Homeostatic and Tissue Reparation Defaults in Mice Carrying Selective Genetic Invalidation of CXCL12/Proteoglycan Interactions

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Background—Interaction with heparan sulfate proteoglycans is supposed to provide chemokines with the capacity to immobilize on cell surface and extracellular matrix for accomplishing both tissue homing and signaling of attracted cells. However, the consequences of the exclusive invalidation of such interaction on the roles played by endogenous chemokines in vivo remain unascertained.

Methods and Results—We engineered a mouse carrying a Cxcl12 gene (Cxcl12Gagt/gagtm) mutation that precludes interactions with heparan sulfate structures while not affecting CXCR4-dependent cell signaling of CXCL12 isoforms (α, β, γ). Cxcl12Gagt/gagtm mice develop normally, express normal levels of total and isoform-specific Cxcl12 mRNA, and show increased counting of circulating CD34+ hematopoietic precursor cells. After induced acute ischemia, a marked impaired capacity to support revascularization was observed in Cxcl12Gagt/gagtm animals associated with a reduced number of infiltrating cells in the ischemic tissue despite the massive expression of CXCL12 isoforms. Importantly, exogenous administration of CXCL12γ, which binds heparan sulfate with the highest affinity ever reported for a cytokine, fully restores vascular growth, whereas heparan sulfate–binding CXCL12γ mutants failed to promote revascularization in Cxcl12Gagt/gagtm animals.

Conclusion—These findings prove the role played by heparan sulfate interactions in the functions of CXCL12 in both homeostasis and physiopathological settings and document for the first time the paradigm of chemokine immobilization in vivo. (Circulation. 2012;126:1882-1895.)

Key Words: angiogenesis-modulating agents ■ chemokine CXCL12 ■ chemokines ■ ischemia ■ proteoglycans

Chemokines control the migration of a large array of cells, thus regulating function and homeostasis of a number of tissues. Glycosaminoglycans (GAGs), the glycanic moiety of proteoglycans, are considered the critical biological structure that determines the immobilization of chemokines on the extracellular matrix and cell surfaces. Immobilization provides chemokines with a robust anchoring against blood flow and, by restraining their diffusion, facilitates both the formation of local gradients and the synchronous coordination of motility and cell adhesion.1-3

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Among GAGs, chemokines bind preferentially to heparan sulfate (HS) through interactions of either canonical BBXB and BBBXXB (B, basic; X, any residue) or discontinuous cationic protein epitopes with the negatively charged sulfated residues of HS.4 Although chemokine binding to GAGs has been well described from a biochemical point of view, the functional aspects of these interactions remain poorly understood, and in vivo investigations to address the importance of chemokine-GAG interactions are limited. In some cases, the experimental approach, eg, the administration of sulfated glycans competing exclusively with HS5 or the genetic interference selectively inhibiting endothelial HS biosynthesis,6 has broad effects and would affect, beyond chemokines, the interaction of GAGs with many other factors. In other

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cases, the overlapping or spatial proximity of chemokine domains involved in receptor-mediated cell signaling and GAG attachment has obscured the interpretation and significance of results obtained by exogenous administration of GAG-binding mutant chemokines with reduced agonist capacity.

In this regard, CXCL12 is unique among chemokines for the spatial separation between the receptor and HS-binding sites, localized on the opposite sides of the molecule,

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thus permitting the evaluation of the contribution of each domain to their biological functions.

In mice, 3 isoforms (CXCL12α, CXCL12β, and CXCL12γ), which show an exceptional degree of interspecies conservation exceeding 95% homology for their amino acid sequences, are generated by alternative splicing.6,10 CXCL12β and CXCL12γ contain the entire amino acid sequence (68Aa) of CXCL12α, which encompasses the CXCR4-binding domain and the protein core BBXB motif (K24H2S2L26K27) critically required for the HS binding. CXCL12β (72Aa) and CXCL12γ (98Aa) differ from CXCL12α by the addition of the last 4 and 30 Aa, respectively, encoding 1 and 4 additional BBXB motifs. Although CXCL12α and CXCL12β display affinities for HS or heparin in the same range of magnitude (Kd = 25 mmol/L, respectively), that of CXCL12γ is 1.5 mmol/L and constitutes the strongest HS affinity ever measured for a cytokine.11,12 This firm interaction with HS relies on the cooperation of the core and the high cationic C-ter domain of the chemokine in which the 4 overlapping BBXB motifs of CXCL12γ mostly account for the slow off-rates from immobilized HS.12

We reported previously that neutralization of the K24H2S2L26K27 cationic charge (K24S and K27S substitutions) is sufficient to drastically reduce binding to HS and complexing of CXCL12α with either immobilized heparin or HS.11-14 Similarly, O’Boyle et al15 recently reported that combined K24S and K27S in CXCL12β are sufficient to impede HS binding of this isoform on the apical surface of endothelial cells and promote a dramatic blood and delayed clearance compared with the wild-type (WT) counterpart. To abrogate HS binding of CXCL12γ, the mutation of K24H2S2L26K27 combined with the C-ter 4-overlapping BBXB motifs is required.12,13 Of note, although all these HS-binding CXCL12 mutants show a preserved global structure defined by nuclear magnetic resonance11,12 and are full CXCR4 agonists in vitro,10-13,15 they have only a marginal capacity to attract leukocytes or endothelial progenitor cells and leukocytes16,17,24 and regulates the BM homing and retention/egress of hematopoietic cells,25,26 as well as the basal trafficking and transendothelial migration of leukocytes.27-29 Aside from homeostasis, CXCL12 is involved in both tumorigenesis and cancer metastasis30 and is a pathogenic factor in systemic disease like rheumatoid arthritis in which it shows proangiogenic and inflammatory effects.31 In addition, CXCL12 plays a role of paramount importance as an essential and nonredundant factor involved in tissue remodeling, particularly in vascular regeneration.32

