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Stromal cell-derived factor 1 (CXCL12) binds to endothelial cells and signals through a receptor different from CXCR4 ☆

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Abstract

Stromal cell-derived factor 1 (CXCL12) is an angiogenic chemokine that is believed to act solely via its cognate receptor CXCR4. Evidence is now provided for the existence of a different CXCL12 binding and signaling receptor on endothelial cells. Bovine aortic endothelial cells (BAECs) strongly expressed CXCR4 and exhibited high binding capacity for fluorescently labeled CXCL12. However, CXCL12 binding was not correlated with the CXCR4 expression level and was virtually unaffected by the specific CXCR4 antagonists AMD3100 or T22. Similar observations were made in endothelial cells of mouse and human origin. Also, AMD3100 failed to block CXCL12 internalization and CXCL12-induced intracellular signal transduction via extracellular signal-regulated kinases 1/2 in BAECs. In contrast, CXCL12 binding and signaling were almost completely inhibited by the CXCR4 antagonist in T-lymphoid SupT1 cells. Together, our data point to the existence of an additional receptor through which CXCL12 exerts its biological effects in endothelial cells.

Keywords: Chemokine binding; Angiogenesis; CXCL12; Endothelial cell; Receptor; Antagonist; Signal transduction

Chemokines exert their action by binding to seven transmembrane G protein-coupled receptors, leading to the activation of intracellular signaling cascades [1]. The chemokine family is divided into four subclasses (i.e., CXC, CC, C or CX_3C) based on the structural arrangement of the conserved aminoterminal cysteine residues [2]. Besides their crucial role in leukocyte migration during immunological responses, the CXC-chemokine receptor CXCR4 and the CC-chemokine receptor CCR5 are the principal entry coreceptors for infection of $CD4^+$ cells by human immunodeficiency virus (HIV) [3,4]. Also, several recent studies have shown the involvement of chemokines and their receptors, most particularly CXCL12/CXCR4, in the outgrowth and metastasis of various tumors, including breast, ovarian, and colon carcinomas, neuroblastomas, gliomas, etc. [5–11] and in certain inflammatory autoimmune disorders such as rheumatoid arthritis [12,13].

The constitutive or inducible expression of various chemokines and chemokine receptors in endothelial tissues also suggests a significant role in vascular (patho)biology [14–19]. Angiogenesis, i.e., the generation of new capillary blood vessels from pre-existing vessels, is a fundamental process in reproduction and wound healing, and a rate-limiting step in solid tumor growth and metastasis [20]. Neovascularization is a complex multistep process relying on an extensive interplay between cells, extracellular matrix components, and soluble factors, including chemokines, which can be either angiogenic or angiostatic [21].

☆ *Abbreviations:* BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; CXCL12, CXC-chemokine ligand 12 (formerly 'stromal cell-derived factor-1'); $CXCL12^{AF647}$, Alexa Fluor 647-conjugated CXCL12; CXCR4, CXC-chemokine receptor 4; Erk, extracellular signal-regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HAEC, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; IC_{50}, 50% inhibitory concentration; (m)Ab, (monoclonal) antibody; MAEC, mouse aortic endothelial cells; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; PE, phycoerythrin; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA.

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Recently, several studies have reported the angiogenic properties of the chemokine CXCL12 [17,22–26]. CXCL12 represents the single natural ligand for CXCR4 [27–29], which, until very recently, was the only known CXCL12 receptor. Because of this unique interrelationship, the chemokine/receptor pair CXCL12/CXCR4 has very long been considered as an exception in the complex chemokine network [2]. Indeed, most other chemokines can bind to several receptors and, *vice versa*, most chemokine receptors recognize more than one ligand. Although it is generally assumed that the angiogenic properties of CXCL12 are also mediated by its cognate receptor CXCR4, we now provide strong evidence that endothelial cells also express an alternative receptor through which CXCL12 may mediate its stimulatory effects. We used bovine aortic endothelial cells (BAEC) as a reliable and reproducible cellular model throughout this study, but our findings were confirmed in human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC).

Materials and methods

Cell cultures. Bovine and mouse aortic endothelial cells (BAECs and MAECs) were obtained from Dr. Marco Presta (Brescia, Italy) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker Europe, Verviers, Belgium) and 0.01 M Hepes (Invitrogen) in culture flasks coated with 0.5% gelatin. Subcultivations were done weekly by digestion of the monolayers with trypsin (Invitrogen). Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were from Cambrex Bio Science (Walkersville, MD) and were cultured in gelatin-coated culture flasks in endothelial growth medium (EGM)-2 (Cambrex). Human T-lymphoid SupT1 and mouse B cell leukemia L1210 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS and 2 mM glutamine (Invitrogen). Human astroglioma U87 cells transfected with CD4 (U87.CD4) or with both CD4 and CXCR4 (U87.CD4.CXCR4) were kindly provided by Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, NY) and were cultured in DMEM (Invitrogen) supplemented with 10% FBS (BioWhittaker Europe), 0.01 M Hepes (Invitrogen), and 0.2 mg/ml geneticin (G-418 sulfate) (Invitrogen). For U87.CD4.CXCR4 cells, the medium also contained 1 μg/ml puromycin (Sigma–Aldrich, St. Louis, MO). Subcultivations were done every 2–3 days. All cell cultures were maintained at 37 °C in a humidified, CO_2-controlled atmosphere.

