Collagen IV and CXC chemokine-derived antiangiogenic peptides suppress glioma xenograft growth

Elena Victoria Rosca[a], Bachchu Lal[b], Jacob E. Koskimaki[a], Aleksander S. Popel[a] and John Laterra[b]

Peptides are receiving increasing attention as therapeutic agents due to their high binding specificity and versatility to be modified as targeting or carrier molecules. Particularly, peptides with antiangiogenic activity are of high interest because of their applicability to a wide range of cancers. In this study, we investigate the biological activity of two novel antiangiogenic peptides in preclinical glioma models. One peptide SP2000 is derived from collagen IV and the other peptide SP3019 belongs to the CXC family. We have previously characterized the capacity of SP2000 and SP3019 to inhibit multiple biological endpoints linked to angiogenesis in human endothelial cells in several assays. Here, we report additional studies using endothelial cells and focus on the activity of these peptides against human glioma cell growth, migration and adhesion *in vitro*, and growth as tumor xenografts *in vivo*. We found that SP2000 completely inhibits migration of the glioma cells at 50 μmol/l and SP3019 produced 50% inhibition at 100 μmol/l. Their relative antiadhesion activities were similar, with SP2000 and SP3019 generating 50% adhesion inhibition at 4.9 ± 0.82 and 21.3 ± 5.92 μmol/l, respectively. In-vivo glioma growth inhibition was 63% for SP2000 and 76% for SP3019 after 2 weeks of administration at daily doses of 10 and 20 mg/kg, respectively. The direct activity of these peptides against glioma cells in conjunction with their antiangiogenic activities warrants their further development as either stand-alone agents or in combination with standard cytotoxic or emerging targeted therapies in malignant brain tumors. *Anti-Cancer Drugs* 23:706–712 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

A recent study by the National Cancer Institute predicts that the societal costs attributed to brain cancer will increase by 0.83 billion dollars by the year 2020 [1]. This increase in cost is predicted despite new medical and therapeutic discoveries, thus supporting the urgent need for more cost effective and efficacious therapies. Peptides are receiving increased attention as therapeutic agents, due to their high binding specificity and versatility to be modified as targeting or carrier molecules [2–4]. Peptides are being increasingly used in therapeutic applications for multiple diseases including cancer. One example under development for multiple cancers is cilengitide, an RGD pentapeptide that inhibits $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [5,6]. Peptides with antiangiogenic activity [7,8] are of particular interest because of the role of angiogenesis as a process central to the development and malignant progression of multiple malignancies [9]. Glioblastoma multiforme (GBM) is one of the most common and aggressive brain tumors, with a dismal median survival of 12–15 months. Invasive gliomas such as GBM are characterized by insidious infiltration of tumor cells into the surrounding brain and robust tumor angiogenesis that together cause tumor growth, morbidity, and death [10,11]. The current standard of care for GBM includes surgical resection of the primary tumor, concurrent adjuvant chemotherapy, and radiation, followed by additional adjuvant chemotherapy. Bevacizumab, an antibody against vascular endothelial growth factor (VEGF), received expedited approved by the US Food and Drug Administration (May 2009) for the treatment of patients with progressive disease following prior therapy. Several studies have demonstrated that bevacizumab leads to an extension of progression-free survival without obviously prolonging the overall survival. The limitations of bevacizumab and other emerging antiangiogenic strategies may relate in part to the compensatory induction of an invasive glioma phenotype and growth pattern. Thus, more effective therapies using agents with multiple mechanisms of action (e.g. antiangiogenic + antiinvasion) are needed [9,12].

In this study, we investigate the application of two antiangiogenic peptides belonging to different classes for glioma therapy. Targeting the disease from different perspectives, angiogenic and tumorigenic, along with inducing these effects through different receptors and thus affecting different signaling and molecular pathways could yield an effective multimodal therapeutic approach.

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Chemokines are cytokines involved in the directed migration of leukocytes and are classified into four major classes: CXC, CC, C, and CX3C. Among these, chemokines belonging to the CXC family have been shown to be involved in tumor angiogenesis [13,14]. We have previously shown that peptides derived from this family are strongly active in inhibiting the migration and proliferation of human umbilical vein endothelial cells (HUVEC) [15,16] and are also capable of inhibiting tumor growth in a breast cancer xenograft model [17].