Overall, the structural characteristics and pleiotropic roles played by CXCL12 make it ideally suited for investigating how the interactions with GAG/HS modulate the biological role of a chemokine in vivo. To this aim, we engineered a transgenic mouse expressing a Cxcl12 gene in which the critical sequences encoding CXCL12 domains involved in HS binding are selectively invalidated while the CXCR4 agonist potency and efficiency of the mutant proteins are preserved intact. The mutant mice show enhanced serum levels of free CXCL12 and an increased number of circulating leukocytes and CD34+ hematopoietic cells.5 Strikingly, and in keeping with the essential role played by CXCL12 in tissue repair, these mice display a dramatically reduced capacity to regenerate vascular growth after acute ischemia that is recovered by expression of WT CXCL12, demonstrating the importance of GAG binding for proper in vivo function. To the best of our knowledge, this is the first description of an animal model in which the HS-binding capacities of an endogenous chemokine were genetically invalidated and the consequences of the selectively impaired function were investigated in both homeostasis and physiopathological settings.

Methods

Experiments were conducted according to the French veterinary guidelines and those formulated by the European Community for experimental animal use (L358-86/609EEEC).

Chemokines: cDNA Expression Vectors and Synthesis

Tissues were obtained by dissection of Cxcl12Gagtm/Gagtm mice or WT littermates previously euthanized. BM was recovered from right tibias by the flushing procedure with 5 mL PBS. Total RNAs were purified with the RNAeasy Kit (Qiagen), and Cxcl12 cDNAs were synthesized with 0.5 µg of the corresponding RNAs with random hexamers. The isoform-specific Cxcl12 cDNAs corresponding to WT and Cxcl12Gagtm/Gagtm mice were generated as described previously13 using the Cxcl12 primers: forward common, 5'-caccatggagccgcaagcgctgctgcc-3'; and reverse, 5'-ctactgttaagaagcttcggagttc-3'. The coding CXCL12γm2 DNA was derived from mutagenesis from the WT sequence. All of them were subcloned in a cytomegalovirus promoter-driven pcDNA3 expression vector (Invitrogen). HEK293 cells were transfected with CXCL12 expression vectors to generate CXCL12-containing cell supernatants. Chemically synthesized chemokines were generated and evaluated for their purity and concentration as previously described.11

Cxcl12 Knock-In and Cxcl12Gagtm Mouse Generation

For animal engineering (iTL, Fresh Meadows, NY; Dr Ailan La), a 14.5-kb genomic DNA was used to construct the targeting vector first subcloned from a positively identified C57BL/6 BAC clone.
The AAG>TGC and AAA>TCT (aa: K>S) mutations within exon 2 and the stop codon TAG insertion in exon 4, which selectively prevents translation of CXCL12y last 30Aas, were generated by 3-step polymerase chain reaction (PCR) mutagenesis. The PCR fragments carrying mutations were then used to replace the WT sequence using conventional subcloning methods. The long homology arm extends ~6.6 kb 5’ to the first set of mutations in exon 2. The LoxP/FRT-Neo cassette was inserted ~2.2 kb downstream of the first set of mutations and ~2.3 kb upstream of the second set of mutations (stop codon insertion in exon 4) in intron 3 to 4. The short homology arm extends 3.3 kb 3’ to the second set of mutations. The targeting vector (backbone; a 2.4-kb pSP72-based vector) was validated by restriction analysis and sequencing after each modification. Targeting vector (10 μg) was linearized by ClaI digestion and then transfected by electroporation of BA1 (C57BL/6×129SvEv) hybrid embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant embryonic stem cell clones, and a secondary confirmation of positive clones identified by PCR was performed by Southern blot analysis. Targeted BA1 (C57BL/6×129SvEv) hybrid embryonic stem cells were microinjected into C57BL6 blastocysts. Resulting chimeras with a high percentage of agouti coat color were mated to WT C57BL/6 mice to generate F1 Cxcl12Gagtm/wt offspring. Tail DNA was analyzed by PCR to confirm the integration of the desired sequences. The obtained Cxcl12Gagtm mice were crossed with transgenic 129sv PGK-Cre for LoxP/FRT-Neo cassette excision. Offspring that do not inherit PGK-CRE and delete LoxP/FRT-Neo cassette were selected, and the colony was amplified. Genotypes of mice encoding Cxcl12Gagtm on excision of Neo cassette were identified by PCR with 5’Tgcagcataaagaagctctg (forward) and 5’Cagcttggtaacagtctggc (reverse) 3’ primers.

