Chemokine (C-X-C Motif) Receptor 4 Blockade by AMD3100 Inhibits Experimental Abdominal Aortic Aneurysm Expansion Through Anti-Inflammatory Effects

Stéphanie Michineau,* Grégory Franck,* Orianne Wagner-Ballon, Jianping Dai, Eric Allaire, Marianne Gervais

Objective—Inflammation plays a critical role in the development of abdominal aortic aneurysms (AAAs). Because stromal cell–derived factor 1 (SDF-1) is known for its ability to attract inflammatory cells, we investigated whether SDF-1/chemokine (C-X-C motif) receptor 4 (CXCR4) axis is expressed in aneurysmal aortic wall and plays a role in AAA physiopathology and asked whether its blockade modulates AAA formation and expansion.

Approach and Results—Quantitative real-time polymerase chain reaction analysis showed that SDF-1α and CXCR4 mRNA levels are increased in both human and CaCl2-induced mouse AAA wall and are positively correlated to the aortic diameter in mice. ELISA quantification and immunostaining demonstrated that, in mice, aortic SDF-1α is rapidly induced during AAA formation, first by apoptotic vascular smooth muscle cells in the injured media and then by adventitial macrophages once AAA is fully established. Using green fluorescent protein-positive (GFP+/−) bone marrow transplantation experiments, we demonstrated that aortic SDF-1 overexpression is implicated in the recruitment of bone marrow–derived macrophages within the AAA wall. Furthermore, in mice, blockade of CXCR4 by AMD3100 decreases the infiltration of adventitial macrophages, inhibits AAA formation, and prevents aortic wall destruction. AMD3100 reduces the mRNA levels of MMP-12 and MMP-14 as well as that of inflammatory effectors MCP-1, MIP-1β, MIP-2α, RANTES, IL-1β, IL-6, TNF-α, and E-selectin. Finally, AMD3100 stabilizes the diameter of formed, expanding AAAs in 2 experimental models.

Conclusions—SDF-1/CXCR4 axis is upregulated in human and mouse AAAs. Blockade of CXCR4 with AMD3100 suppresses AAA formation and progression in two rodent models. Blockade of SDF-1/CXCR4 axis may represent a new strategy to limit progression of small human AAAs. (Arterioscler Thromb Vasc Biol. 2014;34:1747-1755.)

Key Words: aortic aneurysm, abdominal ■ chemokine CXCL12 ■ inflammation ■ pharmacology

Abdominal aortic aneurysms (AAAs) are progressive enlargements of the infrarenal aortas that affect ≈5% of men aged >65 years and spontaneously evolve toward rupture.1 Currently, the management of AAAs relies on open or endovascular surgery when the aortic diameter reaches 5.5 cm. Although noninvasive echography readily enables the screening of small asymptomatic AAAs, no medical therapies are currently available to alter the natural history of small AAAs. Therefore, understanding the initial steps responsible for aneurysmal degeneration and expansion would help in designing new therapeutic options for the third cause of cardiovascular death.

AAA degeneration involves the destruction of fibrillar extracellular matrix proteins and the depletion of medial vascular smooth muscle cells (VSMCs), leading to the weakening of the aortic wall and dilatation of the aorta.2 In this destructive process, the recruitment of inflammatory cells in the adventitia and outer media seems to be an initial and determinant step. Indeed, inflammatory cells, primarily lymphocytes, macrophages, and plasma cells, release proinflammatory mediators and matrix-degrading enzymes (such as matrix metalloproteinases [MMPs]) and generate elastin degradation peptides that promote immune response and accelerate chronic transmural inflammation. Studies in human AAA tissue reveal a strong correlation between degradation of elastin fibers and the extent of inflammatory infiltrates in the outer aortic wall.3

The stimulus for the early recruitment of inflammatory cells in AAAs is still uncertain. One plausible mechanism involves the action of chemoattractant cytokines, which regulate leukocyte trafficking. Among them, the stromal cell–derived factor (SDF)-1, also known as chemokine (C-X-C motif) ligand 12 (CXCL12), is known to mobilize hematopoietic progenitor cells and leukocytes from bone marrow (BM) into circulating blood and, subsequently, into peripheral tissues through interaction with its cognate receptor chemokine (C-X-C motif) receptor 4 (CXCR4).4,5 SDF-1 is highly induced in inflamed
Nonstandard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAA</td>
<td>abdominal aortic aneurysm</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>CXCR</td>
<td>chemokine (C-X-C motif) receptor</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>SDF-1</td>
<td>stromal cell-derived factor 1</td>
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<td>VSMC</td>
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tissues, where it attracts activated CXCR4+ T cells and monocytes, thereby enhancing local inflammatory responses. In this regard, SDF-1 or its receptors CXCR4 have been associated with chronic inflammatory disease pathogenesis, including rheumatoid arthritis, lupus, allergic airway disease, asthma, and inflammatory bowel diseases. This concept has prompted a pharmacological approach, using the selective blockade of CXCR4 by AMD3100 to curb inflammation and its consequences in animal models of inflammatory disorders.

