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VEGF-dependent induction of CD62E on endothelial cells mediates glioma tropism of adult haematopoietic progenitor cells

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Haematopoietic progenitor cells (HPC) are attracted by experimental gliomas *in vivo*. This attraction is further enhanced by irradiation or hypoxic preconditioning of the glioma cells. Adhesive interactions might be critical to the preferential accumulation of HPC within the glioma tissue. Here, we studied the interactions of HPC with endothelial cells. Exposure of human cerebral endothelial cells (SV-HCEC), human microvascular endothelial cells (HMEC) and brain tumour endothelial cells derived from human glioblastomas (BTEC) to supernatants of glioma cells and primary glioma cells (SN-G) induced the expression of E-selectin (CD62E). CD62E expression was further enhanced when the glioma cells had been exposed to irradiation or hypoxia prior to the collection of supernatants, as well as by irradiation or exposure to hypoxia of the endothelial cells. Vascular cell adhesion molecule 1 (VCAM-1) was constitutively expressed on SV-HCEC, HMEC and BTEC, but was not modulated by SN-G, irradiation or hypoxia. Transendothelial HPC migration was enhanced after CD62E induction *in vitro*. Neutralizing antibodies to CD62E strongly reduced the homing of lin$^-$Sca-1$^+$c-kit$^+$ cells to orthotopic SMA-560 gliomas *in vivo*. Tissue microarray sampling normal brain tissue and astrocytomas of WHO grades II–IV revealed a selective expression of CD62E on endothelial cells of tumour vessels. SN-G-induced CD62E expression on endothelial cells *in vitro* required transforming growth factor (TGF)-β signalling in glioma cells and vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGF-R2) signalling in endothelial cells. Further, we observed a nuclear factor kappa B-dependent activation of the CD62E promoter peaking at 12 h after VEGF-R2 activation by glioma-derived VEGF. Taken together, we identify glioma cell-induced CD62E expression on endothelial cells as one mediator of the glioma tropism of HPC.

Keywords: brain tumour; haematopoietic progenitor cells; hypoxia; irradiation; vascular endothelial growth factor, CD62E

Abbreviations: BTEC = brain tumour endothelial cells isolated from human glioblastoma tissue; CD = cluster of differentiation; CD62E = E-selectin; CD62P = platelet selectin; CXCL12 = CXC chemokine ligand 12; DAPI = 4′,6-diamidino-2-phenylindole; ELISA = Enzyme-linked immunosorbent assay; FCS = foetal calf serum; G-CSF = granulocyte colony stimulating factor; HIF = hypoxia-inducible factor; HMEC = human microvascular endothelial cells, HPC = haematopoietic progenitor and stem cells; IL = interleukin; LSK = lin$^-$Sca-1$^+$c-kit$^+$; NFκB = nuclear factor kappa B; NT = no treatment; pVEGF-R2 = phosphorylated VEGF-R2; sKitL = soluble Kit ligand; sCD62E = soluble E-selectin; SCF = stem cell factor; SDF = stromal cell-derived factor; SN-G = supernatant of glioma cell lines LNT-229, LN-308 and primary glioma cultures TI13, TI32, TI59; SN-Gp = supernatant of LNT-229 puro cells; SN-GpSD-208 = supernatant of SD-208-treated LNT-229 puro cells; SN-G-siTGF-β = supernatant of LNT-229 siTGF-$\beta_{1,2}$ cells stably expressing shRNAs

targeting TGF-β_1 and TGF-β_2; SN-FHAS = supernatant of SV-FHAS cells, SN-GRT = supernatant of irradiated glioma cells, SN-GHO = supernatant of hypoxic glioma cells; SFM = serum-free medium; SFI = specific fluorescence index; SV-HCEC = human cerebral endothelial cells; SV-FHAS = human astrocytic cell line; TGF-β = transforming growth factor-β; TNF-α= tumour necrosis factor-α; VCAM = vascular cell adhesion molecule; VEGF = vascular endothelial growth factor; VEGF-R = VEGF receptor

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Introduction

Intracerebral experimental gliomas attract intravenously injected $CD34^+$ haematopoietic progenitor and stem cells (HPC). The underlying molecular pathways leading to the glioma tropism of HPC involve a transforming growth factor (TGF)-β-dependent signalling cascade regulating the release of stromal cell-derived factor (SDF)-1α/CXC chemokine ligand 12 (CXCL12) by glioma cells. CXCL12 interacts with the chemokine receptor CXCR4 on HPC. Further, matrix metalloproteinase 9 cleaves and thereby releases soluble Kit ligand (sKitL), which interacts with the receptor CD117 on HPC (Tabatabai *et al.*, 2005). Cerebral irradiation promotes the homing of HPC to the tumour bulk and to tumour satellites *in vivo*. Supernatants of irradiated or hypoxic glioma cells enhance HPC migration *in vitro* due to a TGF-β-dependent, hypoxia-inducible factor-1α (HIF-1α)-mediated induction of CXCL12 promoter activity in glioma cells (Tabatabai *et al.*, 2006). CXCL12 is also an important mediator for the attraction of mesenchymal stem cells to experimental gliomas (Nakamizo *et al.*, 2005). A systemic gradient of CXCL12 alone, however, does not explain the glioma-mediated attraction of intravenously injected HPC. Moreover, further insight into the mechanism of the glioma tropism is required for the design of a HPC-based cellular therapy that specifically targets gliomas.

The regional expression of endothelial adhesion molecules may contribute to the specificity of HPC homing towards gliomas. Similarities in the trafficking and homing of HPC and leucocytes to particular tissue sites arise. The diapedesis of leucocytes to sites of infection and inflammation is an important step in their recruitment. This process ensures appropriate innate and adaptive immunological responses. Adhesion molecules mediate rolling and arrest of leucocytes on the vessel wall as key steps prior to the transmigration across the endothelial layer (Millan *et al.*, 2006; Nieminen *et al.*, 2006).

Adhesion is also critical for the interaction of HPC within the haematopoietic microenvironment in the bone marrow. Important mediators of this adhesive interaction include vascular cell adhesion molecule 1 (VCAM-1), platelet selectin (CD62P) and endothelial selectin (CD62E) (Frenette *et al.*, 1998; Katayama *et al.*, 2003). CD62P is deposited in storage granules of platelets and endothelial cells. After activation, it is translocated to the surface (Bevilacqua *et al.*, 1989). VCAM-1 and CD62E are solely expressed on endothelial cells (Mazo *et al.*, 1998). In this study, we analysed the role of HPC adhesion to endothelial cells in the glioma tropism of HPC.

Material and Methods

Cell culture

Human adult HPC were isolated by anti-CD34 immunomagnetic microbeads (Bautz *et al.*, 2001). After informed consent, peripheral blood cells were obtained from healthy donors or patients with non-haematological malignancies during G-CSF-induced stem cell mobilization, according to the guidelines of the ethics committee of the University of Tübingen (project number 268/2003-LP). The human CD34 antigen is a reliable marker for identifying a small fraction of human bone marrow and peripheral blood mononuclear cells consisting of primitive uncommitted and pluripotent haematopoietic progenitor cells as well as haematopoietic stem cells (Andrews *et al.*, 1986; Berenson *et al.*, 1988; Terstappen *et al.*, 1991; Van Epps *et al.*, 1994; Gothot *et al.*, 1998; Gao *et al.*, 2001; Albo *et al.*, 2004). The source, generation and culture conditions of the cerebral vascular endothelial cell line SV-HCEC and the astrocytic cell line SV-FHAS have been described (Muruganandam *et al.*, 1997). Brain tumour endothelial cells (BTEC) were isolated from freshly resected human glioblastomas and characterized for their endothelial cell phenotype as described (Miebach *et al.*, 2006). Briefly, the tissue was minced, homogenized and digested enzymatically. The cells were separated by percoll gradient centrifugation. CD31-positive cells were isolated by magnetic beads (Dynal, Hamburg, Germany). Only passage 0–2 cells were used for the experiments. Human microvascular endothelial cells (HMEC) were purchased from Cascade Biologics (Invitrogen GmbH, Karlsruhe, Germany). All glioma cell lines were kindly provided by Dr N. de Tribolet (Lausanne, Switzerland). In contrast to other LN-229 sublines, LN-229 glioma cells cultured in our laboratory exhibit wild-type p53 status (Wischhusen *et al.*, 2003) and are designated LNT-229. The generation of LNT-229 sublines stably depleted from TGF-β_1 to TGF-β_2 by RNA interference (LNT-229 siTGF-$\beta_{1,2}$) and the control cells (LNT-229 puro) has been described (Friese *et al.*, 2004). The following primary glioma cultures were used: T113, T159, derived from glioblastomas and T132, derived from an anaplastic astrocytoma. The primary human glioma cultures were established from samples with high tumour content (Bähr *et al.*, 2003). Supernatants of glioma cells (SN-G) were generated in serum-free medium (SFM): 1.5 million glioma cells were first seeded in serum-containing medium in T75 cell culture flasks. The medium was removed on the following day and the cells were washed three times with PBS. Seven millilitres SFM were added, 48 h later, supernatants were harvested. Supernatant was concentrated using a Centriplus centrifugal filter device YM-3 (3 kD cut-off, Millipore, Eschborn, Germany). Protein quantification was performed using the Bradford assay (BioRad, Munich, Germany).

Treatment of endothelial cells with SN-G was performed for 6, 12, 24, 48 or 96 h. Incubation of cells in hypoxia was performed at 37°C and 1% O_2 for 6, 12, 24, 48 or 96 h (Wick *et al.*, 2002*a*). Cells were irradiated at 2 or 8 Gy γ-cell, Nordion, Kanata, CA, USA) and analysed at 6, 12, 24, 48 or 96 h thereafter (Wick *et al.*, 2002b). Recombinant TGF-β_2 and vascular endothelial growth factor ($VEGF_{165}$), neutralizing antibodies to VEGF (clone 26503) and VEGF-R2 (clone 89106) were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). SD-208 was provided by Scios (Fremont, CA, USA) (Uhl *et al.*, 2004). siRNA pools targeting human TGF-β_1, TGF-β_2 or VEGF containing four selected siRNA duplexes each with a modification pattern that addresses off-target effects caused by both strands (ON-TARGETplus SMARTpool) and the non-targeting control pool (ON-TARGETplus siCONTROL Non-targeting Pool) were designed by and purchased from Dharmacon (Lafayette, CO, USA). The pool of siRNA duplexes targeting TGF-β_1 had the following sense sequences: 5′-AUUG AGGGCUUUCGCCUUAUU-3′, 5′-CCGAGAAGCGGUACCUGA AUU-3′, 5′-GCAGAGUACACACAGCAUAUU-3′, 5′-GTGACUAU CCACCUGCAAGAUU-3′. The siRNA duplexes targeting TGF-β_2 had the following sense sequences: 5′-GGAUUGAGCUAUAUCAG AUUU-3′, 5′-CUGCGUGUCCCAAGAUUUAUU-3′; 5′-GAUGCG GCCUAUUGCUUUAUU-3′, 5′-GAGCAUGCCCGUAUUUAUGU U-3′. The siRNA duplexes targeting VEGF had the following sense sequences: 5′-GCAGAAUCAUCACGAAGUG, 5′-CAACAAAUGUG AAUGCAGA, 5′-GGAGUACCCUGAUGAGAUC, 5′-GAUCAAAC CUCACCAAGGC. Lipophilic transfection was performed with Metafectene Pro (Biontex, Martinsried, Germany).