Chemokines and CXCR4 inhibitors. $CXCL12^{AF647}$ (i.e., human CXCL12 carrying an Alexa Fluor 647® moiety at its second last amino acid) [30] was purchased from CSS-Albachem (East Lothian, Scotland, UK). Unlabeled CXCL12 was synthesized by Dr. I. Clark-Lewis. The bicyclam AMD3100 was obtained from Sigma–Aldrich (St. Louis, MO). The peptide CXCR4 antagonist T22 was purchased from Bachem (Bubendorf, Switzerland).

Flow cytometric analysis of CXCR4 expression. After trypsin digestion, adherent cells were incubated for at least 1 h at room temperature in cell culture medium to allow re-expression of receptor proteins at the cell surface. Then, 0.5×10^6 cells were washed once with PBS containing 2% FBS and were incubated with phycoerythrin (PE)-conjugated anti-CXCR4 mAb clone 12G5 (BD Biosciences, San Diego, CA) for 30 min at room temperature in PBS containing 2% FBS. Thereafter, the cells were washed twice with PBS, fixed in 1% paraformaldehyde in PBS, and analyzed on a FACScalibur flow cytometer equipped with CellQuest software (Becton–Dickinson, San Jose, CA). As a negative control for aspecific background staining, the cells were stained in parallel with Simultest Control γ_1/γ_{2a} (BD Biosciences). To evaluate the effect of the CXCR4 inhibitors on 12G5 mAb binding at the cell surface, the cells were preincubated with AMD3100 or T22 in PBS for 15 min at room temperature and were washed once with PBS containing 2% FBS prior to incubation with PE-conjugated anti-CXCR4 (12G5) mAb.

$CXCL12^{AF647}$ binding assay. Cells were washed once with assay buffer [Hanks' balanced salt solution containing 20 mM Hepes and 0.2% bovine serum albumin (BSA), pH 7.4] and were then incubated for 30 min at room temperature with $CXCL12^{AF647}$ [30] diluted in assay buffer at the indicated concentrations. Thereafter, the cells were washed twice in assay buffer, fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS), and analyzed on a FACSCalibur™ flow cytometer (Becton–Dickinson). To evaluate the inhibitory effect of unlabeled CXCL12 or the CXCR4 antagonists AMD3100 [31] or T22 [32] on the binding of the fluorescent chemokine, the cells were incubated with 25 ng/ml $CXCL12^{AF647}$ in the presence of these blocking agents at different concentrations. The percentages of $CXCL12^{AF647}$ binding were calculated according to the formula $[(MFI - MFI_{NC})/(MFI_{PC} - MFI_{NC})] * 100$, where MFI is the mean fluorescence intensity of the cells incubated with $CXCL12^{AF647}$ in the presence of the inhibitor, MFI_{NC} is the mean fluorescence intensity measured in the negative control (i.e., autofluorescence of unlabeled cells), and MFI_{PC} is the mean fluorescence intensity of the positive control (i.e., cells exposed to $CXCL12^{AF647}$ alone).

CXCR4 gene silencing by siRNA transfection. To suppress CXCR4 expression, BAECs were transiently transfected with small interfering RNA 21-mer duplex [sense: r(GCCUGAAUCCCAUCCUCUA)dTdT and antisense: r(UAGAGGAUGGGAUUCAGGC)dAdG] (designed and synthesized by Qiagen, Germantown, MD) targeting the sequence CTGCCTGAATCCCATCCTCTA (nucleotides 932–952) from bovine CXCR4 mRNA (GenBank Accession No. AF399642). Cells at ~90% confluency in gelatin-coated 24-well plates were transfected with 150 pmoles of siRNA per well using Lipofectamine 2000 and OPTIMEM medium (Invitrogen). After 3 days, the cells were harvested and assessed for $CXCL12^{AF647}$ binding and for CXCR4 expression by anti-CXCR4 antibody (12G5) staining and flow cytometry. CXCR4 knock-down efficiency was usually between 60% and 85%.

Flow cytometric measurement of $CXCL12^{AF647}$ internalization. BAECs were trypsinized and incubated in cell culture medium at room temperature for 1 h prior to the experiment to allow re-expression of receptor proteins at the cell membrane. BAECs and SupT1 cells were washed with assay buffer (see above) and preincubated with or without AMD3100 at 5 μg/ml for 15 min on ice. Then, $CXCL12^{AF647}$ was added at a final concentration of 50 ng/ml and the cells were incubated at 37 °C. At different time points (i.e., 0, 5, 12, 20, 30, and 45 min), two 50-μl aliquots, each containing approximately 3×10^5 cells, were transferred to 250 μl ice-cold PBS, either pH 7 or pH 2. Both samples were immediately analyzed on the FACSCalibur flow cytometer (Becton–Dickinson). At pH 7, total cell fluorescence (i.e., both membrane-bound and intracellular $CXCL12^{AF647}$) is measured. At pH 2, all membrane proteins are removed from the cell surface and the remaining fluorescence exclusively originates from internalized $CXCL12^{AF647}$.