**Characterization of Cxcl12 mRNA and Protein Expression in Normal and Ischemic Tissues**

For quantitative real-time PCR, cerebellum, brain, thymus, heart, and skeletal muscle tissues were obtained by dissection of Cxcl12Gagtm/Gagtm mice or WT littermates. BM was recovered from right tibias by the laser Doppler perfusion imaging. Hind-Limb Ischemia Experimental Model of Surgically Induced Hind-Limb Ischemia

**Induction of Ischemia and Plasmid Electrotransfer**

WT and Cxcl12Gagtm/Gagtm mice and their WT C57BL/6 littermates underwent surgical ligation of the proximal part of the right femoral artery above the origin of the circumflexa femoris lateralis. Expression plasmids (50 μg) encoding for CXCL12y, CXCL12y, or CXCL12ym2 were then injected into both tibial anterior and gastrocnemius muscles of the anesthetized mouse as previously described. Transcutaneous electric pulses (8 square-wave electric pulses of 200 V/cm, 20 milliseconds each, at 2 Hz) were delivered by a PS-15 electroporator (Jouan) using 2 stainless steel plate electrodes placed 4.2 to 5.3 mm apart on each side of the leg. The left leg was not ligated or electrotransferred and was used as an internal control. In additional set of experiments, C57BL/6 mice with surgically induced hind-limb ischemia and treated with expression plasmids also received intravenous injection of 1×10^7 human EPCs.

**Analysis of Neovascularization**

Postischemic neovascularization was evaluated by 3 different methods, as previously described.

**Microangiography**

Mice were anesthetized (pentobarbital) and longitudinal laparotomy was performed to introduce a polyethylene catheter into the abdominal aorta and to inject contrast medium (1 g/mL barium sulfate). Angiography of hind limbs was then performed, and images (2 per animals) were acquired with a high-definition digital x-ray transducer. Images were assembled to obtain a complete view of the hind limbs.

**Capillary and Arteriole Density Analysis**

Frozen tissue sections (7 μm) from calf muscle were incubated with rabbit polyclonal antibody directed against total fibronectin (1:50 dilution; Abcys) to identify capillaries and rabbit anti-mouse α-smooth muscle actin (1:100 dilution; Abcam) to identify arterioles. The capillary-to-myocytes ratio was determined in both ischemic and nonischemic legs.

**Laser Doppler Perfusion Imaging**

Briefly, excess hairs were removed by depilatory cream from the limb, and mice were placed on a heating plate at 37°C to minimize temperature variation. Nevertheless, to account for variables, including ambient light, temperature, and experimental procedures, perfusion was calculated in the foot and expressed as a ratio of ischemic to nonischemic legs.

**Analysis of Cell Infiltration in the Ischemic Tissue**

To evaluate the number of infiltrating CD45.1-positive cells, 10^7 mononuclear cells isolated from the BM of CD45.1 mice were intravenously injected to Cxcl12Gagtm/Gagtm mice and their WT C57BL/6 littermates 1 day after femoral artery ligation. Five days after the injection, the ischemic gastrocnemius muscles were harvested, weighted, minced, and digested in 450 U/mL collagenase I, 125 U/mL collagenase XI, 60 U/mL DNAse I, and 60 U/mL hyaluronidase (Sigma Aldrich) for 1 hour at 37°C. After Ficoll
separation, infiltrating cells were stained with CD45.1-PerCP/Cy5.5, CD34-APC, and CXCRI4-PE and analyzed with an Aria fluorescence-activated cell sorter (BD).

To measure the number of infiltrating EPCs, the ischemic gastrocnemius muscles were digested as described above. The number of cells positive for β2-microglobulin anti-human FITC (1:200; BioLegend) and DAPI was then evaluated on a LSRII Flow Cytometer (Becton Dickinson). In a third set of experiment, 10-week-old WT C57BL/6 mice underwent median laparatomy by total body irradiation (9.5 Gy). BM cells were then isolated from femurs and tibias of green fluorescent protein (GFP) C57BL/6 mice and intravenously injected in irradiated animals. After 8 weeks, mice underwent surgical ligation of the proximal part of the right femoral artery as introduced in the fourth exon to prevent the translation of the GAG interactions in the regenerative process after acute regional ischemia.

No significant differences in the composition of blood, BM, and CD34+ hematopoietic progenitor cells were observed between WT and mutant animals (data not shown). Interestingly, whereas the number of BM hematopoietic cell subpopulations was similar in WT and Cxcl12Gagtm/Gagtm mice, mutant animals displayed higher numbers of circulating total leukocytes, granulocytes, and CD34+ cells (Figure 1E) and no significant differences for both T and B lymphocytes. The numbers of erythrocytes (10.72±0.33 versus 10.32±0.31 per 1 mm3; n=6) and platelets (944±132 versus 1029±197 per 1 mm3; n=6) in WT and Cxcl12Gagtm/Gagtm mice, respectively, were similar.

CXCL12 regulates both the tissue homing and survival of circulating tissue-specific progenitors, hematopoietic progenitor cells, and BM-stromal stem cells32,35; triggers inflammatory cell infiltration; and is required for the periendothelial retention of circulating, BM-derived myeloid cells.32,35,36 These findings prompted us to assess the role of CXCL12/ GAG interactions in the regenerative process after acute regional ischemia.

We observed that in both WT and Cxcl12Gagtm/Gagtm mice, hind-limb ischemia induced massive transcription and expression of Cxcl12 isoforms (Figure 2A). Kinetic analysis of mRNA levels showed that Cxcl12 isoform expression was enhanced as early as day 4 after ischemia and returned to basal levels by day 21 (Figure 2A). The expression ratio between the Cxcl12 isoforms varies between different tissues (Figure IID in the online-only Data Supplement). Of interest, CXCL12γ was the most abundant isoform in skeletal muscle (Figure 2B). We also determined that CXCL12 was expressed in ischemic capillary structure and is colocalized with isolectin B4 staining, a specific marker of endothelial cells, in both WT and Cxcl12Gagtm/Gagtm mice (Figure 2C). In addition, Cxcl12 expression was detected in a number of arterioles, as revealed by the costaining between CXCL12 and α-smooth muscle actin (Figure 2D).

