Lessons from the bone marrow: how malignant glioma cells attract adult haematopoietic progenitor cells

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Stem and progenitor cells (PCs) of various lineages have become attractive vehicles to improve therapeutic gene delivery to cancers, notably glioblastoma. Here we report that adult human and murine haematopoietic PCs display a tropism for intracerebral gliomas but not for normal brain tissue in mice. Organotypic hippocampal slice culture and spheroid confrontation assays confirm a directed PC migration towards glioma cells *ex vivo* and *in vitro*. RNA interference-mediated disruption of transforming growth factor beta (TGF-β) synthesis by the glioma cells strongly inhibits PC migration. We delineate a CXC chemokine ligand (CXCL) 12-dependent pathway of TGF-β-induced PC migration that is facilitated by MMP-9-mediated stem cell factor cleavage *in vitro*. Moreover, neutralizing antibodies to CXCL12 strongly reduce PC homing to experimental gliomas *in vivo*. Thus, we define here the molecular mechanism underlying the glioma tropism of the probably most easily accessible PC population suitable for cancer therapy, that is, adult haematopoietic PC.

Keywords: CXCL12; hippocampal slice culture; metalloproteinase; TGF-β

Abbreviations: CA1 = cornu ammonis 1; CA3 = cornu ammonis 3; CD117 = cluster of differentiation 117; CD34+PC = G-CSF mobilized peripheral blood CD34+ progenitor and stem cells; CFSE = carbofluorescein diacetate succinimidyl ester; CXCL12 = CXC chemokine ligand; CXCR4 = chemokine receptor 4; DG = dentate gyrus; EC = entorhinal cortex; FCS = fetal calf serum; G-CSF = granulocyte colony stimulating factor; HBSS = Hanks’ balanced salt solution; lin− = lineage-depleted; MACS = magnetic activated cell sorting; MMP = matrix metalloproteinase; nu PC = bone marrow-derived murine lin− Sca1+ haematopoietic stem and progenitor and lin− stem and progenitor cells from athymic nude mice; PKB = protein kinase B; RO28-2653 = 5-biphenyl-4-yl-5-[4-(nitro-phenyl)-piperazin-1-yl]-pyrimidine-2,4,6; SC = stem cell; sca1 = stem cell antigen; SCF = stem cell factor; SDF = stromal cell-derived factor; SEM = standard error of the mean; SFM = serum-free medium; sKitL = soluble Kit ligand; TGF = transforming growth factor; VM/Dk PC = bone marrow-derived murine lin− Sca1+ haematopoietic stem and lin− stem and progenitor cells from immunocompetent VM/Dk mice


Introduction

Gliomas are paradigmatic for their deep infiltration of adjacent brain, precluding definitive surgical resection and limiting the efficacy of other local therapies (DeAngelis, 2001). The benefit derived from traditional cancer therapies is low because of defects in the apoptotic machinery of glioma cells, accounting for their resistance to radiation and chemotherapy. Similarly, gene therapy has its limits in the failure of currently available viral vectors to reach infiltrating glioma cells. An easily accessible autologous cellular vector that targets disseminated glioma cells and expresses a therapeutic
transplantation, therefore, represent a major step ahead in the experimental treatment of these tumours. Neural progenitor cells (PCs) from the cortex of C57BL6 mice have been used as vehicles to deliver interleukin 4 to adult bone marrow-derived PC and adult haematopoietic PC as vehicles for glioma therapy. We delineate a cascade for their active glioma homing of haematopoietic stem cells (HSCs) to the bone marrow (Krause et al., 2001). Primary non-transformed adult bone marrow-derived cells with neuronal properties migrate efficiently towards distant sites of brain tumour when injected intracranially (Lee et al., 2003). The homing of haematopoietic stem cells (HSCs) to the bone marrow and chemotaxis involve CXCL12 [stromal cell-derived factor (SDF)-1α] acting on chemokine receptor 4 (CXCR4) expressed on the SC, interleukin 8 and non-peptide leukotriene D4 (Aiuti et al., 1997; Peled et al., 1999; Bautz et al., 2001).

In bone marrow cells, CXCL12 upregulates the expression of MMP-9. The release of soluble Kit ligand (s-KitL)/stem cell factor (SCF) from its membrane-bound form on bone-marrow stromal cells is mediated by MMP-9. Binding to its receptor, CD117 on the haematopoietic SC contributes to their mobilization from a quiescent state in the bone marrow niche (Heissig et al., 2002).

Matrix metalloproteinases (MMPs) are also essential for the invasiveness of malignant gliomas (Kondraganti et al., 2000). Furthermore, glioma cells express SCF mRNA and protein, and the receptor c-Kit (Stanulla et al., 1995). However, the impact of SCF on the growth and motility of glioma cells is unclear. CXCL12/CXCR4 interactions mediate chemotaxis of glioma cells and protect from cell death induced by serum withdrawal via phosphorylation of protein kinase B (PKB/Akt) (Zhou et al., 2002). A small molecule CXCR4 antagonist, AMD 3100, inhibited the growth of experimental glioblastomas and medulloblastomas (Rubin et al., 2003).