Western blot analysis of ERK1/2 phosphorylation. BAECs were seeded in 6-well plates at 50,000 cells/cm^2. After 24 h, the cells were washed and incubated in fresh DMEM-based medium without FBS, supplemented with 0.2% BSA. SupT1 cells were seeded in 6-well plates at 500,000 cells/ml in RPMI-based medium without FBS, supplemented with 0.2% BSA. After 2 days of serum starvation, AMD3100 was added at 10 μg/ml for 30 min, after which the cells were stimulated with 200 ng/ml CXCL12 for 10 min. Next, the cell cultures were washed with ice-cold PBS and lysed in 200 μl lysis buffer [20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA (pH 7.4), 1% Triton X-100, 10% glycerol, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 25 mM glycerophosphate, and 10 μg/ml leupeptin]. Lysates were centrifuged for 20 min at 15,000*g*, and the protein concentration was determined using Bradford reagent (Sigma). SDS–PAGE and Western blot analysis of the cell lysates were performed as described previously [33] using primary antibodies raised against Erk1/2 protein (p44/42 MAPK Ab, 1/1000, Cell Signaling) and phosphorylated Erk1/2 (phospho-p44/42 MAPK [Thr202/

Tyr204] mAb E10, 1/2000, Cell Signaling). Immunoreactive proteins were detected by chemiluminescence (ECLplus, Bio-Rad).

Results

Cell surface expression of CXCR4 in BAEC, SupT1, U87.CD4, and U87.CD4.CXCR4 cells

Bovine aortic endothelial cells (BAECs), human T-lymphoid SupT1 cells, and human astroglioma U87 cells stably transfected with CD4 alone (U87.CD4) or with CD4 and CXCR4 (U87.CD4.CXCR4) were stained with the anti-CXCR4 mAb 12G5 and analyzed by flow cytometry. As shown in Fig. 1, BAECs, SupT1 cells, and U87.CD4.CXCR4 cells strongly expressed the chemokine receptor CXCR4 at their cell membranes, whereas the negative control cell line U87.CD4 was not stained by the anti-CXCR4 antibody 12G5. As expected, 12G5 mAb binding to BAEC, SupT1, and U87.CD4.CXCR4 cells was effectively blocked when the cells had been preincubated with the specific CXCR4 antagonist AMD3100 [31] or with the peptidic CXCR4 inhibitor T22 [32] at 5 μg/ml (Fig. 1). The percentages of inhibition of 12G5 mAb binding in BAECs were 8, 22, 48, 86, 94, and 96 for AMD3100 at 1.6, 8, 40, 200, 1000, and 5000 ng/ml, respectively, yielding an IC_{50} of 44 ng/ml. The respective values for SupT1 cells were 9, 21, 38, 63, 77, and 84, yielding an IC_{50} of 87 ng/ml. Thus, AMD3100 blocked 12G5 mAb binding with comparable potency in BAEC and SupT1 cells.

Binding of $CXCL12^{AF647}$ to BAEC, SupT1, U87.CD4 and U87.CD4.CXCR4, cells and effect of CXCR4 inhibitors

The cells were incubated with increasing concentrations of Alexa Fluor 647-labeled CXCL12 [30] whereafter the fluorescence of the cells was measured with the flow cytometer. No significant binding of the fluorescent chemokine was observed in CXCR4-negative U87.CD4 cells (Fig. 2, black squares in lower panel), attesting the lack of aspecific cell surface binding of $CXCL12^{AF647}$. BAEC, SupT1, and U87.CD4.CXCR4 cells showed a gradual increase in fluorescence after exposure of the cells to increasing concentrations of $CXCL12^{AF647}$ (Fig. 2). In the presence of AMD3100 (5 μg/ml), $CXCL12^{AF647}$ binding was reduced by >75% and by >90% in SupT1 and U87.CD4.CXCR4 cells, respectively. In contrast, the CXCR4 inhibitor