Few study have addressed the role of SDF-1 in aortic aneurysms. Plasma SDF-1α levels have been shown to be positively correlated to the size of ascending aortic aneurysms. Ocaña et al have demonstrated that infiltrating B and T lymphocytes isolated from human AAA walls expressed high level of CXCR4 and that the level of SDF-1α is increased in the adventitia of human AAA wall, suggesting a proinflammatory action of the SDF-1/CXCR4 axis in AAA pathogenesis. But, to date, the effect of SDF-1/CXCR4 axis on the early migration of inflammatory cells within the aortic wall remains unexplored. In the present study, we investigated the aortic expression of SDF-1 (SDF-1α, β, γ) and CXCR4 in human samples and during AAA development in the mouse calcium chloride model. We further evaluated the functional role of the SDF-1/CXCR4 pathway in the recruitment of inflammatory cells within the aortic wall in vivo and the effect of the chronic blockade of CXCR4 by AMD3100 on AAA formation and expansion in 2 experimental models.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

SDF-1/CXCR4 Axis Is Upregulated in Human and Experimental AAAs

We first evaluated whether the expression of the isoforms of SDF-1 and their receptors, CXCR4 and CXCR7, were modulated in the aortic wall of human AAAs (Figure 1A). Quantitative real-time polymerase chain reaction analysis showed a marked increase in SDF-1α mRNA in both medial and adventitial layers of human AAAs, when compared with healthy aortas. The expression of SDF-1β mRNA increased only in the medial layer of human AAAs, whereas the levels of SDF-1γ remained unchanged. In parallel with the increased expression of SDF-1α, the levels of CXCR4 mRNA were upregulated in both medial and adventitial layer of human AAAs but those of CXCR7 were unaffected by the pathology.

We then evaluated the time-course of aortic SDF-1, CXCR4, and CXCR7 mRNA expression during AAA development in mice (Figure 1B–1D). When compared with NaCl-treated mice, perivascular application of CaCl2 to the infrarenal abdominal aorta induced a significant increase of the external aortic diameter from day 2 to day 14 after surgery, with a maximum dilatation reaching 150±19% at day 14 (Figure 1B), in accordance with previous data. The level of SDF-1α, β, γ, and CXCR4 mRNA increased significantly in mouse aortas after CaCl2, a feature of aneurysmal induction, as early as day 2 and up to day 14 (Figure 1C). A positive correlation between aortic mRNA content and aortic diameter was only demonstrated for the SDF-1α isoform and CXCR4 transcripts (Figure 1D). As observed in human samples, the level of CXCR7 mRNA was unaffected by the presence of an aneurysm in mice (Figure 1C).

Using an ELISA test that specifically detects mouse SDF-1-α, we showed that, whereas the aortic level of SDF-1-α protein did not change overtime in NaCl-treated mice, its expression increased significantly after peri-aortic CaCl2 application, reaching a maximum at day 2 and remaining at this level up to 14 days (Figure 2A). As observed for the transcripts, the aortic level of SDF-1-α protein was positively correlated to the external aortic diameter (correlation coefficient, 0.837; P<0.0001; Figure 2B).

Using immunostaining experiments, we showed that, 2 days after CaCl2, aneurysm induction, SDF-1-α is mostly expressed in the injured media, particularly in the region of elastic network destruction where it colocalized with apoptotic α-smooth muscle actin-positive VSMCs (Figure 2C). In contrast, 14 days after CaCl2, aneurysm induction, SDF-1-α accumulated exclusively in the adventitial layer of the AAA wall (Figure 2D). At this later stage, SDF-1-α expression colocalized exclusively with adventitial F4/80+ macrophages and not with α-smooth muscle actin-positive medial VSMCs (Figure 2D). Altogether, these observations demonstrated that SDF-1-α is rapidly expressed during experimental AAA formation, first by apoptotic VSMCs in the injured media and then by recruited macrophages in the adventitia once AAA is fully established.

SDF-1/CXCR4 Pathway Specifically Recruits Macrophages in AAA Lesions

We then assessed whether the SDF-1/CXCR4 axis may determine recruitment of bone marrow (BM)-derived circulating cells within AAA wall, using a green fluorescent protein-positive (GFP+) BM transplantation approach. In wild-type mice irradiated and rescued with BM cells from GFP+/− syngenic mice, the fraction of fluorescent peripheral blood leukocytes 3 weeks after BM transplantation was 89±8%. At this delay, AAAs were generated in GFP−/+ BM transplantation mice by peri-aortic CaCl2 application and mice were treated or not with AMD3100, a potent CXCR4 antagonist. Fourteen days later, few BM-derived GFP-positive cells were detectable in the aortic walls of nonoperated or NaCl-treated mice. In contrast, the number of GFP-positive cells present in the aortic wall significantly increased after CaCl2 application (Figure 3A and 3B) and where almost exclusively confined to the adventitial layer of AAAs (Figure 3A). Chronic treatment with AMD3100 induced a marked mobilization of BM-derived cells, notably polymorphonuclear neutrophils and monocytes in the blood of both NaCl and CaCl2-treated mice (Figure 1 in the online-only Data Supplement), demonstrating the efficacy of the in vivo...
blockade of SDF-1α/CXCR4 interaction in our experiments whatever the presence of an aneurysm. Despite this increased mobilization of BM-derived circulating cells, blockade of CXCR4 by AMD3100 significantly decreased the number of GFP-positive cells recruited within the aneurysmal lesions of CaCl2-treated mice (Figure 3A and 3B), a demonstration of the implication of the SDF-1/CXCR4 axis as a major pathway for the recruitment of BM-derived circulating cells into the injured AAA wall. Using confocal imaging, we further demonstrated that the GFP-positive cells recruited into the AAA wall were exclusively F4/80+ macrophages (Figure 3C). This result is in accordance with the observation that the infiltration of mac-3+ macrophages within the aneurysmal lesion, 14 days after CaCl2 application, was reduced by AMD3100 treatment (Figure 3D). This result is in accordance with the observation that the infiltration of macrophages within the aneurysmal lesion, 14 days after CaCl2 application, was reduced by AMD3100 treatment (Figure 3D).

On the opposite, AMD3100 treatment did not affect the number of T and B lymphocytes recruited within the CaCl2-injured aortic wall in mice (57±32 versus 70±42 CD3+ T cells and 24±7 versus 42±15 CD20+ B cells per mm² in AMD3100-treated and vehicle-treated AAs, respectively; both P=NS; n=6–8). To test whether AMD3100 directly affects the recruitment/migration of macrophage precursors, we performed in vitro experiments using human U937 monocyte cells. We demonstrated that SDF-1α strongly increased U937 cells chemoattraction and that pretreatment of U937 cells with AMD3100 abolished their migration toward SDF-1α (fold increase in cell migration induced by SDF-1 when compared with serum-free medium: 1.5±0.3 versus 17.1±1.5 for cells treated or not with AMD3100, respectively; P<0.001; n=4). Altogether, our data demonstrate that during AAA development, SDF-1/CXCR4 signaling specifically stimulates the recruitment/migration of macrophages, not lymphocytes within the AAA wall.

