New insight into the SDF-1/CXCR4 axis in a breast carcinoma model: Hypoxia-induced endothelial SDF-1 and tumor cell CXCR4 are required for tumor cell intravasation

Fengyan Jin¹, Ulf Brockmeier², Friedrich Otterbach³, and Eric Metzen²

¹ The First Hospital of Jilin University, Changchun, China, present address: Department of Neurology, University of Duisburg-Essen, Essen, Germany, ² Institute of Physiology, and ³ Institute of Pathology and Neuropathology, University of Duisburg-Essen, Essen, Germany

Running title: SDF-1 and CXCR4 are required for tumor cell intravasation

Key words: metastasis, migration, oxygen, hypoxia inducible factor, chemokine receptor

FJ was supported financially by the Deutscher Akademischer Austauschdienst (DAAD) and the Dr. Werner Jackstädt-Stiftung.

Abstract word count: 250

Total word count: 5785

Figures: 6, Tables: None

Corresponding author:

Prof. Eric Metzen, M.D., Institute of Physiology, University of Duisburg-Essen, Hufelandstr. 55, D45147 Essen, Tel: +49 201 7234626, Fax: +49 201 7235661,

Email: eric.metzen@uni-due.de

The authors declare no conflict of interest.
Abstract

The SDF-1/CXCR4 axis has been implicated in breast cancer metastasis. In contrast to its well established role in organ-specific homing and colonization of tumor cells, the involvement in intravasation, especially in a hypoxic environment, is still poorly understood. Initially, we detected both, the chemokine SDF-1 and its receptor CXCR4 in microvessels in invasive ductal cancer samples. To elucidate the role of the SDF-1/CXCR4 axis in vascular endothelium for tumor intravasation, we evaluated the effects of CXCR4 activation in human umbilical vein and dermal microvascular endothelial cells (HUVEC and HDMEC) and in cultured mammary carcinoma cells (MDA MB231 and MCF7). We observed an up-regulation of SDF-1 and CXCR4 in HUVECs in hypoxia which led to proliferation, migration and tube formation. Hypoxia induced adhesion of tumor cells to endothelial cells and stimulated transendothelial migration. The effects of hypoxia were dependent on the activity of the transcription factor hypoxia-inducible factor (HIF). Adhesion to and migration through a HUVEC monolayer were significantly reduced by lentiviral inhibition of CXCR4 in breast carcinoma cells or treatment of endothelial cells with an anti-SDF-1 neutralizing antibody. These data demonstrate that the interaction of SDF-1 secreted by ECs with tumor cell CXCR4 is sufficient to stimulate transendothelial migration of the tumor cells. Our results suggest that the SDF-1/CXCR4 axis is important in angiogenesis and tumor cell intravasation. Because both proteins were readily identifiable in a significant fraction of human breast cancer samples by immunohistochemistry, CXCR4 may constitute a molecular target for therapy when both, SDF-1 and CXCR4 are expressed.
Introduction

In the United States, breast cancer is the most frequent type of cancer with nearly 210,000 new cases diagnosed and almost 40,000 deaths in 2010 (1). Metastasis, i.e. transport of tumor cells to and growth at secondary sites is a major cause for morbidity and mortality (2, 3). The metastatic process involves multiple steps including tumor cell mobilization, angiogenesis, intravasation, survival in the circulation, extravasation, and proliferation in a new tissue environment. Despite increasing knowledge, all these steps are incompletely understood. For example at the primary site, the communication of the tumor cells with their cellular microenvironment which is composed of fibroblasts, neutrophils, mast cells and endothelial cells, not only promotes tumor growth, but also facilitates emigration from the original location (4, 5). These studies strikingly suggest a functionally important interaction between host-stroma and tumor. However, the mechanisms of intravasation are mostly elusive.

In a hypoxic microenvironment, the function of endothelial cells is modified towards an angiogenic phenotype. This response to hypoxia is indeed physiological, for example blood vessel development in the mammalian embryo is also stimulated by hypoxia (6). In tumor biology, this pathway is hijacked by the tumor cells. When a tumor grows beyond a diameter of 2 mm, the tumor cells commonly turn hypoxic. As a response the transcription factor complex hypoxia-inducible factor (HIF) is activated. HIF stimulates secretion of growth factors including vascular endothelial growth factor (VEGF) which acts on endothelial cells to initiate angiogenesis. The developing capillary network in the tumor not only delivers nutrients and oxygen, but also provides the escape route by which detached cancer cells can enter into the circulation.

Stromal cell-derived factor-1 (SDF-1) is a highly conserved chemokine that acts through its cognate receptor CXCR4. Chemokines are low molecular weight signaling peptides frequently involved in leucocyte mobilization and homing. Specifically, the SDF-1/CXCR4
axis plays a critical role in B-cell development (7), angiogenesis (8), HIV infection (9), as well as stem cell mobilization and homing (10). Both, SDF-1 and CXCR4 have been reported to be hypoxia-inducible (11, 12). Several lines of evidence suggest that SDF-1 and CXCR4 are involved in tumor progression and the development of distant metastases: SDF-1 secreted by tumor associated fibroblasts has been reported to be pro-angiogenic (5). In a landmark study it was demonstrated that expression of CXCR4 facilitates orthotopic tumor growth and metastatic potential in SCID mice although, notably, tumor cells were injected i.v., which indicates that the experiments reflect late steps of metastasis (13). In another report CXCR4 was identified in a human breast cancer cell line as one out of five proteins which gives rise to bone metastases after intraventricular injection in mice (14). Correlation of CXCR4 expression to HER2, a well-established prognostic factor in mammary carcinoma was also observed in human breast tumors (15).