It is of note that Cxcl12Gagtm/Gagtm mice displayed a markedly impaired capacity to support efficient postischemic revascularization in a model of hind-limb ischemia. Indeed, laser Doppler imaging showed a reduced paw perfusion in ischemic hind limb of Cxcl12Gagtm/Gagtm compared with WT animals as early as day 14 after the onset of ischemia (Figure 3A). Angiographic scores obtained by microangiographic analysis revealed that vessel density was hampered by 30% in ischemic muscle of Cxcl12Gagtm/Gagtm mice compared with that of WT animals (Figure 3B). Furthermore, analysis of postischemic frozen calf muscle samples showed that regeneration of both capillary and arteriole vessels was reduced in Cxcl12Gagtm/Gagtm animals by up to 44% and 35%, respectively, compared with WT animals (Figure 3C and 3D).

As mentioned, CXCL12/CXCR4 interactions are involved in the recruitment and/or retention of circulating cells in the ischemic tissue. To gain further insights into the cellular and molecular mechanisms associated with the reduction of postischemic vessel growth in Cxcl12Gagtm/Gagtm mice, we analyzed the ability of circulating cells to home to ischemic tissues in Cxcl12Gagtm/Gagtm animals. CD45.1-positive cells were intravenously injected 1 day after the onset of ischemia, and their number and fate were analyzed in the blood and muscular tissues of which is affected in Cxcr4 knockout animals, and skeletal muscle are shown in Figure 1B.
Figure 1. Characterization of Cxcl12<sup>Gagtm/Gagtm</sup> mice. A, Schematic representation of Cxcl12 genomic locus, targeting vector, and recombined Cxcl12 locus (Cxcl12<sup>Gagtm</sup>). Exons 1 through 4 are represented by dark boxes. Mutations incorporating residue substitutions (second exon) and a stop codon (fourth exon) are indicated by vertical arrows and stars. The LoxP-FRT-Neo cassette is inserted in an opposite orientation from the target gene in intron 3 to 4. Long and short homology arm positions and length are indicated. UTR indicates untranslated region. B, Hematoxylin and eosin staining of bone marrow (left), cerebellum (middle), and skeletal muscle (right) tissue sections from Cxcl12<sup>Gagtm/Gagtm</sup> (MUT) and wild-type littermate (WT) animals. Arrows show megakaryocytes; 1 indicates white substance; 2, granular layer; and 3, molecular layer. C, Real-time polymerase chain reaction analysis of Cxcl12. Relative RNA expression levels of Cxcl12 isoforms normalized to HPRT in cerebellum, bone marrow, and skeletal muscle tissues. Cxcl12<sup>α</sup>, Cxcl12<sup>β</sup>, and Cxcl12<sup>γ</sup> ARN levels in MUT animals are expressed relative to the level in WT animals, arbitrarily set to 1 (n=12 per group). D, Blood CXCL12 levels were measured by ELISA in MUT and WT animals. E, Cell populations in peripheral blood collected from MUT and WT animals. Results are mean±SEM. **P<0.01, ***P<0.001 vs WT.
Figure 2. Expression of CXCL12 isoforms in ischemic tissue. A, Quantitative evaluation and relative levels of CXCL12α, CXCL12β, and CXCL12γ mRNA contents 1, 4, 6, and 21 days after ischemia in ischemic and nonischemic legs of Cxcl12Gagtm/Gagtm (MUT) and wild-type littermate (WT) animals. Values are mean±SEM (n=10 per group, representative of 2 independent experiments). Twenty-eight possible comparisons for Bonferroni correction; a value of P<0.0018 was considered significant. **P<0.0003 vs nonischemic at day 1. B, mRNA levels of each Cxcl12 isoform were normalized to that of Cxcl12α in skeletal muscle of nonischemic tissue. Six possible comparisons for Bonferroni correction; a value of P<0.008 was considered significant (n=10 per group). C and D, Histological sections from ischemic skeletal muscles of WT and MUT animals showing CXCL12 expression in capillary (c) and arteriolar (d) structures. Red staining indicates α-smooth muscle actin (α-smooth muscle cells); green staining, Griffonia simplicifolia agglutinin isoelectin B4 (endothelial cells); purple staining, CXCL12. Nuclei were stained with DAPI (blue staining). Bars=20 μm. One representative of 6 experiments is shown.
Figure 3. Postischemic revascularization in Cxcl12<sup>Gagtm/Gagtm</sup> animals. Quantitative evaluation and representative photomicrographs of foot perfusion at different time points (A) and at day 21 for angiographic score (B; vessels in white), capillary density (C; capillaries in green, upper panel: arrows show fibronectin-labeled capillaries and lower panel; arrows show CD31-labeled capillaries), and arteriole density (D; arterioles in green, arrows show α-actin–labeled arterioles) in Cxcl12<sup>Gagtm/Gagtm</sup> (MUT) and wild-type littermate (WT) animals. Results are shown in ischemic (Isc) and nonischemic (N.Isc) legs. Values are mean±SEM (n=15 per group, representative of 3 independent experiments). D indicates day. A, Fifteen possible comparisons for Bonferroni correction; a value of P<0.003 was considered significant. *P<0.03, **P<0.0006 vs WT. B, One possible comparison for Bonferroni correction; a value of P<0.05 was considered significant. ***P<0.001 vs WT (n=15 per group). C and D, Six possible comparisons for Bonferroni correction; a value of P<0.008 was considered significant. **P<0.0016 vs ischemic WT (n=15 per group).
ischemic tissue 6 days after ischemia, a time point associated with a marked upregulation of transcription and expression of Cxcl12 isoforms. The number of CD45.1+/CD34+ was higher in the blood of Cxcl12Gagtm/Gagtm animals compared with WT mice. In contrast, the percentage of CD45.1+/CD34+ and CD45.1+/CXCR4+ was decreased in the ischemic muscle of Cxcl12Gagtm/Gagtm animals compared with WT mice (Figure 4A and 4B). As a consequence, the posts ischemic inflammatory response, a key component of ischemic tissue remodeling, is affected in our experimental conditions, and we showed that the number of Mac3-positive cells was reduced in the ischemic legs of Cxcl12Gagtm/Gagtm mice compared with WT littermates (Figure 4C).

