The Stromal Cell–Derived Factor-1 Chemokine Is a Potent Platelet Agonist Highly Expressed in Atherosclerotic Plaques

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Abstract—Chemokines are chemotactic cytokines that activate and direct the migration of leukocytes. However, their role in modulating platelet function has not been shown. We studied the direct effect of chemokines on human platelets and found that of the 16 tested only stromal cell–derived factor (SDF)-1 induced platelet aggregation, accompanied by a rise in intracellular calcium. Platelets expressed the SDF-1 receptor, CXCR4, and an antibody to CXCR4 and pertussis toxin inhibited SDF-1–induced platelet aggregation, confirming that this effect is mediated through CXCR4, a Gαi-coupled receptor. SDF-1–induced platelet aggregation was also inhibited by wortmannin, LY294002, and genistein, suggesting that phosphatidylinositol 3–kinase and tyrosine kinase are likely involved in SDF-1–induced platelet aggregation. Because chemokines are produced from multiple vascular cells and atherosclerotic vessels are prone to develop platelet-rich thrombi, we examined the expression of SDF-1 in human atheroma. SDF-1 protein was highly expressed in smooth muscle cells, endothelial cells, and macrophages in human atherosclerotic plaques but not in normal vessels. Our studies demonstrate a direct effect of a chemokine in inducing platelet activation and suggest a role for SDF-1 in the pathogenesis of atherosclerosis and thrombo-occlusive diseases. (Circ Res. 2000;86:131-138.)

Key Words: stromal cell–derived factor-1 ■ chemokine ■ platelet ■ atherosclerosis

Although improved medical therapies have led to an overall decrease in mortality from acute coronary events, myocardial infarction remains the leading cause of death in the United States. Numerous studies have demonstrated that the immediate cause of acute coronary syndromes is atherosclerotic plaque disruption inducing platelet activation and acute thrombus formation. Atherosclerosis is a progressive disease in which inflammatory cells, activated smooth muscle cells, lipids, and extracellular matrix accumulate in the arterial wall resulting in growth of plaques. Atherosclerosis is now viewed as an inflammatory disease of the vascular system, with macrophages, lymphocytes, and platelets being important sources of cytokines and growth factors that control the migration, proliferation, and activation of smooth muscle cells and monocytes leading to intimal hyperplasia. Recent data support a critical role of chemokines in the accumulation of macrophages and lipids in atherosclerotic lesions. Chemokines are a superfamily of chemotactic cytokines. They activate and direct the migration of leukocytes by binding to specific G protein–coupled 7-transmembrane cell surface receptors. Expression of several chemokines, including monocyte chemoattractant protein (MCP)-1, MCP-4, RANTES (regulated on activation normal T-cell expressed and secreted), and interleukin-8 (IL-8) is increased in human atherosclerotic plaques compared with normal vessels. In vivo animal studies using genetically modified mouse strains have revealed functional roles for MCP-1 and its receptor CCR2 and the murine homologue of the IL-8 receptor, CXCR2, in monocyte recruitment and retention in atherosclerotic lesions, suggesting that chemokines and their receptors play a critical role in atherogenesis.

Platelets are anucleated cellular fragments that circulate in the blood. In addition to their well-recognized role in hemostasis and acute thrombus formation, platelets are also thought to have proinflammatory and growth-regulatory properties that contribute to progression of atherosclerosis. Platelet activation releases multiple growth factors and inflammatory mediators, including chemokines, into the microenvironment. In fact, the first chemokine described, platelet factor 4, was identified as a heparin-binding protein released from activated platelets and has been used as an in vivo marker of platelet activation. Although platelets contain numerous other chemokines, previous work has not focused on the platelet as a target for chemokines. Because platelets are in contact with cells that produce chemokines, we investigated the effect of chemokines on platelet aggregation and found that of the 16 chemokines tested stromal cell–derived factor-1 (SDF-1), a CXC chemokine and a known chemotactic factor for lymphocytes and...
monocytes, induced platelet activation measured by aggregation and Ca\(^{2+}\) flux. In addition, we found that SDF-1 protein was highly expressed in human atherosclerotic plaques but not in normal vessels. Our data suggest that SDF-1 may be involved in the pathology of atherosclerosis.