Transendothelial migration of HPC

Migration assays were performed in transwell plates (Costar, Cambridge, MA, USA) of 6.5 mm diameter with 5 µm pore filters. SV-HCEC, HMEC or BTEC were plated at 10^5 cells/transwell on filters coated with fibronectin. Non-adherent cells were removed after 24 h. The adherent cells were cultured for 48 h to obtain confluent endothelial monolayers. Confluency of the endothelial monolayers was confirmed by measuring permeability for albumin (Möhle *et al.*, 1997,1998; Naiyer *et al.*, 1999). The monolayers were used unstimulated, stimulated with SN-G or after exposure to irradiation or hypoxia. Prior to the migration experiment, the monolayer was washed three times with PBS. The 10^5 $CD34^+$ HPC in 200 µl SFM were added to the upper compartment. SFM, recombinant CXCL12 or SN-G were added to the lower compartment. In neutralization experiments, the SV-HCEC were pre-incubated for 4 h at 37°C with α-CD62E (R&D, clone BBIG-E4[5D11]) or α-VCAM-1 (R&D, clone BBIG-V1 [4B2]) or appropriate control antibodies. Migration was assessed at 37°C, 5% CO_2 for 16 h. Cells that had migrated to the lower compartment were quantified with the CyQuant Assay (Invitrogen, Karlsruhe, Germany).

Reporter assays

For dual luciferase/renilla assays (Dyer *et al.*, 2000) 200 ng of the respective reporter constructs was cotransfected with 20 ng of pRL-CMV. pGL3b.CD62E contains the full-length promoter of CD62E (Nübel *et al.*, 2004). pNFκB-Luc contains a firefly luciferase gene that is especially designed for the detection of nuclear factor kappa B (NFκB) signal transduction pathway activity. It contains four copies of the NFκB consensus sequence fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter. The binding of endogenous NFκB leads to induction of transcription and activation of the reporter gene in the transfected cells indicating transcriptional activity of NFκB. Ad-IkBM, containing the NFκB repressor, and the control Ad-Mock were kindly provided by Dr P. A. Baker (McGill University, Ottawa, Canada).

TransAM NFκB family

Nuclear extracts were prepared (Nuclear Extract Kit, Active Motif, Rixensat, Belgium) from SV-HCEC, HMEC or BTEC. Raji extracts, provided by the assay, served as positive control. Nuclear extracts from NIH3T3 fibroblasts served as negative control. The transcriptional activity of the NFκB family p50, p65, p52, c-Rel and RelB was analysed by the transcripton factor assay TransAM NFκB family (Active Motif) according to manufacturer's protocol. Briefly, this assay is based on an enzyme-linked immunosorbent assay (ELISA) format using 96-well plates coated with immobilized oligonucleotides containing the NFκB consensus site. The active form of NFκB contained in nuclear extracts of cells binds to the oligonucleotides. Primary antibodies recognizing epitopes on activated p50, p52, p65, c-Rel or RelB and HRP-conjugated secondary antibodies are used for the detection and quantification of active NFκB family members by spectrophotometry.

Immunoblot

Immunoblots were performed as described (Tabatabai *et al.*, 2006). The following antibodies were used: phospho-Smad 2 (Ser 465/467), total Smad 2 (Cell Signaling, Boston, MA, USA), NFκB p50 and p65 (Santa Cruz, CA, USA) and GAPDH (Chemicon, Billerica, MA, USA).

Enzyme-linked immunosorbent assay

The concentrations of VEGF, sCD62E, TGF-β_1 and TGF-β_2 in the supernatants were determined by Quantikine Immunoassays from R&D Systems. A sandwich ELISA was performed to measure the levels of phosphorylated VEGF-R2 and total VEGF-R2 in cell lysates (Duo Set IC, R&D Systems). The proliferation of cells was assessed by a cell proliferation biotrak ELISA system (GE Healthcare, Buckinghamshire, UK).

Flow cytometry

SV-HCEC, HMEC or BTEC were grown in 12-well plates either untreated or stimulated with the indicated supernatant, hypoxia or irradiation for 6, 12, 24, 48 or 96 h. Flow cytometry was performed with a FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) (Eisele *et al.*, 2006). The following antibodies (R&D Systems) were used: anti-human CD62E, VEGF-R2 and VCAM-1. Signal intensity was calculated by dividing mean fluorescence obtained with specific antibody divided by signal intensity obtained with the isotype control antibody (specific fluorescence index, SFI).

Human tissue samples and tissue microarray

We investigated 120 glioblastoma samples (WHO grade IV) obtained from the tumour bank of the Institute of Neuropathology, University of Tuebingen derived from patients who underwent surgical treatment from 1993 to 2003 at the Department of Neurosurgery in Tuebingen or in the Department of Neurosurgery in Seesen. Diffuse astrocytomas WHO grade II ($n = 10$) and

Table 1 Clinical data

Origin of tissue	Number	Median age (range) [years]	Female	Male
Normal brain	33 brains	50.3	15	18
	60 samples	(22–77)		
Diffuse astrocytoma	10 tumours	39.4	3	7
WHO grade II	10 samples	(5–72)		
Anaplastic astrocytoma	10 tumours	44.7	–	10
WHO grade III				
	10 samples	(12–67)		
Glioblastoma	88 tumours	58.1	35	53
WHO grade IV	120 samples	(23–88)		

anaplastic astrocytomas WHO grade III ($n = 10$) were also investigated. All samples from diffuse and anaplastic astrocytomas were taken from primary tumours prior to therapy. Among 120 glioblastoma samples, 88 were derived from primary and 32 from recurrent tumours. Shortly after surgical removal, all specimens were fixed with 4% formalin (pH 7.4) and subsequently embedded in paraffin followed by preparation as tissue microarray (Warth *et al.*, 2007). Further, 60 samples from 33 autopsy cases of a normal brain bank were added. Clinical data are summarized in Table 1. The tumour and normal brain tissues were stained with H&E, mouse anti-human antibodies to CD31 (Clone JC70A, Dako Cytomation, Glostrup, Denmark, 51 mg/ml), goat anti-human CD62E antibodies (Clone BBA18, R&D Systems, diluted 1:50) or the respective isotype control antibodies.

Animal studies

On day 0, 10^4 SMA-560 cells were stereotactically implanted into the right striatum of VM/Dk mice. On day 7, LSK were isolated from the bone marrow of VM/Dk mice as described (Tabatabai *et al.*, 2005). Briefly, femora and tibiae of VM/Dk mice were flushed with 2% FCS-containing PBS, erythrocytes were lysed, bone marrow cells were incubated for 60 min at 4°C with the lineage marker antibodies rat anti-mouse CD4, CD8a, CD45R/B220, Gr-1, CD11b and TER119 (BD Biosciences Pharmingen, San Diego, CA, USA), then washed with PBS prior to the addition of pre-washed sheep anti-rat IgG magnetic beads (Dynabeads, Dynal, Biotech ASA, Oslo, Norway) at a 4 : 1 ratio of beads/cells. After 45 min, beads and attached cells were magnetically removed. The remaining lineage-depleted (lin^-) cells were sorted with anti-$Sca1^+$ beads by MACS (Miltenyi Biotech, Bergisch Gladbach, Germany). The check of purity by flow cytometry revealed that <5% of the isolated LSK expressed CD4, CD8, CD11b, B220 or Gr-1. LSK were between 95% and 98% positive for Sca1 and >99% positive for c-kit (BD Biosciences Pharmingen). The LSK were cultured with murine IL-3 (20 ng/ml), IL-6 (50 ng/ml) and SCF (50 ng/ml) (R&D Systems) over night. On day 8, neutralizing rat anti-mouse CD62E (BD Bioscience, clone 10E9.6) or control antibodies were bolus-injected intraperitoneally (200 µl) and intravenously (5 µl). Four hours later, PKH26 (Sigma-Aldrich, Taufkirchen, Germany) stained LSK were injected intravenously. On day 10, mice were sacrificed. From each experimental group, three brains were analysed by flow cytometry and three brains by histology. For flow cytometry, 5 mm^3 of the tumour-bearing as well as the contralateral hemisphere were dissociated in single cell suspension. For histology, 8 µm cryostat sections were stained with H&E and antibodies to vWF (Dako Cytomation) or CD62E (BD Biosciences). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Vectashield with DAPI, Vector Laboratories, Axxora GmbH, Loerrach, Germany).

Statistical analysis

Quantitative data were obtained for migration, ELISA, flow cytometry and reporter assays as indicated. Data are expressed as mean and SEM. Statistical significance was assessed by one-way ANOVA followed by Tukey's *post hoc* test (Excel, Microsoft, Seattle, WA, USA). The experiments reported here were performed at least three times in triplicate with similar results.

Results

Transendothelial HPC migration is enhanced after pretreatment of endothelial cells with SN-G

The molecular signature of endothelial cells depends on the environment. Endothelial cells from vessels within normal tissue are different from endothelial cells of vessels within inflamed or neoplastic tissue (Frenette *et al.*, 1996*b*). To simulate the conditioning of endothelial cells of vessels within glioblastoma tissue *in vitro*, we cultured cerebral endothelial cells (SV-HCEC) or HMEC in supernatant of LNT-229, LN-308, T113, T159 or T132 glioma cells. Further, we used BTEC from human glioblastoma specimens. HPC migrated through an untreated SV-HCEC-, HMEC (data not shown) and BTEC layer (Fig. 1). The migration was significantly enhanced after the pre-incubation of all three types of endothelial cell layer with different SN-G. Pretreatment of the SV-HCEC, HMEC (data not shown) and BTEC with hypoxia and irradiation also increased the migration of HPC. In contrast, incubation with supernatant of the astrocytic cell line SV-FHAS (SN-FHAS) did not modulate transendothelial migration. Thus, SN-G, hypoxia or irradiation alter the characteristics of endothelial cells and hence promote the transendothelial migration of HPC.