The other peptide that we investigate in this study is derived from collagen IV and it interacts with integrins, specifically with the $\alpha_V\beta_3$ and $\alpha_V\beta_1$ integrins, which are overexpressed on both tumor endothelial and cancer cells [16,18]. We have previously characterized the effect of this peptide on endothelial cells and found that it is capable of strongly inhibiting endothelial cell proliferation and also inhibiting migration (80% inhibition at 30 μg/ml) [19,20]. This peptide and a homologous mimetic peptide were shown to inhibit tumor growth in breast cancer [17,21] and lung cancer [22] xenograft models.

In this study, we focus on the characterization of the activity of the peptides on glioma cells and test their activity *in vivo* in glioma xenografts using U87 cells.

Materials and methods

Cell culture

HUVEC were purchased from Lonza (Lonza Inc., Allendale, New Jersey, USA) and were grown according to the manufacturer's recommendation using endothelial basal media (EBM-2) supplemented with the Bullet Kit [endothelial growth medium (EGM-2); Lonza]. Cells of passages 2–7 were used for experiments. U87 cells were originally purchased from the American Type Culture Collection and maintained in minimum essential medium with Earle Salts and L-glutamine (MEM 1 ×; Mediatech Inc., Manassas, Virginia, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Grand Island, New York, USA), 2 mmol/l sodium pyruvate (Gibco), 0.1 mmol/l MEM non-essential amino acids (Gibco), and penicillin–streptomycin (Gibco). Cells were grown at 37°C in a humidified incubator with 5% CO_2.

Peptide synthesis

The peptides were synthesized using solid-phase synthesis and were supplied as trifluoroacetic acid salts with an amidated C terminus by Abgent (San Diego, California, USA). The purity of the peptides was more than 95% and the supplier provided product characterization (MALDI-TOF and HPLC traces) as proof of molecular weight and purity. The 20-mer collagen IV derived peptide, which we call SP2000 (sequence LRRFSTMPFMFCNINNVCNF), was solubilized in 5% dimethyl sulfoxide (DMSO) and water due to its hydrophobic profile, whereas the 24-mer CXCL1 chemokine-derived peptide, which we call SP3019 (sequence NGRKACLNPASPIVKKIIEKMLNS), was solubilized in PBS. The pH of solubilized peptides was found to be around pH 7. For all experiments, the DMSO percentage was maintained at a nontoxic threshold (determined by toxicity curves of DMSO on cells) with a final DMSO percentage (< 0.2%) that was used as a control in all experiments.

Proliferation assays

A colorimetric-based proliferation assay using a water soluble tetrazolium (WST-1) (Roche Diagnostics Operations Inc., Indianapolis, Indiana, USA) proliferation reagent was used to assess the antiproliferative effect. Two thousand cells per well were plated in 96-well plates and allowed to adhere overnight. The next day, the medium was exchanged with a serum-containing medium containing either peptide or the solubilization vehicle for the control. Three days later, the medium with peptides was replaced with serum-free endothelial basal media-2 containing WST-1 reagent, and incubated for 4 h as per the manufacturer's protocol. The change in color is due to the formazan dye, which is the result of the cleavage of the tetrazolium salt WST-1 by the mitochondrial succinate-tetrazolium reductase. Absorbance measurements at 450 nm were carried out using a Victor V fluorescence plate reader (Perkin Elmer, Waltham, Massachusetts, USA). Dose–response curves of percent live cells (in comparison with untreated cells but incubated in complete media with 0.2% DMSO) were created. Assays were performed in at least two independent replicates and each replicate was performed using three experimental replicates.

Migration assay

Migration inhibition was investigated using the Oris Pro Migration assay (CMA 1.101; Platypus Technologies, Madison, Wisconsin, USA). Briefly, 40 000 cells/well in complete medium were added to a 96-well plates containing well stoppers to block the migration of cells to the center region of the wells. Cells were allowed to adhere for 4 h, after which the stoppers were removed and fully supplemented media with or without an experimental peptide were added to the wells. After 18 h, cells were stained with calcein AM (0.5 μg/ml) (Invitrogen, Grand Island, New York, USA) and the cells that migrated to the center of the well were quantified by reading fluorescence using a Victor V plate reader (Perkin Elmer) and also imaged using a Nikon microscope (Eclipse T-100); images were acquired with the CCD Sensicam mounted on a Nikon microscope (Cooke Company, Michigan, USA). The detection of the cells that migrated into the previously restricted region is possible due to the addition of a detection mask at the bottom of the plate, which obstructs from measurement the cells that did not migrate.

