Platelets Release CXCL4L1, a Nonallelic Variant of the Chemokine Platelet Factor-4/CXCL4 and Potent Inhibitor of Angiogenesis

Sofie Struyf, Marie D. Burdick, Paul Proost, Jo Van Damme, Robert M. Strieter

Platelet factor-4 (PF-4)/CXCL4 was the first chemokine described to inhibit neovascularization. Here, the product of the nonallelic variant gene of CXCL4, PF-4<sup>alt</sup>/PF-4<sup>var1</sup>, designated CXCL4L1, was isolated for the first time from thrombin-stimulated human platelets and purified to homogeneity. Although secreted CXCL4 and CXCL4L1 differ in only three amino acids, CXCL4L1 bound heparin with lower affinity than CXCL4 but was more potent in inhibiting chemotaxis of human microvascular endothelial cells toward interleukin-8 (CXCL8) or basic fibroblast growth factor (bFGF). In vivo, CXCL4L1 was also more effective than CXCL4 in inhibiting bFGF-induced angiogenesis in rat corneas. Thus, activated platelets release CXCL4L1, a potent regulator of endothelial cell biology, which affects angiogenesis and vascular diseases.

The chemokine family consists of proinflammatory cytokines, primarily involved in chemoattraction and activation of specific leukocytes in various immunoinflammatory responses. In addition, chemokines activate blood platelets, important components of hemostasis, contributing to wound healing by forming thrombi and to the initiation of repair processes. However, a recent study indicated that circulating activated platelets and platelet–leukocyte aggregates promote formation of atherosclerotic lesions. Finally, chemokines influence tumor growth by regulating angiogenesis. Net angiogenesis is determined by a balance between angiogenic and angiostatic factors within the local microenvironment. The CXC chemokine family is unique because it comprises angiogenic and angiostatic chemokines.

The first chemokine described as a regulator of angiogenesis is platelet factor-4 (PF-4)/CXCL4. Although this angiostatic platelet-derived chemokine has been the subject of extensive research as a candidate anticancer drug, its nonallelic human gene variant PF-4<sup>alt</sup>/PF-4<sup>var1</sup> has not been investigated previously. In addition to post-translational processing (eg, by proteolytical cleavage), gene duplication is another means to increase the biological diversification of the human chemokine family. For example, we identified the chemokine LD78β/CCL3L1, a nonallelic gene variant of CCL3, as the most potent monocyte chemoattractant. More than a decade ago, PF-4<sup>alt</sup>/PF-4<sup>var1</sup>, the gene of a nonallelic CXCL4-variant was identified. The predicted mature proteins differ in only three amino acids located in the COOH terminus (P58→L, K66→E, and L67→H) in CXCL4 and CXCL4L1, respectively. This region is involved in CXCL4–heparin interaction. Here we describe the isolation of natural CXCL4L1 protein from thrombin-stimulated platelets and demonstrate for the first time that the SCYB4V1 gene is translated into biologically active protein. We characterized this chemokine as a potent inhibitor of angiogenesis, which is more effective than authentic CXCL4.

Materials and Methods

An extended Materials and Methods section can be found in an online supplement available at http://circres.ahajournals.org.

Purification, Identification, and Measurement of CXCL4L1

For large-scale CXCL4 production, platelets were stimulated with thrombin. The conditioned medium was subsequently purified as described. For large-scale CXCL4 production, platelets were stimulated with thrombin. The conditioned medium was subsequently purified as described. For large-scale CXCL4 production, platelets were stimulated with thrombin. The conditioned medium was subsequently purified as described.

Biological Assays

Endothelial cell chemotaxis assays were performed as described. The total number of migrated cells was counted in 15 separate high-power fields (HPFs; ×400). Specific migration was expressed as the number of endothelial cells that migrated per HPF after subtracting the background (unstimulated control).

In vivo angiogenesis was assessed using the rat cornea micro-pocket assay as described.

Results and Discussion

The release of chemokines from the α-granules of outdated platelet preparations was stimulated with thrombin. The conditioned media were concentrated and subsequently fractionated by heparin–Sepharose affinity chromatography (data not shown). The major peak of CXCL4 immunoreactivity eluted from this column at high salt concentrations (1.6 to 1.8 mol/L NaCl) and contained authentic CXCL4 as determined by mass spectrometry. The CXCL4 immunoreactivity detected in fractions with intermediate heparin affinity (eluting at 1 to 1.4 mol/L NaCl) was further purified by reverse-phase high-performance liquid chromatography (RP-HPLC) and eluted in two separate peaks between 32% and 34% acetonitrile (Figure 1A), both corresponding to pure 8-kDa protein bands on SDS-PAGE (Figure 1B). As deduced from a combination of sequence analysis and mass spectrometry (supplemental Table A and supplemental Figure A, available online at http://circres.ahajournals.org), the RP-HPLC frac-
tions N° 57 to 60 contained authentic CXCL4. The NH₂-termin al sequence and detected average Mr of the CXCL4 immunoreactivity (fractions N° 53 to 55) eluting at 32% acetonitrile, corresponded to CXCL4L1. 13 Authentic CXCL4 with high affinity for heparin was purified in parallel with CXCL4L1, and homogenous preparations of the mature proteins were compared for angiostatic activity.