On the other hand, neither SDF-1 nor CXCR4 has been identified as a prognostic marker in large microarray DNA screens published by several independent groups (16, 17). A low molecular weight CXCR4 antagonist did not inhibit growth in a murine tumor model (18). In one study it was found that expression of SDF-1 reduced the metastatic potential of breast carcinoma cell lines (19) and, furthermore, an immunohistochemical analysis of SDF-1 expression in human breast cancer patients came to the result that SDF-1 expression was a marker of longer disease-free and overall survival (20). Collectively, these reports demonstrate that the role of SDF-1 and CXCR4 in human tumors is still controversial, and in particular, that the effect of SDF-1/CXCR4 activation in tumor-host interaction at the primary site is elusive.

It is still an open question whether tumor cells enter into the vasculature passively or whether they migrate through blood vessels actively. Evidence for active metastasis is provided by the observation that the tumor microenvironment can induce migration-related pathways (21, 22) or collaboration of stromal and tumor cells in metastasis (4, 23). However, currently there is
no study available in which the whole sequence of events is followed in a single co-culture model. Therefore, it was the aim of our study to provide mechanistic insight into the role of the SDF-1/CXCR4 axis in the early steps of metastasis with particular emphasis on the effects of hypoxia in a co-culture system of breast cancer and endothelial cells. We observed that endothelial SDF-1 stimulated active adhesion of CXCR4 expressing breast carcinoma cells to endothelial cells and transendothelial migration. Importantly, we confirmed endothelial SDF-1 and tumor cell CXCR4 expression in a significant fraction of samples from mammary tumors which pinpoints the relevance of our data in a clinical setting.

Materials and Methods

Immunohistochemistry and immunofluorescence

Staining of SDF-1 and CXCR4 was performed in 140 samples of primary breast carcinomas chosen from a collection of our Institute of Pathology. The patients presented between 1989 and 1993, were operated with curative intent at the Department of Obstetrics and Gynaecology, University Hospital Essen. The study was performed according to the Declaration of Helsinki and approved by the local Ethics Committee. Immunohistochemistry was conducted as described previously (24). Following individually optimised heat-based antigen retrieval for each antibody, SDF-1 was stained with a monoclonal anti-CXCL12/SDF1 [clone 79018 (R&D Systems, Inc, Minneapolis, USA) dilution 1:1000, antigen retrieval: 20 min 95°C water bath, citrate buffer, pH 6.0]. CXCR4 immunohistochemistry was done with a rabbit monoclonal antibody generously provided by Prof. Stefan Schulz, Jena, Germany, dilution 1:30, antigen retrieval as for SDF-1. CXCR7 was stained with a monoclonal antibody (MAB42273, R&D Systems). Immunofluorescence was performed with the same CXCL12/SDF-1 antibody and human von-Willebrand-Factor
(vWF) antibody (dilution 1:100, rabbit polyclonal, Dako). SDF-1 and vWF were detected with an Alexa Fluor488 goat anti-mouse antibody and Alexa Fluor568 goat anti-rabbit antibody (Invitrogen), respectively.

Cell culture

Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC), both from PromoCell (Heidelberg, Germany) were grown in endothelial cell growth medium (ECGM, PromoCell) containing the recommended additives. ECs were used for experiments from passage 3-6. HEK-293T, MDA-MB 231 and MCF7 cells were obtained from ATCC. MDA-MB 231 were maintained in DMEM (Invitrogen), MCF-7 cells were grown in RPMI1640 (Invitrogen), supplemented with 10 % fetal calf serum, penicillin and streptomycin. Hypoxic culture conditions were achieved in a hypoxic chamber (Toepffer Lab Systems, Göppingen Germany) by inflation of N₂ and CO₂ into the air-filled chamber until 1 % O₂ and 5 % CO₂ were reached.

Chemokines, antibodies and reagents

Recombinant CXCL12/SDF-1α, CXCL12/SDF-1 monoclonal antibody (MAb) (Clone 79018, Mouse IgG1), CXCR4 (Fusin) MAb (Clones 12G5 and 44716), and affinity-purified HIF-2α antibody were purchased from R&D Systems (Wiesbaden, Germany). CXCR4 polyclonal antibodies were from Abcam (Cambridge, UK). HIF-1α MAb was from BD Transduction Laboratories (Heidelberg, Germany). Mouse Ig G2a, (Clone G155-178) was purchased from BD Biosciences. Human α-tubulin MAb was from Santa Cruz Biotechnology (Heidelberg, Germany). Human actin antibody was from Sigma (Munich, Germany). Dimethyloxalylglycine (DMOG) was purchased from Alexis Biochemicals (Lörrach, Germany).
Bacterial strains, plasmids, lentiviral transduction and siRNA transfection

