Anti-inflammatory therapy by intravenous delivery of non-heparan sulfate-binding CXCL12

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ABSTRACT Interaction between chemokines and heparan sulfate (HS) is essential for leukocyte recruitment during inflammation. Previous studies have shown that a non-HS-binding mutant form of the inflammatory chemokine CCL7 can block inflammation produced by wild-type chemokines. This study examined the anti-inflammatory mechanism of a non-HS-binding mutant of the homeostatic chemokine CXCL12. Initial experiments demonstrated that mutant CXCL12 was an effective CXCR4 agonist. However, this mutant chemokine failed to promote transendothelial migration in vitro and inhibited the haptotactic response to wild-type CCL7, CXCL12, and CXCL8, and naturally occurring chemoattractants in synovial fluid from the rheumatoid synovium, including CCL2, CCL7, and CXCL8. Notably, intravenous administration of mutant CXCL12 also inhibited the recruitment of leukocytes to murine air pouches filled with wild-type CXCL12. Following intravenous administration, wild-type CXCL12 was cleared from the circulation rapidly, while the mutant chemokine persisted for >24 h. Chronic exposure to mutant CXCL12 in the circulation reduced leukocyte-surface expression of CXCR4, reduced the chemotactic response of these cells to CXCL12, and inhibited normal chemokine-mediated induction of adhesion between the α4β1 integrin, VLA-4, and VCAM-1. These data demonstrate that systemic administration of non-HS-binding variants of CXCL12 can mediate a powerful anti-inflammatory effect through chemokine receptor desensitization.—O’Boyle, G., Mellor, P., Kirby, J. A., Ali, S. Anti-inflammatory therapy by intravenous delivery of non-heparan sulfate-binding CXCL12. *FASEB J.* 23, 3906–3916 (2009). www.fasebj.org

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CHEMOKINES ARE A FAMILY OF SMALL (8–10 kDa) proteins that play important roles in both normal homeostatic processes and the recruitment of inflammatory leukocytes. Chemokines function through binding and activating specific members of a family of G-protein-coupled chemokine receptors (GPCRs). While these receptors can be stimulated by soluble ligands, it is clear that chemokines in vivo are largely bound to heparan sulfate (HS) components of proteoglycans on cell surfaces and within the extracellular matrix (1). This binding occurs between cationic consensus heparin-binding domains in the protein sequence and anionic, sulfated regions in HS (2). Previous studies by our group and others (3, 4) have shown that specific mutation to disrupt the heparin-binding domain of the proinflammatory chemokines CCL5 and CCL7 does not compromise specific GPCR ligation. However, the mixture of either of these mutants with wild-type chemokines can abrogate the potential of the latter to induce transendothelial leukocyte migration in vitro and normal chemokine-mediated inflammation in vivo (4). Although offering tantalizing potential for development of systemic anti-inflammatory therapies (2), the mechanism by which non-HS-binding chemokines acts is unclear (5). Interactions with HS are known to promote oligomerization of certain chemokines (6), and disruption of this biology has been postulated as a mechanism for their actions in in vivo models (2).

A number of approaches for manipulation of chemokine biology are currently undergoing investigation (7), including small-molecule-specific chemokine receptor antagonists (8), virus-derived chemokine-binding proteins (9), and nonsignaling mutant chemokine “cell-jammers” (10). In each case, these strategies are specific for the blockade of single ligands or receptors. Given the multiple redundancy within both chemokines and their receptors, it seems likely that a strategy to simultaneously modulate a number of chemokines will have a clinical advantage (11); indeed, naturally occurring chemokine “scavenger” receptors such as D6 and DARC can perform this function in vivo by clearance of groups of chemokines (12). Notably, GPCR agonists, including the chemokines, can induce both homologous and heterologous receptor tolerance, suggesting that single agents might negate the potential of

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a cell to respond to multiple chemokines (5). Our group used this argument to explain the observation that addition of a non-HS-binding, mutant form of CCL7 to mixed chemokine populations within murine air pouches limited the inflammatory action of wild-type chemokines (4). However, simultaneous introduction to the air pouch of both mutant and wild-type chemokines does not allow definition of spatial aspects of the anti-inflammatory activity.

A more realistic strategy is to study the potential of systemic administration of non-HS-binding mutant chemokines to block site-specific recruitment of immune cells induced by local administration of wild-type chemokines. However, CCL7 is a powerful inflammatory chemokine, and the non-HS-binding form shares the potential of the wild-type sequence to interact with CCR1, CCR2, CCR3, and CCR5, which are expressed in variable proportions by most leukocytes (13). Systemic administration of inflammatory chemokines is tolerated but can be associated with nonspecific immune activation and inflammation. Indeed, some direct tissue damage may be caused through widespread induction of a respiratory burst in responsive phagocytes (14).

The chemokine CXCL12 is a prototypical HS-binding chemokine; indeed, the less common CXCL12β isoform is extended at the carboxyl terminus to include an additional series of BBxβ consensus HS-binding domains that confer one of the highest measured chemokine affinities for HS (900 pM; ref. 15). This chemokine directs homoeostatic stem-cell and leukocyte trafficking through the stimulation of CXCR4; reports (16) suggest that CXCL12 can also play a role in directing the site-specific metastatic spread of some cancer cells. Under normal conditions, CXCL12 is expressed by specific tissues, including lung, liver, and bone marrow (17). The constitutive nature of the expression of this chemotactic ligand is consistent with reports that stimulation of CXCR4 generally induces little toxic respiratory burst activity. Notably, a mutant CXCL12 sequence has been designed that has minimal affinity for HS (18).