The predominant expression of CXCL12γ in muscle, particularly its massive accumulation in ischemic tissues, suggests that this isoform might play a critical role in neovascularization through an HS-binding-dependent mechanism. Thus, if the angiogenic default observed in Cxcl12Gagtm/Gagtm was due to the selective invalidation of HS-binding capacity of Cxcl12 products, exogenous admin-
istration of WT CXCL12 proteins with full HS-binding activity should restore vascular regeneration in ischemic tissues with the highest efficiency.

To assess this hypothesis, we first compared the capacity of both WT CXCL12α and CXCL12γ, which differ notably in their affinity capacity to bind cell surface in a GAG-dependent manner, to induce posts ischemic vascular regeneration in control animals. Electrotransferred expression of DNA plasmids encoding for CXCL12α or CXCL12γ (Figure 5A) revealed a dramatically increased potency of CXCL12γ to promote neovascularization as evaluated by tissue perfusion, angiographic score, capillary density, and attraction of inflammatory macrophages in the ischemic tissue (Figure 5B–5E). To obtain direct and formal evidence that the superior efficiency of CXCL12γ was actually related to the interaction of the chemokine with HS, we evaluated the proangiogenic capacities of CXCL12γ and mutant CXCL12γm2, which carries the K24S and K27S substitutions combined with the neutralization of 9 of the 17 positively charged residues encompassed in the C-ter domain of this isoform (Figure IIIA in the online-only Data Supplement). CXCL12γm2 displays no detectable HS binding to cell surface HS, whereas it activates CXCR4 with preserved efficiency and even increased potency compared with the WT control (Figure IIIB and IIIC in the online-only Data Supplement). CXCL12γm2 failed to induce posts ischemic vessel growth beyond baseline levels (Figure 5B, 5C, and 5E).

To further investigate the mechanism of the increased proangiogenic effect of CXCL12γ, we compared the capacity of each isoform to regulate both the number of cells infiltrating the ischemic tissue and their fate. To this aim, hind-limb ischemia was induced in mice that had been lethally irradiated and reconstituted with BM-derived cells isolated from GFP + mice. Electrotransfer of CXCL12γ plasmid enhanced by 300% and 150% the number of GFP + cells infiltrating the ischemic muscle compared with mice treated with empty plasmid or CXCL12α, respectively (Figure 6A). The failure of CXCL12γm2 to induce a significant effect further proved the involvement of an HS-dependent mechanism in the robust neovascularization induced by CXCL12γ. Of note, the percentages of GFP+/BS1-lectin + cells of the endothelial lineage and GFP+/Mac3 + revealing the macrophage phenotype were similar in mice treated with CXCL12γ or CXCL12α. Further experiments using human EPCs labeled with carboxyfluorescein succinimidyl ester revealed that CXCL12γ, but not CXCL12γm2, increased the number of EPCs infiltrating ischemic hind-limb muscle (day 4 after ischemia) by 150% and subsequently vessel growth by ~50% (day 14 after ischemia) compared with mice treated with CXCL12α (Figure IV in the online-only Data Supplement).

Collectively, these results suggest that CXCL12γ regulates the accumulation of BM-derived cells in ischemic tissues with the highest efficiency compared with CXCL12α and in a strictly HS-dependent manner, although it does not differ from CXCL12α in terms of induction of cell differentiation. The increased number of EPCs homing to the injured muscle early after ischemia allows it to acribe its prominent proangiogenic effect to a primary attraction of endothelial cell precursors.

Finally, to demonstrate that the deficient neoangiogenesis observed in Cxcl12Gagtm/Gagtm animals was related to the expression of CXCL12 HS-binding mutants and their inability to promote efficient vascular reparation, we electrotransferred a plasmid encoding WT CXCL12γ plasmid in the ischemic hind limb. Expression of this isoform virtually normalized vascular regeneration in ischemic tissues, as proved by the significant increase in both vessel density and infiltration of inflammatory macrophages (Figure 7). This finding conclusively links the default of tissue reparation observed in these animals to the reduced capacity of mutant CXCL12 proteins to form complexes with HS proteoglycans.