*E. coli* XL1-blue (Stratagene, Heidelberg) served as the general host for plasmid preparation, *E. coli* Stbl3 (Invitrogen) were used for production of pLKO.1 (8453, Addgene), pLKO.1-shRNA-HIF-1α-1 (Mission® TRC shRNA TRCN0000003810, Sigma), pLKO.1-shRNA-HIF-1α-2 (Mission® TRC shRNA TRCN0000003811, Sigma) pLKO.1-shRNA-HIF-2α-1 (Mission® TRC shRNA TRCN0000003803, Sigma), pLKO.1-shRNA-HIF-2α-2 (Mission® TRC shRNA TRCN0000003806, Sigma) and pLKO.1-shRNA-CXCR4 (Mission® TRC shRNA TRCN0000256864, Sigma). Generation of recombinant lentivirus was performed as described previously (25). Lentivirus infected MDA-MB 231 and MCF7 cells were selected by treatment with 1 µg/ml and 3 µg/ml puromycin, respectively. CXCR4 siRNA transfection of MCF7 cells was performed according to the DharmaFECT siRNA transfection protocol (Thermo Fisher Scientific, Waltham, MA). 2×10^5 MCF7 cells were transfected, the final concentration of the siRNA was 25 nM. The cells were incubated with the transfection solution for 72 hours. Gene silencing was monitored by Western blotting.

Western blot

Whole cell lysates from HUVECs, MDA-MB 231 and MCF-7 were prepared and Western blotting was performed as reported (25).

Fluorescent labeling and flow cytometry

The fluorescent labeling of HUVECs, MDA-MB 231 and MCF7 cells with calcein-AM (Invitrogen) was conducted according to the manufacturer’s protocol. Fluorescence was detected using a Zeiss Axiovert 200M confocal microscope (Carl Zeiss, Jena, Germany). For FACS analysis HUVECs or MDA-MB 231 cells were detached with 2 mM EDTA in PBS. For CXCR4 determination and membrane SDF-1 expression, the unstained cells were fixed by incubation with 1 % paraformaldehyde (PFA) for 5 minutes. The cells were incubated with
20 µl PE-conjugated human CXCR4 MAb (Clone 12G5) for 30 minutes at 4 °C. For intracellular SDF-1 detection, the cells were permeabilized with Cytofix/Cytoperm (BD Biosciences) and incubated with 25 µg/ml CXCL12/SDF-1 MAb (Clone 79018) for 30 minutes at 4 °C. SDF-1 was detected with a PE-conjugated goat anti-mouse antibody (BD Pharmingen). Cells were analysed with a Beckman Coulter FACSCalibur. A total of 10,000 events were assessed. Data were analyzed by CELLQuest software (BD Biosciences).

Tube formation assay
HUVECs (2×10⁴/well) were transduced with pLKO.1-shRNA-HIF-1α, pLKO.1-shRNA-HIF-2α or pLKO.1-shRNA-CXCR4 and seeded into Matrigel-coated wells of a 96 well plate. 24 hours later photographs were taken with a Leica DFC 290 camera (Leica Microsystems, Wetzlar, Germany). Only perfectly continuous tubes between 2 branching points were regarded as a tube.

Proliferation assay
To assess HUVEC proliferation 1×10⁴ HUVECs were seeded into 24 well plates in the absence or presence of recombinant human SDF-1 (1-100 ng/ml). CXCR4 neutralizing antibodies (10 µg/ml) or control mouse IgG (BD Biosciences) were added 12 hours prior to treatment with SDF-1. The viable cells were counted at day 3 and day 6. When the effects of endogenous SDF-1 were to be blocked, 1×10⁴ HUVECs were seeded into a 24 well plate and cultured in serum-free ECGM in the absence or presence of an SDF-1 neutralizing antibody for 3 days. For proliferation assays after HIF-1α, HIF-2α or CXCR4 knockdown, 8×10⁴ HUVECs were transduced with the appropriate vectors and cultured for 3 days. To quantify tumor cell proliferation, 1×10⁵ MDA-MB 231 were plated and grown for 4 days.

Tumor cell-to-endothelium adhesion assay
5×10⁴ HUVECs transduced with pLKO.1-shRNA-CXCR4 or pLKO.1 were exposed to normoxia or hypoxia for 12 hours after the monolayer had formed. 1×10⁴ calcein labelled MDA-MB 231 cells were added, adhesion was allowed to occur for 2 hours at 37 °C. Non-adherent cells were washed off, adherent MDA-MB 231 cells were quantified.

Adhesion of tumor cells to endothelial tubes.
1×10⁴ calcein labelled MDA-MB 231 or MCF7 cells were co-cultured with 2×10⁴ HUVEC or HDMEC on a Matrigel-coated 96 well plate. 10 µg/ml CXCR4 neutralizing antibody or 6-12 µg/ml SDF-1 antibody (both from R&D Systems) was added to block CXCR4 or SDF-1, respectively. After 24 hours, the adherent tumor cells were quantified.