The current study was designed to define the potential of the non-HS-binding mutant form of CXCL12 (mtCXCL12) to bind CXCR4 and induce normal signal transduction. A further series of in vitro experiments was performed to determine how addition of mtCXCL12 can alter the normal migration of leukocytes across monolayer endothelium in response to wild-type chemotactants. After demonstration of limited toxicity together with prolonged retention in the circulation, mtCXCL12 was used in combination with wild-type CXCL12 to examine the potential for systemic blockade of site-specific inflammation in vivo. A final series of experiments was performed to assess the possibility that persistence of the mutant ligand induces a state of chemokine unresponsiveness that results in the failure of integrin activation following normal leukocyte stimulation by HS-bound, wild-type chemokines at the focus of developing inflammation.

**MATERIALS AND METHODS**

**Chemokines**

Human CXCL12 chemokines were synthesized, purified, and validated by Albachem (East Lothian, UK). Wild-type CXCL12 contains lysine, histidine, and lysine at sequence positions 24, 25, and 27, respectively; each of these residues was changed to serine in the mtCXCL12 sequence (18). The mutant sequence is as follows, with the variant residues underscored: KPVSLSYRPHFRESFHRVARANVSSTSLNTPICALQIQLQRKLKNNSNROVCIGDIPKLKWQYELKALNRFPK. Human CCL7 and CXCL8 were obtained from R&D Systems (Abingdon, UK).

**Chemokine receptor activation assays**

Radioligand competition assays were performed as described previously (4), using Chinese hamster ovary (CHO) cells stably transfected with CXCR4 by our group (19). Briefly, 2 × 10⁵ CHO-CXCR4 cells were pelleted and resuspended in binding buffer containing chemokine. A tracer concentration of 100 pM [¹²⁵I]CXCL12 in 50 μl was then added, and the assay was incubated for 2 h on ice with shaking. The cells were then washed twice with 0.5 M NaCl in binding buffer before being transferred to fresh tubes for counting in a micro-beta scintillation counter (LKB Wallac; Perkin Elmer, Cambridge, UK). Go, activation, calcium flux, pAKT, and pERK assays were performed as described previously (20).

**Chemotaxis assays**

Peripheral blood mononuclear cells (PBMCs) were extracted from venous blood taken from healthy volunteers. Following heparinization, blood was diluted in an equal volume of serum-free RPMI and subjected to density gradient centrifugation using Ficoll-Paque density gradient medium (specific gravity 1.077±0.001g/ml; Amersham Pharmacia, Little Chalfont, UK). The interfacial layer was harvested and washed by centrifugation. The human endothelial cell hybridoma EAhy926 was cultured as described previously (21).

The chemotactic potential of leukocytes was assessed using a 3-μm Transwell filter system developed using polyethylene terephthalate chemotaxis filters (Falcon; Fahrenheit Laboratory Supplies, Rotherham, UK) and corresponding 24-well companion plates. In some cases, transendothelial chemotaxis was assessed by first culturing 5 × 10⁴ EAhy926 cells on the filter for 72 h before the migration assay. PBMCs (2×10⁶) were placed in upper chamber of a 3-μm filter, whereas the lower chamber contained serum-free RPMI supplemented with chemokine. A 1:10 dilution of synovial fluid drained from the knee of a patient with rheumatoid arthritis was used in transendothelial chemotaxis assays, as described previously (4). To assess the chemotactic factors present in synovial fluid, a 1:10 dilution was analyzed using a chemokine antibody array (Raybiotech, Norcross, GA, USA), according to the manufacturer’s instructions.

The assay was incubated for 90 min at 37°C before removal of excess cells and medium from both chambers. The upper surface of the filter was gently swabbed to remove nonmigrating cells before fixation in 100% ice-cold methanol for 1 h. Each filter was then stained using an α-naphthyl acetate esterase staining kit (Sigma, Poole, UK). Finally, the filters were dehydrated and mounted. High power microscopy (×400) was used to count migrant cells in 9 randomly chosen fields per labeled filter. To assess migration of nonadherent cells (typically lymphocytes), the medium from the lower chamber was collected, the cells were pelleted by centrifugation, and counting was performed by flow cytometry using 3907 ANTI-INFLAMMATORY ACTION OF NON-HS-BINDING CXCL12
Flow-Count beads (Becton-Dickinson, Cowley, UK). Chemo
tactic indexes were normalized to express the migration
results as a percentage of the response to wild-type CXCL12.

Partitioning of chemokine in this assay was examined by
ELISA, as described previously (22). Samples were taken
above and below the chemotaxis filter at intervals up to 90
min. Different plates and filters were used for each time point
to minimize disruption to any gradient during sampling. Goat
anti-human CXCR4 antibodies, anti-goat biotin conjugated
secondary antibodies, and streptavidin-horseradish peroxi
dase were used to perform the ELISA (R&D Systems).