**Blockade of CXCR4 by AMD3100 Inhibits AAA Formation in Mice**

In human AAs, destruction of extracellular matrix components is triggered by inflammation and proteolysis.216 Because blockade of SDF-1α/CXCR4 axis specifically decreased macrophage infiltration after CaCl2 aneurysmal induction, we tested whether AMD3100 reduces AAA formation in mice. As expected, periaortic CaCl2 application (n=23) generated an aortic aneurysm after 14 days (79±8% increase in the aortic diameter) when compared with nonoperated aortas (n=10; Figure 4A and 4B). Of interest, chronic blockade of CXCR4 by AMD3100 (n=23) significantly reduced the CaCl2-induced
aortic dilatation in mice (aortic diameter at D14, 0.66±0.02 mm versus 0.82±0.04 mm in AMD3100- and vehicle-treated CaCl2 mice, respectively; P<0.0001; Figure 4A and 4B). In this mouse model, as in human AAAs, aortic dilatation is characterized by the thinning of the aortic wall with destruction of the aortic elastin network (Figure 4C) and rarification of α-smooth muscle actin–positive VSMCs within the media (Figure 4D). Chronic treatment with AMD3100 preserved the medial aortic elastic lamellae (Figure 4C) and VSMC contents (Figure 4D) in CaCl2-treated mice.

Figure 3. Stromal cell–derived factor 1 (SDF-1) is rapidly expressed in the aortic wall during abdominal aortic aneurysm (AAA) formation in mice, first by injured vascular smooth muscle cells (VSMCs) and then sustainably by adventitial macrophages. A, SDF-1α protein level in the aortic wall during AAA formation was quantified by ELISA over time and reported to the total protein content. Time course expression of SDF-1α protein on aortic extracts from NaCl-treated (white columns) or CaCl2-treated mice (black columns) is shown (n=4–6 per time point in each group); *P<0.01 vs corresponding NaCl-treated group; #P<0.001 vs corresponding group at day 0. B, Correlation between aortic SDF-1α protein expression level and aortic external diameter, 14 days after AAA generation (n=23). r, correlation coefficient. C, Representative images of triple immunofluorescence for elastin (green), α-smooth muscle actin (SMA)–positive VSMCs (red), and SDF-1 or cleaved-caspase 3 (cl-casp3; yellow, top and bottom, respectively) performed on aortic cross section from CaCl2-treated mice at day 2 (D2). D, Representative images of triple immunofluorescence for elastin (green), SDF-1 (yellow), and αSMA+VSMCs or F4/80+ macrophages (red, top and bottom, respectively) performed on aortic cross section from CaCl2-treated mice at day 14 (D14).

vehicle at day 14. D, Computer-assisted quantification of Mac3-positive macrophages from aortic cross sections of NaCl-treated (n=5) or CaCl2-treated mice that received subcutaneous infusion of vehicle (n=8) or AMD3100 (240 μg/d; n=8) at day 14. Scale bar, 50 μm. **P<0.01 vs NaCl-treated mice; ###P<0.001 vs CaCl2-treated mice receiving vehicle.
Blockade of CXCR4 by AMD3100 Decreases the Expression of Inflammatory and Proteolytic Mediators

After 14 days, periaortic CaCl2 application resulted in an increased expression of all tested inflammatory mediators (Figure 5). Chronic treatment with AMD3100 inhibited the CaCl2-mediated increase in MIP-2α, RANTES, IL-1β, TNF-α, inducible nitric oxide synthase (iNOS), and E-selectin mRNA (Figure 5A). Despite a trend toward a reduction in MCP-1, MIP-1α, MIP-1β, and IL-6 gene induction in the AMD3100-treated group, changes did not reach statistical significance (Figure 5A).

Regarding proteolysis, we showed that mRNA levels of MMP-12, 14, and urokinase-type plasminogen activator (uPA) and their endogenous inhibitor, TIMP-1, increased at day 14 in the AAA wall of CaCl2-treated mice and that AMD3100 treatment significantly reduced MMP-12 expression and tended to reduce MMP-14, TIMP-1, and uPA expression (Figure 5B). Plasminogen activator inhibitor-1 (PAI-1) mRNA decreased after CaCl2-treatment, but AMD3100 had no effect on its expression level. Finally, there was no induction of tPA nor MMP-9 and TIMP-2 transcripts after CaCl2 application or any effect of AMD3100 treatment (data not shown).

Blockade of CXCR4 by AMD3100 Stabilizes Already-Formed AAAs in Mice and Rats

We then addressed whether chronic CXCR4 blockade would stop further expansion of formed AAAs. For this purpose, we used the CaCl2 model in mice and alternately used the xenograft model in rats that offers the advantage to mimic important features of human AAAs, including constant expansion, the presence of inflammatory and proteolytic burden, and intraluminal thrombus.

In the mouse CaCl2 model, chronic AMD3100 administration starting 4 days after CaCl2 application (ie, on established AAAs; Figure 1B) significantly inhibited aortic diameter expansion on the long term (external aortic diameter at D14, 0.82±0.05 versus 1.09±0.10 mm in AMD3100-treated (n=9) and vehicle-treated (n=8) CaCl2 mice, respectively; P<0.05; Figure II in the online-only Data Supplement). In rats, 14 days after xenograft implantation (D14; ie, at the time of treatment initiation), we showed that the external diameter of abdominal aortas had significantly increased by 109% and was not significantly different between randomized vehicle-treated (n=12) and AMD3100-treated (n=9) rats (external aortic diameter, 2.9±0.2 versus 3.2±0.2 mm; respectively; P=NS).