Cell migration assays
All cell migration assays were conducted in a modified Boyden chamber, i.e. with 24 well plates in which transwell inserts (polycarbonate membrane insert with 6.5mm diameter and 8.0 µm pores, Corning, Schiphol, The Netherlands) were placed. To assess endothelial cell migration, 2-5×10⁴ HUVECs were seeded into the upper compartment and incubated for 24 hours. The non-migrated cells on the top side of the surface were removed. The migrated cells on the bottom side of the membrane were counted in 12 standardized fields. To test endothelial effects on tumor cell migration, 1×10⁵ HUVECs or HDMECs were seeded in the lower compartment of the chamber. When the cells had formed a monolayer, they were treated with SDF-1 neutralizing antibodies (R&D Systems) before calcein labelled MDA-MB 231 cells were seeded into the upper compartment. Exposure to hypoxia was for 24 hours. Migrated cells in the lower compartment were counted and photographs were taken. For transendothelial migration assays, HUVECs were cultured in the upper compartment which was precoated with 0.1 % gelatine. Leakiness of the endothelial monolayer was tested with FITC dextran before addition of tumor cells. 2×10⁴ calcein labelled MDA-MB 231 cells were
added to the upper compartment and incubated for 24 hours. The migrated cells were quantified. Since only the tumor cells were labelled, this assay allows discrimination of tumor cells from migrated endothelial cells.

Statistical analysis

Data are expressed as the mean ± s.d. Studies involving more than 2 groups were analyzed by one-way ANOVA coupled to post-hoc comparison tests for multiple pairwise comparisons. All other experiments were analyzed with an unpaired two-tailed Student’s t-test. In all figures * indicates $P<0.05$ while ** indicates $P<0.001$.

Results

SDF-1 and CXCR4 are expressed in microvessels in human breast carcinoma tissues.

SDF-1 and CXCR4 are expressed in a number of tissues including bone marrow, lung, and lymph nodes. Since these factors were implicated in breast cancer progression and metastasis we regarded it as a prerequisite that they are indeed expressed in breast cancer samples. Immunohistochemistry showed that SDF-1 was expressed in the tumor cells in approximately 50 % of 140 samples chosen from a collection of mammary tumor samples. We performed double immunofluorescence by staining SDF-1 and vWF in 10 of these samples. A significant fraction of the cells stained positive for SDF-1 but negative for vWF (Fig. 1A, case 1). These cells are most likely tumor cells. Interestingly, we detected SDF-1 also in the endothelium of microvessels. In 3 out of the 10 samples, SDF-1 was predominantly expressed in endothelial cells (Fig. 1A, case 2). Within the 140 samples, CXCR4 was expressed in 13 cases in the tumor cells (Fig. 1B, top). Interestingly, 23 samples showed necrotic areas, in 7 of these cases CXCR4 was apparently induced in the proximity of the necroses (Fig. 1B, centre). Necroses
are likely to be hypoxic which is in line with the idea that CXCR4 is hypoxia-inducible. From the 140 samples, a subgroup of 24 samples showed CXCR4 expression in blood vessels (Fig. 1B, bottom). Which molecular events or pathways govern CXCR4 expression in tumor cells as opposed to endothelial cells is unknown. There was no apparent correlation between CXCR4 and SDF-1 expression. In 13 out of 130 cases, tumor cells stained positive for CXCR7. Again, expression of CXCR7 did not correlate to SDF-1 or CXCR4 expression. Tumor cells stained positive for CXCR4 and CXCR7 simultaneously in only 2 cases.

**Autocrine SDF-1/CXCR4 signaling in hypoxia is required for ECs angiogenesis.**

In initial experiments, we observed that hypoxia induced proliferation, tube formation and migration in HUVECs (data not shown). To analyse the regulation of CXCR4 under hypoxic conditions, we assessed the expression of CXCR4 by FACS (Suppl. Fig. 1A). As expected, CXCR4 increased markedly under hypoxia as compared to normoxia. Hypoxic induction was confirmed by Western blotting (Suppl. Fig. 1B). As shown in Suppl. Fig. 1C, an intracellular SDF-1 up-regulation by hypoxia was detected by FACS which probably reflects SDF-1 being processed before secretion since storage of chemokines is uncommon. Importantly, secretion was also elevated as shown by ELISA in samples of the conditioned media (Suppl. Fig. 1D). In both, HUVEC and HDMEC, CXCR7 was neither detectable by Western blotting nor by immunofluorescence in our hands. Next, we analyzed whether SDF-1 induced proliferation and angiogenesis. To do so, HUVECs were cultured in the absence or presence of increasing concentrations of SDF-1 (1 – 100 ng/ml), CXCR4 neutralizing antibody (10 µg/ml) or Ig G control antibody. SDF-1 stimulated HUVEC proliferation (Suppl. Fig. 2A) and tube formation (Suppl. Fig. 2B) in a dose-dependent manner. Anti-CXCR4 neutralizing antibodies inhibited proliferation and tube formation significantly.

To assess the importance of CXCR4 in the endothelial cells, HUVECs were transduced with pLKO.1-shRNA-CXCR4. Expression of CXCR4 was significantly reduced as compared to
empty vector (Fig. 2A). Strikingly, reduction of CXCR4 retarded HUVEC proliferation (Fig. 2A). We next tested whether CXCR4 also plays a role in migration. As shown in Fig. 2B, HUVECs expressing shCXCR4 exhibited attenuated migration under normoxia and hypoxia as compared to controls. To demonstrate the involvement of CXCR4 in capillary morphogenesis, we analyzed tube formation in HUVEC. After CXCR4 knockdown the tube numbers were greatly reduced (Fig. 2C and D). We observed that serum deprived HUVECs died at day 3 in normoxia. However, many of the HUVECs survived when kept in hypoxia. An SDF-1 neutralizing antibody partly abrogated this effect in a dose-dependent manner (Fig. 2E and F). Furthermore, we performed tube formation assays with the SDF-1 neutralizing antibody. We observed that blocking of the SDF-1 signal reduced tube numbers under normoxia and under hypoxia (Fig. 2G and H).