Adhesion assay

The adhesion inhibition activity of the peptides in cellular adhesion was assessed using the RT-CIM technology [23,24]. A total of 25 000 cells/well were plated in 16-well E-plates

(Roche Diagnostics Operations Inc.) in the presence or absence of the peptide. The adhesion was monitored over time (3 h) by measuring changes in electrical impedance, which is a direct measure of the cells adhering on the electrodes. Half maximal inhibitory concentration (IC_{50}) curves were created for each cell type and peptide. Assays were performed in at least two independent replicates and each replicate was performed using two experimental duplicates.

Tube formation assay

The compounds were tested for their ability to inhibit tube formation, an in-vitro correlate of angiogenesis; the protocol was described by Arnaoutova *et al.* [25,26] and it includes plating HUVEC on top of the basement membrane extract, and after incubation at 37°C, the cells naturally rearrange themselves in a network of tubes. Briefly, 50 µl/well of Matrigel (BD Biosciences, Sparks, Maryland, USA) was plated in a cold 96-well plate and incubated at 37°C for 30 min for polymerization. A total of 15 000 cells/well were added to the top of the gel and incubated in complete media in the presence or absence of a peptide for 19 h. Images were captured using the CCD Sensicam mounted on a Nikon microscope (Eclipse T-100). Assays were performed in at least two independent replicates and each replicate was performed using three experimental replicates and one image of a randomly chosen field was acquired per well.

Tumor xenografts

Animals were housed and treated according to the approved animal protocol of the Institutional Care and Use Committee at Johns Hopkins Medical Institution. Glioma xenografts were generated as described previously [27]. Female 6–8-week nude mice (National Cancer Institute, Frederick, Maryland, USA) were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Subcutaneous xenografts were generated by injecting 4×10^6 of cells in 0.05 ml of media subcutaneously into the dorsal flank of nude mice. When tumors reached $\approx 100\,mm^3$ in size, the mice were randomly divided into groups ($n = 5$ per group) and treatment was commenced. Peptides were administered once per day (for 14 days) intraperitoneally at doses of 10 mg/kg for SP2000 and 20 mg/kg for SP3019; these doses were based on our previous work [17]. Tumor volumes were estimated by measuring two dimensions [length (a) and width (b)] and calculating the volume as $V = ab^2/2$.

Data analysis

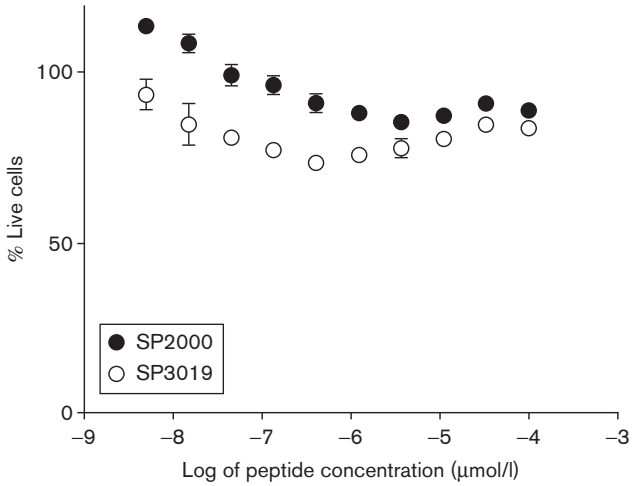
Statistical significance ($P < 0.05$) was determined within each experiment using the independent Student's t-test and analysis of variance along with Dunnett's test if we were comparing different sets of data with one group.

Results

Proliferation

Previous work from our laboratory has shown that both peptides (SP2000 and SP3019) inhibit the proliferation of HUVEC cells almost completely at 40 µg/ml [15,19]. In this study, we analyzed the effect of each peptide on the proliferation of human glioma cells. The results are presented in Fig. 1 and show that the peptides had little effect on U87 cancer cell proliferation.