SN-G induces the expression of CD62E on SV-HCEC and HMEC

To understand the increase in transendothelial HPC migration, we performed flow cytometry for the detection of VCAM-1 and CD62E on SV-HCEC, HMEC or BTEC either un- or pretreated with SN-G, irradiation or hypoxia. Untreated SV-HCEC, HMEC and BTEC constitutively expressed VCAM-1 at SFI values of 1.8, 1.7 and 1.9. The expression of VCAM-1 was not altered after pretreatment of the endothelial cells with SN-G, irradiation or hypoxia (data not shown). In contrast, CD62E was expressed on untreated BTEC but not on untreated SV-HCEC or HMEC. However, SN-G enhanced or induced the expression of CD62E as did hypoxia or irradiation (Fig. 2). Pretreatment with $VEGF_{165}$ served as a positive control.

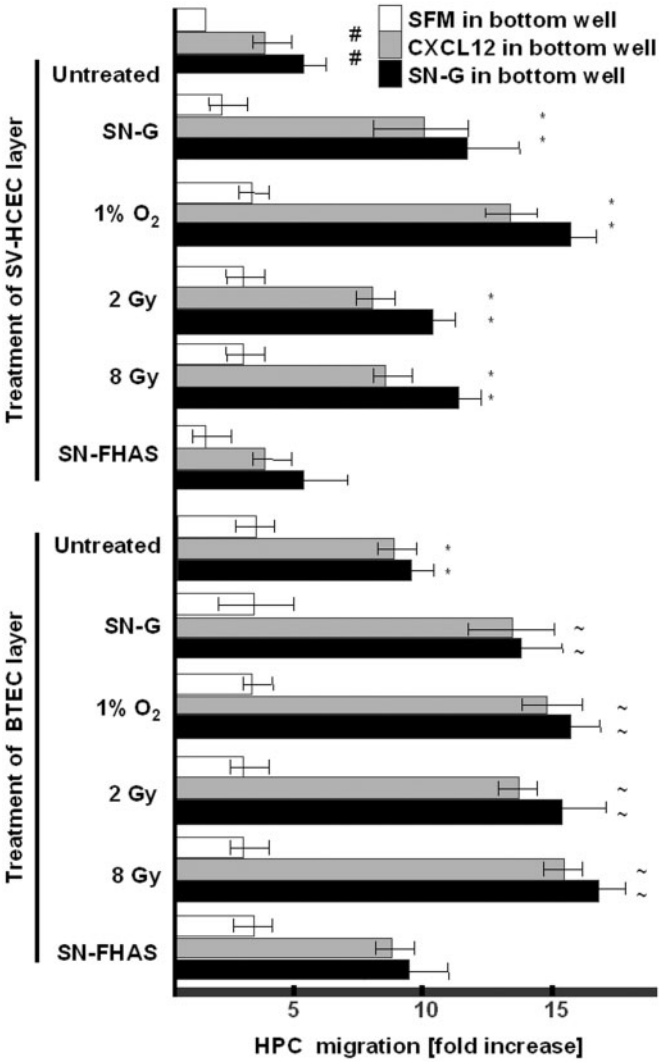

Fig. 1 Transendothelial migration of HPC through an SV-HCEC and BTEC layer. SV-HCEC or BTEC, either untreated or pretreated with SN-G, hypoxia at 1% O_2 for 24 h or irradiation at 2 and 8 Gy, were seeded on the transwells of a chemotaxis chamber. HPC (10^5) were added to the upper chamber. SFM, CXCL12 or supernatant of LNT-229 were added to the lower compartment. HPC migration was assessed 16 h later. The bars indicate mean HPC migration relative to migration towards SFM through an untreated SV-HCEC layer and SEM ($n = 3$, $^{\#}P < 0.05$ compared with untreated SV-HCEC layer/SFM bottom well; $^{*}P < 0.05$ compared with untreated SV-HCEC layer/CXCLI2 bottom well; $P < 0.05$ compared with untreated BTEC layer/CXCLI2 in bottom well).

Endothelial cells of tumour vessels in human astrocytomas but not normal brain vessels highly express CD62E

To confirm the relevance of CD62E induction on endothelial cells *in vitro*, we performed immunohistochemistry on human tissue microarray sampling normal brain sections, diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III) and glioblastomas (WHO grade IV). Normal brain tissue contains small capillaries and vessels whereas the vessels in glioblastomas are characteristically composed of enlarged and thickened endothelial cell layers. CD31 staining labelled endothelial cells within the vessel walls of normal and neoplastic tissue. CD62E immunoreactivity was absent on endothelial cells of blood vessels within normal human brain samples. In contrast, CD62E was present in all neoplastic samples. CD62E strongly correlated with blood vessel morphology. Large vascular proliferations in glioblastoma samples showed the strongest CD62E immunoreactivity whereas smaller, more regular blood vessels found in the same tumours displayed a weaker CD62E staining intensity (Fig. 3). There was no difference between CD62E expression in samples derived from primary or recurrent glioblastomas. A slight cytoplasmic CD62E staining was detected in tumour cells of most tumour samples. This prompted us to determine the concentration of soluble CD62E (sCD62E) in SN-G and in supernatants of endothelial cells *in vitro* by ELISA. Supernatant of BTEC harvested after 96 h contained sCD62E (70 ± 10 pg/ml) whereas SN-G and supernatant of untreated SV-HCEC and HMEC did not. In contrast, 96 h after stimulating SV-HCEC and HMEC with SN-G, sCD62E was detected in the supernatant of these endothelial cells (50–70 pg/ml). Thus, CD62E shedding and release of sCD62E may account for the apparent cytoplasmic staining observed in astrocytoma samples (Fig. 3).

Enhanced migration of HPC through an SV-HCEC layer *in vitro* depends on CD62E

We next investigated the role of HPC adhesion to CD62E for transendothelial HPC migration through an SN-G-conditioned endothelial cell layer. We studied the transendothelial migration in the absence or presence of neutralizing antibodies to CD62E either with or without pretreatment of the endothelial cell layer. Neutralizing CD62E reduced the transendothelial HPC migration significantly (Fig. 4A, first panel). Because of the important regulatory role of TGF-β for HPC glioma tropism (Tabatabai *et al.*, 2006), we also investigated the role of TGF-β in the transendothelial HPC migration by targeting TGF-β expression and TGF-β signalling in glioma cells. We used stably transfected LNT-229 siTGF-$\beta_{1,2}$ cells (Friese *et al.*, 2004; Eisele *et al.*, 2006) or siRNA pools targeting TGF-β_1 and TGF-β_2, respectively, for transient transfection of LN-308 before collection of SN-G.

Transendothelial HPC migration through cells that had been pretreated with supernatant of LNT-229 siTGF-$\beta_{1,2}$ (SN-GsiTGF-β), was reduced and neutralizing α-CD62E did not modulate HPC migration (Fig. 4A, second panel). Further, transient transfection of LN-308 glioma cells with siRNA pools targeting TGF-β_1 and TGF-β_2 inhibited TGF-β_1 and TGF-β_2 release into the supernatant (Fig. 4B). Combined targeting of TGF-β_1 and TGF-β_2 in

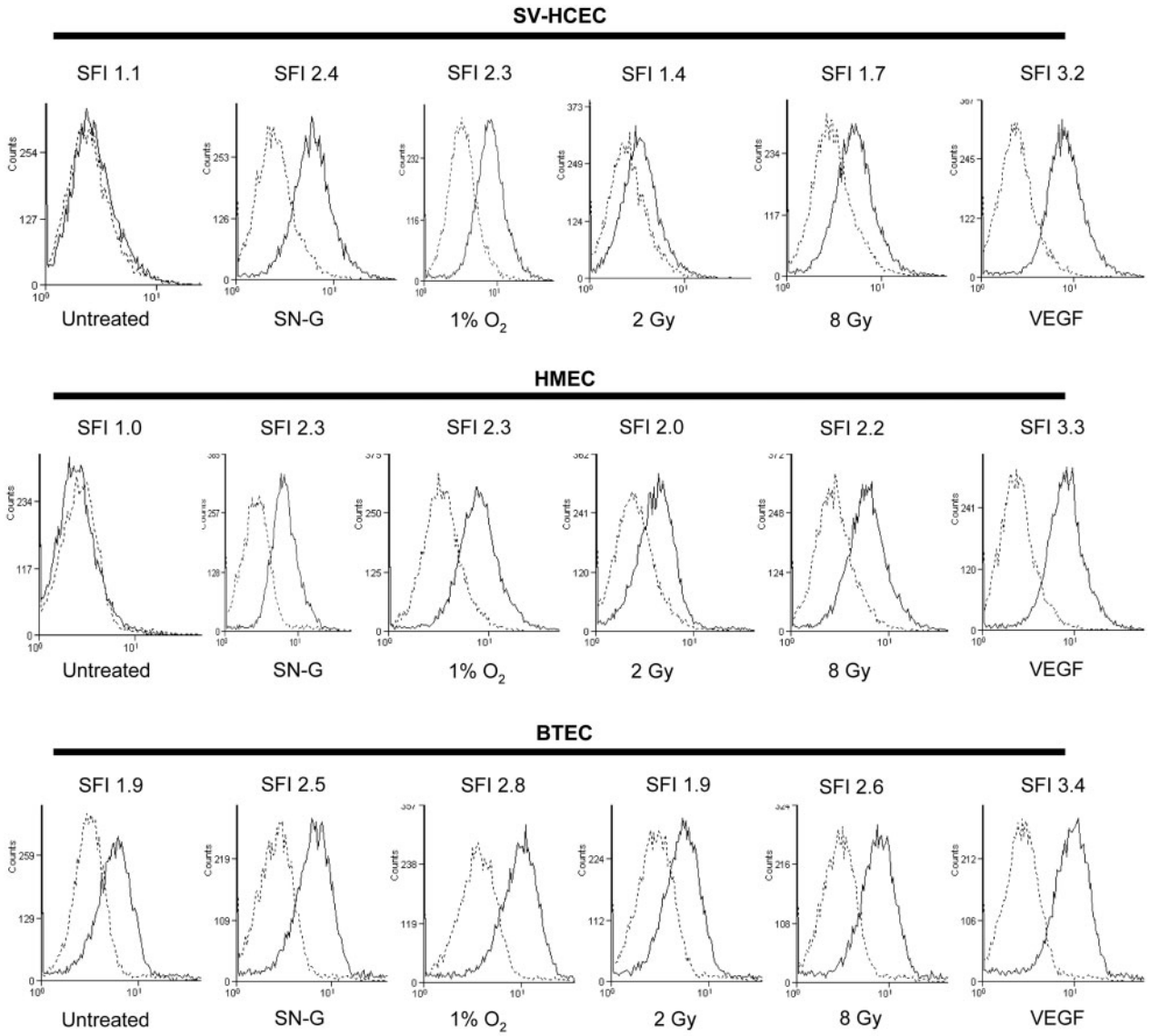

Fig. 2 Flow cytometry demonstrates modulation of endothelial CD62E expression by SN-G, hypoxia or irradiation. CD62E expression was analysed on the surface of endothelial cells without treatment, after stimulation with supernatant of LNT-229 for 48 h, 1% O_2 for 24 h, 24 h after irradiation at 2 and 8 Gy or exposure to $VEGF_{165}$ (40 ng/ml) for 24 h.