To examine the distribution of chemokine on the endothelium,
the filter was fixed in methanol, and the apical surface of the
filter was stained for CXCL12 using the same reagents as above
and streptavidin-FITC for analysis by confocal microscopy with a
DAPI nuclear counterstain. Confocal sensitivity was adjusted
such that no signal was seen in filters incubated with secondary
anti-goat-biotin and tertiary streptavidin-FITC reagents.

Leukocyte recruitment to murine air pouches

Eight-week-old female BALB/c mice (Charles River, Margate,
UK) were used for generation of air pouches as described
previously (4) in full compliance with UK Home Office
regulations for animal experimentation. Briefly, air pouches
were induced by injecting 3 ml of sterile air subcutaneously
into the back of each animal, followed by 1 ml of air on 3
further occasions (d 2, 4, and 5, respectively); this produced
stable fluid-filled pouches. On d 6, each pouch was injected
with 1 ml of PBS containing either 10 μg of wild-type
CXCL12, mtCXCL12, or a combination of 5 μg of both
chemokine sequences. Age- and sex-matched control mice
were injected with PBS alone.

After 24 h, recruited cells were recovered by gently lavaging
the pouch twice with 0.75 ml of PBS containing 1 mM EDTA.
The exudates were centrifuged at 500 g for 5 min, and the
supernatants were removed. The cell pellets were resus
pended in 1 ml of PBS, and 200 μl of each cell suspension was
then smeared by cytocentrifugation (Shandon, Runcorn, UK)
and stained with Diff Quick (Sigma) for differential cell
counting, 3 × 10⁶/ml, with 9 fields/cytopsin counted. In all
cases, groups of 5 animals were examined, and samples were
counted in a masked procedure by 2 separate investigators.

Splenocytes were isolated by trituration of splenic tissue
before filtration through a 70-μm filter. Leukocytes were then
purified by density gradient centrifugation using Histopaque
1083 (Sigma).

Intravenous administration of chemokine

An additional series of air-pouch experiments was performed to
examine how intravenous administration of chemokine modu
lated immune cell recruitment to the air pouches. Air pouches
were generated as above, and 20 μg of wild-type CXCL12 or
mtCXCL12 was injected into the tail vein immediately before
instillation of chemokines into the air pouch. Twenty micro
grams was injected to approximate the chemokine concentra
tion within the air pouch (10 μg/ml). To assess the clearance of
chemokine, 40 μl of blood was collected from each mouse for
up to 24 h following administration. Following 1 h of clotting,
the serum was harvested by centrifugation, and the CXCL12-
specific ELISA was performed as above.

To test the ability of serum from these mice to modulate
CXCR4 expression, MOLT-16 T-cells were incubated with a
1:1 dilution of serum from each group of animals. After 90
min, CXCR4 expression was measured by flow cytometry.
As a negative control, cells were incubated in the serum of mice
that had only received PBS IV. For a positive control, 10 nM of
wild-type CXCL12 was used to supplement serum from PBS
mice.

To examine the oxidative burst activity of whole blood, a
Burst Test kit (Opregen Pharma, Heidelberg, Germany) was
used. A volume of 100 μl of heparinized mouse blood was
stimulated with either chemokine, 50 mM fMLP, or 2
× 10⁻⁷ M opsonized Escherichia coli for 10 min at 37°C. On stimulation,
leukocyte production of reactive oxygen metabolites, such as
superoxide anion, hydrogen peroxide, and hypochlorous acid,
was monitored by the addition and oxidation of dihydroroho
dammine 123 to rhodamine 123 and measured by flow cytometry.

Integrin activation assay

Changes in integrin affinity for the ligand VCAM-1 were
measured using a VCAM-1.Fc fusion protein-binding assay
that was modified from a previous report for use with
MOLT-16 T cells (23). Briefly, 10 μl of 400 μg/ml VCAM-1.Fc
fusion protein (R&D Systems) was added to 8 × 10⁶ cells in 20
μl of PBS containing 0.5% BSA at 37°C; in some cases the fusion
protein was cross-linked with a FITC-conjugated anti-Fc anti
body for 30 min to form a fluorescent oligomeric immune
complex before addition to the cells. Then, 10 nM chemo
kine or, for positive control, either 1 mM MnCl²⁺ or 10 nM
PMA was added, and the cells were fixed after appropriate
incubation periods by addition of 500 μl prewarmed 4%
paraformaldehyde in PBS. After fixation for 5 min, 3 ml
ice-cold PBS was added. If necessary, the cells were then
washed and stained with a FITC-conjugated anti-Fc secondary
antibody (Sigma) before analysis by immunofluorescence
flow cytometry.

Statistical analyses

All results are expressed as means ± se of replicate samples.
The significance of changes was assessed by the application of an
ANOVA with Tukey’s posttest. All data were analyzed using
Prism 3 software (GraphPad, San Diego, CA, USA).

RESULTS

HS affinity does not alter agonist activity

An initial series of experiments was performed to study
the agonist activity of mtCXCL12. Heparin-Sepharose
chromatography demonstrated that mtCXCL12 had a
lower affinity than the wild-type chemokine for hepa
rin, being eluted from heparin-Sepharose columns by
0.55 and 0.8 M NaCl, respectively (not shown).