Although AAA diameter continued to expand in vehicle-treated rats, AMD3100 treatment starting at day 14 suspended AAA expansion up to 28 days after treatment initiation (aortic diameter increase at D14+28, −3.7±4.5 versus 16.1±4.7% in AMD3100- and vehicle-treated rats, respectively; P<0.01; Figure 6A and 6B). In rats, the stabilizing effect of AMD3100 was associated with the inhibition of macrophage infiltration within the AAA wall (Figure 6C), whereas the aortic recruitment of T-lymphocytes remained unaffected (604±101 versus 559±62 T cell receptor-positive T-cells per mm² in AMD3100- and vehicle-treated rats,

Figure 4. Blockade of chemokine (C-X-C motif) receptor 4 (CXCR4) by AMD3100 inhibits abdominal aortic aneurysm (AAA) formation in mice. A and B, Macroscopic pictures of representative infrarenal aortas (A) and quantification of aortic external diameter (B) from sham-operated (n=10), NaCl-treated (n=18), or CaCl2-treated mice that received subcutaneous infusion of vehicle (n=23) or AMD3100 (240 μg/d; n=23) at day 14. ○, Individual values; red thick bars, mean±SEM. C and D, Representative staining (left) and computer-assisted quantification (right) of elastic (orcein staining; G) or VSMCs (anti-α-smooth muscle actin [α-SMA] staining; D) from aortic cross sections of NaCl-treated (n=10) or CaCl2-treated mice that received subcutaneous infusion of vehicle (n=10) or AMD3100 (240 μg/d; n=9) at day 14. Scale bar, 50 μm. *P<0.05, **P<0.01, ***P<0.001 vs NaCl-treated mice; #P<0.05, ##P<0.01, ###P<0.001 vs CaCl2-treated mice; ¥P<0.05, ¥¥P<0.01, ¥¥¥P<0.001 vs CaCl2-treated mice receiving vehicle.
respectively; \( P = \text{NS; } n=9–10 \). Hence, after AMD3100 treatment, the reduction of macrophage accumulation in rat AAA walls coincided with the parallel significant decrease in the number of adventitial isolectin B4+ neovessels at day 28 (Figure 6D).

**Discussion**

We show that SDF-1\( \alpha \) isoform and its receptor CXCR4 are upregulated in both mice and human AAA wall. SDF-1\( \alpha \) is rapidly induced during AAA formation in mice, first by

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**Figure 5.** Blockade of chemokine (C-X-C motif) receptor 4 (CXCR4) by AMD3100 decreases the expression of inflammatory and proteolytic mediators in abdominal aortic aneurysm (AAA) lesions in mice. **A** and **B,** Relative expression at day 14 of inflammatory (**A**) and proteolytic (**B**) mediators’ mRNA in the aortic wall of NaCl-treated mice (n=6; NaCl) or CaCl\(_2\)-treated mice that received subcutaneous infusion of vehicle (n=6; CaCl\(_2\), vehicle) or AMD3100 (240 \( \mu \)g/d; n=4; CaCl\(_2\), AMD3100). Data are mean±SEM. *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \) vs NaCl-treated mice; \#\( P<0.05 \), \##\( P<0.01 \), \###\( P<0.001 \) vs CaCl\(_2\)-treated mice receiving vehicle. iNOS indicates inducible nitric oxide synthase; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; RANTES, regulated on activation, normal T-cell expressed, and secreted; TNF, tumor necrosis factor; and uPA, urokinase-type plasminogen activator.

**Figure 6.** Blockade of chemokine (C-X-C motif) receptor 4 (CXCR4) by AMD3100 stabilizes already-formed abdominal aortic aneurysm (AAA) in rats. **A,** Representative macroscopic pictures of AAAs from rats that received subcutaneous infusion of vehicle or AMD3100 (240 \( \mu \)g/d) at D14+28. Dotted lines represent the delimitations of both anastomosis. **B,** External diameter change in vehicle- (n=12) or AMD3100-treated (n=9) rats. ○, individual values; red thick bars, means±SEM. **\( P<0.01 \) vs vehicle. **C,** Macrophage and endothelial cell staining with anti-CD68 antibody and isolectin B4, respectively, on aortic cross sections of vehicle and AMD3100-treated rats at D14+28. Scale bars, 50 \( \mu \)m. **D,** Computer-assisted quantification of CD68-positive macrophages and isolectin B4-positive microvessels in the AAA wall of vehicle and AMD3100-treated rats at D14+28. *\( P<0.05 \) vs vehicle, n=7 to 10.
apoptotic VSMCs in the injured media and then in the adventitia where it colocalizes with macrophages. We demonstrate that SDF-1α is implicated in the recruitment of BM-derived inflammatory cells. Furthermore, the blockade of CXCR4 by AMD3100 decreases the infiltration of adventitial macrophages within the AAA wall, inhibits AAA formation, and prevents aortic wall destruction probably by reducing the release of proteolytic and inflammatory effectors. Finally, using a rat model of AAs, we demonstrate that AMD3100 stabilizes expanding formed AAs in rats.

Our study is the first to focus on differential expression of the 3 major SDF-1 splicing variants, SDF-1α, β, and γ and their 2 specific receptors, CXCR4 and CXCR7. Studying the whole chemokine axis is of particular interest because of the differences in pharmacological and biochemical properties of the different isoforms and because CXCR7 modulates SDF-1/CXCR4 signaling. We show that SDF-1γ level is unaffected in human AAs. Both SDF-1α and β expressions are increased in human and mouse AAs. However, if more investigations would be needed to address the specific role of each isoforms in AAA, our finding that SDF-1α is 10-fold more expressed than SDF-1β and that CXCR4, but not CXCR7, is strongly induced in both human and experimental lesions points out the importance of the SDF-1α/CXCR4 axis in AAA pathogenesis. In support of this view, we also demonstrate that SDF-1α and CXCR4 mRNA levels, as well as SDF-1α protein level, positively correlate with the aortic diameter in the mouse CaCl₂ model. This result is in accordance with a previous report showing a positive correlation between plasma level of SDF-1α and aortic diameter in ascending aortic aneurysm.