**HIF-1α and HIF-2α are essential for angiogenesis.**

In general, hypoxia significantly stimulates angiogenesis. As in HUVEC, both HIF-α subunits were detectable and induced by hypoxia in HDMEC. We also confirmed that CXCR4 is hypoxia-inducible in HDMEC (Supl. Fig. 4A). The relative contribution of HIF-1α as opposed to HIF-2α to the transcriptional response of endothelial cells in hypoxia is controversial (26, 27). To elucidate the role of HIF-1α or HIF-2α in endothelial CXCR4 expression and angiogenesis, HUVECs were transduced with pLKO.1-shRNA-HIF-1α or pLKO.1-shRNA-HIF-2α alone or in combination. We then induced HIF activity chemically using the prolyl hydroxylase inhibitor DMOG. Analyzing CXCR4 expression by Western blotting, we demonstrated that both HIF-1α and HIF-2α regulated CXCR4 expression in HUVECs (Fig. 3A). Apparently, both HIF α-subunits affected CXCR4 expression although the precise contribution of each subunit is difficult to quantify because knockdown of one subunit affected also the other. HIF-1α− and HIF-2α− knockdown reduced proliferation (Fig. 3B). HIF-2α appeared to have a more pronounced effect. Apparently, there was some degree
of synergy between the two factors since double knockdown of HIF-1α and HIF-2α had the
most striking effect. Similarly, migration of HUVECs after HIF-1α or HIF-2α knockdown
was reduced markedly, particularly after HIF-1α/2α double knockdown (Fig. 3C). In addition,
transduction of HIF-1α shRNA or HIF-2α shRNA abrogated hypoxic induction of tube
formation (Fig 3D and E). Again, the most significant suppression was achieved by combined
HIF-1α/2α knockdown.

**Hypoxia induces CXCR4 expression in MDA-MB 231 and MCF7, and stimulates
transendothelial migration towards an SDF-1 gradient.**

Hypoxia was shown previously to enhance CXCR4 expression in cultured tumor cells. While
CXCR4 expression was readily detectable, we were unable to demonstrate expression of
CXCR7 in normoxia or hypoxia by Western blotting or cell surface expression by
immunofluorescence or FACS. To test regulation of CXCR4 by hypoxia we exposed two
breast carcinoma cell lines, MDA-MB 231 (aggressive type) and MCF7 (moderately
aggressive) to hypoxia or DMOG treatment. In the case of MDA-MB 231, 24 hours of
hypoxia or DMOG led to a comparable increase in CXCR4 expression (Fig. 4A and B).
However, 4 hours in hypoxia were insufficient to increase CXCR4 levels (Fig. 4B). Similarly,
CXCR4 expression of MCF7 showed a marked increase after treatment with DMOG or
hypoxia (Suppl. Fig. 3). Knockdown of HIF-1α or HIF-2α led to decreased CXCR4
expression in MDA-MB 231 (Fig. 4C) and MCF7 (Suppl. Fig. 3). Most efficient attenuation
of CXCR4 expression was observed by combined knockdown of HIF-1α/2α (Fig. 4C). Next,
we observed that hypoxia facilitated transendothelial migration of MDA-MB 231 cells, in
particular when an SDF-1 gradient was present (Fig. 4D).
Collaboration of endothelial and breast carcinoma cells mediates active adhesion and migration through the SDF-1/CXCR4 axis.

To analyse whether hypoxia, CXCR4 and SDF-1 affect the interaction of tumor cells with endothelial cells, a tumor-to-endothelium adhesion assay was developed. Hypoxia promoted the adhesion of MDA-MB 231 cells to HUVECs. This interaction was disrupted by selectively blocking CXCR4 in MDA-MB 231 cells, not by blocking CXCR4 in HUVECs (Fig. 5A and B). This result indicated that CXCR4 expression by tumor cells is important for the adhesive function. Next, calcein labelled MDA-MB 231 were co-cultured with HUVECs in Matrigel for 24 hours. We observed that MDA-MB 231 were closely associated with the tubes formed by HUVECs. Again, blocking CXCR4 in MDA-MB 231 cells, but not in HUVECs inhibited this association (Fig. 5C and D). We performed transendothelial migration assays to investigate whether CXCR4 influences transendothelial migration. We observed that CXCR4 knockdown in MDA-MB 231 cells, but not in HUVECs reduced transendothelial migration under normoxia and hypoxia (Fig. 5E and F). CXCR4 knockdown using siRNA in MCF7 significantly reduced migration (Fig. 5G and H). Because CXCR4 in HUVECs did not regulate these processes we asked whether HUVEC-derived SDF-1 regulates these steps. The presence of an SDF-1 neutralizing antibody attenuated the association of MDA-MB 231 as well as MCF7 with HUVECs remarkably (Fig. 6A - D). Importantly, adhesion of MDA-MB 231 to HDMEC tubes was also inhibited by addition of an SDF-1 antibody (Supl. Fig. 4B). When we blocked the activity of the SDF-1 secreted by HUVECs or HDMECs with an SDF-1 neutralizing antibody for 12 hours, migration of MDA-MB 231 or MCF7 was substantially reduced in hypoxia (Fig. 6E – F, Supl. Fig. 4C)). Taken together, these findings strongly indicate that SDF-1 expressed in HUVECs or HDMECs promotes tumor-to-endothelium adhesion and transendothelial migration of tumor cells.
Discussion