Fig. 1

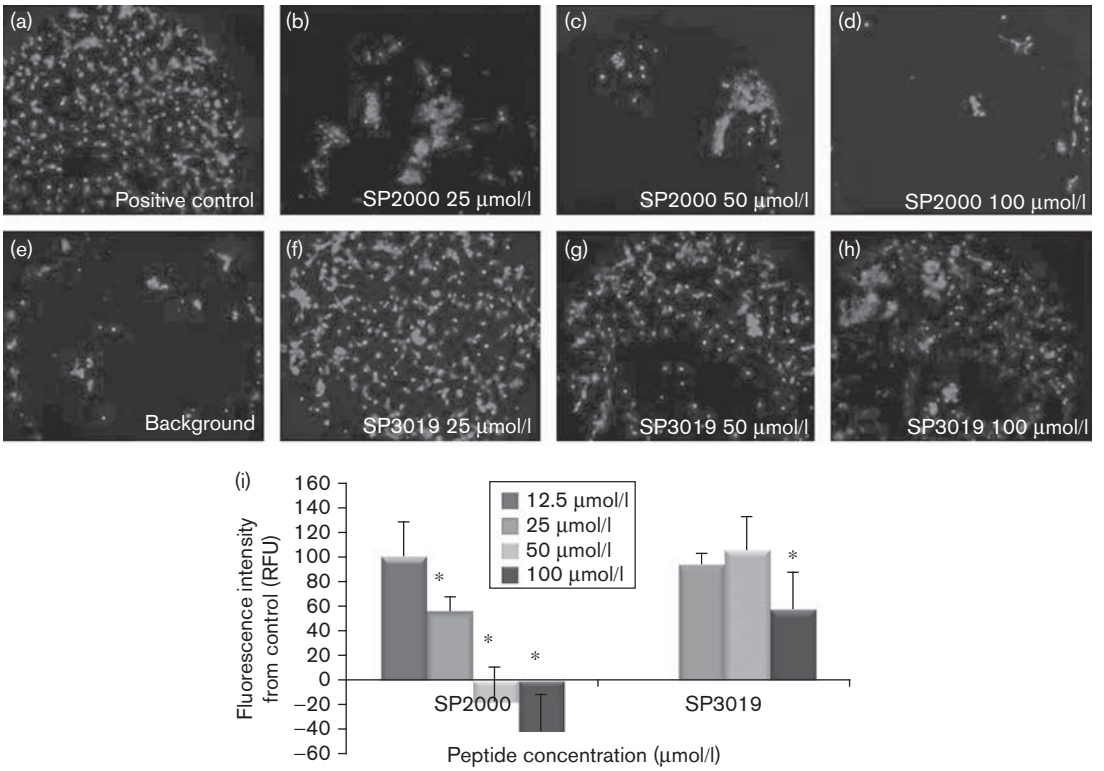


Proliferation activity: the activity of the peptides on U87 glioma cell proliferation. The cells were incubated in the presence of peptides and the percentage of live cells was calculated in comparison with cells incubated with media containing the solubility vehicle. Error bars depict SD.

Migration

The capacities of these peptides to inhibit endothelial cell migration have been characterized previously. SP2000 and SP3019 inhibited HUVEC migration by 80% at 30 µg/ml and 50% at 20 µg/ml, respectively [15,19]. We analyzed the potential activity of the peptides on U87 glioma cell migration using the Platypus assay. Cells were exposed to peptides for 18 h and allowed to migrate into a previously restricted area. The results presented in Fig. 2 show strong inhibition of glioma cell migration. The SP2000 peptide displayed very strong inhibitory activity (completely inhibits migration of the glioma cells at 50 µmol/l) consistent with its molecular target integrins that are critical to cellular migration [16]. SP3019 was substantially less potent in this assay, showing 50% inhibition at 100 µmol/l. The quantification of the migration was performed by measuring the fluorescence intensity of the calcein-labeled cells and it is shown in Fig. 2i; the negative values are a result of the subtraction of the background. In some cases, the alignment of stoppers is not quite exact, thus allowing some cells to enter the occlusion zone, leading to negative measurements when the average of the background is subtracted from the experimental value. However, the pictures captured show that the treatment and the background conditions are very similar, thus indicating no migration in the case of the treatment.