The molecular mechanisms underlying the phenomenon of a possible lesion- or tumour-directed migration of either primary embryonic or adult SC, primary neuronal or glial precursor cells have remained unknown. Here we characterize the possible in vitro and in vivo use of adult peripheral CD34+ human haematopoietic PC and adult bone marrow-derived murine lin-Scal- haematopoietic PC as vehicles for glioma therapy. We delineate a cascade for their active glioma tropism which involves two transforming growth factor (TGF)-β-dependent pathways, (i) CXCL12 synthesis and (ii) MMP-9-mediated cleavage of SCF, facilitating the promigratory effect of CXCL12.

### Materials and methods

#### Isolation of haematopoietic PC from mice

Nude mice were purchased from Charles River Laboratories, Sulzfeld, Germany. VM/Dk mice were originally purchased from TSE Research Center (Berkshire, UK) and are now bred in our laboratory. Nude or VM/Dk mice that were 4 to 6 weeks-old were killed. Femurs and tibiae were flushed with phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS). Bone marrow cells (10^6/ml) were incubated in PBS containing 2% FCS with the lineage marker antibodies from BD Biosciences Pharmingen (San Diego, CA), rat antimouse CD4, CD8α, CD45R/B220, Gr-1, CD11b and TER119 at 4°C for 60 min, and washed three times with PBS containing 2% FCS. Prewashed sheep anti-rat IgG magnetic beads (Dynabeads, Dynal Biotech ASA, Oslo, Norway) were added at a 4:1 ratio of beads/cells in PBS containing 2% FCS and incubated on a rotating platform for 45 min at 4°C. Beads and attached cells were magnetically removed. The remaining lineage-depleted (lin-) cells were MACS-isolated with anti-SC antigen (Sca)1 beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Flow cytometry revealed that Lin-Sca1 cells were between 97.5 and 98.5% positive for Sca1 (FITC-conjugated antibody) and 99% positive for c-kit (PE-conjugated antibody, BD Biosciences Pharmingen). Also, <4% of the isolated Lin-Sca1 cells expressed CD4, CD8α, CD11b, B220 or Gr-1. Isolated cells were used directly for the in vitro experiments. For the in vivo experiments, isolated cells were pretreated with murine IL-3 (20 ng/ml), IL-6 (50 ng/ml) and SCF (50 ng/ml) (R&D Systems, Minneapolis, MN) for 24 h.

#### Isolation of human CD34+ haematopoietic PC

After informed consent, peripheral blood mononuclear cells were obtained. Mononuclear cells were separated by Ficoll density gradient centrifugation. CD34+ cells were isolated using immunomagnetic microbeads (MACS system; Miltenyi Biotech). The purity of CD34+ cells isolated by positive selection was between 95.1 and 98%.

### Cell culture and primary cells

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 depleted from TGF-β1 and TGF-β2 by RNA interference has been described (Friese et al., 2004). T189 and T204 are short term cultures from glioblastoma biopsy samples (Bahr et al., 2003). Staining of glioma cells with Vybrant™ carbofluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Invitrogen, Carlsbad, CA), staining of haematopoietic SC with PKH26 or DiI and staining of the DNA on frozen sections with 4,6-diamidino-2-phenylindole (DAPI) were performed according to manufacturer’s protocols.

#### Chemotaxis assay

Lin- cells were suspended in RPMI containing 10% FCS and incubated in tissue culture flasks at 37°C for 1 h for resensitization of potentially desensitized chemokine responses (Bowman et al., 1998). Cells that had been washed three times with PBS were placed into a transwell chemotaxis chamber (24 wells, 5 μm pore size; Corning Inc., Acton, MA) (Wright et al., 2002). Chemokines or conditioned media were added to the bottom well. Murine lin- cells (10^5 per top
well) or $10^5$ human CD34$^+$ or $5 \times 10^5$ Lin$Sca1^+$ cells per top well were added in 200 μl serum-free medium (SFM) and allowed to migrate for 4 h at 37°C. The following antibodies, chemicals and proteins were used in these assays: anti-human/mouse CXCL12; anti-human SCF, monoclonal anti-human CXCR4 (clone 12G5) (all R&D Systems); anti-human TGF-β2, 5-biphenyl-4-yl-5-[4-(nitro-phenyl)-piperazin-1-yl]-pyrimidine-2,4,6 (RO28-2653) (15 μM) (Roche Diagnostics, Penzberg, Germany); and human recombinant CXCL12, SCF (R&D Systems), active MMP-9 (Oncogene; Merck Biosciences GmbH, Darmstadt, Germany) and TGF-β2 (PeproTech, London, UK).