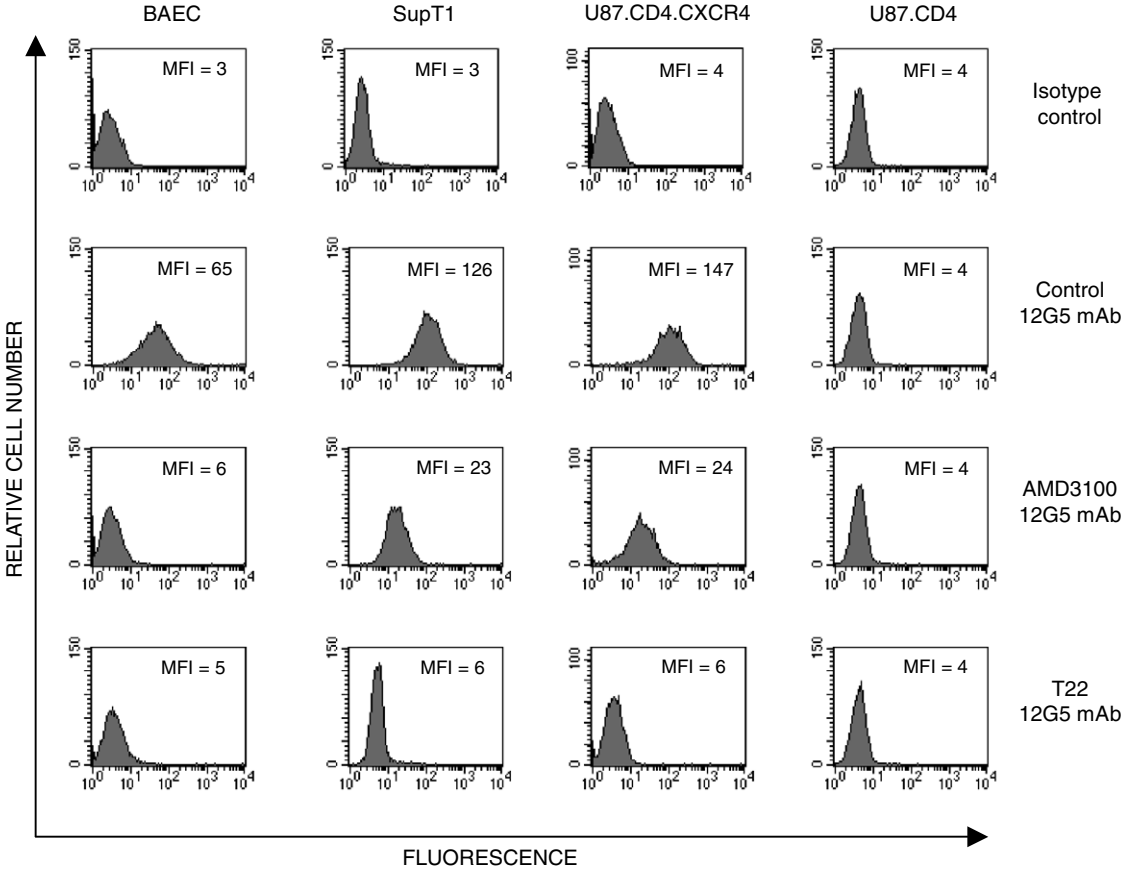

Fig. 1. Analysis of CXCR4 expression and inhibitory effect of CXCR4 antagonists on anti-CXCR4 mAb binding to BAEC, SupT1, U87.CD4, and U87.CD4.CXCR4 cells. The cells were preincubated with the CXCR4 antagonists AMD3100 or T22 at 5 μg/ml and were then stained with anti-CXCR4 mAb 12G5. The mean fluorescence intensity (MFI) values are indicated in each histogram. The aspecific background fluorescence of the cell population was measured by an isotype control mAb. Cell debris was excluded from the analysis by dot plot gating. These are the data from one representative experiment, which was repeated several times with similar results.

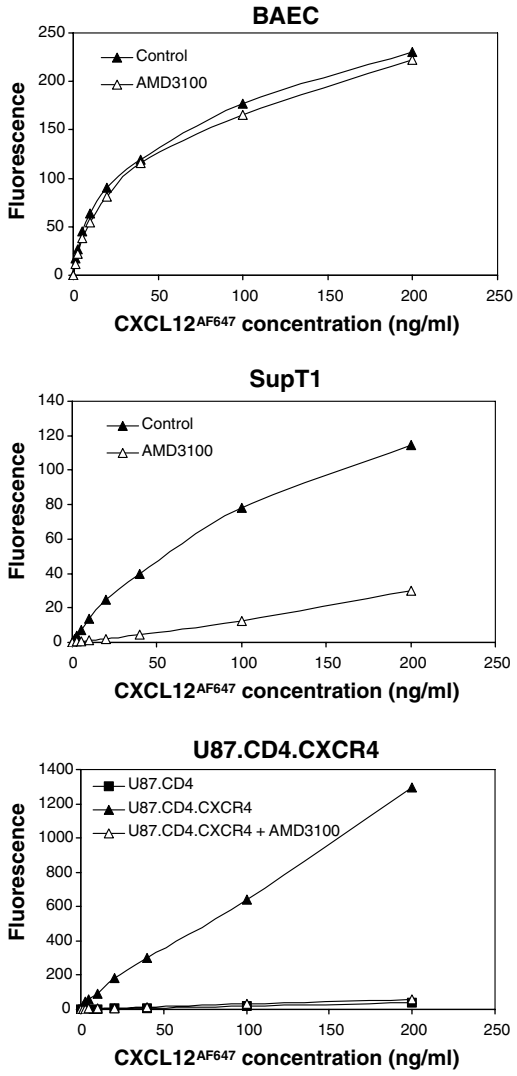

Fig. 2. Binding curve of $CXCL12^{AF647}$ in BAEC, SupT1, U87.CD4.CXCR4, and U87.CD4 cells. The cells were incubated with increasing concentrations of the fluorescent chemokine in the presence (open symbols) or absence (closed symbols) of the CXCR4 antagonist AMD3100 at 5 μg/ml and were then analyzed by flow cytometry. The data from one representative experiment are shown.