**Discussion**

The animal model we have engineered allows the selective characterization of proteoglycan-chemokine interactions in vivo separately from interactions of a defined chemokine to its specific receptor. Cxcl12Gagtm/Gagtm mice exhibit normal expression of isoform-specific Cxcl12 mRNAs encoding for CXCR4 agonist chemokines. No detectable developmental anomalies were observed in Cxcl12GagtmGagtm animals. This is in sharp contrast to Cxcl12- and Cxcr4-deficient mice, which show abnormal hematopoiesis and multiple organogenesis default associated with perinatal mortality, and Cxcr7 knockout mice, which show disrupted cardiac development. Manifold reasons could explain the lack of detectable anatomic anomalies in the mutant animals: the preserved expression levels of total and isoform-specific Cxcl12 mRNA in Cxcl12GagtmGagtm mice tissues; the intact cell signaling capacities of mutant chemokines, leading to orientated cell migration, as previously shown for mutated CCL19 and CCL21; and the fact that the high-affinity oligosaccharidic HS structure recognized by CXCL12, which remains unknown, could not be displayed on HS during embryogenesis and appears later during adult life, thus explaining the apparent lack of developmental effects. Thus, a binding-specificity switch of brain HS proteoglycans from fibroblast growth factor-2 to -1 has been proved, demonstrating that indeed the binding capacity of embryonic HS for a given protein is not necessarily present and is acquired during development.

The Cxcl12GagtmGagtm mice nevertheless display a subtle anomaly revealing an increased number of circulating white blood cells, including CD34 + hematopoietic progenitor cells. This phenomenon could be explained by a reduced attraction/retention in lymphoid tissues and is probably related to impaired HS-dependent tissue adhesiveness of the CXCL12 mutant proteins. Given the crucial role played by CXCL12 and CXCR4 in both the homing of hematopoietic cells to the BM and the inability of the HS-binding CXCL12 mutants to adhere to the cell surface and the surrounding extracellular matrix of CXCL12-producing stromal cells forming CXCL12-BM niches, could facilitate their egress from the BM. In this regard, the accumulation of circulating CD34 + cells, which normally reside in BM, strongly argues in favor of reduced retention of these cells in BM. The BM egress of the mature leukocytes could also be due to the same mecha-
nism. Alternatively, the increased number of these cells in the periphery could be ascribed to the gradient generated by the blood accumulation of free, HS-binding, disabled CXCL12 proteins and/or their failure to firmly immobilize on the apical surface of sinus and postcapillary endothelial cells, which would inhibit the return of leukocytes to the BM.

Whereas basal coronary flow was impaired in Cxcr4−/− mice, paralleled by reduced angiogenesis and myocardial vessel den-
sity, we did not detect changes in the basal number of capillary and arterioles in skeletal muscle of $\text{Cxcl12Gagtm/Gagtm}$ animals. In contrast, $\text{Cxcl12Gagtm/Gagtm}$ animals show a marked default in angiogenesis and neovascularization of ischemic tissues, revealing a biological role for CXCL12/GAG interactions in tissue repair after ischemia. It has previously been reported that recruitment of CXCR4-positive progenitor cells to regenerating tissues is mediated by hypoxic gradients via hypoxia-inducible factor-1–induced expression of CXCL12. Furthermore, CXCL12 attracts EPCs, induces angiogenesis, and increases the number of newly formed vessels and blood flow in models of hind-limb ischemia when overexpressed either alone or in combination with cell therapy. Recruitment and retention of BM-derived circulating inflammatory cells mediated by CXCL12 participate in vascular remodeling and arteriole growth. In addition, CXCL12 seems to have a direct effect on the migration of smooth muscle cells and smooth muscle progenitor cells.

Figure 6. CXCL12γ controls bone marrow–derived cells infiltration in ischemic tissues. Quantitative evaluation and representative photomicrographs of the percentage of green fluorescent protein (GFP)–expressing cells (A), GFP-expressing and BS1-labeled cells (B), and GFP-expressing and Mac3-labeled cells (C) in the ischemic leg (Isc) of irradiated wild-type (WT) mice reconstituted with bone marrow of WT GFP+ animals and treated with empty DNA plasmid (CONT) or DNA expression vectors encoding for CXCL12α, CXCL12γ, or the heparan sulfate–binding mutant CXCL12γ-m2. Value quantification is represented in histograms. Values are mean ± SEM ($n=10$ per group, representative of 2 independent experiments). A through C. Twenty-eight possible comparisons for Bonferroni correction; a value of $P<0.0017$ was considered significant. *$P<0.0017$ vs ischemic CONT; †$P<0.0017$ vs ischemic CXCL12α; #$P<0.0017$ vs ischemic CXCL12γ. N.Isc indicates nonischemic leg.
However, all previous studies focused on deciphering the capacity of CXCL12α to promote angiogenesis without dissecting the respective contribution of receptor activation and GAG binding. Furthermore, only the therapeutic capacity of exogenous WT chemokines was investigated, whereas the role played by endogenous CXCL12/GAG interactions in the spontaneous postischemic recovery remains unknown. The defective postischemic neovascularization observed in Cxcl12Gagtm/Gagtm animals thus brings new light to the original evidence that, beyond CXCL12-induced receptor cell signaling, the endogenous CXCL12/GAG interactions play a critical role in tissue regeneration, particularly vascular growth. Indeed, the efficiency of CXCL12γ to rescue the default in Cxcl12Gagtm/Gagtm animals contrasts with the lack of effect of CXCL12γm2 and reveals the key role of this mechanism in the reparative process. This finding is in keeping with the efficiency of CXCL12γ and the inability of CXCL12γm2 to promote homing of BM-derived circulating inflammatory cells and circulating EPCs into ischemic tissues and to modulate vessel growth in this setting.