Quantification of migrated cells
The principle of the CyQuant Assay (Molecular Probes) is a proprietary green fluorescent dye, CyQuant GR dye, which exhibits fluorescence when bound to cellular nucleic acids. The CyQuant Assay has a linear detection range from 50 or fewer to at least 5 $\times 10^4$ cells in 200 μl volumes using a single dye concentration. A reference standard curve for haematopoietic PC was established for converting sample fluorescence values into cell numbers. Migrated cells were carefully collected from bottom wells, centrifuged and resuspended in 200 μl freshly prepared working solution containing CyQuant cell lysis buffer and CyQuant GR dye. The samples were transferred to a microplate and fluorescence was measured at 480 nm excitation and 520 nm emission. Samples without cells as well as samples with defined cell numbers were included as controls.

Organotypic entorhino-hippocampal slice cultures
Slice cultures were prepared and maintained as described (Ullrich et al., 2001; Eyupoglu et al., 2003). Seven-day-old Wistar rats were used for explantation. After decapitation, the brains were rapidly dissected in 2% agarose gels and visualized by ethidium bromide staining. The β-actin cDNA fragment was amplified as an internal control for equal amplification (Fries et al., 2004). A water control was run in each amplification to control for cross-contamination between tubes.

ELISA
Glioma cell supernatants generated in SFM for 48 h were concentrated with the Centriplus centrifugal filter device YM-3 (3000 Da cut-off; Millipore, Eschborn, Germany). CXCL12 and SCF levels were measured by ELISA (R&D Systems).

Flow cytometry
The levels of CXCR4 protein at the cell surface were quantified by flow cytometry using the specific monoclonal antibodies MAB173 or isotype IgG2B (R&D Systems). The cells were incubated with MAB173 or mouse IgG2B isotype control (10 μg/ml) antibodies for 1 h at 4°C. After three washes with ice-cold PBS containing bovine serum albumin (0.2%), the cells were stained with PE-labelled goat anti-mouse IgG (Sigma) for 30 min at 4°C and washed three times. Fluorescence intensity was measured using a Becton Dickinson FACSCalibur (Heidelberg, Germany). The specific fluorescence index was calculated by dividing the mean fluorescence intensity of cells stained with the specific antibody by the mean fluorescence intensity obtained with an isotype-matched control antibody.

Animal studies
Stereotactic implantation of 5 × 10³ murine SMA-560 or 5 × 10⁴ human LNT-229 glioma cells into the right striatum of 6- to 12-week-old VM/Dk mice (SMA-560) or athymic mice (CD1 nu/nu, Charles River) was carried out for tumour for matrix. After implantation it takes 5 (VM/Dk mice) or 7 days (athymic mice) to reliably generate a tumour. To resemble the clinical situation of larger tumour bulks, we let the tumours grow for 15 (VM/Dk mice) or 22 (athymic mice) days. PKH26-stained VM/Dk (SMA-560 model)

Collagen gel spheroid invasion assay
Glioma spheroids were generated by incubating 1.5 × 10³ cells for 120 h in 96-well plates precoated with 20% Noble Agar (Difco Laboratories, Detroit, MI). Spheroids were embedded into a collagen matrix containing collagen type I (Vitrogen 100, Cohesion Technologies, CA), 10% FCS and 10% NaHCO₃ in a 12-well plate. The sprouting of the spheroids was monitored by time-lapse video. To monitor the invasion of PC into the glioma spheroids, 10⁵ Dil-stained 2 × 10⁵ nu PC or 5 × 10⁵ VM/Dk PC were embedded into the collagen matrix with or without a glioma spheroid. Invasion of the spheroids by Dil-stained PC was monitored by fluorescence microscopy.

RT–PCR
RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). First strand synthesis was performed using Superscript II reverse transcriptase (Gibco, Gaithersburg, MD). The primer sequences were as follows: CXCL12 forward GAGAATTCTGAA-CCGCAAGGTCTTGG (nucleotides 235–261), reverse GATCTAGATCACATCTGAACTGCTT (nucleotides 522–496), yielding a 287 bp product. The PCR conditions included predenaturation at 95°C for 5 min and 35 cycles consisting each of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, synthesis at 72°C for 1 min, and a post-synthesis run of 72°C for 7 min. The PCR products were separated in 2% agarose gels and visualized by ethidium bromide staining. The β-actin cDNA fragment was amplified as an internal control for equal amplification (Friese et al., 2004). A water control was run in each amplification to control for cross-contamination between tubes.
or CD34<sup>+</sup> (LNT-229 model) PC (10<sup>6</sup>) were injected into the tail vein on days 15 (SMA-560) or 22 (LNT-229). Neutralizing CXCL12 antibody (50 μg) or control antibody was injected intraperitoneally within 10 min. Mice were killed 48 h after injection of the PC. Cryostat sections were DAPI-stained. The number of PKH26-labelled PC in brain or tumour tissue was assessed on serial 8 μm sections by fluorescence microscopy by two independent observers (G.T., W.W.). CD34 expression was determined using a FITC-conjugated anti-human CD34 antibody (DAKO, Glostrup, Denmark; clone QBEnd 10, F7166). Immunohistochemistry on frozen sections was performed to determine GFAP (DAKO, clone AB5622, dilution 1:1000) staining. FITC-conjugated IgG was used as isotype-control for CD34-FITC-staining. Negative controls were performed without primary antibody. The experiments were performed according to the German animal protection law.