LN-308 cells inhibited the phosphorylation of Smad2 (Fig. 4B) indicating abrogation of TGF-β signalling. HPC migration through endothelial cells that had been pre-treated with supernatant of these cells was accordingly reduced and neutralizing CD62E did not modulate the migration (Fig. 4A, third and fourth panel).

Pre-irradiation of the endothelial cells resulted in an enhancement of transendothelial HPC migration (Fig. 1) that was also reduced by neutralizing CD62E antibodies (Fig. 4C). In contrast, neutralizing VCAM-1 antibodies did not significantly reduce HPC migration through an untreated SV-HCEC- or BTEC or an SN-G-treated SV-HCEC layer (Table 2). Exposure to hypoxia, irradiation, supernatant, neutralizing or control antibodies did not affect viability or proliferation of the endothelial cells or glioma cells (data not shown).

Homing of murine HPC to orthotopically implanted SMA-560 gliomas *in vivo* is reduced after neutralization of CD62E

Subsequently, we investigated the role of CD62E for the glioma tropism of murine HPC in an orthotopic syngeneic glioma model *in vivo*. The 10^4 SMA-560 glioma cells were implanted into the right striatum of VM/Dk mice. On day 8, 10^6 PKH26-stained LSK cells were injected intravenously. Four hours before and 4 h after the injection of LSK, CD62E-neutralizing antibodies (six mice) or control

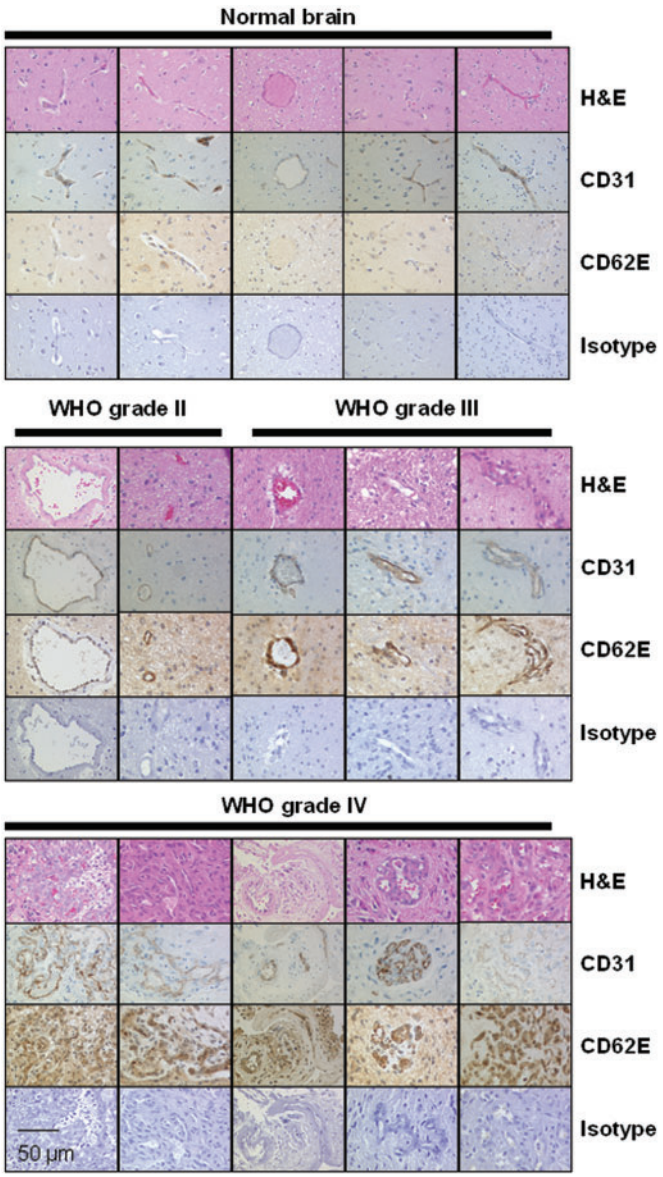

Fig. 3 Endothelial cells of human astrocytoma vessels highly express CD62E. Representative samples of normal brain tissue, diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III) and glioblastoma are shown. CD31 staining was used to detect the vessels within the normal brain or the tumour tissue. Depicted are H&E staining, immunoreactivity for CD31 and CD62E and the isotype control for CD62E (size bar bottom left: 50 µm).

antibodies (six mice) were bolus-injected both intraperitoneally and intratumourally in parallel in the same animals to block adhesion. On day 10, the brains were removed. Three brains per group were used for assessment by flow cytometry, three brains were analysed by histology. Injection of CD62E and control antibodies did not affect tumour morphology, tumour growth or vessel density as assessed by H&E or vWF staining (Fig. 5A). The endothelial cells of tumour vessels expressed CD62E (Fig. 5A). Fluorescence staining showed reduced numbers of PKH26-labelled cells within the SMA-560 gliomas after pretreatment with anti-CD62E antibodies (Fig. 5B). Single cell suspensions of freshly removed samples of the SMA-560 gliomas or of the contralateral hemisphere were analysed by flow cytometry to quantify the numbers of PKH26-labelled cells. Co-treatment with anti-CD62E antibody strongly reduced the amount of PKH26-positive LSK in orthotopic SMA-560 gliomas (Fig. 5C).

TGF-β signalling in glioma cells is required for SN-G-mediated induction of CD62E expression on SV-HCEC

We then characterized the time course of CD62E induction in SV-HCEC and HMEC. TNF-α treatment (100 U/ml) served as a positive control. This treatment resulted in high expression of CD62E by SV-HCEC (Fig. 6A) and HMEC (Fig. 6B) after 6 h, then declining over the next 90 h. Similarly, treatment with $VEGF_{165}$ (40 ng/ml) resulted in high CD62E expression after 6 h, which was no more detectable 96 h after treatment. Culturing of SV-HCEC or HMEC in supernatant of the astrocytic cell line SV-FHAS did not induce CD62E expression. Treatment of SV-HCEC or HMEC with SN-G resulted in a maximum CD62E expression at 12 h (Fig. 6A and B).

Next, we analysed the role of TGF-β signalling for the induction of CD62E. The treatment of SV-HCEC with recombinant TGF-β_2 (10 ng/ml) did not induce CD62E expression (Fig. 7A). We also collected supernatant of glioma cells that had been pretreated with the TGF-β receptor I kinase inhibitor, SD-208 (0.1 µmol/l and 1.0 µmol/l). SD-208 treatment blocked TGF-β signalling in LN-308, T113 and T132 cells (Fig. 7B). The exposure of SV-HCEC to supernatant of these SD-208-treated glioma cells did not induce CD62E expression (Fig. 7A). In accordance, exposure of SV-HCEC to SN-308 siTGF-$\beta_{1,2}$ (Fig. 4A and B) did not induce CD62E (Fig. 7A). Finally, we also treated LNT-229 siTGF-$\beta_{1,2}$ cells stably depleted from TGF-β_1 and TGF-β_2 (Friese *et al.*, 2004) with recombinant TGF-β_2 (10 ng/ml) for 7 days to rescue TGF-β signalling in these glioma cells (Friese *et al.*, 2004; Tabatabai *et al.*, 2005; Eisele *et al.*, 2006). Supernatant from these cells again induced CD62E on SV-HCEC (Fig. 7A). In contrast, the treatment of SV-HCEC with SN-G in the presence of SD-208, which abrogates TGF-β signalling pathway in SV-HCEC and HMEC (Fig. 7C), did not influence the SN-G-mediated induction of CD62E. The exposure of endothelial cells to SN-GRT or SN-GHO led to the induction of CD62E, too. This effect was again prevented by SD-208 treatment of the glioma cells before harvesting the supernatant (data not shown).

The experiments involving the addition of recombinant TGF-β_2 or SD-208 indicate that (i) TGF-β signalling in glioma cells is required for the induction of CD62E on endothelial cells; but (ii) TGF-β signalling in SV-HCEC is