Radioligand-binding experiments indicated that
mtCXCL12 had an affinity for CXCR4 that was identical
to the wild-type sequence (Fig. 1A). The trend in compe
ition was similar for the wild-type and mtCXCL12,
indicating similar affinities for CXCR4, with IC₅₀ values of
6.3 nM (95% confidence interval between 4.8 and
8.2 nM) and 7.8 nM (95% confidence interval between
4.8 and 12.6 nM), respectively. Cell-activation assays
demonstrated that mtCXCL12 was able to fully activate the
receptor and elicit complete signal transduction, including
adenylate cyclase inhibition (Fig. 1B), generation
of a cytoplasmic calcium flux (Fig. 1C), and
phosphorylation of AKT (Fig. 1D) and ERK (Fig. 1E).
Both wild-type and mtCXCL12 were able to induce
migration of PBMCs in a GAG-independent transfilter chemotaxis assay (Fig. 1F). Together these data demonstrate that mtCXCL12 does not differ from wild-type CXCL12 in its ability to activate CXCR4 and subsequent chemokine receptor signal transduction pathways.

Non-HS-binding receptor agonist has a dominant inhibitory effect on transendothelial migration

Wild-type CXCL12 elicited significant migration of PBMCs across an endothelial monolayer (P<0.001), whereas mtCXCL12 was unable to induce the migration of either adherent monocytes or nonadherent lymphocytes across a monolayer of endothelial cells (Fig. 2A). Further experiments demonstrated the potential of mtCXCL12 to antagonize the normal response to the wild-type chemokine. When used at equimolar concentrations, a mixture of 5 nM wild-type CXCL12 and 5 nM mtCXCL12 did not elicit any significant migration of nonadherent cells across the endothelial monolayer (Fig. 2B); a similar result was observed when 2.5 nM mtCXCL12 was mixed with 7.5 nM wild-type CXCL12 (P<0.05, compared with wild-type CXCL12 alone).

A 1:10 dilution of synovial fluid drained from the knee of a patient with rheumatoid arthritis provides a broad-spectrum stimulus in leukocyte chemotaxis assays (4). The chemokines present in synovial fluid were examined by antibody array, with chemokines including CCL7, CCL8, and CXCL8 detectable (Fig. 3A). These are consistent with a number of published studies describing the chemokine milieu within the inflamed synovium (24). Addition of synovial fluid to the lower chamber of a transendothelial chemotaxis assay induced a significant migration of PBMCs through the filter (Fig. 3B). However, addition of either 10 or 1 nM concentrations of mtCXCL12 to the diluted synovial fluid in the lower chamber reduced the response to background levels; addition of wild-type CXCL12 did not significantly diminish the response to this synovial fluid. Further experiments demonstrated a significant reduction in transendothelial PBMC migration toward either CCL7 or CXCL8 following the addition of mtCXCL12 to the lower chamber (in both cases, P<0.05; Fig. 3C).

Non-HS-binding CXCL12 does not partition to the endothelium

There was no difference between the wild-type and mtCXCL12 in their capacity to diffuse across a Transwell filter (Fig. 4A). When a monolayer of endothelial cells was cultured on the filter, little wild-type CXCL12 was found in free solution on the apical surface at time points up to 90 min. In contrast, significant levels of
Non-HS-binding CXCL12 can limit inflammation in vivo

A murine model of inflammation was used to assess the in vivo modulation of CXCL12 activity by mutation of the GAG-binding domain. Air pouches were formed by subcutaneous injection of air into the back of mice; these pouches develop a lining that resembles the synovial membrane (25). Injection of 10 μg of wild-type CXCL12 into air pouches induced significant recruitment of lymphocytes ($P<0.05$) and mononuclear phagocytes ($P<0.01$) after 24 h (Fig. 5). At this time, more than twice as many lymphocytes were recruited to the air pouches by wild-type CXCL12 than by PBS, demonstrating that this homeostatic chemokine can induce significant inflammation in this model. The recruitment of cells in response to mtCXCL12 was not significantly greater than that produced by injection of PBS ($P>0.05$); the failure of the mutant sequence to recruit leukocytes in vivo was apparent for all the leukocyte populations enumerated after differential staining. Notably, injection of a 1:1 mixture of wild-type CXCL12 and mtCXCL12 elicited no more lymphocyte infiltration than was observed with PBS alone ($P>0.05$); this effect extended to all leukocyte subpopulations (Fig. 5).

Modulation of inflammatory recruitment by intravenous chemokine

The ability of intrapouch mtCXCL12 to antagonize leukocyte migration into the air pouch is consistent with the results from previous studies in which the non-HS-binding mutant chemokine CCL7 was mixed with the wild-type chemokine at a single site (4). In the current study, a further series of experiments was performed to assess the effect of intravenous mtCXCL12 on the intra-air pouch inflammatory response.

A series of experiments contrasted the effect of intravenous chemokine on leukocyte recruitment to wild-type CXCL12-filled air pouches (Fig. 6). Positive

Figure 2. mtCXCL12 can antagonize the response to wild-type CXCL12 in vitro. A) PBMCs were stimulated to migrate across monolayers of endothelial cells toward 10 nM wild-type (wt) or mtCXCL12. Data are expressed as chemotactic indices after normalization to the maximal response. B) Transendothelial migration assays were performed using a range of concentrations of mixed wild-type and mtCXCL12; nonadherent cells in the lower chamber were counted, and data are expressed as chemotactic indices. In both cases, results represent independent experiments; data are means ± s.e.