**Statistical analysis**

Quantitative data were obtained for chemotaxis, ELISA, flow cytometry and the number of PC within the volume of the experimental glioma 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). Interrelationships among PC migration and CXCL12 levels were determined using the Pearson product–moment correlation. All experiments reported here were performed at least three times in triplicate with similar results.

**Results**

**Adult haematopoietic PC home into orthotopic experimental gliomas**

To investigate the homing of adult haematopoietic PC to gliomas *in vivo*, we used G-CSF-mobilized peripheral blood human CD34<sup>+</sup> cells (CD34<sup>+</sup> PC) in a xenograft nude mouse paradigm. PKH26-stained CD34<sup>+</sup> PC were injected intravenously on day 22 after the implantation of human LNT-229 glioma cells into the brains of nude mice. Two days after PC injection, the mice were killed and liquid nitrogen-frozen brains were cut into 10 μm sections. Immunofluorescence microscopy revealed CD34<sup>+</sup> PC within the tumour on day 24 (Fig. 1A, Panels 1 and 2) but no cells within the normal homolateral or contralateral brain parenchyma (Fig. 1A, Panel 4). Similar results were obtained using Lin<sup>−</sup> stem and progenitor cells of immunocompetent VM/Dk mice (VM/Dk PC) administered to SMA-560 glioma-bearing VM/Dk mice (Fig. 1A, Panels 5–8). We further questioned whether the injected CD34<sup>+</sup> PC still expressed CD34 or whether the PC that homed to the glial tumour expressed glial or neuronal markers. Figure 1B shows a strong CD34 signal on PKH26-positive PC (Panels 1 and 2) but not the astrocytic marker, glial fibrillary acidic protein (GFAP) (Panel 3) nor microtubule-associated protein (MAP)2 (Panel 4) that is expressed on adult neurons. The slight positivity for MAP2 surrounding the PC is due to LNT-229 glioma cells that express this marker.

**PC are responsive to chemotactic cues in vitro**

VM/Dk and CD34<sup>+</sup> PC were analysed in chemotaxis assays to examine the molecular mechanisms underlying this glioma tropism. These assays revealed increased migration of VM/Dk or CD34<sup>+</sup> PC, but not LNT-229 glioma cells (data not shown), towards CXCL12, the major cytokine for SC homing to the bone marrow niche, and towards supernatant from human LNT-229 or SMA-560 cells. Heat-denatured LNT-229 or SMA-560 glioma supernatant did not enhance migration, suggesting that migration is stimulated by a heat-labile molecule, presumably a protein. In fact, the impact of LNT-229 and SMA-560 supernatant on migration was blocked by a neutralizing antibody to CXCL12 (Fig. 2A), suggesting that gliomas may use bone marrow cues to attract PC. RT–PCR confirmed that CXCL12 mRNA expression is common in human glioma cell lines including LNT-229 (Fig. 2B). We next correlated the attraction of glioma cell supernatants for PC with CXCL12 levels. Figure 2C shows that all glioma cell lines and two primary *ex vivo* glioblastoma cell cultures released CXCL12 protein in the supernatant and that all supernatant attracted CD34<sup>+</sup> PC. T98G, T189 and T204 supernatant contained the highest levels of CXCL12 and induced the greatest increase of PC migration. There was a significant correlation between migration towards the supernatant and CXCL12 levels (*r* = 0.69, *P* < 0.05). SMA-560 cells contained 1350 pg/ml CXCL12 and enhanced CD34<sup>+</sup> PC 3-fold as did T204 cells. Similar results were obtained with bone marrow-derived murine lin<sup>−</sup>Sca1<sup>−</sup> haematopoietic stem and progenitor cells from athymic nude mice (nu PC) (data not shown). CXCL12 interacts with CXCR4 and is its only known ligand. CXCL12-neutralizing antibodies to CXCR4 did not affect basal migration, but blocked CXCL12-induced migration of human CD34<sup>+</sup> PC in a concentration-dependent manner (10–40 μg/ml). Moreover, these antibodies also blocked the promigratory effect of T98G supernatant (Fig. 2E).