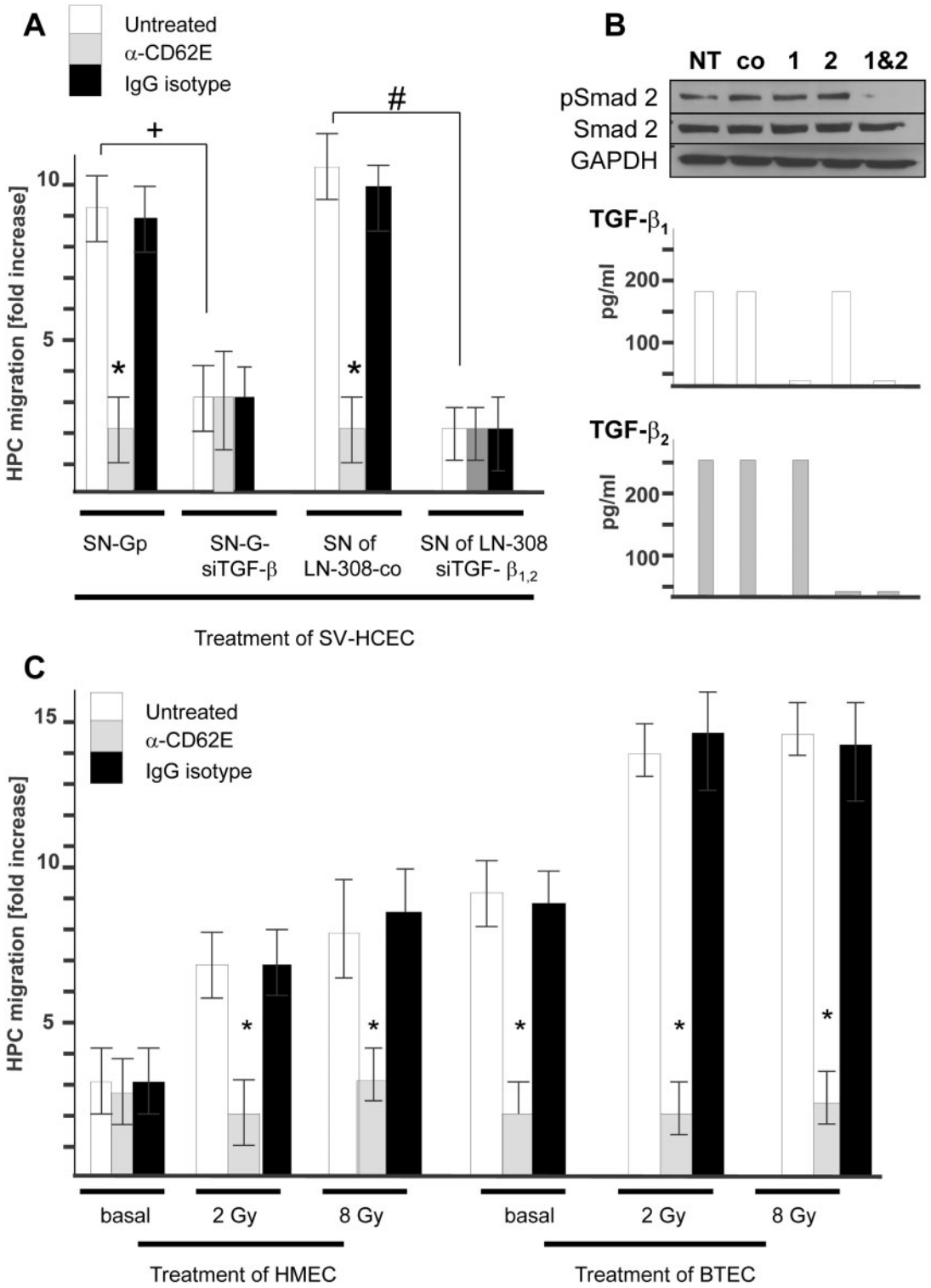

Fig. 4 Neutralizing CD62E antibodies diminish transendothelial HPC migration *in vitro*. (**A**) HPC migration through SV-HCEC layer treated with SN-Gp-, SN-GsiTGF-β-, supernatant of LN-308 co or siTGF-β1,2 towards CXCLI2 in the bottom well after addition of neutralizing CD62E antibodies (grey bars) or of the respective IgG isotype control (black bars) was assessed ($^{*}P < 0.05$ compared with IgG; $+P < 0.05$ compared with SN-Gp; $^{\#}P < 0.05$ compared with supernatant of LN-308 co; co = non-targeting control siRNA pool). (**B**) Immunoblot for detection of phospho Smad2, total Smad2 and GAPDH loading control; ELISA detecting TGF-β_1 or TGF-β_2 levels (NT = not treated, co = non-targeting control siRNA pool, 1 = transfected with siRNA pool targeting TGF-β_1, 2 = transfected with siRNA pool targeting TGF-β_2, 1&2 = co-transfection with siRNA pools targeting TGF-β_1 and TGF-β_2). (**C**) HMEC or BTEC were untreated or irradiated. HPC migration was assessed after the addition of neutralizing CD62E antibodies (grey bars) or the corresponding IgG isotype control (black bars) ($^{*}P < 0.05$ compared with IgG).

Table 2 Transendothelial HPC migration: no modulation by VCAM antibodies

Treatment of monolayer	Chemoattractant	HPC migration [fold increase]
SV-HCEC, untreated	SFM	1
	CXCLI2	3.1
	SN-G	7.1
SV-HCEC, α-VCAM-1	SFM	1
	CXCLI2	3
	SN-G	6.9
SV-HCEC, SN-G	SFM	1.6
	CXCLI2	6.5
	SN-G	11.6
SV-HCEC, SN-G + α-VCAM-1	SFM	1.2
	CXCLI2	6.1
	SN-G	11.3
BTEC, untreated	SFM	1
	CXCLI2	8
	SN-G	8.5
BTEC, α-VCAM-1	SFM	1
	CXCLI2	7.5
	SN-G	8

$CD34^+$ HPC (10^5) were added to the upper compartment of an endothelial cell monolayer-covered transwell insert. The monolayer was either untreated or stimulated with SN-G. SFM, CXCLI2 (100 ng/ml) or supernatant of LNT-229 cells were added to the bottom wells. Relative transendothelial migration, i.e. migration related to basal migration towards SFM through the untreated monolayer, was determined 16 h later with or without neutralizing α-VCAMI-antibodies (mean, $n = 3$, SEM < 10%).

not involved in SN-G-mediated induction of CD62E expression.

Induced CD62E expression on SV-HCEC is mediated by VEGF-R2 signalling

We next analysed the levels of VEGF, a candidate inducer of CD62E expression, in concentrated SN-Gp and SN-GsiTGF-β from cells treated with or without SD-208 or TGF-β_2 (Table 3). VEGF release was reduced by abrogated TGF-β signalling. In contrast, addition of recombinant TGF-β_2 to LNT-229 siTGF-$\beta_{1,2}$ cells for 7 days before generation of supernatant restored VEGF levels in the supernatant. Further, irradiated and hypoxic SV-HCEC released increased levels of VEGF (Table 3). SV-HCEC, HMEC and BTEC expressed the corresponding receptor VEGF-R2 (Fig. 8A). Therefore, we analysed the role of VEGF/VEG-R2 signalling in endothelial cells for CD62E induction. Treatment of SV-HCEC, HMEC or BTEC with $VEGF_{165}$, SN-G, hypoxia or irradiation increased the levels of phosphorylated VEGF-R2. NIH3T3 cells were used as negative controls (Fig. 8B). The levels of total VEGF-R2 in untreated SV-HCEC (597 pg/ml) did not increase by exposure of SV-HCEC to $VEGF_{165}$ (590 pg/ml) or supernatant of LN-308 (572 pg/ml). Pretreatment with hypoxia, 2 Gy or 8 Gy, however, increased the levels of total VEGF-R2 in SV-HCEC (807, 992 and 2670 pg/ml). Similarly, the levels of total VEGF-R2 in untreated HMEC (664 pg/ml) was not increased by exposure to $VEGF_{165}$ (579 pg/ml), supernatant of LN-308 (660 pg/ml), whereas hypoxia (772 pg/ml), irradiation at 2 Gy (1222 pg/ml) or 8 Gy (2982 pg/ml) led to elevated levels of total VEGF-R2 protein in HMEC. In BTEC, we detected levels of total VEGF-R2 in untreated (502 pg/ml), hypoxic (452 pg/ml) or irradiated cells (2 Gy: 459, 8 Gy 449 pg/ml). These data indicate that VEGF-R2 signalling activity in SV-HCEC, HMEC and BTEC is enhanced in response to SN-G, hypoxia or irradiation. Targeting of VEGF in glioma cells by siRNA pools led to a reduced VEGF release by LN-308 glioma cells or T113 primary cultures (Fig. 8C). VEGF levels were also are reduced in supernatant of LN-308 siTGF-$\beta_{1,2}$ (Fig. 8C) and supernatant of LNT-229 after inhibition of TGF-β signalling (Table 3). The SN-G-mediated *de novo* expression of CD62E was prevented by VEGF gene silencing and by neutralizing VEGF or VEGF-R2 antibodies (Fig. 8D). Further, the exposure of SV-HCEC to irradiation or SN-G, SN-GRT or SN-GHO supplemented with neutralizing α-VEGF and α-VEGF-R2 antibodies prevented CD62E induction (data not shown). Thus, (i) SN-G enhances VEGF-R2 signalling activity in endothelial cells; (ii) VEGF from SN-G, SN-GHO or SN-GRT is required for the SN-G-induced expression of CD62E in SV-HCEC; (iii) VEGF release from SV-HCEC is required for CD62E induction after exposure to hypoxia or irradiation.

Transcriptional activation of CD62E in endothelial cells is mediated by NFκB

The promoter of CD62E contains multiple NFκB-binding sites (Schindler *et al.*, 1994). We detected NFκB p65 and p50 in whole cell lysates of SV-HCEC (Fig. 9A). Protein levels were not modulated by irradiation, hypoxia or exposure to SN-Gp, SN-GsiTGF-β or SN-GRT. Futher, we analysed the activation of the NFκB transcription family in nuclear extracts of BTEC, SV-HCEC and HMEC (Fig. 9B). Raji extracts served as positive control, nuclear extracts from NIH3T3 cells were used as negative controls. The NFκB transcription factor family is activated in untreated endothelial cells. We next analysed the activity of p50 in nuclear extracts of endothelial cells after treatment with $VEGF_{165}$ or SN-G. TNF-α served as positive control (Fig. 9C). $VEGF_{165}$ and SN-G further increased the transcriptional activity of p50 whereas treatment with supernatant of LN-308 siVEGF did not. In addition, we performed reporter assays with pNFκB-luc. This plasmid contains four copies of the NFκB consensus sequence and detects the transcriptional activity of NFκB. Treatment with TNF-α (100 U/ml) served as a positive control (Figs 9 and 10). The relative luciferase activity of pNFκB-luc in untreated BTEC, SV-HCEC and HMEC demonstrates the baseline transcriptional activity (Fig. 9D) in accordance with the data of the transAM assay (Fig. 9B and C).