Fig. 2



Migration activity: the inhibitory activity of the peptides on the migration of the glioma cells U87. (a) Positive control (untreated cells); (b–d) inhibition activity of the SP2000 at several concentrations; (e) typical background measurement (image of the well with the stopper removed immediately preceding the imaging); and (f–h) inhibitory activity of the SP3019 peptide. (i) Quantification of the images. The negative values are due to the subtraction of the background values, which can vary significantly between the wells depending on the alignment of the stopper in the well. Error bars depict SD. * indicates statistical significance ($P<0.05$) from the control.

Adhesion

The strong inhibitory activity of SP2000 against both HUVEC and U87 cell migration predicted that it would likely also inhibit HUVEC and glioma cell adhesion. Thus, we tested these peptides using the RT-CIM adhesion assay and the results are presented in Fig. 3. The SP2000 peptide was effective at inhibiting the adhesion of both cell types as depicted by the IC_{50}: SP2000 1.7 ± 0.67 µmol/l on HUVEC and 4.9 ± 0.82 µmol/l on U87 cells. SP3019 displayed a lower potency, with an IC_{50} of 23.59 ± 15.5 µmol/l on HUVEC and 21.3 ± 5.92 µmol/l on U87 cells.

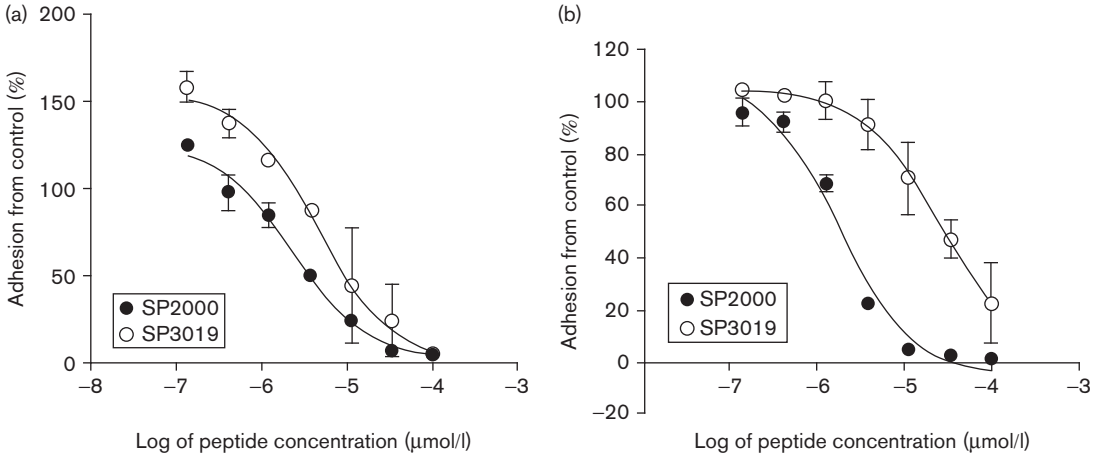
Tube formation

Tube formation is a typical comprehensive in-vitro assay to assess angiogenesis. This assay incorporates the adhesion and migration dynamics and should thus yield a more realistic measurement of the activity of compounds *in vivo*, where the interplay between all effects is important in suppressing tumor growth. Figure 4 shows that both peptides are capable of inhibiting tube formation of HUVEC when plated on a Matrigel extract. We have tested the activity of the peptides at a high concentration of 100 µmol/l because at this concentration, the effects of the peptides are drastic, leading to complete inhibition of the tube formation, thus not requiring quantification of the effect, which may be subjective and difficult to interpret.