We next studied the molecular mechanism of PC attraction by glioma supernatant in more detail. MMP-9 contributes to the mobilization of haematopoietic PC from the bone marrow to the circulation (Heissig et al., 2002). TGF-β promotes MMP-9 expression by glioma cells (Wick et al., 2001). Consequently, we determined the possible modulation of glioma-directed chemotaxis of PC by TGF-β.

RNA interference targeting TGF-β reduces chemotactic activity of LNT-229 glioma cells for adult haematopoietic PC in organotypic culture. We confronted LNT-229 puro or siTGF-β<sub>1/2</sub> glioma cells (green) and CD34<sup>+</sup> PC (red) on 7-day-old adult rat hippocampal slices in an organotypic brain environment (Fig. 3A) (Ullrich et al., 2001). Tumour and SC migration were monitored up to 4 days after implantation using fluorescence microscopy. PC migration did not occur at random, but was directed towards the glioma.
Moreover, PC migration was reduced when LNT-229 cells were TGF-β-depleted (Fig. 3B).

TGF-β-dependent attraction of PC involves CXCL12, MMP-9 and SCF

To translate the results from the slice cultures into a three-dimensional paradigm using nu PC, the invasion of PC into glioma cell spheroids in a collagen matrix was examined (Fig. 4A). Nu PC were used in the following experiments instead of CD34+ PC because of the high numbers of cells needed. The distribution of PC in empty gels was diffuse (Panels 1 and 5), whereas LNT-229 spheroids strongly attracted the PC (Panels 2 and 6). In contrast, spheroids formed by LNT-229 siTGF-β1/2 showed much less attraction for nu PC (Panels 3 and 7). Spheroids formed by SMA-560 cells were similarly chemoattractive for VM/Dk PC in this assay (data not shown). Furthermore, supernatant from siTGF-β1/2-transfected LNT-229 cells displayed significantly less chemoattraction for nu PC than control supernatant. Reduced chemotaxis in siTGF-β1/2 cells was overcome by exposure of the LNT-229 siTGF-β1/2 cells to exogenous TGF-β1 (2 ng/ml) for 7 days but not for 2 days. Previously, zymography had revealed reduced MMP-9 activity in the LNT-229 siTGF-β1/2 supernatant (Friese et al., 2004). Here, the reduction of PC migration was rescued by exogenous MMP-9. Moreover, HPC migration significantly decreases after treatment of supernatants from LNT-229 with neutralizing antibodies against TGF-β for 2 days (Fig. 4B). However, neither the addition of neutralizing TGF-β antibodies nor

**Fig. 1** Glioma tropism of human haematopoietic PC. (A) LNT-229 cells were implanted into the right striatum of nude mice and 10⁶ CD34⁺ PC were injected into the tail vein, 22 days later. SMA-560 cells were implanted into the right striatum of VM/Dk mice and 10⁶ VM/Dk PC were injected into the tail vein 15 days later. Immunofluorescence was used to visualize the nuclei by DAPI and the CD34⁺ PC by PKH26 (Panels 1 and 2) (Panels 5 and 6) after tumour implantation (magnification ×40 in Panels 1 and 5 and ×100 in Panels 2 and 6). H&E stains are included to show the represented tumour areas at ×40 (Panels 3 and 7). The left striatum is visualized as a control at ×40 (4, 8). (B) LNT-229 cells and 10⁶ CD34⁺ PC were implanted as in (A). Immunofluorescence was used to visualize the nuclei by DAPI and the CD34⁺ PC by PKH26 (Panel 1) or a FITC conjugated CD34 antibody (Panel 2). GFAP (Panel 3) and MAP2 (Panel 4) immunohistochemical staining was used to determine glial or neuronal markers.
the addition of exogenous recombinant TGF-β to SFM lead to any change in HPC migration as compared with SFM alone. These data suggest an indirect effect of TGF-β in PC migration. In fact, there were also lower CXCL12 protein levels in cells treated with a neutralizing TGF-β antibody or transfected with siTGF-β1/2, and higher CXCL12 protein levels were observed in the LNT-229 siTGF-β1/2 supernatant after exposure to exogenous TGF-β for 7 days but not 2 days nor by exposure to MMP-9 (Fig. 4C).

Similarly, SCF levels were reduced in the supernatant of siTGF-β1/2 cells, and the loss of SCF was reversed by 7 days exposure to TGF-β (Fig. 4D), whereas TGF-β1 had no effect on SCF levels in LNT-229 puro cells (data not shown). In contrast to CXCL12, SCF levels in LNT-229 siTGF-β1/2 supernatant were also restored by MMP-9 (Fig. 4D).