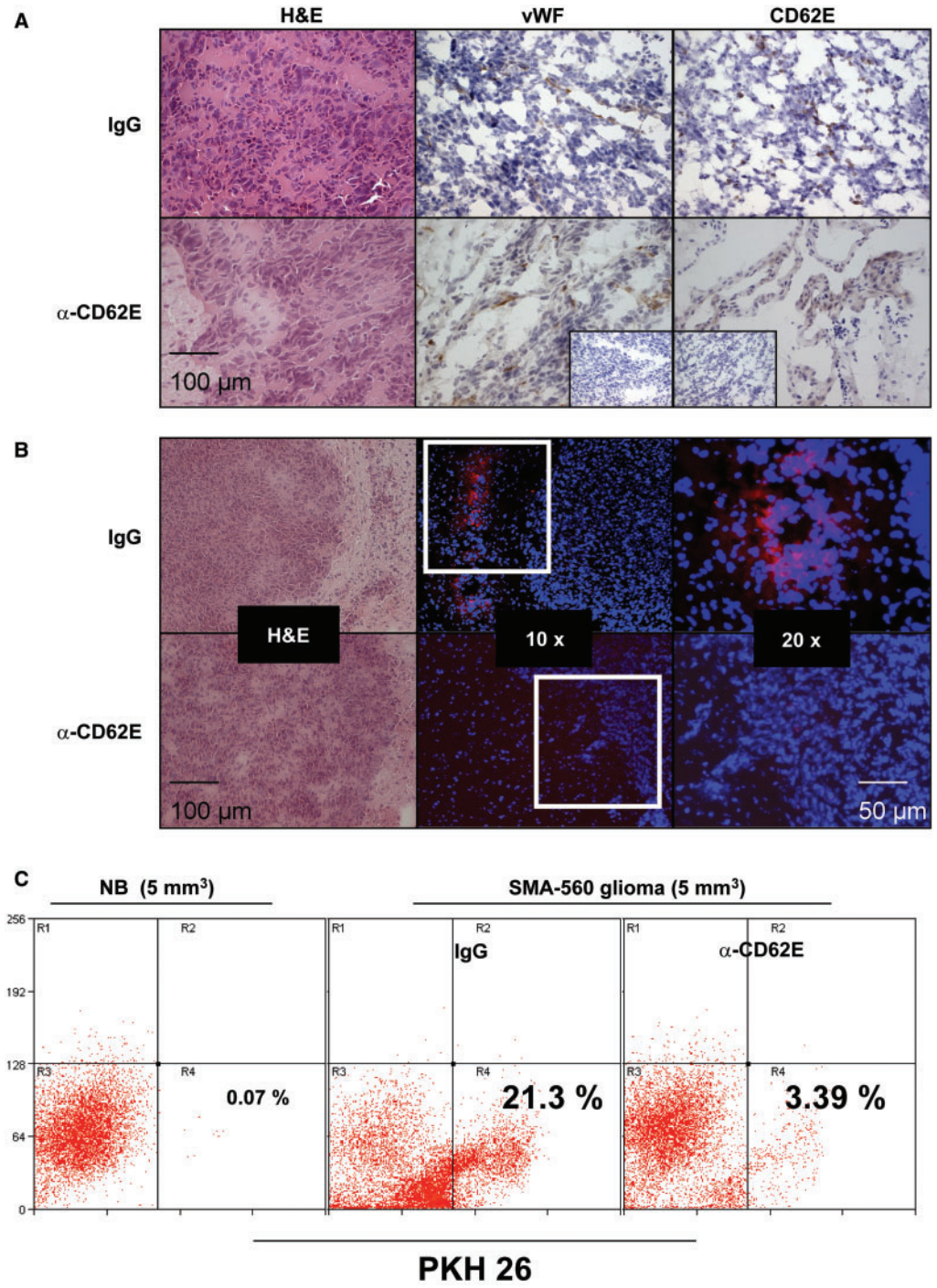

Fig. 5 Neutralizing CD62E antibodies diminish the attraction of murine HPC by orthotopic SMA-560 gliomas *in vivo*. (**A**) Morphology of SMA-560 glioma was assessed by H&E. vWF and CD62E proteins were visualized by immunohistochemistry. CD62E is expressed by tumour endothelial cells. Isotype controls for vWF and CD62E are displayed as inserts. H&E and corresponding vWF and CD62E stainings of one mouse per experimental group are displayed. Size bar bottom left: 100 µm. (**B**) Fluorescence microscopy after nuclear counterstaining with DAPI for the detection of glioma cells (blue) and PKH26 stained LSK (red). Size bars bottom left: 100 µm and bottom right: 50 µm. (**C**) The numbers of PKH26-stained LSK in the SMA-560 gliomas or in the contralateral hemisphere (NB) were quantified by flow cytometry. In (**A**), (**B**), (**C**), α-CD62E = treatment with neutralizing CD62E antibodies, IgG = treatment with control antibodies.

After exposure to TNF-α, $VEGF_{165}$ or SN-G, NFκB transcriptional activity significantly increased. Neutralizing antibodies to VEGF and VEGF-R2 reduced SN-G-induced NFκB transcriptional activity (Fig. 10).

In parallel, we analysed the activity of the CD62E promoter by measuring the relative luciferase activity of pGL3basic.CD62E. In this plasmid, the reporter gene is driven by the CD62E promoter and indicates the activity of

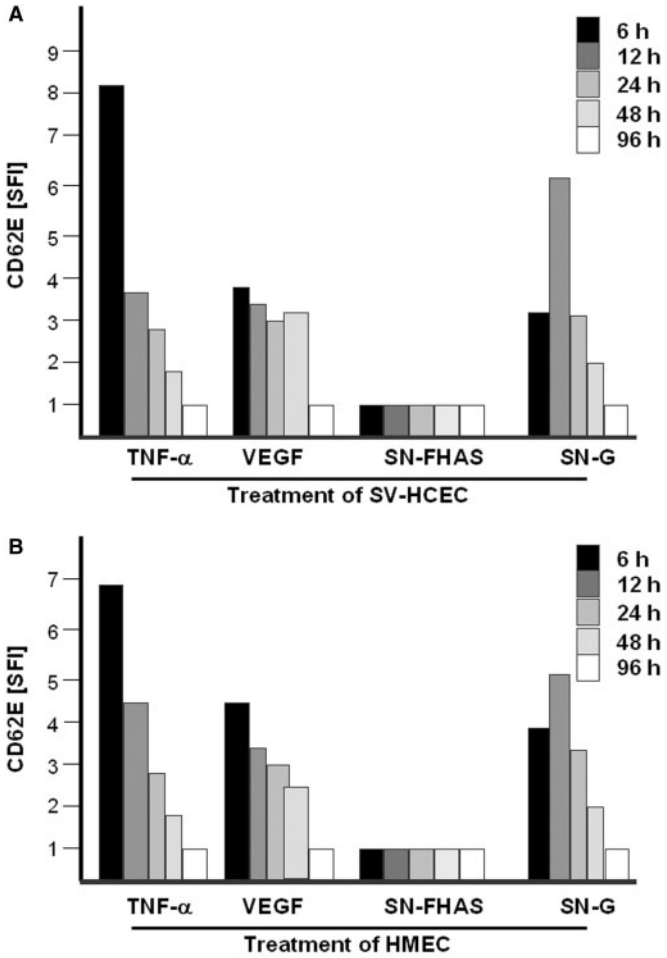

Fig. 6 Time-course of induced CD62E expression in SV-HCEC and HMEC. Flow cytometry was performed to investigate CD62E expression of SV-HCEC (**A**) or HMEC (**B**) at 6, 12, 24, 48 and 96 h after pretreatment as indicated. Note that an SFI of 1 indicates the absence of protein expression.

the CD62E promoter. We detected relative luciferase activity in untreated BTEC indicating a baseline promoter activity in accordance with the data from flow cytometry (Fig. 2). In contrast, luciferase activity was absent in untreated SV-HCEC or HMEC, indicating a lack of CD62E promoter activity in this cell population (Fig. 9D). After treatment with TNF-α, $VEGF_{165}$ or SN-G, however, the relative luciferase activity of pGL3.basic.CD62E increased (Fig. 10). Irradiation with 2 Gy, 8 Gy or hypoxia also increased pGL3.basic.CD62E activity (data not shown). NFκB transcriptional activity and CD62E promoter activity were reduced by neutralizing α-VEGF and α-VEGF-R2 antibodies (Fig. 10). To analyse whether NFκB transcriptional activity is required for CD62E promoter activity, we performed reporter assays after targeting NFκB by the NFκB-super-repressor Ad-IKBM. We transduced SV-HCEC with 100 MOI of Ad-IKBM. Ad-Mock served as control. Relative luciferase activity of pGL3.basic.CD62E in SV-HCEC was not induced by SN-G in the presence of the NFκB super repressor (Fig. 10). These experiments place VEGF signalling and NFκB upstream of the transcriptional activation of CD62E in SV-HCEC. The presumptive signalling cascade leading to the induction of CD62E in SV-HCEC by SN-G, irradiation or hypoxia is summarized in Fig. 11.

Discussion

Adult HPC migrate towards experimental gliomas. Therefore, this adult stem cell population might be a promising cellular vector for the delivery of therapeutic molecules to experimental gliomas. Before applying therapeutic approaches, however, the underlying molecular mechanisms of the glioma-mediated HPC attraction should be better understood. The objective in characterizing the glioma tropism of HPC is to achieve an optimal seeding of experimental gliomas with HPC. Adhesion molecules expressed on endothelial cells serve as keys to allow the entrance of circulating cells at specific tissue sites (Frenette *et al.*, 1996*a*). Bone marrow- and umbilical cord blood-derived $CD34^+$ HPC adhere to CD62E on bone marrow microvasculature (Najyer *et al.*, 1999; Dimitroff *et al.*, 2001; Hidalgo *et al.*, 2002; Greenberg *et al.*, 2000). P- and E-selectin-deficient mice exhibit severe deficiencies in haematopoiesis (Frenette *et al.*, 1996). Intravital microscopy studies show that P- and E-selectin contribute to HPC rolling in the bone marrow microvasculature. Blocking both selectins and VCAM-1 inhibits the rolling interaction almost completely (Mazo *et al.*, 1998). Hidalgo *et al.* (2002) demonstrated by fluorescence intravital microscopy and homing assays in NOD/SCID mice that endothelial selectins are necessary for $CD34^+$ HPC homing: rolling on bone marrow endothelium and retention in the bone marrow compartment are severely reduced in CD62E-deficient NOD/SCID mice. Thus, homing of HPC to the bone marrow is mediated by HPC interactions with multiple adhesion receptors. Among these, CD62E and VCAM-1 are expressed preferentially on endothelial cells. Therefore, we focused on investigating the possible contribution of CD62E and VCAM-1 to HPC glioma tropism.

CD62E expression in glioma-conditioned endothelial cells *in vitro* and *in vivo*

We used three different endothelial cell types: the cerebral endothelial cell line SV-HCEC (Muruganandam *et al.*, 1997), the endothelial cell line HMEC and BTEC (Miebach *et al.*, 2006). These endothelial cells constitutively express VCAM-1, but not CD62E. In the bone marrow microvasculature, CD62E and VCAM-1 are constitutively expressed (Schweitzer *et al.*, 1996). In most other tissues, however, CD62E is transcriptionally regulated and expressed on the surface after exposure to specific inflammatory stimuli (Bevilacqua *et al.*, 1989). We found CD62E expression on untreated BTEC. *De novo* CD62E expression by SV-HCEC