Figure 3. mtCXCL12 can antagonize a range of chemotactic factors. PBMCs were stimulated to migrate across monolayers of endothelial cells toward a 1:10 dilution of synovial fluid (SF) drained from the knee of a patient with rheumatoid arthritis. A) Chemotactic factors present in SF were examined by membrane array, and densitometric analysis was performed to examine the relative abundance of a range of chemokines. B) Varying concentrations of wild-type or mtCXCL12 were added to the lower chamber in combination with the diluted SF. Results represent 1 of 3 experiments performed using SF from different donors. C) Wild-type or mtCXCL12 (2.5 nM) was added to the lower chamber in combination with 7.5 nM CXCL8 or CCL7. Data are mean ± s.e. chemotactic indices for mononuclear cells.

mtCXCL12 were found in free solution ($P<0.05$). Immunofluorescent staining and confocal microscopy were employed to examine the presentation of CXCL12 in this assay. The mtCXCL12 was not bound to the endothelial surface; however, apical presentation of the wild-type chemokine was observed (Fig. 4B).

Figure 5. mtCXCL12 can antagonize a range of chemotactic factors. PBMCs were stimulated to migrate across monolayers of endothelial cells toward a 1:10 dilution of synovial fluid (SF) drained from the knee of a patient with rheumatoid arthritis. A) Chemotactic factors present in SF were examined by membrane array, and densitometric analysis was performed to examine the relative abundance of a range of chemokines. B) Varying concentrations of wild-type or mtCXCL12 were added to the lower chamber in combination with the diluted SF. Results represent 1 of 3 experiments performed using SF from different donors. C) Wild-type or mtCXCL12 (2.5 nM) was added to the lower chamber in combination with 7.5 nM CXCL8 or CCL7. Data are mean ± s.e. chemotactic indices for mononuclear cells.
and negative control groups, with intravenous PBS and intrapouch PBS, or intravenous PBS and intrapouch wild-type CXCL12, showed levels of infiltrating leukocytes that were similar to those in earlier experiments (Fig. 5). Interestingly, intravenous administration of wild-type CXCL12 promoted an increased infiltration of PBS-filled air pouches. This increase in infiltration of PBS-filled air pouches was not observed following intravenous administration of mtCXCL12; indeed, in these animals the level of lymphocyte infiltration of PBS-filled air pouches was slightly lower than that observed in animals that received intravenous PBS ($P<0.05$).

Notably, intravenous administration of mtCXCL12 to animals with wild-type CXCL12 in their air pouches resulted in a significant decrease in the number of both lymphocytes and mononuclear phagocytes entering the venous administration of mtCXCL12; indeed, in these animals the level of lymphocyte infiltration of PBS-filled air pouches was slightly lower than that observed in animals that received intravenous PBS ($P<0.05$).

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Figure 4. mtCXCL12 is not presented by the apical surface of endothelium. A) Soluble chemokine levels were measured by ELISA for up to 90 min following addition to the lower chamber of a chemotaxis assay with and without an endothelial monolayer. Black bars, wild-type CXCL12 across a filter; open bars, mtCXCL12 across a filter; hatched bars, wild-type CXCL12 across a monolayer; cross-hatched bars, sampling from mtCXCL12 across a monolayer. Data are means ± se. B) Partitioning of chemokine on the apical surface of the endothelium was examined by confocal microscopy. CXCL12 is labeled green with blue DAPI nuclear counterstain. Scale bars = 25 μm.

Figure 5. mtCXCL12 can antagonize the response to wild-type CXCL12 in vivo. Comparison of the potential of wild-type and mtCXCL12 to recruit cells using a murine air pouch model. Air pouches were injected with 10 μg of wild-type (wt) or mt CXCL12, or a 1:1 mixture of both wild-type and mtCXCL12 (5 μg of each protein), or PBS; in each case, $n = 5$. Number of recruited lymphocytes and mononuclear phagocytes was evaluated 24 h after administration of the chemokines. Representative results are shown from 1 of 3 separate experiments; data are means ± se.
pouch compared with groups that received only intravenous PBS (in both cases, P < 0.01). In contrast, intravenous administration of wild-type CXCL12 slightly increased the mean number of cells infiltrating air pouches filled with wild-type CXCL12. Together, these data suggest that systemic administration of non-HS-binding CXCL12 is able to inhibit localized, CXCL12-mediated inflammation within the air pouch.

To assess the kinetics of chemokine clearance from the blood after intravenous administration of the wild-type and mutant sequences, 40 μl blood samples were taken from the tail vein for up to 24 h. The serum was collected, and the CXCL12 levels were analyzed by ELISA; wild-type CXCL12 was cleared within 1 h of injection (Fig. 7A), whereas mtCXCL12 remained above detectable levels in the serum for up to 24 h after injection.