SDF-1α is a chemotactic cytokine known to attract inflammatory cells (ie, lymphocytes, monocytes, macrophages, and mast cells), as demonstrated in vitro using chemotaxis assays or in vivo during wound-healing or inflammatory diseases. We have explored the functional and pathological implications of SDF-1α in AAs. Using a BM transplantation approach, we demonstrate that increased aortic expression of SDF-1α paralleled the recruitment of BM-derived inflammatory cells in the injured aorta in mice. Moreover, blockade of SDF-1α signaling using AMD3100 specifically decreases macrophage infiltration within AAA adventitia without affecting lymphocyte trafficking and reduces aortic destruction. In parallel, AMD3100 limits AAA formation and curbs diameter progression of formed AAs in mouse and rat models. These results clearly implicate the SDF-1α/CXCR4 pathway in the pathogenesis of aneurysmal disease. To gain further mechanistic insight on the role of SDF-1α in AAA pathogenesis, we explore which cellular compartments are involved in the increased aortic expression of SDF-1α in AAs. We show that, at the onset of AAA formation, SDF-1α is mainly secreted by apoptotic VSMCs present in the injured media, suggesting that VSMC-derived SDF-1α has a role in initiating macrophage recruitment and AAA development. In contrast, later on, aortic SDF-1α expression is exclusively relayed by infiltrated macrophages themselves that may amplify self-recruitment and exacerbate local inflammation once AAA is fully established. This result is in accordance with recent data demonstrating that in human AAA samples, SDF-1α expression is confined to the vicinity of inflammatory cell infiltrates. Altogether, our data clearly implicate the SDF-1/CXCR4 pathway in the formation and expansion of AAs, through the accumulation of macrophages, and not lymphocytes within the AAA wall.

In human and experimental models, infiltration of inflammatory cells into the injured aortic wall plays a key role in orchestrating AAA expansion. Once recruited, inflammatory cells exacerbate local inflammation through the secretion of proinflammatory mediators. Among these inflammatory effectors, monocyte chemotactic protein-1, regulated on activation, normal T-cell expressed and secreted (RANTES), interleukin-1β, interleukin-6, or tumor necrosis factor-α have been clearly implicated in the development of experimental AAs and are upregulated in human AAA walls. In our study, we show that MCP-1, MIP-1β, MIP-2α, and RANTES mainly produced by macrophages, as well as IL-1β, IL-6, and TNF-α, are overexpressed in the wall of CaCl₂-induced AAs. Interestingly, CXCR4 blockade by AMD3100 abrogates the increase of all tested proinflammatory mediators. Besides, E-selectin expression, an adhesion molecule that facilitates the recruitment of inflammatory cells within injured tissues, was also reduced in AMD3100-treated aortas. Our data suggest that SDF-1α/CXCR4 axis governs a loop of self-amplification of the inflammatory process that can be interrupted by a pharmacological approach.

Destruction of the aortic extracellular matrix components and induction of VSMC apoptosis in human AAs are triggered by MMP-dependent proteolysis and by oxidative stress. In our study, there was no induction of MMP-9 transcripts 14 days after CaCl₂ application, whereas MMP-12 and MMP-14 and iNOS transcripts were upregulated. Chronic AMD3100 treatment inhibited the aortic increase in MMP-12 and MMP-14 as well as iNOS mRNA levels. In human AAs, iNOS is a major source of reactive oxygen species that are known to increase aortic MMP expression and activation. In AAs, the extent of MMP release is spatially linked to the development of neovessels in the adventitia/media, and increased adventitial neovascularisation is associated with AAA degeneration or rupture. Our present data show that AMD3100 treatment decreased adventitial angiogenesis in established rat AAs. Altogether, our data suggest that the inhibition of the aortic destruction by AMD3100 results, at least in part, from a decreased generation of MMPs and reactive oxygen species into AAA wall together with a reduction of macrophage infiltration and adventitial angiogenesis. As a marker of a decrease in inflammatory, proteolytic, and oxidative stress burdens, elastin network and VSMC content were preserved in the residual aortic wall of AMD3100-treated mice.

In humans, current treatments of AAs rely on elective surgery of large aneurysms (ie, replacement [prosthesis] or strengthening [stentgraft] of the aorta), when the rupture risk balances that of surgery. In fact, these 2 surgical techniques are associated with a high postoperative mortality rate and a limited durability, respectively. Alternative strategies aimed at controlling further expansion of AAs before surgical threshold are needed. Pharmacological approaches able to stop small AAA expansion would lower the need for surgery and allow for AAA detection by echography in targeted populations.
The average delay between small AAA diagnosis and elective surgery frequently lasts more than 5 years. Over the past decade, our laboratory has promoted cell35–37 or gene38 therapy to stop further AAA expansion, but these 2 approaches are hampered by technical difficulties and potential high cost for a frequent disease. To this regard, 1 major result of our study is that the blockade of CXCR4 by AMD3100 limits AAA formation in mice but also stabilizes already-formed AAAs in 2 experimental models. In humans, short-term AMD3100 delivery is approved by the Food and Drug Administration for mobilizing hematopoietic stem cells from BM to blood for transplantation in cancer. A recent report has shown preliminary evidence for the safety and clinical efficacy of long-term AMD3100 treatment in immunodeficient patients, arguing for a promising interest of AMD3100 as a pharmacological agent to stabilize small AAAs in humans.