In the current study we demonstrate that the chemokine SDF-1 and its cognate receptor CXCR4 are expressed in breast cancer \textit{in vivo} and analyze the effects of manipulating these proteins in a co-culture model of mammary carcinoma and vascular endothelial cells. The evidence presented suggests that activation of the SDF-1/CXCR4 axis in a growing tumor contributes to angiogenesis and the initial steps of metastasis. CXCR4 activation had surprisingly similar effects on tumor and endothelial cells: it induced proliferation and migration. In addition, SDF-1 binding to CXCR4 led to specific effects on endothelial and tumor cells: in endothelial cells CXCR4 stimulated tube formation which may point to pro-angiogenic activity \textit{in vivo} while in the tumor cells activation of this receptor resulted in adhesion to endothelial cells and transendothelial migration.

In addition to their hematopoietic activities, SDF-1 and CXCR4 have been implicated in the regulation of angiogenesis in a number of (patho)physiological situations: the retina, the gastrointestinal tract and the myocardium following infarction have been shown to rely on SDF-1 and CXCR4 for angiogenesis (28-30). Tumors also depend on angiogenesis since diffusion of oxygen and nutrients is insufficient to sustain tumor growth beyond a size of approximately 2 mm. Therefore, the anti-angiogenic antibody bevacizumab (Avastin®) has been developed and is used to antagonize the pro-angiogenic molecule VEGF. With respect to breast cancer, however, the use of this antibody is discussed controversially (31). It is important to note that our data do not simply suggest that the SDF-1/CXCR4 axis is pro-angiogenic. This is only one component which we have observed \textit{in vitro}, as we have clearly shown that SDF-1 stimulates proliferation, migration and tube formation of endothelial cells. In addition, and importantly, CXCR4 signaling also fostered growth of cultured tumor cells (data not shown) and the interaction between tumor and endothelial cells which resulted in
transendothelial migration. Transposed to the in vivo situation these processes may result not only in angiogenesis but also in intravasation and metastasis. The in vivo sequence of events which we propose and which we have followed meticulously in vitro is as follows: rapid proliferation of tumor cells leads to areas of hypoxia within the primary tumor. Hypoxia then activates the ubiquitous transcription factor HIF which results in activation of an array of HIF target genes including SDF-1 and CXCR4 which contribute to tumor cell migration and adhesion to endothelial cells. Eventually, activation of the SDF-1/CXCR4 axis results in transendothelial migration. Within this scenario, angiogenesis provides the escape route for tumor cells. The secretion of SDF-1 by endothelial cells (potentially also by tumor and stroma cells) and CXCR4 expression by the tumor cells results in entering of the tumor cells into the blood vessels. In principle, CXCR4 expression of tumor cells also favors extravasation in tissues where SDF-1 is expressed. Indeed this effect has been reported previously (14, 32) and is regarded as a reason for the organ specific pattern of metastasis seen in many cases of breast cancer. Since our experiments focused on endothelial SDF-1 secretion and also demonstrated effects on tumor cell migration our data suggest that in particular in hypoxic regions of the tumor an SDF-1 gradient develops which attracts tumor cells to the blood vessels and results in tumor cell intravasation at the site of primary tumor growth.

Hypoxia is an important feature in many solid tumors. Effects of hypoxia are associated with several aspects of tumor biology including angiogenesis, metastasis as well as resistance to radiation and chemotherapy (33-35). In a hypoxic environment, angiogenesis is induced by a number of genes including vascular endothelial growth factor-A (VEGF-A), VEGFR1, Angiopoietin-2 (Ang-2) and others (36, 37). Hypoxia also stimulates expression of CXCR4 in different cell types. We observed that both SDF-1 and CXCR4 in HUVECs are hypoxia-inducible. The key regulators of the cellular responses to low oxygen concentrations are two distinct α-subunits termed HIF-1α and HIF-2α. The individual contributions of HIF-1α and
HIF-2α to angiogenesis are under debate. We observed that HUVECs expressed both HIF-1α and HIF-2α when cultured under hypoxic conditions. In our hands, both HIF-1 and HIF-2 contributed to migration and tube formation in response to hypoxia because inhibition of either HIF-1α or HIF-2α provoked partial loss of induction of CXCR4 in response to hypoxia. Previously a reciprocal interaction between the two α-subunits has been reported in tumor cells of renal origin (38). We could not observe this effect in our cells but frequently noticed that inhibition of one α-subunit led to a parallel decrease of the other. The molecular basis of this discrepancy is unclear. Since the effects of HIF-1α and HIF-2α knockdown were additive we believe that both subunits contribute to CXCR4 induction by hypoxia.