To identify a possible mechanism for the anti-inflammatory activity of the mtCXCL12 sequence, splenocytes were isolated from chemokine-treated animals and analyzed by immunofluorescence flow cytometry to quantify cell surface expression of the CXCL12 receptor CXCR4 (Fig. 7B). Twenty-four hours following intravenous administration of 20 μg mtCXCL12, there was a significant reduction in the expression of cell-surface, immunoreactive CXCR4 (P = 0.01). A small reduction in CXCR4 was also seen at this time after administration of 20 μg wild-type CXCL12, although this did not reach statistical significance (P = 0.08). Splenocytes from mice that received intravenous mtCXCL12 were isolated after 24 h and applied to standard chemotaxis assays to assess their potential to migrate toward wild-type CXCL12 and were less responsive to CXCL12 than splenocytes from PBS control animals (data not shown). To test whether the residual mtCXCL12 as measured in Fig. 7A was sufficient to influence receptor expression, MOLT-16 T cells were incubated for 90 min with serum from each group. A significant reduction in surface CXCR4 was seen following incubation with serum from mtCXCL12-treated mice compared with those incubated with serum from wild-type CXCL12- or PBS-treated mice (Fig. 7C; P < 0.05).

To examine possible side effects of intravenous chemokine delivery, oxidative burst activity of murine peripheral leukocytes was examined. There was no significant burst response following treatment with 10 μg/ml of either wild-type or mtCXCL12; this dose corresponds to that initially delivered by injection. In contrast to this, the proinflammatory chemokine CCL7 did elicit a mild flux in 4% of cells, although this was significantly less than that seen in samples stimulated with opsonized E. coli (Fig. 7D; P < 0.05). No IFN-γ release was detectable from peripheral murine leukocytes up to 6 h following treatment with 10 μg/ml CXCL12 (data not shown).

Integrin activation is perturbed by chronic chemokine exposure

A series of experiments was performed to assess the effect that chronic (6 h) exposure of leukocytes to mtCXCL12 in the circulation would have on CXCL12-mediated activation of the α4β1 integrin VLA4. Activation of the adhesive potential of this integrin has previously been investigated using an immunofluorescence flow cytometric assay to measure the binding of a soluble human adhesion moleculeFc fusion proteins to appropriate immune cells (23). In this study, the VLA-4- and CXCR4-expressing MOLT-16 T cell line (26) was stimulated with wild-type CXCL12 and within 1 min showed a transient increase in the potential to bind VCAM-1.Fc (Fig. 8A). There was no difference between the potential of wild-type CXCL12 and mtCXCL12 to activate this integrin (Fig. 8B), with both chemokines producing an increase in adhesion of comparable magnitude to that induced by the positive control reagent (1 mM MnCl₂). In further experiments, T cells that encountered CXCL12 for the first time were contrasted with those that had been washed by centrifugation after chronic exposure for 6 h to 10 nM of wild-type or mtCXCL12; chronic exposure was found to abolish the potential of further exposure to wild-type CXCL12 to enhance the adhesion of either monomeric or oligomeric VCAM-1.Fc fusion proteins (Fig. 8C, D, respectively). Notably, restimulation of cells that had been previously exposed transiently to 10 nM of wild-type or mtCXCL12 for 20 min before washing and resting for 5 h and 40 min bound the VCAM-1.Fc fusion protein as well as T cells acutely stimulated with CXCL12 (Fig. 8C).
CXCL12 is a well-studied chemokine that is expressed constitutively by a number of tissues to maintain homeostatic body functions (27). A previous study of the interaction between this chemokine and glycosaminoglycans led to the development of a mutant sequence variant with reduced affinity for HS (18). Non-HS-binding variants of the inflammatory chemokines CCL5 (2) and CCL7 (4) are known to have immunoregulatory properties. The current study was designed to assess the potential of the non-HS-binding form of the homeostatic chemokine CXCL12 to modulate normal chemokine-mediated inflammation.

**DISCUSSION**

CXCL12 is a well-studied chemokine that is expressed constitutively by a number of tissues to maintain homeostatic body functions (27). A previous study of the interaction between this chemokine and glycosaminoglycans led to the development of a mutant sequence variant with reduced affinity for HS (18). Non-HS-binding variants of the inflammatory chemokines CCL5 (2) and CCL7 (4) are known to have immunoregulatory properties. The current study was designed to assess the potential of the non-HS-binding form of the homeostatic chemokine CXCL12 to modulate normal chemokine-mediated inflammation.

**Antimicrobial Action of Non-HS-Binding CXCL12**

**Figure 7.** mtCXCL12 persists in the circulation and affects both CXCR4 expression and function. A) After intravenous administration of 20 μg of wild-type (closed bars) or mtCXCL12 (open bars), blood was sampled for up to 24 h for analysis of chemokine concentration by ELISA; broken line is the background seen in PBS treated mice (n=5). B) After 24 h, splenic mononuclear cells were purified from each group of animals and from a PBS-treated control group (n=5) and labeled with anti-CXCR4. Median immunofluorescence values are shown; broken line indicates background isotype control fluorescence. C) CXCR4 staining performed on MOLT-16 T cells 90 min following incubation with serum from mice treated with PBS, wild-type, or mtCXCL12. Serum from the PBS-treated group was supplemented with 10 nM CXCL12 as a positive control. Median immunofluorescence values are shown; broken line indicates background isotype control fluorescence. D) Oxidative burst activity of whole mouse blood was examined by a dihydrorhodamine 123 assay following 10 min stimulation with 10 μg/ml chemokine, 50 mM fMLP or 2 × 10^7 opsonised E. coli for 10 min at 37°C. Data are means ± se.