In-vivo tumor growth suppression

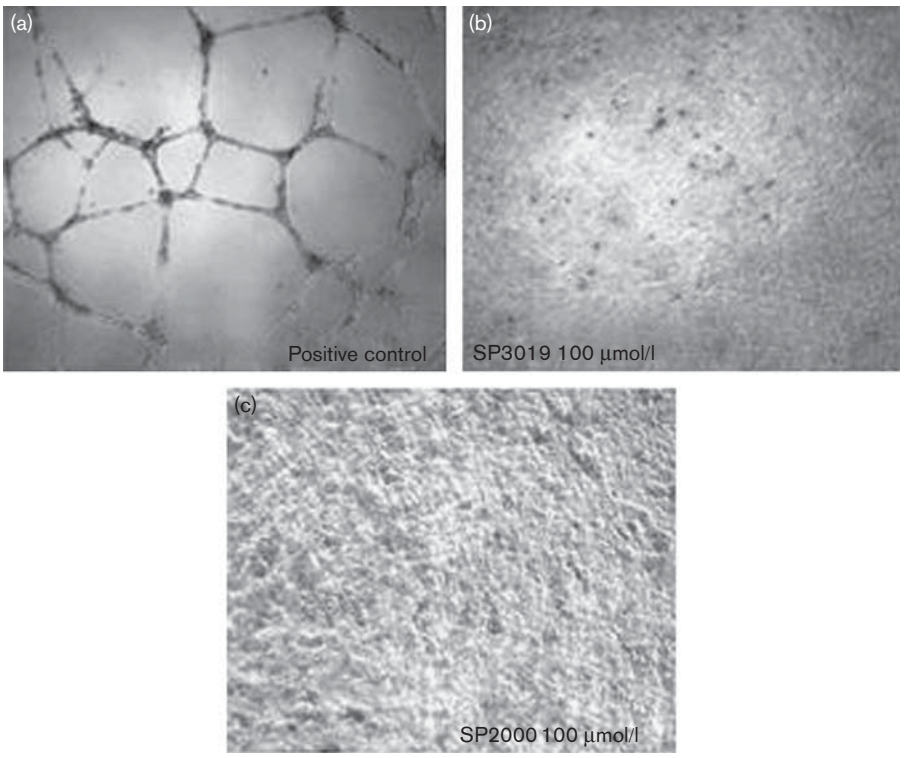
On the basis of the in-vitro peptide activities on both endothelial and tumor cells, we tested their therapeutic potential using the U87 human glioma xenograft model. Immune-deficient Nu/nu mice received U87 cells by a subcutaneous injection and daily treatment with peptides was initiated after tumors reached a volume of $\sim 100\,mm^3$. Peptides were administered intraperitoneally at 10 mg/kg for SP2000 and 20 mg/kg for SP3019 on the basis of our previous experiments with other types of cancer [17,21,22]. Both peptides were active in inhibiting tumor growth, as shown in Fig. 5. Tumor volumes in the treated groups were significantly different from those in the control group starting with day 3 and continued throughout the experiment ($P < 0.05$). At day 14, the tumors in the treated groups displayed a size reduction of 62.5 and 75.7% for SP2000 and SP3019, respectively. Treatment with the scrambled peptide was tested for SP3019 in this study and for SP2000 in a previous study [17]; the results were not statistically different from the control but were statistically different from the treatment groups.

Fig. 3



Inhibition of adhesion. (a) Inhibition activity on the adhesion of human umbilical vein endothelial cell and (b) activity of both peptides at inhibiting the adhesion of U87 glioma cells. Error bars depict SD.

Fig. 4

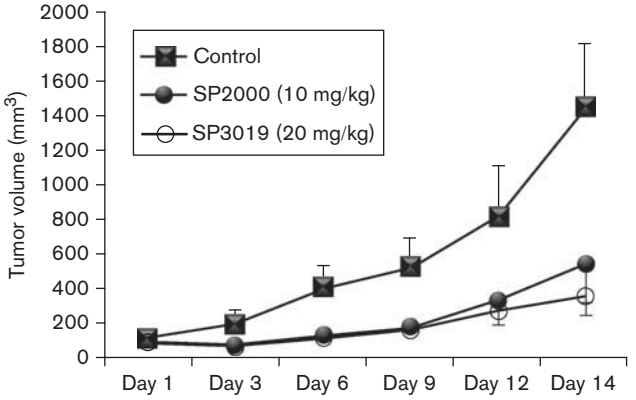


Tube formation inhibition. (a) Positive control, human umbilical vein endothelial cell on Matrigel in complete media without any peptide treatment; (b) the inhibition of tube formation with SP3019; and (c) with SP2000.