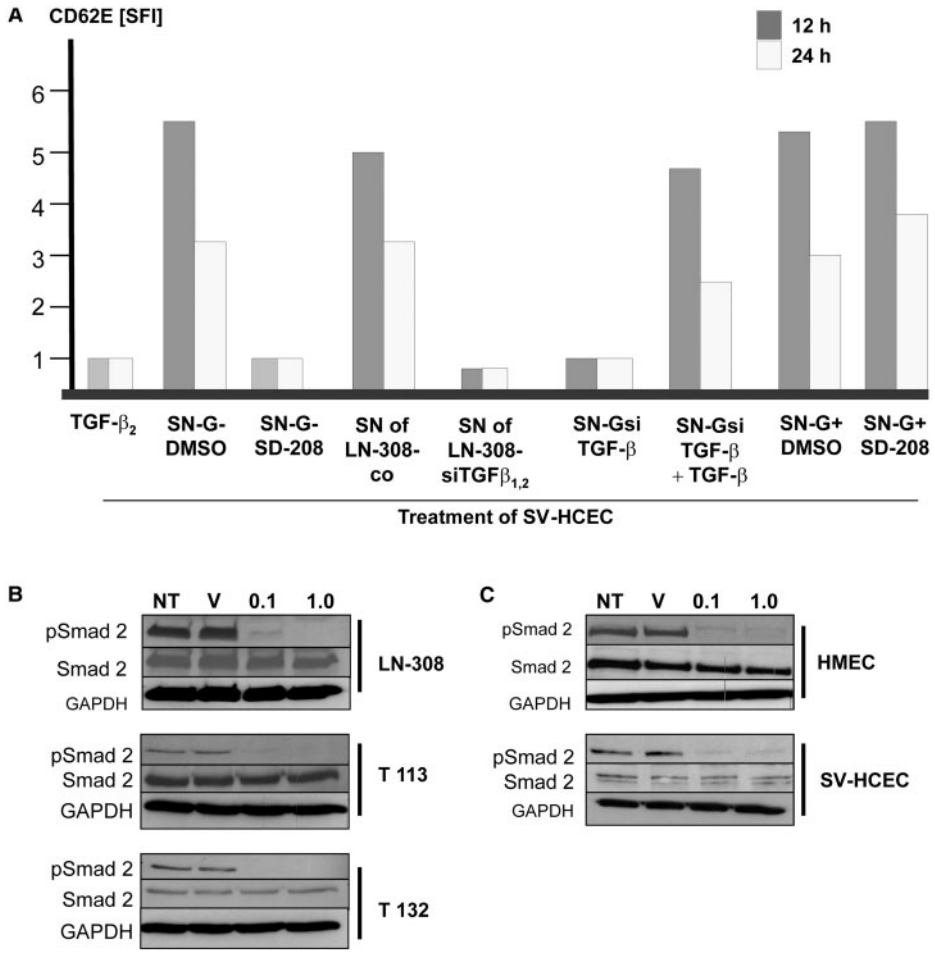

Fig. 7 The role of TGF-β signalling for the induction of CD62E expression. (**A**) Flow cytometry was performed to analyse CD62E expression of SV-HCEC after the indicated treatments. (**B**) Immunoblot for the detection of phospho Smad2, total Smad2 and GAPDH in lysates of LN-308, T113 or T132 after treatment with SD-208. (**C**) Immunoblot for the detection of phospho Smad2, total Smad2 and GAPDH in lysates of SV-HCEC or HMEC after treatment with SD-208. In (**B**) and (**C**) NT = not treated, V = vehicle, 0.1 = SD-208 0.1 μmol/l, 1.0 = SD-208 1.0 μmol/l.

Table 3 Modulation of VEGF release by LNT-229 and SV-HCEC

Concentrated Supernatants	VEGF [pg/ml]
LNT-229	390
LNT-229 puro	410
LNT-229 puro, pretreated with DMSO	400
LNT-229 puro, pretreated with SD-208	85
LNT-229 siTGF-$\beta_{1,2}$	94
LNT-229 siTGF-$\beta_{1,2}$ treated with recombinant TGF-β_2	370
SV-HCEC	50
SV-HCEC, 1% O_2, 12 h	610
SV-HCEC, 1% O_2, 24 h	920
SV-HCEC, 2 Gy	680
SV-HCEC, 8 Gy	750

VEGF protein levels were determined in concentrated supernatant by ELISA. The cells were either untreated, preincubated with SD-208 (1 μmol/l, 24 h) or vehicle, TGF-β_2 (10 ng/ml, 7 days), exposed to 1% O_2 for 12 h and 24 h or preirradiated (2 Gy, 8 Gy, 48 h before harvest of supernatant) (mean, $n = 5$, $p < 0.01$, SEM < 10%).

and HMEC was induced after stimulation with SN-G, SN-GHO or SN-GRT, as well as treatment with hypoxia (Fig. 2) or irradiation. The constitutive VCAM-1 expression on the endothelial cells was not altered by these treatments. These results differ from studies with bone marrow microvasculature: total body irradiation of mice increased VCAM-1 expression, but the constitutive expression level of CD62E within the bone marrow microvasculature was unchanged (Mazo *et al.*, 2002). Having observed an SN-G-induced CD62E expression by SV-HCEC and HMEC *in vitro*, we analysed CD62E expression *in situ*. The endothelium of vessels within astrocytoma tissue of WHO grades II–IV highly expressed CD62E whereas CD62E staining was absent in normal brain (Fig. 3). In addition to the well-defined CD62E staining of tumour endothelium, there was a diffuse extracellular and cytoplasmic staining in the neoplastic specimens. This might indicate the shedding of CD62E and the presence of the soluble form of CD62E (Oh *et al.*, 2007). In that regard, we also detected sCD62E

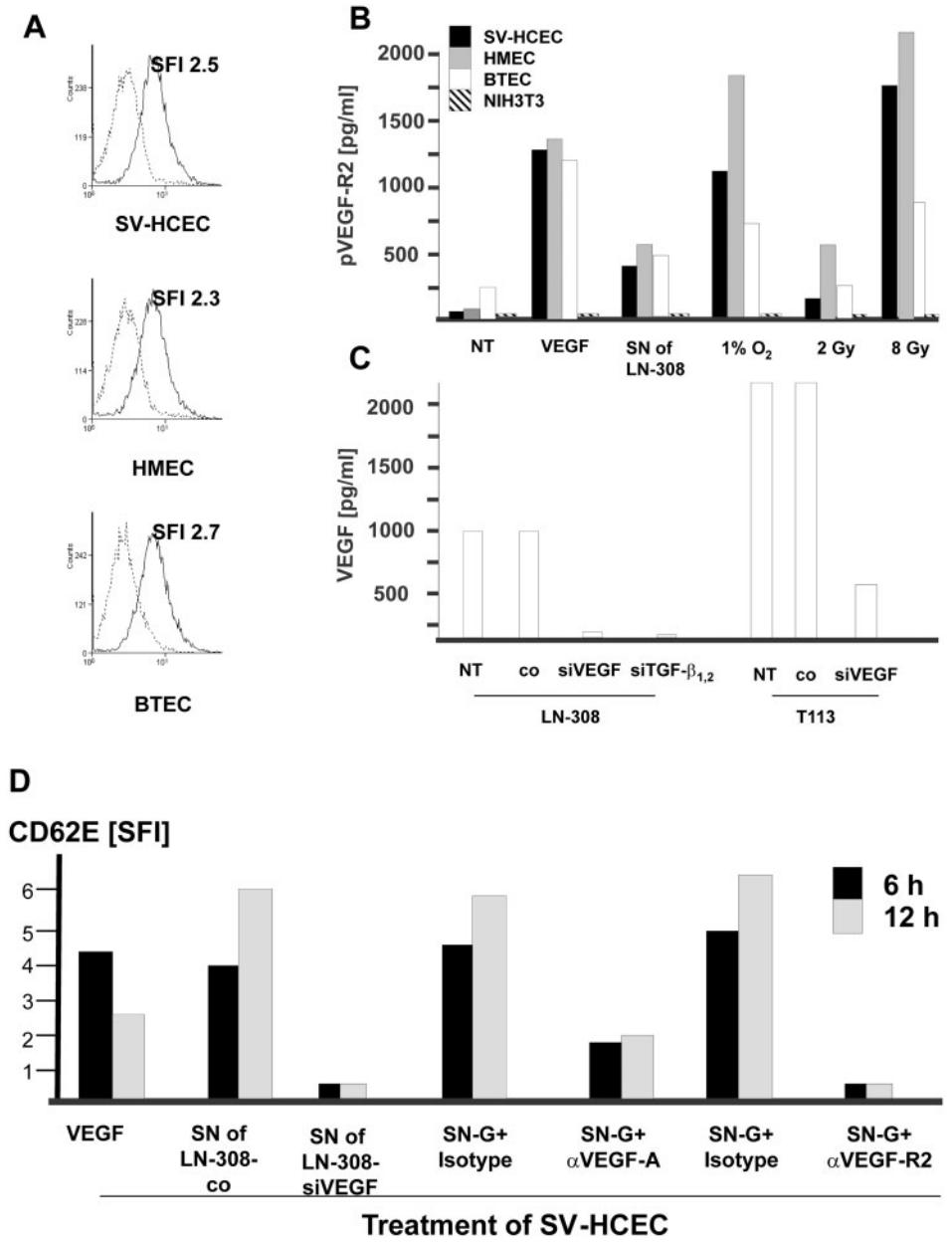

Fig. 8 The role of VEGF/VEGF-R2 signalling for the induction of CD62E expression. (**A**) Flow cytometry was performed to determine VEGF-R2 expression of SV-HCEC, HMEC and BTEC. (**B**) The levels of phosphorylated VEGF-R2 in SV-HCEC, HMEC, BTEC or NIH3T3 cells were asssessed after the indicated treatments. (**C**) ELISA was performed to evaluate the release of VEGF after the indicated treatments. (**D**) CD62E expression was analysed by flow cytometry after the indicated treatments.

in supernatant of stimulated SV-HCEC, HMEC and in supernatant of untreated BTEC *in vitro*.

CD62E is one mediator of the glioma-mediated HPC attraction *in vitro* and *in vivo*

The transendothelial migration of HPC was significantly diminished in the presence of neutralizing CD62E antibodies (Fig. 4) whereas neutralizing VCAM-1 antibodies had no such effect. *In vivo*, administration of a CD62E-blocking antibody prevented the accumulation of PKH26-positive LSK in orthotopic SMA-560 gliomas (Fig. 5), indicating that CD62E is required for the glioma tropism of LSK *in vivo*. In a model of chronic kidney disease, Gong *et al.* (2006) demonstrated *de novo* CD62E expression in renal vascular endothelium mediating leucocyte adhesion. The bolus injection of neutralizing CD62E antibodies diminished interstitial inflammation and macrophage sequestration in the kidney *in vivo*. Recently, Nishiwaki *et al.* (2007) demonstrated a crucial role for CD62E in the interaction between circulating endothelial progenitor cells and vessel

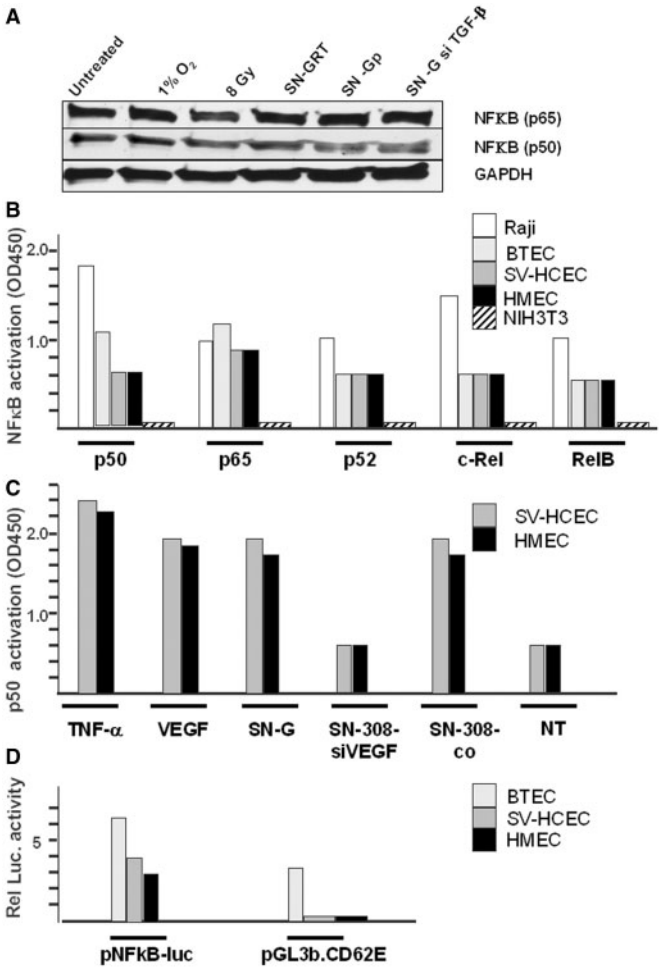

Fig. 9 NFκB protein levels and transcriptional activity of NFκB family members in endothelial cells. (**A**) Immunoblots show the expression of NFκB in lysates of SV-HCEC either untreated or after exposure to 1% O_2, irradiation at 8 Gy, treatment with SN-GRT, SN-Gp or SN-GsiTGF-β. GAPH was used as a loading control. (**B**) Profiling of the transcriptional activity of the NFκB family in endothelial cells. (**C**) Transcriptional activity of p50 was assessed in SV-HCEC or HMEC 24 h after the indicated treatments. (**D**) Untreated BTEC, SV-HCEC or HMEC were transfected with NFκB-luc or pGL3b.CD62E. The bars indicate relative luciferase activity.