Basic fibroblast growth factor (bFGF; 50 ng/mL) and CXCL8 (80 ng/mL) induced human microvascular endothelial cell (HMVEC) chemotaxis (Figure 2; supplemental Figure B, available online at http://circres.ahajournals.org), which was completely blocked by CXCL4L1 (1 to 100 ng/mL; □) compared with CXCL4 (1 to 300 ng/mL; △) for its capacity to induce migration of HMVECs. In addition, chemotaxis of HMVEC toward bFGF (50 ng/mL) or CXCL8 (80 ng/mL) was inhibited in the presence of different concentrations of CXCL4L1 (□ and ○) or CXCL4 (■ and ○). To demonstrate specific migration, background (unstimulated control) migration (on average, 38 cells per HPF) was subtracted.

The molecular mechanism for CXCL4 angiostatic function is still a matter of debate. It has been suggested that CXCL4 is a unique chemokine that does not bind to a G-protein-coupled receptor but activates cells (ie, neutrophils) and platelets through binding to cell surface glycosaminoglycans (GAG).18,19 The fact that GAG binding is important for CXCL4 interaction is supported by another study demonstrating that CXCL4 and CXCL10 share a GAG binding site on endothelial cells through which these chemokines inhibit FGF-induced endothelial cell growth.20 However, it is not clear whether CXCL4 binding to GAG alone is necessary and sufficient to trigger endothelial cell signaling.21,22 CXCL4 function is not abrogated in heparan sulfate-deficient cells, and CXCL4 mutants or peptides lacking heparin affinity are capable of inhibiting angiogenesis.11 Recently, a splice variant of CXCR3, designated CXCR3B, was identified that binds CXCL4.23 Finally, others have reported that the inhibitory effect of CXCL4 is mediated through complex formation with bFGF or CXCL8.24,25

In this study, we identified CXCL4L1 as another angiostatic factor stored in platelets, which undergoes degranulation at sites of vascular injury or thrombosis and modulates net angiogenesis within the local microenvironment. We demonstrated that CXCL4L1, compared with CXCL4, is a more potent inhibitor of endothelial cell chemotaxis in vitro and has more profound effects in blocking angiogenesis in vivo. This discovery may have significant implications for the use of CXCL4L1 as a therapeutic tool to inhibit aberrant angiogenesis in a variety of diseases.
This work was supported by the Fund for Scientific Research of Flanders (FWO-Vlaanderen), the Concerted Research Actions (GOA) of the Regional Government of Flanders, the Interuniversity Attraction Poles Programme-Belgian Science Policy, the Quality of Life Program of the European Commission, and National Institutes of Health grants CA87879, HL66027, P50CA90388, and GOA of the Regional Government of Flanders, the Interuniversity. This work was supported by the Fund for Scientific Research of Flanders and the Life Program of the European Commission, and National Institutes of Health grants CA87879, HL66027, P50CA90388, and GOA of the Regional Government of Flanders, the Interuniversity.

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Extended materials and methods

Measurement of human CXCL4 by ELISA

For the detection of human CXCL4, a classical sandwich ELISA was developed as described previously for other chemokines using mouse monoclonal anti-human CXCL4 (R&D Systems, Abingdon, UK) and rabbit polyclonal anti-human CXCL4 (Peprotech, Rocky Hill, NJ) as primary and secondary antibodies, respectively. Purified natural CXCL4 (vide infra) was used as a standard. This ELISA could not discriminate between different NH2-terminal forms of CXCL4 and CXCL4L1.