Bound to HS structures on the apical surface of endothelial cells,47 CXCL12 expressed by autocrine or juxtacrine mechanisms could be determinant for vascular targeting of circulating progenitor and inflammatory cells and induction, in an integrin- and CXCR4-dependent manner, of both firm adhesion and transendothelial migration of infiltrating BM-derived cells.48 Among CXCL12 isoforms, CXCL12γ binds to microvascular endothelial cell surface with the highest efficiency13,47 and is ideally suited to accomplish this role. Abundantly expressed and induced in injured tissues by hypoxia, CXCL12γ could delimit a static field of chemokine, keeping cells motile (haptokinesis) in a restrained tissue compartment. This may contribute to the reparative effect of EPCs by enhancing cell adhesiveness and survival.49 It could be hypothesized that, released progressively on cleavage of its C-ter domain, the pool of immobilized CXCL12γ could provide a gradient of active, diffusible chemokines that would promote long-range chemotaxis toward the injured tissue. An analogous mechanism contributes to the regulation by CCL21 of lymphocyte T-cell homing in secondary lymphoid organs.1

In terms of the potential of CXCL12 in regenerative medicine, the continuous delivery or administration in an immobilized form of CXCL12α is required to provide therapeutic effect in postischemic treatment.50 CXCL12γ could overcome this limitation, thus efficiently activating the proangiogenic mechanisms. Collectively, our findings unravel the contribution of endogenous CXCL12/HS complexes to the biological functions of this chemokine. The prevalence of CXCL12γ expression in hypoxic conditions and its superior capacity to promote neovascularization further highlight the importance of HS-binding mechanisms in the tissue reparative activity of CXCL12.

These findings pave the way for investigating the contribution of CXCL12/HS interactions to other homeostatic and physiopathological functions played by this unique chemokine and could eventually have broad relevance for other chemokine systems.

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**Figure 7.** CXCL12γ restores the defective angiogenic phenotype in Cxcl12Gagtm/Gagtm animals. Quantitative evaluation of foot perfusion (A), capillary density (B), arteriole density (C), and Mac3-positive cells (D) in Cxcl12Gagtm/Gagtm (MUT) and wild-type littermate (WT) animals treated with or without DNA expression vector encoding CXCL12γ. Results are shown in ischemic (Isc) and nonischemic (N.Isc) legs. Values are mean±SEM (n=10 per group). A, Three possible comparisons for Bonferroni correction; a value of P<0.016 was considered significant. *P<0.016 vs WT. B through D, Fifteen possible comparisons for Bonferroni correction; a value of P<0.003 was considered significant. **P<0.0006 vs ischemic WT; #P<0.003, ##P<0.0006 vs ischemic MUT.
Disclosures

None.

References


**CLINICAL PERSPECTIVE**

CXCL12 regulates with unchallenged capacity both the tissue homing and survival of circulating tissue-specific progenitors, hematopoietic cells, and bone marrow stem cells and triggers inflammatory cell infiltration. Beyond the binding to its cognate receptor CXCR4, we speculate that the interaction of CXCL12 with proteoglycans, particularly heparan sulfate, enables the formation of chemokine gradients that determine the orientated migration and tissue recruitment of circulating cells. We engineered a mouse carrying a Cxcl12 gene (Cxcl12Gagtm) mutation that precludes interactions with heparan sulfate structures while not affecting CXCR4-dependent cell signaling of CXCL12 isoforms. After induced acute ischemia, a marked impaired capacity to support revascularization was observed in Cxcl12Gagtm/Gagtm animals associated with a reduced number of infiltrating cells in the ischemic tissue. Of interest, Cxcl12γ was the most abundant isoform in both the normal and ischemic muscle. Importantly, exogenous administration of CXCL12γ, which binds heparan sulfate with the highest affinity ever reported for a cytokine, fully restored vascular growth, whereas heparan sulfate–binding CXCL12γ mutants failed to promote revascularization in Cxcl12Gagtm/Gagtm animals. In terms of the potential of CXCL12 in regenerative medicine, the continuous delivery or administration in an immobilized form of CXCL12α is required to provide therapeutic effect in posts ischemic treatment. CXCL12 could overcome this limitation, thus efficiently activating the proangiogenic mechanisms. The findings presented here pave the way for investigating the contribution of CXCL12/heparan sulfate interactions to other homeostatic and physiopathological functions played by this unique chemokine and could eventually have broad relevance for other chemokine systems.
Homeostatic and Tissue Reparation Defaults in Mice Carrying Selective Genetic Invalidation of CXCL12/Proteoglycan Interactions

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Supplementary Figure 1. Construction and sequencing validation of point mutation targeting vector of Cxcl12. (a) Point mutations in 2\textsuperscript{nd} and 4\textsuperscript{th} exons were generated by 3-step PCR mutagenesis using PT1-PT4 and PT5-PT8 primers. The PCR fragments carrying mutations were then used to replace the correspondent wild type sequence using conventional sub cloning methods. (b) The boundaries of the two homology arms (LA , SA) were confirmed by sequencing with P6 and T7 primers that read through both sides of the backbone vector. The floxed Neo cassette was confirmed by sequencing with N1 and N2 primers that read from the 5’- and 3’- ends of the Neo cassette, respectively, into the genomic sequences. The mutations were confirmed by sequencing with PT1, SQ1 and PT8 primers. The sequencing results also proved that no other mutations were introduced into exon 2 and exon 4 before the natural stop codon.