Importantly, we have demonstrated by immunohistochemistry that SDF-1 and CXCR4 are expressed in a significant fraction of mammary tumors. We observed that SDF-1 is expressed in approximately 50% of our samples. Within these samples SDF-1 was mainly expressed by the tumor cells but frequently also in endothelial cells as demonstrated by colocalization with vWF. SDF-1 has also been detected in endothelial cells of human breast cancer samples in a previous study (5, 20). Interestingly, none of our tumor cell lines (MDA MB231 and MCF-7) expressed SDF-1. On the other hand, CXCR4 expression in breast cancer samples was more difficult to assess than anticipated. Several commercial antibodies led to nuclear staining of almost all tumor cells which we regarded as unreliable. Using another antibody which has been characterized extensively very recently (39) we observed that nearly 10% of the tumors we analysed were positive for CXCR4. From our data it is indeed conceivable that these patients would benefit from antagonizing CXCR4 signaling. Of note, very recently an in vitro study was published which implies that the effects of CXCR4 and CXCR7 inhibition may act synergistically (40). Importantly, CXCR4 and CXCR7 positive tumours can be identified on a routine basis by immunohistochemistry. For some tumor entities it was reported that another chemokine receptor termed CXCR7 can also bind SDF-1 and thus modulate CXCR4 signaling. Since expression of both receptors was only detectable in 2 out of approximately...
130 cases in our cohort, functional interaction is of course possible but seems to be a rare event. On the other hand, CXCR7 could also transmit proliferative or angiogenic signals. Overall, our data suggest that inhibition of CXCR4 may reduce growth and metastasis in breast carcinoma. Interestingly, a low molecular weight compound termed AMD3100 which antagonizes CXCR4 function is already in clinical use to mobilize hematopoietic stem cells and has been reported to have a tolerable side effect profile (41).

Collectively, we discovered a crosstalk mechanism between breast carcinoma and endothelial cells. Hypoxia-dependent induction of the SDF-1/CXCR4 pathway links metabolism to tumor angiogenesis and active transendothelial migration. Inhibition of the activated SDF-1/CXCR4 signaling pathway in a primary tumor environment may therefore attenuate angiogenesis and carcinoma intravasation. Our data are primarily applicable to breast cancer because we have analysed cultured breast carcinoma cell lines and confirmed SDF-1 and CXCR4 expression in samples of mammary carcinoma. Since importance of CXCR4 and SDF-1 has already been proposed for tumors of other origin, our data may also be relevant for other tumor types.

Acknowledgements

The authors would like to thank Kirsten Goepelt, Gabriele Ladwig and Frank Splettstoesser for excellent technical assistance and Petra Liebig for help with the manuscript. We are also grateful to Professor Dirk Hermann, Department of Neurology, for continuous support and many helpful discussions. Professor Stefan Schulz, Department of Pharmacology, Jena, Germany, provided us with the invaluable CXCR4 antibody which is greatly appreciated.
Grant Support

FJ was supported financially by the Deutscher Akademischer Austauschdienst (DAAD) and the Dr. Werner Jackstädt-Stiftung.

References


Figure legends

Figure 1. Vascular SDF-1 and CXCR4 immunoreactivity in human breast carcinoma tissues. A, Immunofluorescent SDF-1 staining in examples of human primary breast carcinoma tissue. Case 1 is a ductal carcinoma in situ (DCIS). Case 2 is an invasive ductal carcinoma. Endothelial cells were detected with an anti-vWF antibody (red), SDF-1 is stained green. Cell nuclei are stained with DAPI (blue). B, CXCR4 immunohistochemistry. Invasive ductal mammary carcinoma samples were stained for CXCR4, the signal was detected with a secondary antibody and DAB. top, diffuse CXCR4 staining of the tumor cells, centre, perinecrotic induction, bottom, endothelial staining.

Figure 2. Effects of hypoxia on HUVEC are dependent on SDF-1 and CXCR4. A, top: CXCR4 knockdown detected in whole cell lysates by Western blot. Bottom: proliferation assay in HUVECs after shRNA CXCR4 knockdown. Control cells were transduced with pLKO.1. HUVECs were cultured for 4 days, viable cells were counted. The result gives means
of 4 independent experiments (* versus normoxic control, \( p<0.05; \) † versus hypoxic control, \( p<0.05 \)). **B**, *In vitro* transwell migration assay: HUVECs were plated into the upper compartment and incubated for 24 hours. The migrated cells on the bottom of the surface were counted in 12 standardized fields. Data are from 4 independent experiments. **C**, tube formation assay after CXCR4 knockdown: HUVECs were seeded into Matrigel-coated wells and incubated for 24 hours under normoxia or hypoxia. Representative photographs of tube-like structures were taken. **D**, quantification of tube numbers in the whole field, data represent means of 3 independent experiments. **E**, hypoxia survival assay: \( 1\times10^4 \) of serum deprived HUVECs were incubated in hypoxia in the presence or absence of SDF-1 neutralizing antibodies for 3 days. Normoxic serum-free HUVEC served as additional control. Representative photographs come from 4 independent experiments. **F**, viable HUVECs were labelled with calcein and counted at day 3. The data give the mean of 3 independent experiments. **G**, tube formation assays in the presence or absence of SDF-1 neutralizing antibodies. The results are from 4 independent experiments. **H**, quantification of tube numbers in the whole field from 3 independent experiments.