**Figure 8.** Chronic exposure to CXCL12 prevents chemokine-mediated, proadhesive activation of the α4β1 integrin. A flow cytometric assay was used to measure changes in the binding of VCAM-1.Fc fusion protein following CXCL12 stimulation of a T-cell line. A) Treatment with 10 nM wild-type CXCL12 rapidly and transiently increases the binding of monomeric VCAM-1.Fc. B) T-cell line was treated for 1 min with 10 nM wild-type (wt) or mtCXCL12 before analysis of monomeric VCAM-1.Fc binding; stimulation with PBS or Mn^2+ were used for negative and positive control respectively. C) T-cell line was untreated (acute), treated with 10 nM wt or mt CXCL12 for 6 h (chronic), treated with wild-type or mtCXCL12 for 20 min, then washed and cultured with no ligand for 5 h 40 min (transient). Each of the cell samples was then washed and stimulated with 10 nM wild-type CXCL12 for 1 min before measurement of monomeric VCAM-1.Fc binding; stimulation with PBS or Mn^2+ were used for negative and positive control respectively. D) Assay was performed as in C except antibody-crosslinked, oligomeric VCAM-1.Fc was used to determine integrin avidity changes; in this case PMA stimulation was used for positive control. In all cases, results represent 1 of 3 independent experiments; data are means ± se.
No significant difference was found between wild-type CXCL12 and mtCXCL12 with respect to their receptor affinities or their abilities to induce G-protein coupling, cytoplasmic Ca$^{2+}$ fluxes, Akt activation, or ERK activation. Furthermore, both chemokine sequences induced similar leukocyte migration in a free-diffusion model of chemotaxis. However, unlike wild-type CXCL12, the mtCXCL12 was unable to stimulate the migration of either mononuclear phagocytes or lymphocytes across a monolayer of endothelial cells. Notably, the mtCXCL12 variant had a powerful inhibitory effect on leukocyte migration induced by wild-type CXCL12 or the mixture of chemokines (24) and other chemoattractant factors (28) present in synovial fluid drained from the rheumatoid synovium. This is consistent with results that have been reported for a non-HS-binding form of the inflammatory chemokine CCL7 (4) and may be a consequence of heterologous receptor desensitization (5, 29). To investigate this possibility, wild-type or mtCXCL12 were separately mixed with mononuclear cell prototypical CC and CXC chemokines (30), CCL7 and CXCL8, in the lower chamber of transendothelial chemotaxis assays. It was found that the normal chemotactic response was inhibited by mtCXCL12 but not by the wild-type chemokine. These data suggest that treatment with mtCXCL12 induces desensitization of receptors other than CXCR4; this phenomenon is not fully understood but may be of significant value for the development of anti-inflammatory therapies (5, 31).

Experiments were performed to examine the differences between the wild-type and mtCXCL12 in the in vitro chemotaxis assays. Both chemokines diffused equally across a bare filter; however, only the wild-type chemokine was efficiently sequestered and presented by the apical surface of the endothelial monolayer. After 90 min, significant levels of mtCXCL12 were observed in the medium above the endothelial monolayer. This suggests a mechanism for our earlier observations and is consistent with our earlier report that significant levels of addition of a chemokine to the upper chamber of this assay can result in receptor cross desensitization and the importance of apical presentation (22, 32).

These experimental observations were extended to a murine model. The air-pouch system has been used for >20 yr to assess inflammation and provides a well-established method for studying chemokine-mediated leukocyte infiltration (4, 33). This model was used for an in vivo chemotaxis assay in which introduction of wild-type CXCL12 to air pouches was found to elicit significant lymphocyte and mononuclear phagocyte recruitment. These results are consistent with a previous report (34) that subcutaneous injection of CXCL12 elicited specific infiltration by mononuclear leukocytes; <1% of the infiltrating cells were neutrophils. Notably, the presence of mtCXCL12 within the pouches did not induce leukocyte recruitment. Furthermore, mixture of wild-type CXCL12 with the non-HS-binding mutant sequence resulted in abrogation of the chemotactic potential of the wild-type chemokine; a similar observation was made with the in vitro chemotaxis assay.

A series of experiments was performed to assess the potential of wild-type CXCL12 and mtCXCL12 for systemic application. Initial observations demonstrated that wild-type CXCL12 was effectively cleared from murine blood within 1 h of intravenous administration. This rapid clearance is consistent with the reported 25.8 min half-life of CXCL12 in the circulation of rats (35) and may reflect normal sequestration to appropriately sulfated domains in HS on the apical surface of endothelial cells (22); such sequestered CXCL12 can be remobilized and maintained in the blood by injection of soluble heparin-like molecules (36). The failure of mtCXCL12 to bind intravascular HS proteoglycans provides a likely explanation for the retention of 60% of this variant chemokine in the blood of treated mice after 6 h. Significant levels of the mtCXCL12 were detectable up to 24 h following intravenous administration. This observation is consistent with the duration of receptor internalization and leukocyte desensitization toward CXCL12 that we observed in splenocytes from these animals. Notably, the residual mtCXCL12 present in serum taken 24 h following intravenous administration was sufficient to cause internalization of CXCR4 when applied to a T-cell line. The level of CXCR4 internalization observed was similar to that observed when fresh CXCL12 was applied.