Hence, in addition to its effect on inflammation, proteolysis, and oxidative stress, the impact of AMD3100 on AAA growth may be potentiated by its strong mobilizing properties for BM CXCR4+ precursors, notably endothelial progenitors and mesenchymal stem cells, and thereby may promote AAA repair. We have previously shown that increasing endothelial progenitors and mesenchymal stem cells by local cell therapy is able to slow the progression of experimental AAAs. In our study, we did not address whether CXCR4+ cell levels were increased into the peripheral blood after chronic AMD treatment. However, AMD3100 was able to mobilize BM-derived circulating cells and may have facilitated the release and homing of other putative BM-derived CXCR4+ vascular progenitors.

As a conclusion, we have demonstrated that SDF-1α and its receptor CXCR4 are induced in AAA walls in human and mice and that their expression level correlates positively with the aortic diameter in mice. We also showed that AMD3100 inhibits AAA formation and prevents aortic wall destruction in mice and slows the progression of established AAAs in mice and rats, by decreasing inflammation, adventitial angiogenesis, proteolysis, and oxidative stress. We propose that SDF-1/CXCR4 axis blockade has a great potential as a pharmacological treatment to reduce further expansion of small AAAs.

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Disclosures
None.

References
Abdominal aortic aneurysms (AAAs) are aortic enlargements that result from extracellular matrix destruction and vascular smooth muscle cell apoptosis, triggered by inflammation and proteolysis. We investigated whether stromal cell–derived factor 1α, a chemokine known for its ability to attract leukocytes, and its receptors chemokine (C-X-C motif) receptor 4 (CXCR4) play a role in AAA pathogenesis. We show that stromal cell–derived factor 1α and CXCR4 transcripts are upregulated in human and mouse AAAs and that their aortic levels are positively correlated to the aortic diameter in mice. We further demonstrate that the stromal cell–derived factor 1/CXCR4 axis is implicated in AAA development in mice by mediating bone marrow–derived inflammatory cell recruitment and that chronic CXCR4 blockade by AMD3100 inhibits experimental AAA formation and progression through anti-inflammatory properties. Our data provide the first demonstration that stromal cell–derived factor 1/CXCR4 axis blockade has a great potential as a pharmacological strategy to slow the rate of expansion of small AAAs and may delay the need for surgery.

Significance

Abdominal aortic aneurysms (AAAs) are aortic enlargements that result from extracellular matrix destruction and vascular smooth muscle cell apoptosis, triggered by inflammation and proteolysis. We investigated whether stromal cell–derived factor 1α, a chemokine known for its ability to attract leukocytes, and its receptors chemokine (C-X-C motif) receptor 4 (CXCR4) play a role in AAA pathogenesis. We show that stromal cell–derived factor 1α and CXCR4 transcripts are upregulated in human and mouse AAAs and that their aortic levels are positively correlated to the aortic diameter in mice. We further demonstrate that the stromal cell–derived factor 1/CXCR4 axis is implicated in AAA development in mice by mediating bone marrow–derived inflammatory cell recruitment and that chronic CXCR4 blockade by AMD3100 inhibits experimental AAA formation and progression through anti-inflammatory properties. Our data provide the first demonstration that stromal cell–derived factor 1α/CXCR4 axis blockade has a great potential as a pharmacological strategy to slow the rate of expansion of small AAAs and may delay the need for surgery.
Chemokine (C-X-C Motif) Receptor 4 Blockade by AMD3100 Inhibits Experimental Abdominal Aortic Aneurysm Expansion Through Anti-Inflammatory Effects
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Supplemental Material

Supplemental Figures

Supplemental Figure I. Chronic blockade of CXCR4 increases circulating neutrophile and monocyte count in mice. Quantification of circulating neutrophile (CD11b+ Ly6G+ 7/4+) and monocyte (CD11b+ Ly6G- 7/4+) subsets by flow cytometry in NaCl- or CaCl2-treated mice that received subcutaneous infusion of vehicle or AMD3100 (240 µg/day) for 14 days. Results are expressed as mean±SEM (n=5-10 per group). *P<.01 vs corresponding value.

Supplemental Figure II. Blockade of CXCR4 by AMD3100 stabilizes already-formed AAAs in mice. A, Representative macroscopic pictures of AAAs from mice that received subcutaneous infusion of vehicle or AMD3100 (240 µg/kg) starting at day 4 (i.e., on established AAAs) until day 14. B, External diameter in vehicle- (n=8) or AMD3100-treated (n=9) mice at day 14. Represents individual values; red thick bars represent means SEM. *P<.05 vs vehicle.
Supplemental Tables

**Supplemental Table I. Primers used for quantitative RT-PCR using SYBR Green detection tools**

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<th>gene</th>
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<td>5’-AGTGGTCTAGCGGAAAGTC-3’</td>
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**Supplemental Table II. Candidate genes and their assay ID for quantitative RT-PCR using Taqman detection tools**

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METHODS

Human AAA explants
Fragments from the maximum dilation area of human AAAs (asymptomatic; diameter >50 mm) (n=5) were collected during elective surgery in the Department of Vascular Surgery of the HenriMondor hospital in agreement with the local ethics committee. Healthy abdominal aortic samples were a kind gift from Jean-Baptiste Michel (INSERM U698 research unit, Bichat Hospital, Paris, France) (n=5). For each aortic samples, the medial and adventitial layers (n=5) were carefully separated, individually snap-frozen and stored at -80°C before RNA extraction.