**Figure 3. HIF-1α and HIF-2α contribute to the effects of hypoxia on HUVECs.**

Endothelial cells were transduced with pLKO.1-shRNA-HIF-1α or pLKO.1-shRNA-HIF-2α. **A**, CXCR4 expression in HUVECs after HIF-1α or HIF-2α knockdown. HUVECs were treated with 1 mM DMOG for 4 hours. Data represent at least 3 independent experiments. **B**, hypoxia survival assay: HUVEC were cultured in serum-free ECGM under hypoxia for 3 days. Representative photographs from 2 independent experiments are shown. **C**, HUVEC migration assay after HIF-1α or HIF-2α knockdown. Columns give average of 3 independent experiments. **D**, tube formation assay: HUVECs were exposed to hypoxia for 24 hours. Representative data from 3 independent experiments are shown. **E**, Quantification of tube numbers shows means of 3 independent experiments.
Figure 4. CXCR4 is induced by hypoxia in breast carcinoma cells. A, CXCR4 expression on the cell surface in MDA-MB 231 cells after 24 h of exposure to hypoxia or to DMOG as detected by flow cytometry. Representative data are shown from at least 3 independent experiments. B, DMOG dose response curve, incubations were for 4 hours. C, MDA-MB 231 cells expressing shHIF-1α, shHIF-2α or both. The cells were treated with 1mM DMOG for 4h. D, transendothelial migration assay: MDA-MB 231 cells were added to a HUVEC monolayer in the upper compartment in the presence or absence of SDF-1 and incubated for 24 hours. Representative photographs of MDA-MB 231 in the lower chamber from 3 independent experiments are shown.

Figure 5. CXCR4 in breast carcinoma cells is responsible for tumor-to-endothelium adhesion and transendothelial migration. MDA-MB 231 and HUVEC were transduced by pLKO.1-shRNA-CXCR4 or pLKO.1. A, the adhesion of MDA-MB 231 (control or shCXCR4) cells to a HUVEC monolayer (control or shCXCR4). HUVECs were exposed to normoxia or hypoxia for 12 hours prior to the plating of calcein-labelled MDA-MB 231 cells. Non-adherent cells were thoroughly washed off after 2 hours. Representative photographs from 4 independent experiments. B, quantification of adherent MDA-MB 231 counted in 12 continuous fields. C, adhesion of calcein labelled MDA-MB 231 (control or shCXCR4) to HUVEC (control or treated with a CXCR4 neutralizing antibody before the experiment). D, quantification of MDA-MB 231 associated with endothelial tubes. E, transendothelial migration assay: MDA-MB 231 (control or shCXCR4) cells were added to HUVECs (control or shCXCR4) on a transwell insert, and exposed to normoxia or hypoxia for 24 hours. F, quantification of migrated MDA-MB 231. G and H, transendothelial migration assay with MCF-7 cells (control or siRNA) performed as with MDA-MB 231 in panels E and F.
Figure 6. Adhesion and transendothelial migration of breast carcinoma cells in the presence or absence of neutralizing SDF-1 antibodies. A and B, adhesion of calcein labelled MDA-MB 231 to endothelial tubes in the presence or absence of SDF-1 neutralizing antibody. A, representative photographs. B, quantification of MDA-MB 231. C and D, adhesion of calcein labelled MCF7 to endothelial tubes in the presence or absence of SDF-1 neutralizing antibody after 24 hours of co-culture. C, representative photographs. D, quantification of MCF7. E and F, migration assay: calcein labelled MDA-MB 231 migrated towards a HUVEC monolayer in the presence or absence of neutralizing SDF-1 antibodies in the bottom chamber. E, representative photographs. F, quantification of migrated MDA-MB 231.
Figure 1A. Figure 1B.
Figure 2.

A) Western blot analysis showing CXCR4 expression under normoxia and hypoxia conditions for control and shCXCR4 groups.

B) Graph showing migration of cells under normoxia and hypoxia conditions for control and shCXCR4 groups.

C) Images showing NOX under control and shCXCR4 conditions.

D) Images showing HOX under control and shCXCR4 conditions.

E) Images showing effect of SDF-1 Ab on cell numbers under 1% O2 condition for control and 3µg/ml SDF-1 Ab.

F) Graph showing cell numbers under different SDF-1 neutralizing Ab concentrations for control and 3µg/ml SDF-1 Ab.

G) Images showing NOX under control and different SDF-1 Ab concentrations.

H) Graph showing tube numbers under control and different SDF-1 neutralizing Ab concentrations.
Figure 3.

A

B

C

D

E

1% O2 (serum-free)

control  shHIF-1α  shHIF-2α  shHIF-1α  shHIF-2α

migration (% of control)

control  shHIF-1α  shHIF-2α  shHIF-1α  shHIF-2α

1% O2

control  shHIF-1α

shHIF-2α  shHIF-1α+shHIF-2α

tube numbers

control  shHIF-1α  shHIF-2α  shHIF-1α  shHIF-2α

Downloaded from mcr.aacrjournals.org on January 6, 2018. © 2012 American Association for Cancer Research.
New insight into the SDF-1/CXCR4 axis in a breast carcinoma model: Hypoxia-induced endothelial SDF-1 and tumor cell CXCR4 are required for tumor cell intravasation

Eric Metzen, Fengyan Jin, Ulf Brockmeier, et al.

Mol Cancer Res  Published OnlineFirst July 5, 2012.