an improved in vitro model system for the tumor-microenvironment interaction. The method demonstrated has several advantages over the current model systems. First, we can study tumor cells and microvascular cells from the same species. It is also possible to co-isolate microvascular cells and GPCs from the same patient and thus evaluate the effects of a microenvironment that co-evolved with the tumor. Further, we utilize tumor microvasculature instead of the commonly used HUVECs. We and others have shown that these 2 cell types show significant differences with respect to their phenotype14 (Fig. 4b). Further, our analysis of a traditional, FCS-cultured glioma line, U87, revealed a different requirement for interaction as compared to GPCs. While U87 were stimulated by tMVEC-derived ECM alone, GPCs required a direct contact to the tMVECs. How could that difference be explained? While the GPCs are adapted to grow in a serum free medium in suspension, U87 usually grow adherent to the culture dish in FCS-containing medium. As serum contains ECM-components, most notably fibronectin,22 one could imagine that U87 underwent a selection for cells which, at least partially, depend on ECM anchorage and the addition of FCS-derived growth factors. Adhesion to substrate, withdrawal of growth factors and the addition of FCS has been shown to induce the differentiation of GPCs. Thus there would be no selective pressure for this phenotype in GPC cultures.

What could be a potential mechanism by which the tMVECs stimulate the proliferation of GPCs? While our current studies do not reveal a detailed mechanism, we show that substrate-adhesion or the presence of soluble, tMVEC-derived factors alone is not sufficient. We hypothesize that factor(s) that require close proximity between these 2 cell types could mediate the effects we observed. Such factors could be, for example, short range soluble factors that require close contact to function. Alternatively, the molecules involved in this interaction can provide novel targets to fight this disease.

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