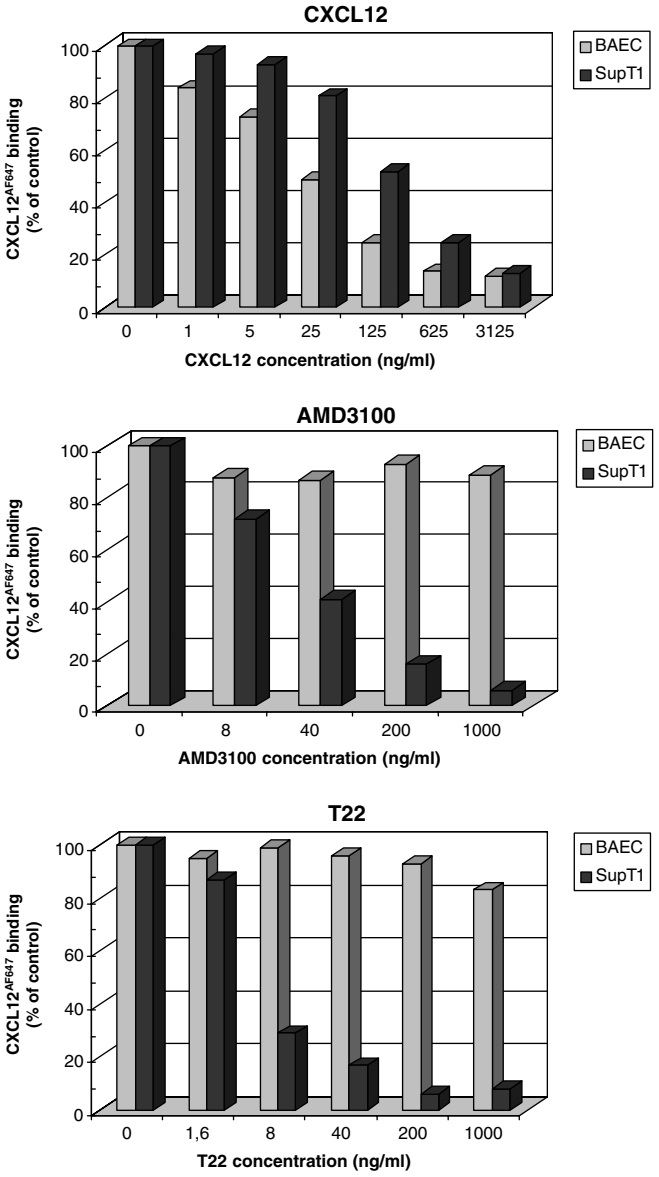

Fig. 3. Concentration-dependent effect of unlabeled CXCL12, AMD3100, and T22 on the binding of $CXCL12^{AF647}$ to BAECs (gray bars) and SupT1 cells (black bars). The cells were incubated with the fluorescent chemokine at 25 ng/ml in the presence of increasing concentrations of unlabeled CXCL12 (upper panel), the bicyclam AMD3100 (middle panel) or T22 (bottom panel) and were then analyzed by flow cytometry. The bars represent the percentages of $CXCL12^{AF647}$ binding in the presence of the inhibitor, relative to the positive control where the cells were exposed to 25 ng/ml $CXCL12^{AF647}$ alone.

completely failed to inhibit chemokine binding to BAECs (Fig. 2).

In a next set of experiments, BAECs and SupT1 cells were incubated with 25 ng/ml $CXCL12^{AF647}$ in the presence of increasing concentrations of either the unlabeled chemokine, the small-molecule CXCR4 antagonist AMD3100 or the peptidic CXCR4 inhibitor T22. As expected, unlabeled CXCL12 and $CXCL12^{AF647}$ competed for binding to both SupT1 cells and BAECs: the fluorescence of the cells, resulting from $CXCL12^{AF647}$ binding, gradually decreased in the presence of increasing amounts of unlabeled chemokine (Fig. 3, upper panel). Conversely, the CXCR4 inhibitors AMD3100 and T22 dose-dependently blocked the binding of the fluorescent chemokine in T-lymphoid SupT1 cells but were unable to do so in BAECs (Fig. 3, middle and lower panels).

It should be noted that the $CXCL12^{AF647}$ binding experiments were performed at room temperature in order to increase the sensitivity range of the assay and, thus, to improve the significance of the data. When the experiments were performed at 4 °C, the fluorescence values were about 4-fold lower (data not shown), making it difficult to detect minor differences between inhibitor-treated and untreated cells. At room temperature, the fluorescence measured should not only be attributed to membrane-bound chemokine but also to internalized chemokine. However, for our

purposes, this is not relevant and does not compromise the interpretation of our results. Indeed, at 4 °C, AMD3100 also completely failed to inhibit $CXCL12^{AF647}$ binding in endothelial cells (data not shown).

CXCL12 binding is not correlated with CXCR4 expression in BAECs

To further investigate whether CXCL12 binding to endothelial cells is CXCR4-dependent, we examined the effect of CXCR4 knock-down via RNA interference on the ability of BAECs to bind $CXCL12^{AF647}$. Three days after transfection of BAECs with small interfering RNA that specifically targets CXCR4 mRNA, the expression level of CXCR4 at the cell membrane was reduced by 72% in the experiment shown in Fig. 4A (see inset). However, CXCR4 down-regulation did not result in marked differences in chemokine binding capacity, as shown by the $CXCL12^{AF647}$ binding curves obtained with siRNA-transfected *versus* untransfected BAECs (Fig. 4A).