No obvious toxic effects were observed after intravenous administration of 20 μg of either wild-type or mtCXCL12. Furthermore, studies in vitro showed that neither high doses of wild-type nor mtCXCL12 induced an oxidative respiratory burst in blood-derived murine leukocytes, promulgating the absence of a damaging systemic inflammatory response in response to these agents. The observed failure of CXCL12 to induce an oxidative respiratory burst is consistent with results of a previous study (37) and contrasts with the potential of inflammatory chemokines such as CCL7 and CXCL8 to induce such a burst (13, 38). The absence of apparent toxicity of exogenous CXCL12 is compatible with the expression of high levels of this chemokine within many tissues under normal conditions, including the brain, lungs, bone marrow, and liver (17).

Following demonstration of the safety of intravenous administration of wild-type CXCL12 and mtCXCL12, a series of experiments was performed to assess the potential of both these chemokines to modulate the infiltration by mononuclear leukocytes of CXCL12-filled air pouches. Notably, it was found that intravenous administration of wild-type CXCL12 enhanced infiltration of the air pouches, while administration of mtCXCL12 effectively prevented the recruitment of mononuclear leukocytes to air pouches.

To examine a potential mechanism for the anti-inflammatory activity of mtCXCL12, splenocytes were isolated from animals 24 h after intravenous administration of PBS, wild-type CXCL12, or mtCXCL12. It was found that mononuclear splenocytes from animals that had been treated with the non-HS-binding mtCXCL12
showed both reduced expression of CXCR4 and chemotaxis toward CXCL12. By contrast, splenocytes from animals treated 24 h earlier with either PBS or wild-type CXCL12 showed levels of CXCR4 expression that were not significantly different. These data are consistent with the reported kinetics of ligand-induced CXCR4 phosphorylation, followed by desensitization and internalization with a T-cell surface half-life of 5 min (39).

The transient availability of wild-type CXCL12 in the blood will allow efficient CXCR4 recycling; indeed, it has been reported that this process can be 75% complete within 2 h (40).

In addition to down-regulation of CXCR4 expression and desensitization of the chemotactic response toward CXCL12, chronic exposure to CXCL12 was shown to prevent the transient increase in adhesion to either monomeric or oligomeric (antibody cross-linked) VCAM-1. Fc fusion protein produced by treatment of VLA4-expressing T cells with CXCL12. The changes in adhesion observed using the 2 variants of this assay are consistent with increased integrin affinity and avidity, respectively (23). Notably, transient stimulation of T cells with wild-type chemokine followed by a period of rest allowed the cells to respond normally by transient adhesion to VCAM-1 following secondary exposure to CXCL12. Activation of the adhesive functions of the αβ1 integrin VLA4 plays an important role in mediating the transition from normal leukocyte rolling to static adhesion onto the apical surface of chemokine-expressing endothelial cells. The transient nature of adhesive activation and the potential for desensitization of this process both suggest that efficient intravascular adhesion of VLA-4-expressing cells can only occur when both CXCL12 and VCAM-1 are encountered at the site of leukocyte to endothelial cell contact (41). While this is the case when wild-type chemokine is bound to HS on the surface of VCAM-1-expressing endothelial cells, the non-HS-binding mtCXCL12 has the potential to trigger an inappropriate transient increase in adhesive potential followed by desensitization of this process at a site distant from the endothelial surface.

These data are consistent with a model in which non-HS-binding mtCXCL12 persists in the circulation and chronically stimulates CXCR4 in blood-borne leukocytes, while wild-type CXCL12 is only present transiently leading to equally transient chemokine receptor stimulation. Previous studies (42) have demonstrated internalization of CXCR4 following ligand binding. The lower level of CXCR4 observed in leukocytes from animals treated with mtCXCL12 suggests that this ligand maintains chronic receptor internalization in vivo. This leads to receptor desensitization that can explain the failure of leukocytes from these animals to respond as efficiently as cells from animals treated with wild-type CXCL12 in chemotaxis assays. Notably, chronic but not transient stimulation of CXCR4 also resulted in the failure of normal activation of the important proadhesive integrin VLA-4 following restimulation with CXCL12.

While this report demonstrates a potential to induce homologous tolerance of wild-type CXCL12, the possibility that a wider, heterologous GPCR tolerance is also produced cannot be discounted. Such tolerance could explain both the blockade of the chemotactic response to synovial fluid from joints of patients with rheumatoid arthritis and the reduction of background inflammation in PBS-filled air pouches produced by mtCXCL12. Heterologous desensitization is a well-established feature of GPCRs (31) and has been proposed as an explanation for anti-inflammatory activity of other non-GAG-binding chemokines receptor agonists (5). The potential of a non-HS-binding variant of the homeostatic, non-respiratory-burst-inducing chemokine CXCL12 to induce such tolerance suggests that appropriate CXCR4 agonists may be developed as powerful anti-inflammatory agents.

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REFERENCES


ANTI-INFLAMMATORY ACTION OF NON-HS-BINDING CXCL12