Purification and identification of CXCL4L1

For large scale CXCL4 production, outdated platelets (Red Cross blood transfusion centre, Belgium) were stimulated with 1 U/ml thrombin (Sigma, St. Louis, MO) for 2 h at 37°C in PBS. The conditioned medium was subsequently purified by a purification procedure previously described. The conditioned media (1 to 5 liters) were first concentrated and partially purified by adsorption to silica (“Silica Matrex”; pore size 100 Å, particle size 35-70 µm; Millipore, Billerica, MA; 10 g/l conditioned medium). After 2 h of magnetic stirring at 4°C, the silicic acid was sedimented by centrifugation and subsequently washed once with PBS and once with PBS containing 1 M NaCl pH 7.4. Elution of proteins from the silica matrix was done by magnetically stirring three consecutive times with 100 ml of PBS containing 1.4 M NaCl and 50% ethylene glycol pH 7.4 during 30 min at 4°C. A second elution with 0.3 M glycine/HCl pH 2.0 buffer (2x 100ml) was performed to improve recovery of chemokines. Subsequently, the pooled eluates (500 ml) were dialyzed against equilibration/loading buffer (50 mM Tris/HCl-50 mM NaCl, pH 7.4) before fractionation by heparin-Sepharose chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted from the column in a linear NaCl gradient (0.05-2 M NaCl in the loading buffer; 5 ml-fractions). For all fractions, the protein concentration was determined by a Coomassie blue G-250 binding assay using the Bio-Rad commercial kit (Bio-Rad Laboratories, Hercules, CA). After adjustment of the pH (from pH 7.4 to below pH 4.0 with 1% trifluoroacetic acid (TFA) in H2O), pooled heparin-Sepharose fractions containing CXCL4 immunoreactivity (ELISA described above) were injected on a RP-HPLC column equilibrated with 0.01% TFA in
H₂O (C-8 Aquapore RP-300 column; PerkinElmer, Norwalk, CT or Source 5 RPC column; Amersham Biosciences) and eluted in an acetonitrile (0-80%) gradient (0.4 or 1 ml-fractions). Sometimes an additional chromatographical step was needed to obtain homogenous chemokine preparations. In that case RP-HPLC fractions were further purified by Mono S (Amersham Pharmacia Biotech) cation-exchange fast protein liquid chromatography (FPLC). A linear NaCl (0-1 M) gradient in 50 mM formate pH 4.0 was used to elute proteins (0.2 ml-fractions). Purified fractions were analyzed by SDS-PAGE under reducing conditions to determine purity. Pure proteins were identified by NH₂-terminal amino acid sequence analysis on a Procise 491 cLC protein sequencer (Applied Biosystems, Foster City, CA). For mass spectrometry, RP-HPLC fractions were diluted 1/5 or 1/10 with 0.1% acetic acid in 50% acetonitrile/50% H₂O and sprayed at 5 µl/min in the source of an electrospray ESQUIRE ion trap mass spectrometer (Bruker/Daltonics, Bremen, Germany).

**Endothelial cell chemotaxis**

Human lung microvascular endothelial cells (HMVEC-L) were obtained from Clonetics (Walkersville, MD), and were cultured following the manufacturer’s instructions in EBM-2 medium supplemented with the EGM-2-MV “bulletkit” containing FCS, ascorbic acid, hydrocortisone, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF). Endothelial cell chemotaxis assays were performed as previously described ⁴. Cells were harvested by trypsinization, resuspended in EBM-2 medium without growth factors with 2% FCS added. An aliquot of 160 µl containing 5x10⁵ cells/ml was added to each of the lower wells of a 12-well chemotaxis chamber (Neuro Probe Inc., Cabin John MD). The chambers were assembled (by placing 0.1% gelatin coated 5 µm pore size filters over the lower wells, followed by a gasket and the upper chamber) and incubated at 37°C in a CO₂ incubator for 2 h in an inverted position. The chambers were then turned upright, and 100 µl aliquots of sample dilutions were added to the upper wells of the chamber. As endothelial cell chemoattractants CXCL8 and bFGF (both from Peprotech) were used. The chambers were placed in a 37°C CO₂ incubator for 2 h at which time the filters were removed, and subjected to Diff-Quik staining. The total number of migrated cells was counted in 15 separate fields of view under 400X power. Results were expressed as the number of endothelial cells that migrated per high power field (HPF) after subtracting the background (unstimulated control) to demonstrate specific migration.

**Rat Corneal Micropocket Assay for Angiogenesis**

*In vivo* angiogenesis was assessed using the rat cornea micropocket assay as previously described ⁴. Briefly, reagents were diluted in PBS plus 0.25% serum albumin to their final concentration per
pellet of Hydron casting solution (Hydro Med Sciences, New Brunswick NJ). 5 µl aliquots were pipetted onto the flat surface of a sterile polypropylene specimen container and were allowed to polymerize overnight under UV light in a laminar flow hood. Prior to implantation, the pellets were rehydrated with normal saline. Animals were given 150 mg/kg ketamine and 250 µg/kg atropine, and the corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket (1-2 mm from the limbus). Six days after implantation, animals received heparin (1000 U) and ketamine (150 mg/kg) i.p. 30 min prior to sacrifice, followed by perfusion with 10 ml of colloidal carbon via the left ventricle. Corneas were then harvested and photographed. No inflammatory response was observed in any of the corneas treated with the cytokines tested. Sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were considered positive neovascularization responses. Negative responses were characterized by either no vessel growth or by the presence of only an occasional hairpin loop or sprout that displayed no evidence of sustained growth.
Table A. Biochemical characterization of natural CXCL4 and CXCL4L1 protein shown in Figure 1A and B