The supernatant of LNT-229 puro and siTGF-β1/2 cells enhanced PC migration when supplemented with CXCL12 (Fig. 5A). In contrast, MMP-9 (Fig. 4B) and SCF had a specific stimulating effect on migration induced by the supernatant of TGF-β depleted but not puro control cells, consistent with a critical loss of MMP-9 and SCF resulting from TGF-β depletion. Neutralizing antibodies to CXCL12 or SCF, or the specific MMP inhibitor RO28-2653, reduced migration stimulated by control or siTGF-β1/2 supernatant. The combination of CXCL12 and SCF neutralization exerted no further effect (Fig. 5A).

We next ordered the interrelations between TGF-β, CXCL12, SCF and MMP-9 in that process. Importantly, neither SCF nor MMP-9 retained their activity when CXCL12 was neutralized, indicating that CXCL12 is essential for the chemotaxis towards LNT-229 cell supernatant. Exogenous CXCL12 but not MMP-9 compensated for the neutralization of SCF, indicating that SCF is dispensable when CXCL12 levels are high. The effects of RO28-2653 were overcome by CXCL12 or SCF (Fig. 5B).

**Fig. 2** Attraction of adult haematopoietic PC towards glioma cells. (A) The migration of $10^5$ CD34+ PC (left panel) or $5 \times 10^5$ lin’Scal1’ PC (right panel) was analysed in a transwell assay. SFM, CXCL12 (100 ng/ml), 100 μg LNT-229 or SMA-560 supernatant, heat-denatured LNT-229 or SMA-560 supernatant, or control IgG or neutralizing α-CXCL12 antibodies (100 ng/ml) were added to the bottom well. Data are expressed as mean and SEM of fluorescence of migrated PC analysed by CyQuant assay relative to SFM (n = 3, **p < 0.01 compared with SFM, ***p < 0.001 compared with native supernatant). (B) CXCL12 expression was analysed by RT–PCR. β-actin expression served as a reference. (C) CD34+ PC migration was analysed in a transwell assay using SFM or 100 μg conditioned supernatant in the bottom well. Data are expressed as in (A) (n = 3, upper axis). CXCL12 protein levels in the supernatant were determined by ELISA (mean and SEM, n = 3, lower axis). (D) CXCR4 expression was analysed by flow cytometry and expressed as specific fluorescence index. (E) CD34+ PC migration was analysed in a transwell assay with or without preincubation with control (open bars, 40 μg/ml) or α-CXCR4 antibodies at the indicated concentrations (grey bars) for 30 min. SFM, CXCL12 or T98G supernatant (100 ng/ml) were placed in the bottom well. Data are expressed as in Fig. 1B (*P < 0.05 compared with SFM, *P < 0.05 compared with control IgG).
These data suggest that CXCL12 is absolutely essential for glioma-directed PC migration and that this pathway is positively modulated by MMP-9 and SCF. In fact, CXCL12 alone enhanced migration towards LNT-229 siTGF-β1/2 and control supernatant, and SCF alone enhanced migration towards LNT-229 siTGF-β1/2 but not control supernatant in a concentration-dependent manner, and their combination displayed synergy (Fig. 5C). Altogether, these data place (i) TGF-β upstream of CXCL-12, MMP-9 and SCF, (ii) MMP-9 upstream of SCF and (iii) CXCL12 parallel to MMP-9 and SCF. Furthermore, CXCL12 is indispensable, whereas SCF needs CXCL12 to induce migration and in fact facilitates CXCL12-induced migration.

**Homing of adult haematopoietic PC into orthotopic SMA-560 gliomas in vivo critically depends on CXCL12**

Finally, we investigated the role of CXCL12 for PC homing in an intracranial syngeneic glioma model in vivo. VM/Dk PC (10⁶) were administered intravenously on day 15 after SMA-560 glioma tumour inoculation into the right striatum.

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**Fig. 3** LNT-229 glioma cells attract PC in a TGF-β-dependent manner. (A) Green LNT-229 glioma cells were transplanted into the entorhinal cortex EC. Red CD 34⁺ PC were transplanted into the the stratum lacunosum moleculare of the layer CA1. CA3 and DG are labelled for orientation. (B) Dil-stained CD34⁺ PC (1.4 × 10⁴) or CFSE-stained LNT-229 puro or siTGF-β1/2 cells (5 × 10⁴), or combinations thereof, were transplanted on a rat hippocampal slice and monitored by fluorescence microscopy (magnification ×40) at days 1–4.
Injection of PC was followed by the intraperitoneal administration of neutralizing CXCL12 or control antibodies within 10 min. PC were found within the tumour but neither in the adjacent brain tissue nor elsewhere distant from the visible tumour mass. Co-treatment with anti-CXCL12 antibodies significantly reduced PC homing to the tumour (Fig. 6).