Also, the CXCR4 expression level may largely vary in different cell culture conditions. As shown in Fig. 4B, CXCR4 was strongly upregulated when BAECs were serum-deprived. CXCR4 expression, as measured by 12G5 mAb staining, was >3-fold higher in BAECs that had been cultured in the absence of FBS during 2 days (MFI = 145) than in BAECs that were kept in the presence of FBS (MFI = 39). Yet, $CXCL12^{AF647}$ was bound to virtually the same extent in both conditions, the respective MFI values being 67 and 76 (Fig. 4B). Similar results were obtained in HUVECs (data not shown). Together, these data indicate that CXCR4 expression and CXCL12 binding are not related in endothelial cells.

CXCL12 is internalized in BAECs via a CXCR4-independent route

In addition, internalization of $CXCL12^{AF647}$ was studied in BAECs and SupT1 cells by means of a flow cytometric assay as described in Materials and methods. Briefly, total $CXCL12^{AF647}$ (i.e., membrane-bound plus internalized) is detected when the cells are analyzed at neutral pH, whereas in acidic circumstances (pH 2), all membrane-bound proteins are removed from the cell surface and only the proportion of intracellular (i.e., internalized) chemokine is measured. In BAECs exposed to the fluorescent chemokine at 50 ng/ml, the ratio of intracellular *versus* total $CXCL12^{AF647}$ gradually increased in function of time to reach a plateau level of ~75% after 20–30 min (Fig. 5, upper panel, open *versus* closed squares). The percentages of internalized chemokine were 51%, 66%, 73%, and 77% after 5, 12, 20, and 30 min, respectively. The corresponding values in SupT1 cells were 30%, 59%, 72%, and 79%, respectively (Fig. 5, lower panel, open *versus* closed squares). However, no significant intracellular accumulation of $CXCL12^{AF647}$ occurred in SupT1 cells that had been pretreated with the CXCR4 antagonist AMD3100 (Fig. 5, lower panel, open triangles), whereas AMD3100 pretreatment only afforded a minor reduction in chemokine internalization in BAECs (Fig. 5, upper panel, open triangles *versus* open squares). These data point to a different route of CXCL12 internalization in both cell lines.

CXCL12 activates the Erk1/2 signaling cascade in a CXCR4-independent manner in BAECs

BAECs and SupT1 cells were serum-starved for 48 h and were then stimulated with CXCL12, either in the presence or in the absence of AMD3100, as described under

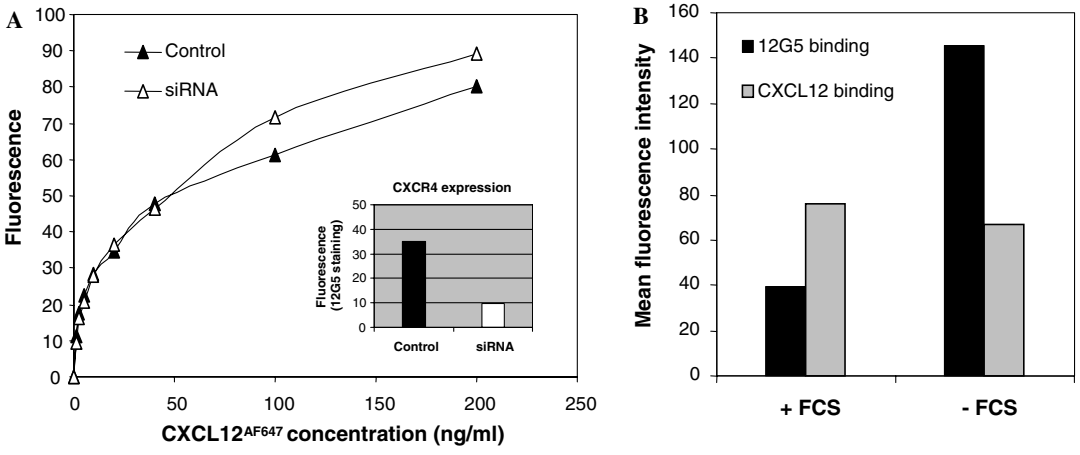

Fig. 4. (A) Effect of CXCR4 gene silencing on $CXCL12^{AF647}$ binding to BAECs. The cells were transfected with siRNA specifically targeting CXCR4, and $CXCL12^{AF647}$ binding was assessed after 3 days, when cell surface expression of CXCR4 was suppressed by 72%, as ascertained by anti-CXCR4 mAb 12G5 staining and flow cytometry. (B) Effect of serum starvation on CXCR4 expression and $CXCL12^{AF647}$ binding in BAECs. The cells were cultured for 48 h in the presence or absence of FBS, whereafter anti-CXCR4 mAb 12G5 staining or $CXCL12^{AF647}$ binding, and subsequent flow cytometric analysis were performed. Black and gray bars display the mean fluorescence intensities of the cell populations for 12G5 antibody (phycoerythrin)-associated and chemokine (Alexa Fluor 647)-associated fluorescence, respectively.