Primers used to generate the point mutations (mutations are in bold and underlined)

PT1 (\textbf{Acc651}): 5’- ACTTTGGTACCACTTTCTGTACCATCCATCTGC -3’

PT2: 5’- AGACAGATGC\textbf{GA}GACGGTGGCTCTGGCGATGTGGCTC -3’

PT3: 5’- ACATCGCCAGAGCCAACGTC\textbf{T}GCATCTG\textbf{T}CTATCCTCAACACTCCAAACTGTG -3’

PT4 (\textbf{Acc651}): 5’- ACTTTGGTACCCTCTGAGCAAGTGAGTGCAAGTG -3’

PT5 (\textbf{Acc651}): 5’- ACTTTGGTACCTCTGAGCAAGTGAGTGCAAGTG -3’

PT6: 5’- \textbf{CT}ACCTTAGATAAAAATTAGTAGAACC -3’
PT7: 5’- GTTCTACTAATTTATCTAAGGTAGGGCGCAGAGAAAAAGTGGA-3’

PT8 (Acc65I): 5’- ACTTGGTACCAGAGTTTACCCTGAGCTTCTAGGC-3’

**Sequencing Primer Sequences**

Primer N1  5’-TGCAGGAGCAGAGCCACTTTGGATGCTAGC-3’

Primer N2  5’-TTCCTCGTGCTTTACGGTGATCG-3’

Primer P6  5’-GAGTGACCATATGGACATATTGTC-3’

Primer T7  5’-TAATGCGAGGTTAACCTGGCTTATCG-3’

Primer PT1  5’-ACTTGGTACCACTTTCTGTAACCATTCCTGC-3’

Primer SQ1  5’-ACAGGACACATCTACAGCTACCTGCAAGTC-3’

Primer PT8  5’-ACTTGGTACCAGAGTTTACGTCAGGTTTGGAC-3’

**Supplementary Figure 2. Characterization of Cxcl12Gagtm/Gagtm animals.** (A) Schematic diagram of Cxcl12 isoforms structures and sequence alignment of the corresponding mature proteins. Mutations introduced in Cxcl12Gagtm are indicated (stars). In the amino-acid sequence the substituted residues corresponding to mutations are underlined. The incorporation of the non-sense mutation in the open reading frame of Cxcl12γ generates a truncated protein of 68Aa-length identical to Cxcl12α, which last aminoacid (K68) is marked by a star. (B) PCR amplification on genomic DNA from wild type (WT), Cxcl12Gagtm/wt (Het) and Cxcl12Gagtm/Gagtm (Mut) animals. (C-D) Real-time PCR analysis of Cxcl12 products. (C) RNA expression levels of each isoform normalized to HPRT in brain and thymus tissues from WT and
Cxcl12\textsuperscript{Gagtm/Gagtm} animals. (D) mRNA levels of each Cxcl12 isoform were normalized to that of Cxcl12\textsubscript{a}

**Supplementary Figure 3. Functional properties of WT and HS-binding mutant CXCL12 used for post-ischemic revascularization treatment or expressed from Cxcl12\textsuperscript{Gagtm} animals.** (a) Sequence alignment of CXCL12 WT and mutant chemokines used in experiments described in b and c. In bold, HS-binding motifs; underlined, substituted amino acids. (b) Cell surface (CHO cells) binding activity and (c) chemotactic properties of synthetic WT and HS-binding mutants CXCL12\textsubscript{am} and CXCL12\textsubscript{gm2} assessed in CD4\textsuperscript{+} T lymphocytes. (d) Quantitative evaluation of EPC migration through transwell membrane in response to treatment with supernatant of HEK293 T transfected cells with cDNA coding either for WT or mutant isoforms isolated from Cxcl12\textsuperscript{Gagtm} animals. CXCL12 content in supernatants was measured by ELISA and for each WT/mutant couple equal amounts (ranging from 0.5 to 1nM) were used. Open bars: synthetic CXCL12\textsubscript{a}, 1nM. AMD3100 (5mM) is a low molecular, synthetic CXCR4 antagonist. PBS, Phosphate buffered saline.**p<0.01 versus PBS, †p<0.05 versus conditions without AMD3100. n = 10 per group, representative of 2 independent experiments.

**Supplementary Figure 4. CXCL12\textsubscript{g} controls EPC infiltration in ischemic tissues.** (a) Quantitative evaluation of EPC migration through transwell membrane in response to VEGF, CXCL12\textsubscript{a} or CXCL12\textsubscript{g}, in presence or absence of the synthetic CXCR4 antagonist, AMD3100 (5mM). n = 6 to 8 per group, representative of 2 independent experiments.
*p<0.05, **p<0.01, ***p<0.001 versus PBS, ††† p<0.001 versus CXCL12α, ## p<0.01 versus CXCL12γ. (b) Number of CFSE-stained human EPC (EPC) in the ischemic leg of C57BL/6 mice treated with intramuscular administration of cDNA expression vector encoding CXCL12α, CXCL12g or CXCL12gm2, 4 days after the onset of ischemia. Quantitative evaluation of foot perfusion (c), microangiography (d) and capillary density (e), 14 days after ischemia in C57BL/6 mice treated with intravenous administration of EPC only or along with intramuscular cDNA expression vector encoding CXCL12α, CXCL12g or CXCL12gm2. Empty DNA plasmid (CONT). Values are mean ± SEM. n = 10 per group, representative of 2 independent experiments. 10 possible comparisons for Bonferroni correction, a value of p<0.005 was considered significant. *p<0.005, **p<0.001, versus CONT, † p<0.005, †† p<0.001 versus CXCL12α+EPC, ##p<0.001 versus CXCL12γ+EPC.
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3