Discussion

The insufficient delivery of therapeutic molecules to deeply infiltrating glioma cells is the major reason for the failure of novel local treatment strategies for malignant gliomas. There is, therefore, a great need for the evaluation of versatile cellular vectors that specifically approach tumour cells to deliver therapeutic molecules. Recent studies have demonstrated the potential of PC, transformed neuronal precursors, neuronally differentiated bone marrow cells and adult neural SC to engraft the lesioned brain (Kim et al., 1998; Aboody et al., 2000; Priller et al., 2001; Lee et al., 2003). The molecular mechanisms underlying this apparent attraction of the lesioned brain for PC and other SC have not been elucidated.

In the present study, we explored how a readily available PC type, adult haematopoietic PC, are attracted by glioma cells in vitro and in vivo. Haematopoietic PC reside in the adult bone marrow and are easily mobilized by G-CSF (Kimura et al., 2004). They possess the unique ability to self-renew and are an unlimited source of potential cellular vectors. We first confirmed the observation made with other SC types (Aboody et al., 2000; Lee et al., 2003) that haematopoietic PC administered systemically can be traced within intracerebral gliomas, but not within normal brain (Fig. 1A). Careful examination with immunohistochemistry revealed no expression of glial or neuronal markers but sustained expression of CD34 on the PC residing for 2 days in the LNT-229 gliomas (Fig. 1B). In contrast, haematopoietic PC in the regenerating chicken embryo spinal cord displayed expression of neuronal markers between days 4 and 9 after implantation. This neuronal differentiation was paralleled by a loss in CD34

Fig. 4 TGF-β-dependent signalling pathway controlling PC attraction. Dil nu PC were detected by fluorescence microscopy. Glioma spheroids were visualized by light microscopy (×100) 48 h after random PC implantation into gels containing no glioma cells (Panels 1 and 5), LNT-229 puro (Panels 2 and 6) or siTGF-β1/2 glioma spheroids (Panels 3 and 7). Panels 4 and 8 show LNT-229 puro spheroids without PC. (B) Nu PC migration (105 cells) was analysed with supernatant from control cells or cells preexposed to recombinant TGF-β, or control or neutralizing TGF-β antibodies (dark grey bars), with recombinant TGF-β, or control or neutralizing TGF-β antibodies alone (light grey bar) (**P < 0.01, α-TGF-β compared with IgG or siTGF-β1/2 compared with puro). CXCL12 (C) or SCF (D) protein levels were determined by ELISA in the supernatant from control cells or cells preexposed to recombinant TGF-β (10 ng/ml) for 2 or 7 days or MMP-9 for 24 h. (**P < 0.01, siTGF-β1/2 compared with puro PC).
Fig. 5  TGF-β acts upstream of CXCL12 and MMP-9/SCF and CXCL12 and SCF act synergistically to promote PC migration.  

(A) Nu PC migration (10^5 cells) was analysed with supernatant of control cells or cells preexposed to CXCL12 (100 ng/ml), SCF (10 ng/ml), neutralizing CXCL12 antibody (100 μg/ml), RO28-2653 (15 μM) neutralizing SCF antibody (10 μg/ml) or isotype control antibodies in the bottom well ("P < 0.05 or ++P < 0.01, CXCL12, SCF, α-CXCL12, α-SCF or RO28-2653 compared with controls).  

(B) Nu PC migration was analysed in a transwell assay with SFM containing CXCL12 (100 μg/ml), LNT-229 puro or siTGF-β1/2 supernatant containing neutralizing CXCL12 antibody (100 μg/ml), RO28-2653 (15 μM) or SCF antibody (10 μg/ml) and the indicated combinations with SCF (10 μg/ml), MMP-9 (10 ng/ml) or CXCL12 (100 ng/ml) in the bottom well (***P < 0.01, α-CXCL12, α-SCF or RO28-2653 compared with controls).  

(C) Nu PC migration was analysed as in (B) using the indicated concentrations of CXCL12 or SCF or combinations thereof (**P < 0.01, siTGF-β1/2 compared with puro, +P < 0.05, effect of CXCL12 or SCF compared with controls or CXCL12 and SCF compared with CXCL12 or SCF alone).
expression (Sigurjonsson et al., 2005). Hence, neuronal or glial differentiation of CD34 PC might be influenced by the different microenvironment in the regenerating chicken embryo spinal cord compared with a human xenograft glioma, or the interval between administration and analysis. Others have also failed to show glial differentiation of bone marrow-derived cells (Wehner et al., 2003). Cell culture assays indicated that the migration of PC towards glioma supernatant in vitro involves CXCL12/CXCR4 interactions (Fig. 2), recapitulating the mechanisms mediating PC homing to the bone marrow, extravasation of mature leukocytes to sites of tissue damage and differentiation-dependent thymocyte migration in the thymus during T lymphopoiesis (Aiuti et al., 1997; Kim et al., 1998; Peled et al., 1999). CXCL12/CXCR4 interactions have also been proposed to stimulate glioma cell survival and proliferation (Zhou et al., 2002; Rubin et al., 2003). Although all glioma cells examined here express CXCL12 and CXCR4, CXCL12 was not chemoattractive for these glioma cell lines (Fig. 2B and C and data not shown). Thus the chemoattractive effect of CXCL12 acting on CXCR4 (Fig. 2E) may be limited to specific target cell populations. Apart from glioma and bone marrow stromal cells, there is constitutive (Rostasy et al., 2003) and diseas-enhanced expression of CXCL12 in astrocytes and neurons. For instance, human immunodeficiency virus-infected astrocytes, and astrocytes and neurons of patients with Alzheimer’s disease show increased CXCL12 release (Zhang et al., 2003). Hence, our experiments do not rule out a contribution of the lesion induced by the implantation of the glioma cells to the PC tropism. At the same time, the exclusive localization of the PC within the tumour as well as the in vitro data support a major role for PC attraction by the glioma cells.