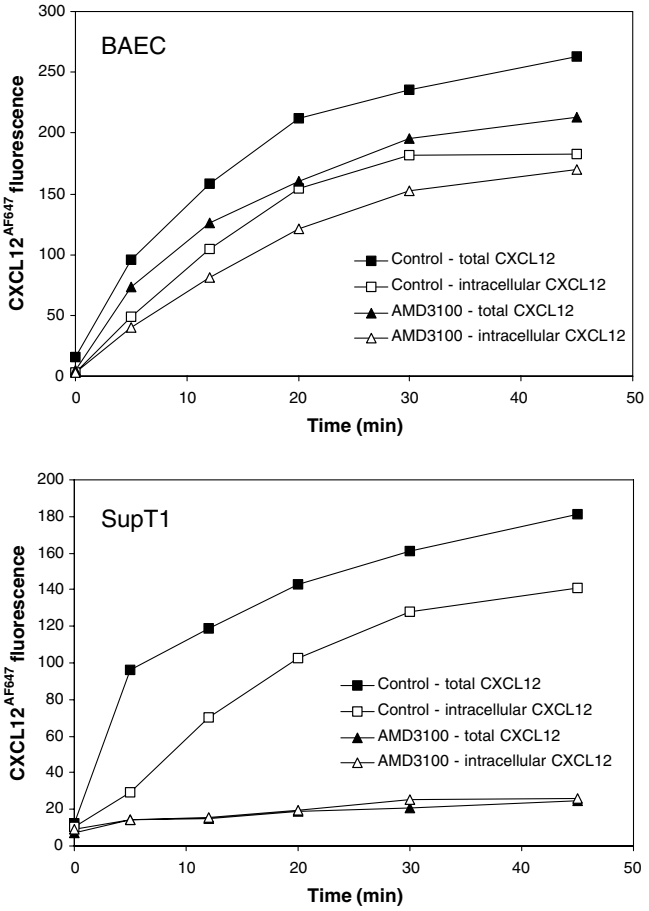

Fig. 5. Effect of the CXCR4 antagonist AMD3100 on time-dependent internalization of $CXCL12^{AF647}$ in BAECs and SupT1 cells. The cells were exposed to the fluorescent chemokine at 50 ng/ml in the absence (squares) or presence (triangles) of AMD3100 (5 μg/ml). At the indicated time points, total (closed symbols) and intracellular (open symbols) fluorescence of the cells were measured by use of the flow cytometer as described in Materials and methods.

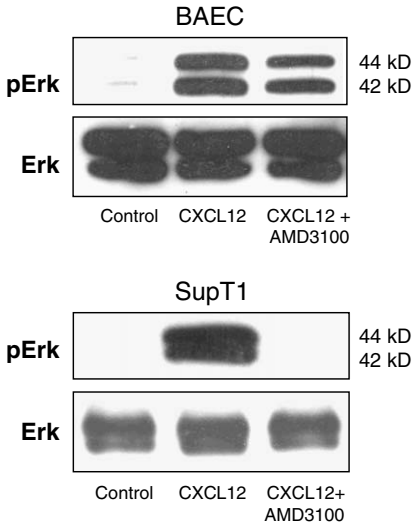

Fig. 6. Effect of the CXCR4 antagonist AMD3100 on CXCL12-induced Erk1/2 phosphorylation in BAECs and SupT1 cells. After serum starvation for 48 h, the cells were preincubated for 30 min in the absence or presence of AMD3100 (10 μg/ml) and stimulated with the chemokine (200 ng/ml) for 10 min. The cells were lysed and the cell extracts were subjected to SDS–PAGE. Total Erk1/2 protein (44 and 42 kDa isoforms) (bottom) and phosphorylated Erk1/2 (top) were detected by immunoblotting with anti-Erk1/2 antibody and anti-phospho(Thr202/Tyr204)-Erk1/2 mAb E10 and subsequent visualization by chemiluminescence. Experiments were repeated with similar results.

Materials and methods. Total and phosphorylated Erk1/2 were detected by Western blotting. As shown in Fig. 6, CXCL12 strongly induces phosphorylation of Erk1/2 in both cell lines after 10 min. In AMD3100-pretreated BAECs, Erk activation was not considerably diminished. Conversely, the CXCR4 antagonist totally abolished CXCL12-induced Erk signaling in SupT1 cells (Fig. 6). These observations suggest that CXCL12 signals through CXCR4 in SupT1 cells but through a different receptor in BAECs.

Discussion

Numerous recent reports have highlighted the important role of the chemokine stromal cell-derived factor-1 (SDF-1)/CXCL12 in angiogenesis [17,22–26]. It has generally been assumed that CXCL12 exerts its biological effects via exclusive binding to, and signaling through, its cognate chemokine receptor CXCR4. Here, we present evidence for the existence of an alternative CXCL12 binding and signaling receptor on endothelial cells.