Mouse model of AAA, time-course study and AMD3100 treatment
AAAs were generated in 8 week-old male C57Bl/6 male mice by periaortic application of CaCl₂, as previously described. Briefly, mice anesthetized with pentobarbital (50 mg/kg, IP) underwent laparotomy and the abdominal aorta between the renal and the iliac arteries was isolated from the surrounding peritoneal structures. A 0.5x0.2 cm gelatin sponge (Curaspon, Curamedicals) immersed in 0.5M CaCl₂ was then applied on the external surface of the aorta for 15 min. Sponges immersed in 0.9% NaCl were used for sham-operated animals. The sponge was then removed, the aorta was rinsed with sterile saline and the incision was closed. Animals were allowed to recover on a homeothermic blanket at 37°C. At time of sacrifice, mice were anesthetized with pentobarbital (50 mg/kg, IP). After intracardiac perfusion of cold 0.9% NaCl, infrarenal aortas were cleared of surrounding tissue, photographed in situ and cut into two pieces, one being fixed in 4% paraformaldehyde and decalcified overnight in 0.5M EDTA before paraffin embedding, the other snap-frozen in liquid nitrogen and kept at -80°C.

Time course of SDF-1 expression during AAA formation. NaCl- or CaCl₂-treated mice were sacrificed immediately (day 0) or 2, 7, 14 or 28 days after surgery (n=5 per group and time point).

AMD treatment. To study the effect of CXCR4 blockade on AAA formation, CaCl₂-treated mice received AMD3100 (Sigma-Aldrich), a CXCR4 receptor antagonist (240 µg/day, n=23) or vehicle (n=23) administered subcutaneously using a mini-osmotic pump (Alzet model 2002, Charles River), starting immediately after surgery and until sacrifice. Unoperated (n=10) and NaCl-treated mice (n=18) were also included. To study the effect of CXCR4 blockade on AAA expansion, CaCl₂-treated mice received AMD3100 (n=9) or vehicle (n=8) administered subcutaneously using a mini-osmotic pump, starting 4 days after surgery and until sacrifice. All mice were sacrificed 14 days after surgery.

Bone Marrow Transplantation (BMT)
BMT was performed as previously described. GFP⁻/⁻ bone marrow (BM) cells were harvested from femurs of 8 week-old male GFP⁺/⁻ C57Bl/6 mice (n=35). Seven week-old male WT C57Bl/6 mice were lethally X-irradiated with a total dose of 9 Gy (⁶⁰Cobalt source, n=40). One day later, male recipient mice (n=34) received unfractionated GFP⁺/⁻ BM cells (4-8.10⁶ cells per host) in 0.2 mL saline by retro-orbital injection. Three weeks after BMT, BMT GFP⁺/⁻ mice were anesthetized and underwent periaortic NaCl (n=10) or CaCl₂ application (n=19). In the CaCl₂-treated group, BMT GFP⁺/⁻ mice received AMD3100 (240 µg/day, n=9) or vehicle (n=10) administered subcutaneously using a mini-osmotic pump immediately after surgery and for 14 days. At this delay, mice were sacrificed. Five age-matched unoperated BMT GFP⁺/⁻ mice were used as controls (n=5).
**Xenograft model of expanding AAA in rats and AMD3100 treatment**

AAAs were generated in 8 week-old male Fischer 344 rats (Charles River Laboratories, France) by implanting an aortic xenograft from guinea pig, as previously described. Briefly, guinea pig infrarenal aortas were decellularized using 0.1% sodium dodecyl sulfate to obtain intact tubes of aortic extracellular matrix that were subsequently orthotopically grafted into rats using 10-0 sutures. Fourteen days later, a chimeric AAA (>50% diameter increase) had developed from the degraded guinea pig extracellular matrix, colonized with cells and thrombus of rat origin. Rats with formed AAAs (*i.e.* 14 days after xenograft implantation) received AMD3100 (240 µg/day, n=9) or vehicle (n=12), by continuous subcutaneous delivery using mini-osmotic pump for 4 weeks. The subrenal aorta was photographed *in situ* under beating heart in anesthetized rats, at the time of AMD treatment initiation, and four weeks later, before euthanasia. AAAs were then fixed in 70% ethanol and embedded in paraffin.

**Quantification of circulating monocytes and neutrophils by flow cytometry**

40 µl of peripheral blood per mice were added to bead-containing TruCount tubes (BD Biosciences). After addition of Fc Blocking reagent (BD Biosciences), each tube was incubated for 5 min at room temperature. Blood cells were then stained with a cocktail of antibodies (anti-CD11b-APC Cy7, anti-Ly6B.2-FITC, anti-Ly6G-PE) used to enumerate neutrophil (CD11b<sup>+</sup>Ly6B.2<sup>+</sup>Ly6G<sup>+</sup>) and monocyte (CD11b<sup>+</sup>Ly6B.2<sup>+</sup>Ly6G<sup>−</sup>) subset populations. After red blood cell lysis (BD Lysis Buffer), cell suspensions were run for 7-color fluorescence staining on a fluorocytometer (CyAn<sup>TM</sup> ADP, Beckman Coulter). The absolute count of leukocyte subpopulation was calculated from appropriate regions gated by flow cytometry analysis using the formula 

\[\text{Absolute count} = \frac{\text{no. of events in region containing selected cell subpopulation}}{\text{no. of events in absolute-count bead region}} \times \left[\frac{\text{total no. of absolute-count beads}}{\text{test volume [40 µl]}}\right].\]

**Total RNA extraction, cDNA synthesis and Quantitative Real-time Polymerase Chain Reaction**

Total, medial or adventitial aortic walls from human or mice were ground to a fine powder in liquid nitrogen and total RNA was extracted in Trizol reagent using PureLink RNA Micro Kit (Invitrogen, Cergy Pontoise, France). The purity and concentration of total extracted RNA were evaluated using a spectrophotometer (NanoDrop Technologies, Montchanin, DE). The RNA was reverse transcribed using the high-capacity VILO SuperScript reverse transcriptase (Invitrogen, France). Quantitative real-time PCR (QRT-PCR) was performed with the StepOnePlus real-time PCR system (Life Technologies, France) using SYBR Green or TaqMan detection tools. Custom TaqMan expression assays (Lifetechnologies, France) or designed primer sets (Eurogentec, France) are listed for each human or murine candidate gene on Supplemental Tables I and II.