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Chemokine</th>
<th>Relative Abundancy</th>
<th>Determined NH2-terminal sequence&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>CXCL4L1</td>
<td>20%</td>
<td>FARAEAEEDGDLQ…</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7%</td>
<td>AEAEEDGDLQ…</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63%</td>
<td>EAEEEDGDLQ…XVKTTSQVR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10%</td>
<td>AEEDGL</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Mass Spectrometry&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Fraction Number</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
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<td>fr54</td>
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<td></td>
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<td>fr58</td>
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<td>fr59</td>
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<td></td>
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<tr>
<td>fr60</td>
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</table>

<sup>a</sup> Edman degradation (determined NH2-terminal sequence) and <sup>b</sup> mass spectrometry analysis results (deduced NH2-terminal sequence (in italic) and experimental Mr) of C8 RP-HPLC purified natural CXCL4L1 and CXCL4 (also shown in Figure 1). <sup>c</sup> CXCL4L1 was present in different molecular forms: a protein containing one (A) and four (FARA) extra NH2-terminal residues from the signal peptide, the predicted mature protein (EAEEDG…) and a shorter protein missing the NH2-terminal glutamine (AEEDG…). Biological characterization was performed with fractions containing predominantly the mature CXCL4L1 protein (EAEEDG…) in comparison with mature CXCL4 (EAEEDG…) obtained after the same purification procedure starting from the major CXCL4 immunoreactivity peak with high affinity for heparin.
### Table B. *In vivo* angiostatic activity of CXCL4L1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nº of pos pellets&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nº of neg pellets&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% inhibition&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer (vehicle control)</td>
<td>0/5</td>
<td>5/5</td>
<td>0%</td>
</tr>
<tr>
<td>bFGF (50 ng)</td>
<td>25/25</td>
<td>0/25</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CXCL4 (50 ng)</td>
<td>5/22</td>
<td>17/22</td>
<td>NA</td>
</tr>
<tr>
<td>CXCL4 (50 ng) + bFGF (50 ng)</td>
<td>17/22</td>
<td>5/22</td>
<td>23%</td>
</tr>
<tr>
<td>CXCL4L1 (50 ng)</td>
<td>1/22</td>
<td>21/22</td>
<td>NA</td>
</tr>
<tr>
<td>CXCL4L1 (50 ng) + bFGF (50 ng)</td>
<td>6/22</td>
<td>16/22</td>
<td>73%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rat cornea neovascularization in response to 50 ng of CXCL4, CXCL4L1, bFGF, or combinations of these cytokines. Numbers indicate the number of pellets (“positive pellets”) in which an angiogenic response was observed (for criteria see materials and methods section) or “negative pellets” in which no neovascularization occurred, relative to the total number of pellets implanted.

<sup>b</sup> The percentage inhibition of bFGF-induced angiogenesis in the pellets in which CXCL4 or CXCL4L1 was combined with bFGF.

<sup>c</sup> NA, not applicable.
Figure A. Mass spectrometrical analysis of natural CXCL4L1 protein.

The average $M_r$ of RP-HPLC fraction N° 54 (Fig. 1A and 1B) was determined on an electrospray ion trap mass spectrometer. The m/z values for the differently charged ions in the unprocessed spectrum (calculated from the average of 1500 spectra) are indicated, as are the numbers of protons they carry. Four different NH$_2$-terminal isoforms of CXCL4L1 are present, of which the NH$_2$-terminal sequence can be deduced based on the difference between the four proteins in the charge-deconvoluted spectrum (insert). This deduced NH$_2$-terminal sequence is indicated in the supplemented Table A (vide supra).
FIGURE B. CXCL4L1 strongly inhibits bFGF-induced and CXCL8-induced endothelial cell chemotaxis.

CXCL4L1 (50ng/ml) was compared with CXCL4 (50ng/ml) for its capacity to reduce migration of HMVEC toward bFGF (50ng/ml) or CXCL8 (80ng/ml). To demonstrate specific migration, background (unstimulated control) migration (on average 68 cells/high power field) was subtracted. Results (mean ± SEM) are derived from 2 independent experiments in triplicate.

References