For practical reasons, i.e., avoidance of the heterogeneity that is known to exist among different preparations of human endothelial cell cultures (e.g., HUVEC), we have chosen bovine aortic endothelial cells (BAECs) as a reproducible and reliable study model to investigate CXCL12 binding and signaling in endothelial cells. We showed that BAECs strongly express CXCR4 and exhibit high CXCL12 binding capacity. However, the bicyclam AMD3100 and the peptide T22, two potent and specific CXCR4 inhibitors with a totally different structure [31,32], were unable to block specific binding of fluorescently labeled CXCL12 to BAECs. In contrast, both compounds were very potent inhibitors of CXCL12 binding to human T-lymphoid SupT1 cells. A possible explanation could have been that AMD3100 and T22 do not efficiently bind to endothelial CXCR4 due to a slightly different receptor protein conformation in endothelial cells. However, this is very unlikely since both CXCR4 inhibitors effectively inhibited anti-CXCR4 12G5 mAb binding to BAECs with comparable potency as to SupT1 cells. Thus, it remains unclear why AMD3100 and T22 do not even afford a partial reduction of CXCL12 binding to BAECs, despite the abundant CXCR4 expression in these cells.

Also, the ability of BAECs to bind CXCL12 remained unchanged when CXCR4 protein expression was upregulated by serum starvation. Similar observations were made in HUVEC (data not shown). In addition, CXCL12 binding to BAECs was not impaired when CXCR4 expression was knocked down by RNA interference. This is in agreement with our findings on mouse aortic endothelial cells

(MAECs), which showed high CXCL12 binding despite being CXCR4-negative both at the mRNA and at the protein level (data not shown). Like in BAECs, CXCL12 binding to MAECs could not be blocked by CXCR4 inhibitors. Conversely, CXCL12 binding to CXCR4-positive murine leukemic L1210 cells could be completely blocked by the CXCR4 antagonists (data not shown), which is in line with our observations in human SupT1 cells.

Finally, CXCL12 internalization and intracellular signaling via Erk1/2 were strongly inhibited by AMD3100 in SupT1 cells but virtually unaffected in BAECs. Thus, CXCL12 is counteracted by CXCR4 antagonists in tumor cells from leukocytic lineages, such as human SupT1 and mouse L1210 cells, but not in cells from endothelial origin.

Taken together, our results indicate that cell surface binding of CXCL12 and downstream signaling events are predominantly mediated by a receptor other than CXCR4 in our endothelial cell systems. It should be noted that this conclusion is not restricted to BAECs but can be extended to endothelial cells from other species. Indeed, both MAECs (murine) and HUVECs (human), as well as human aortic endothelial cells (HAECs), showed strong and CXCR4-independent (i.e., CXCR4 antagonist-resistant) CXCL12 binding capacity (data not shown).

The generally accepted hypothesis that CXCL12 acts through CXCR4 in endothelial cells originates from several studies pointing to an important role for CXCR4 in angiogenesis. For instance, the angiogenic factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2) have been shown to enhance CXCL12-induced neovascularization by upregulating the expression of CXCR4 on endothelial cells [22]. It was also reported that inflammatory cytokines like IFN-γ and IL-1β regulate the transcription of CXCR4 in endothelial cells and correspondingly influence the chemotactic response of these cells to CXCL12 [18]. Furthermore, Guleng *et al.* [35] found that blockade of CXCR4 by neutralizing antibodies attenuates *in vivo* tumor growth by inhibiting angiogenesis. A principal role for CXCR4 in organ vascularization was most convincingly demonstrated by the finding that mice lacking this receptor die *in utero* and are defective in vascularization of the gastrointestinal tract [36].

Our present findings that CXCL12 binding and subsequent downstream events could not be prevented by the well-known specific CXCR4 inhibitors AMD3100 and T22 in various endothelial cell types from different species are in apparent contradiction with these previous reports. However, our results do not exclude CXCR4 as a key player in angiogenesis but indicate that, at least in our experimental cell systems, an alternative receptor exists which recognizes CXCL12 as a ligand and which may also transmit CXCL12-induced signals.

Several proteins may fulfill the role of this alternative CXCL12 receptor in endothelial cells. (i) Heparan sulfate is abundantly present on endothelial cells [37,38] and has previously been reported to act as a binding anchor for CXCL12 [34]. However, we found that heparitinase treatment did not markedly affect the CXCL12 binding potential of BAEC (data not shown). This argues against heparan sulfate as the unknown CXCL12 receptor. (ii) The orphan CXC-chemokine receptor RDC1 has recently been demonstrated to bind and functionally respond to CXCL12 in primary T cells and has therefore been renamed CXCR7 [39]. We have examined mRNA expression of this receptor in different cell types by RT-PCR and could indeed demonstrate strong expression of RDC1/CXCR7 in endothelial cells (i.e., BAEC, MAEC, HAEC, and HUVEC), whereas no or very little RDC1/CXCR7 mRNA could be detected in the non-endothelial cells (i.e., L1210, SupT1, U87.CD4, and U87.CD4.CXCR4) (data not shown). These observations might point to RDC1/CXCR7 as a plausible candidate to fulfill the function of alternative CXCL12 receptor in endothelial cells. On the other hand, Infantino *et al.* [40] reported that CXCL12 is not a ligand for RDC1 in all cells. Thus, further studies have to be undertaken to uncover the exact nature of the CXCL12 binding protein(s) in endothelial cells. The identification and characterization of the alternative endothelial cell receptor for CXCL12 may provide new insights into the mode of action of this chemokine as an angiogenic factor and may offer a new target for the design of anti-angiogenic drugs.

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