Amounts of cDNA of interest were normalized to that of GAPDH (ΔCt = CT gene of interest − CT GAPDH). Results are reported as relative gene expression (2<sup>−ΔΔCt</sup>).

**Quantification of SDF-1 in mouse aortas by ELISA**

Proteins from mouse aortas were prepared by homogenizing tissues with a potter in a ice-cold extraction buffer (1% Nonidet P-40 in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 1 mM EDTA, 50 mM NaF, and protease inhibitors (Sigma-Aldrich)) after aorta pulverization with a MultiSample Bio-Pulverizer (Biospec, Bartlesville, USA). Commercially available ELISA kits (Quantikine, R&D system) were used to measure the protein level of SDF-1 in aortic extracts and results were normalized to total protein content, determined by BCA quantification.
**Monocytes chemotaxis assay**

Human U937 monocytes (ATCC) were cultured in suspension in RPMI medium containing 10% SVF. For migration assay, U937 cells were washed in PBS, centrifuged and resuspended in RPMI 1640 containing 0.1% bovine serum albumin. Monocytes were seeded onto the upper chamber of transwell membrane (8 μm pores) (BD Biosciences, France) at a concentration of 4.105 cells/well. In some experiments, cells were preincubated with AMD3100 (20 µM) for 30 minutes at 37°C, prior seeding. The bottom chambers were filled with 0.5 mL of serum-free medium with or without recombinant human SDF-1α (50 ng/mL, R&D system, France). Cells were allowed to migrate for 4 hours at 37°C and cells that had migrated toward the lower compartment were counted using a Neubauer chamber.

**Histological Analysis**

Five µm thick paraffin-embedded cross sections of AAAs were used. For histological analysis, sections were stained with orcein for visualization of elastic fibers. For immunohistochemical analysis, sections were incubated with the following antibodies: mouse anti-alpha smooth muscle actin (αSMA, Sigma-Aldrich), rabbit anti-CXCL12α subunit (eBioscience), rabbit anti-GFP (Life Technologies), rat anti-mac3 and rat anti-mouse CD45R (both from BD Biosciences), rabbit anti-mouse CD3 antibody (Abcam), mouse anti-rat CD68 or mouse anti-rat TCR (both from Serotec), goat anti-rat CD20 (Santacruz) antibodies. When a mouse primary antibody was used on mouse sections, the Vector M.O.M kit was used, according to the manufacturer’s instructions. After incubation with a biotin-conjugated anti-species antibody (Vector Laboratories), immunostaining was amplified using peroxydase-conjugated streptavidin complexes (Vector Laboratories) and peroxydase was detected using VIP or DAB (Vector Laboratories). For neovessels identification, sections were incubated overnight at 4°C with biotinylated isoelectin-B4 (Vector Laboratories) and revealed as described above. Sections were counterstained with hematoxylin, mounted in Eukitt and examined with a bright field microscope (Zeiss).

The colocalization of SDF-1, GFP or cleaved caspase 3 with macrophages or VSMCs was performed by double immunofluorescence staining. Briefly, sections were incubated overnight at 4°C with anti-SDF-1, anti-cleaved caspase 3 (Cell Signaling, France) or anti-GFP antibody. These primary antibodies were revealed by incubation with a biotinylated secondary antibody and then an Alexa 647-conjugated streptavidin. Macrophage staining was then performed by incubating sections overnight at 4°C with the rat anti-F4/80 antibody (Serotec). The signal was revealed by incubation with an Alexa 555-conjugated anti-rat and sections were mounted in Mowiol. For colocalization of SDF-1 or cleaved caspase 3 with VSMCs, VSMC staining was then performed with the M.O.M kit using a Cy3-conjugated anti-αSMA antibody. Fluorescence was examined with a Leica TCS SP5 confocal (Leica Microsystems) or a fluorescence (AxioImager D1, Zeiss) microscope and analyzed in sequential scanning mode for triple detection of Alexafluor 555, Alexafluor 647 and elastin green-autofluorescence. Images were obtained with a 63x oil immersion or 20x objective lens.

**Computer-Assisted Morphometric Analysis**

Histological images were digitally captured using the Axiovision 4.8 Software (Zeiss). Customized programs were used to quantify the remodeling of the vessels, the GFP-positive cells, the VSMC densities and the mac-3 stained area. The observer was blinded to treatment allocation.

**Remodeling of the vessels**

Maximal external diameters were measured from in situ images of infrarenal aortas using
Axiovision.

**Elastic fiber content**
The orcein-stained surface was quantified on histological cross-sections using AxioVision. Briefly, the software allows for the selection and subsequent quantification of pixel intensities in a chosen color spectrum (red-brown corresponding to orcein staining). All microscopic slides were stained simultaneously in the same orcein bath and the same range of pixel intensity was used for all the quantifications. Results were expressed as a percentage of total aortic surface.

**Cell recruitment on aorta and medial VSMC density**
The number of GFP positive cells, macrophages, T and B lymphocytes or VSMCs in aortic section was evaluated by counting the number of cells immunostained by anti-GFP, anti-CD68, anti-CD3 or anti-TCR, anti-CD20 or anti-CD45R or anti-αSMA antibody, respectively. The mac-3 stained surface was quantified using the same principle as the orcein-stained surface quantification. The number of neovessels in aortic sections was evaluated by counting the number of isolecitin-B4 positive structures surrounding a lumen. The total aortic surface was determined in parallel and results were expressed as the number of cells per mm² or as the percentage of total aortic surface.

**Statistics**
Results were expressed as means±SEM. All statistical analysis were performed using GraphPad Prism Software (version 4.02). Comparison between more than two groups was done by a one-way analysis of variance (ANOVA) followed, if significant (P<.05) by the Newman-Keuls post-hoc test for multiple comparisons. For analysis between two groups, student-t test was used. Linear regression and Pearson’s correlation coefficient were obtained for all correlation studies. P<.05 was considered as statistically significant.

**REFERENCES**