TGF-β has become a major target of immunotherapy for malignant glioma (Friese et al., 2004; Uhl et al., 2004). There is also an evolving role for TGF-β in the migration and invasion of malignant gliomas (Wick et al., 2001). However, the local release of TGF-β by glioma cells may also be required to attract PC in vivo. Since TGF-β-depleted LNT-229 siRNA cell are non-tumourigenic in nude mice (Friese et al., 2004), we adopted the organotypical slice culture paradigm to study a role for TGF-β for PC homing to gliomas. Here, PC attraction was reduced by RNA interference targeting TGF-β. These assays allowed us to conclude that PC truly migrated preferentially towards tumour cells and appeared to rule out a preferential utilization of identical substrate pathways for migration as an alternative explanation for the co-localization of implanted tumour cells and PC (Fig. 3). Similarly, the supernatant of LNT-229 siTGF-β1/2 cells showed a strong reduction in PC attraction (Fig. 4). Since the promigratory activity of this supernatant was not rescued by TGF-β and since TGF-β itself was not chemoattractive for PC (Fig. 4B), we postulated an indirect pathway leading from TGF-β to PC migration. In fact, we obtained evidence to suggest that the TGF-β-dependent synthesis and release of CXCL12 and MMP-9 mediate PC migration. This is because PC migration and CXCL12 are reduced in LNT-229 siTGF-β1/2 cells and only long-term TGF-β supplementation sufficient to restore CXCL12 and MMP-9 mediated PC migration. This is because PC migration and CXCL12 are reduced in LNT-229 siTGF-β1/2 cells and only long-term TGF-β supplementation sufficient to restore CXCL12 and MMP-9 (Friese et al., 2004), or exogenous CXCL12 rescue the TGF-β depletion (Fig. 4B–D and Fig. 5A). Furthermore, neutralization of CXCL12 abolished migration towards TGF-β-containing control supernatant,
placing CXCL12 downstream of TGF-β. In addition, MMP-9 expression and activity were reduced by RNA interference targeting TGF-β (Friese et al., 2004), and neutralization of MMP-9 by RO28-2653 reduced migration towards TGF-β-containing and TGF-β-depleted supernatant (Fig. 5A). The target of MMP-9, in turn, was probably SCF because MMP-9 augmented SCF release and migration of PC in LNT-229 siTGF-β1/2 cells (Fig. 4B and D). Furthermore, in the presence of CXCL12, SCF synergistically enhanced PC migration towards supernatant of TGF-β-depleted and control cells (Fig. 5C). The MMP-9-mediated release of s-KitL and binding to its receptor, CD117, contribute to the mobilization of haematopoietic PC from the bone marrow to the circulation (Hattori et al., 2002). In addition to our finding that SCF is involved in glioma-directed haematopoietic PC migration, SCF is important for the migration of neuronal PC to areas of freeze-damaged brain (Sun et al., 2004). Figure 7 summarizes that the TGF-β-dependent release of CXCL12 and MMP-9/SCF are not sequential but parallel pathways because MMP-9 does not enhance the release of CXCL12 (Fig. 4C) and because MMP-9 and SCF are unable to overcome CXCL12 neutralization (Fig. 5B).

In summary, we demonstrate the central role of TGF-β and CXCL12 in the glioma-induced and -directed migration of adult peripheral CD34+ human and adult bone marrow-derived murine haematopoietic PC. CXCL12 is the essential mediator of PC migration, and the CXCL12 attraction is enhanced by MMP-9-induced SCF cleavage. However, the use of these PC as cellular vectors for gene therapy in glioblastoma may not be successfully combined with therapeutic strategies targeting TGF-β-mediated immunosuppression and motility.

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References


Bautz F, Denzinger C, Kanz L, Möhle R. Chemotaxis and transendothelial migration of Cd34+ haematopoietic progenitor cells induced by the inflammatory mediator leukotriene D4 are mediated by the 7-transmembrane receptor CysLT1. Blood 2001; 97: 3433–40.


Haematopoietic stem cells homing to gliomas