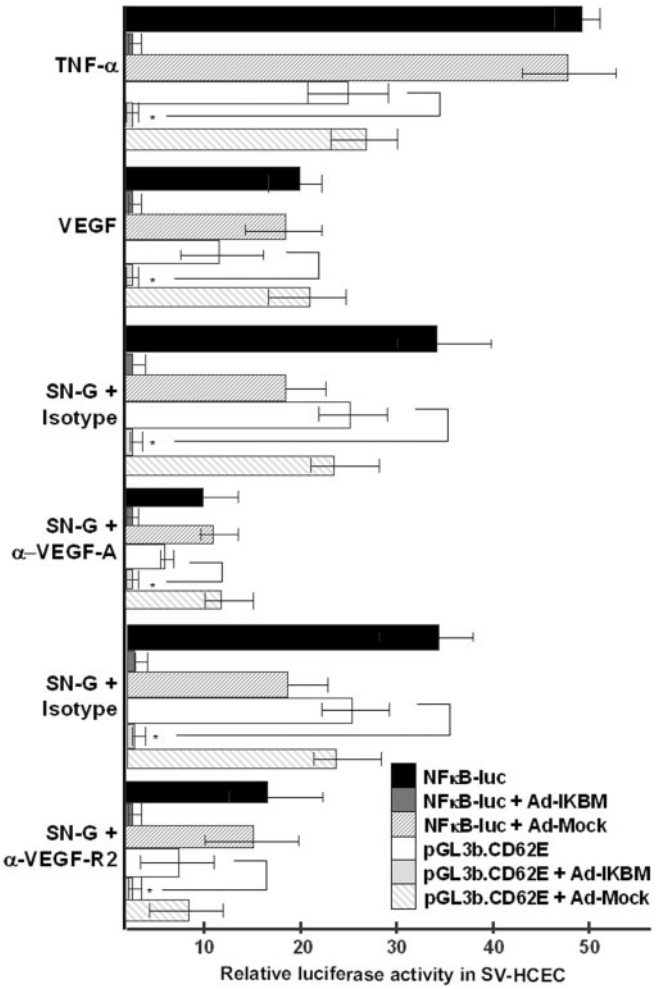

Fig. 10 Transcriptional activation of CD62E by SN-G is mediated by NFκB. SV-HCEC were transfected with NFκB-luc or pGL3b.CD62E either alone or transduced with the NFκB super repressor Ad-IKBM or Ad-Mock. The cells were then treated with TNF-α, $VEGF_{165}$ or supernatant as well as supernatant with neutralizing VEGF or VEGF-R2 antibodies. The bars indicate relative luciferase activity ($n = 3$, $^{*}P < 0.01$ compared with pGL3b.CD62E).

endothelium in the mouse hind-limb ischaemia paradigm. Oh and colleagues (2007) demonstrated that CD62E is a pivotal molecule for endothelial progenitor cell homing to the ischaemic site and for vasculogenesis in an ischaemic limb model. Based on these data, a novel cell-based therapy for ischaemic atherosclerosis might be designed.

SN-G-mediated induction of CD62E requires TGF-β signalling in glioma cells and VEGF/VEGF-R2 signalling in endothelial cells

We next investigated the molecular mechanism mediating the *de novo* expression of CD62E in SV-HCEC or HMEC. Culturing of SV-HCEC with SN-GsiTGF-β did not induce CD62E expression (Fig. 7). HPC migration through an SN-GsiTGF-β-treated SV-HCEC layer was not altered by neutralizing α-CD62E. The reduction of relative HPC migration (Fig. 4A) observed after pretreating SV-HCEC with SN-GsiTGF-β can be explained by the lack of CD62E expression (Fig. 7). Based on this observation, we analysed the role of TGF-β signalling in the glioma-mediated induction of CD62E. When TGF-β signalling was inhibited in LNT-229 glioma cells before supernatant collection—either by SD-208 or by siRNA—these supernatants did not induce CD62E expression (Fig. 7). Inhibition of TGF-β signalling in SV-HCEC or HMEC by SD-208, however, did not modulate SN-G-mediated CD62E induction. Further, treatment of SV-HCEC with recombinant

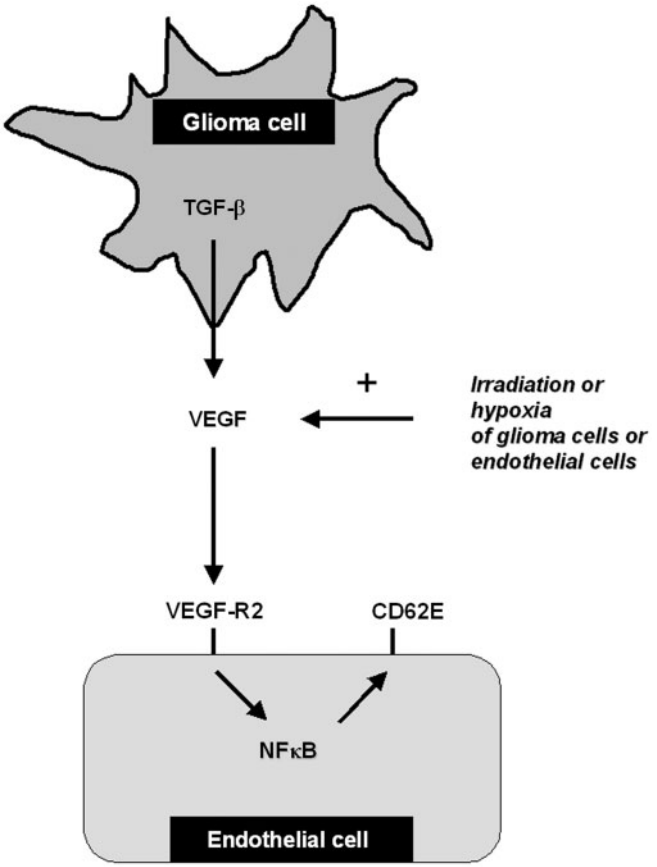

Fig. 11 Schematic overview of results. *De novo* expression of CD62E on endothelial cells in response to a TGF-β-dependent VEGF release by glioma cells involves the activation of VEGF-R2 in endothelial cells, resulting in NFκB-mediated CD62E expression. VEGF also mediates the irradiation- or hypoxia-induced *de novo* expression of CD62E.

TGF-β_2 alone did not induce CD62E (Fig. 7). We concluded that glioma-mediated CD62E induction requires intact TGF-β signalling in the glioma cells, but not in SV-HCEC. Inhibition of TGF-β signalling in glioma cells also led to diminished VEGF levels (Table 3, Fig. 8C). Irradiation with 2 or 8 Gy and pre-exposure to hypoxia on the other hand increased VEGF secretion by SV-HCEC and HMEC (Table 3). We therefore analysed the influence of VEGF/VEGF-R2-signalling on CD62E induction in SV-HCEC. Treatment of SV-HCEC and HMEC with supernatant of glioma cells that had been transfected with siRNA pools targeting VEGF did not induce CD62E (Fig. 8D). Neutralization of VEGF or VEGF-R2 bioactivity impaired SN-G-mediated CD62E induction (Fig. 8D) indicating a pivotal role of this signalling pathway in SV-HCEC or HMEC in the induction of CD62E expression. Recently, Stannard *et al.* (2007) demonstrated that VEGF primes endothelial cells by sensitizing them to cytokines, leading to enhanced selective pro-inflammatory responses, including upregulation of CD62E.

De novo expression of CD62E by endothelial cells is mediated by NFκB

To further characterize the downstream events after VEGF-R2 activation, we performed reporter assays. SN-G, irradiation or hypoxia induced CD62E promoter activity in SV-HCEC. Since the CD62E promoter contains three NFκB binding sites (Schindler *et al.*, 1994), we analysed the role of NFκB in our paradigm. NFκB is expressed and transcriptionally active in SV-HCEC, HMEC and BTEC (Figs 9 and 10). NFκB is also activated in pre-sheared HUVEC leading to NFκB-dependent cytoprotective responsiveness (Patridge *et al.*, 2007). Consequently, we investigated the upstream control of CD62E promoter activity with the NFκB superrepressor, Ad-IkBM. Inhibition of NFκB acitivity strongly reduced CD62E promoter activity (Fig. 10). This central role of NFκB activity for CD62E induction in our studies parallels results in other models: endothelial cell activation by HIV infection results in CD62E expression via a Tat-induced NFκB-mediated pathway (Cota-Gomez *et al.*, 2002). CD62E induction within an atherosclerotic lesion is evoked by the platelet-specific chemokine 4 derived from platelets and activation of NFκB was crucial for CD62E induction on endothelium by platelet-specific chemokine 4 (Yu *et al.*, 2005).

In summary, we define a signalling cascade leading to CD62E induction on endothelial cells in response to treatment with SN-G, SN-GHO, SN-GRT, irradiation or hypoxia. This cascade involves a TGF-β-dependent release of VEGF by glioma cells and the subsequent activation of VEGF-R2 in endothelial cells resulting in NFκB-mediated CD62E expression. We observed a selective expression of this adhesion molecule in human astrocytoma samples of WHO grades II–IV and demonstrated the functional relevance of CD62E for transendothelial HPC migration *in vitro* and *in vivo*. Therefore, we conclude that adhesion to CD62E on tumour endothelium might be a pivotal first step in the glioma tropism of HPC. Given the central role of VEGF in the glioma-mediated induction of CD62E on endothelial cells, a putative HPC-based therapy against glioblastomas might not be effective in combination with antiangiogenic strategies targeting VEGF, e.g. enzastaurin or bevacizumab.

Acknowledgements

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