Discussion

GBM or gliomas account for ~40% of malignant brain tumors and 10 000 GBM cases are diagnosed annually, with a median survival time of 15 months and a 5-year survival of less than 5% (National Cancer Institute). GBM are highly vascularized tumors, which makes tumor vasculature a primary therapeutic target. A *milieu* of molecules that play a role in GBM tumorigenesis and angiogenesis have been identified and some of them have been investigated as drug targets [28]. Among the

Fig. 5



In-vivo tumor xenograft inhibition. Tumor volume of a subcutaneous glioma xenograft measured every third day. Squares depict the control group, which received an intraperitoneal injection of the solubility vehicle (PBS); the solid circle represents the tumor growth of the group treated with daily intraperitoneal injections of SP2000 and the open circles show tumors treated with SP3019. Error bars depict SD.

molecules that serve as primary targets for antiangiogenic therapy are VEGF and their receptors VEGFRs; platelet-derived growth factor and its receptors; matrix metalloproteinase, especially MMP2 and MMP9; integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, transcription factor hypoxia-inducible factor 1α; and chemokines and their receptors [28]. The peptides investigated in this study target two of the above categories: the integrin and chemokines families. We have shown that both peptides are active at inhibiting proliferation, migration, and adhesion and tube formation of endothelial cells and are also have the ability to inhibit the adhesion and migration of tumor cells. The collagen-derived peptide, SP2000, is capable of strongly inhibiting the migration of U87 cells *in vitro*. One of the reasons why gliomas are so difficult to treat is because the tumor cells are highly migratory, and at the time of surgical resection, many cells have already disseminated from the primary tumor, giving rise to secondary tumors at a later point [29]. Thus, therapeutic approaches that inhibit the migration of tumor cells are promising for not only being effective in inhibiting primary tumor growth but also in reducing the development of anatomically distant secondary tumors. In-vitro tumor spheroids have been shown to be characterized by two regions: core and rim regions. Cells in the core are categorized as proliferative, whereas cells in the rim are highly migratory. These two cell phenotypes also show differential sensitivity to conventional chemotherapeutic treatment, with the cells in the core region being responsive and the rim cells not being responsive, indicating that the migratory phenotype might be responsible for the low therapeutic response observed clinically [29,30]. Thus, the peptides presented here show a strong ability to inhibit migration; hence, perhaps as therapeutic agents in conjunction with chemotherapy, they could alter the migratory phenotype to a proliferating profile, thus increasing the sensitivity to chemotherapy. This, in conjunction with ability to reduce tumor vasculature, could result in a considerable change in the overall response to therapy.

In-vivo tumor growth inhibition was strong; there were 62.5 and 75.7% reductions in sizes for SP2000 and SP3019, respectively. These activities correlated well with the in-vitro activity and it may be the result of a dual targeting approach: endothelial and tumor cells. Previously, we have demonstrated that treatment with the SP2000 peptide leads to a decrease in vasculature and in proliferative cells, both mouse and human, in comparison with the control, whereas the SP3019 peptide led to a decrease in microvascular density but an increase in the proliferation of mouse cells, which could be due to a stimulation of macrophages [17]. Nude and SCID are immunocompromised mice with a loss in either T or both T and B cells; however, they have normal or even higher levels of macrophages and natural killer cells [31]. The previous results, indicating an increase in proliferation of mouse cells, coupled with a higher CD31 staining present in this study (results not shown), are indicative of the stimulation of macrophages and natural killer cells (both cells types express the CD31 marker). Thus, treatment with the peptide derived from chemokines could also represent a multimodality approach by stimulating the infiltration of immune cells into the tumors, which could lead to tumor growth suppression [32,33]; these aspects warrant further investigation.

In conclusion, in this study, we present the novel application of two peptides, one derived from collagen IV (SP2000) and the other derived from the CXCL1 chemokine (SP3019), for glioma growth inhibition. The peptides showed strong activity in in-vitro screening, which translated into strong tumor growth inhibition in a glioma xenograft. These peptides show multimodal potential that warrants further study in combination with standard chemotherapeutic approaches.

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Conflicts of interest

There are no conflicts of interest.

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