**Materials and Methods**

**Blood Collection and Platelet Preparation**

Human blood was collected from antecubital veins of healthy, male or female, aspirin-free volunteers into syringes containing heparin (10 U/mL final concentration) for flow cytometry studies and sodium citrate (0.38% final concentration) for aggregation studies or acid citrate dextrose (1.5 mL of acid citrate dextrose for 8.5 mL of blood) for Ca\(^{2+}\) flux studies. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 150g for 15 minutes. Platelet-poor plasma was prepared by centrifugation of PRP at 1200g for 15 minutes.

**Aggregation Studies**

Experiments were performed using a Chrono-Log model 560vs or 490-2D aggregometer. Aliquots of PRP (0.50 or 0.45 mL each, with a platelet concentration of 1 to 3x10\(^{10}\) platelets/mL) were incubated at 37°C and stirred at 1000 rpm. Reombinant chemokines were obtained from PeproTech Inc, and SDF-1β was obtained from 2 different sources, PeproTech Inc and Genetics Institute. Aggregation was measured as a percentage change in optical density, with the instrument calibrated to yield 0% change in optical density for PRP and with the platelet-poor plasma 100% standard for change in optical density. Aggregation scale was set so that maximal light transmittance of the sample after the addition of SDF-1 in the presence or absence of inhibitory antibodies (Abs) or chemical inhibitors. The data are expressed as mean±SD.

Inhibition experiments were done using a CXCR4 monoclonal Ab (mAb) 12G5 (R&D), goat anti–SDF-1 polyclonal Ab (R&D), pertussis toxin, wortmannin, LY294002, genistein, and aspirin (Sigma). DMSO was used as vehicle for wortmannin, LY294002 and the third level of activation, is associated with proteins per lane) by standard SDS-PAGE under reducing conditions and blotted onto polyvinylidene difluoride membranes (Bio-Rad) using a semidry blotting apparatus (0.8 mA/cm\(^2\), 30 minutes; Bio-Rad). Blots were blocked, and dilution of first and second Ab was made in 5% dry skim milk/PBS/0.1% Tween. After 1 hour of incubation with the primary goat anti-human SDF-1 Ab (R&D), blots were washed 3 times (PBS/0.1% Tween), and the secondary peroxidase-conjugated rabbit anti-goat Ab (Jackson ImmunoResearch) was added for another hour. Blots were washed 3 times, and detection of the antigen was carried out using the enhanced chemiluminescent detection method (Dupont-NEN).

**Immunohistochemistry**

Atherosclerotic plaques from human carotid arteries and nonatherosclerotic arteries were obtained at endarterectomy or from transplant donors or autopsies by protocols approved by the Human Investigation Review Committee at the Brigham and Women’s Hospital. Serial cryostat sections (6 mm) were cut, air-dried onto microscope slides (Fisher Scientific), and fixed in acetone at −20°C for 5 minutes. Sections preincubated with PBS containing 0.3% hydrogen peroxideactivity were incubated (60 minutes) with the primary goat anti-human SDF-1 Abs (R&D and Santa Cruz Biotechnology) or control Ab, diluted in PBS supplemented with 5% appropriate serum. Finally, sections were incubated with the respective biotinylated secondary Ab (45 minutes, Vector Laboratories) followed by avidin-biotin-peroxidase complex (Vectastain ABC kit, and Ab binding was visualized with 3-amino-9-ethyl carbazole (Vector Laboratories). Cell types were characterized by double immunofluorescence staining using anti–muscle α-actin mAb specific for smooth muscle cells (Enzo Diagnostics), anti-CD31 mAb specific for endothelial cells (Dako), and anti-CD68 mAb specific for macrophages (Dako), using streptavidin conjugated by FITC (cell-specific Ab) and Texas Red (SDF-1α specific Ab).

**Results**

**SDF-1 Induces Human Platelet Aggregation**

We studied the in vitro aggregation of human platelets in response to 16 chemokines (SDF-1α and SDF-1β; interferon-inducible protein of 10 kDa [IP-10]; neutrophil-activating peptide-2 [NAP-2]; IL-8; epithelial cell– derived neutrophil-activating protein [ENA-78]; growth-regulated oncogene-α [GRO-α]; monokine induced by interferon-γ [Mig]; MCP-1, MCP-2, MCP-3, and MCP-4; eotaxin; RANTES; macrophage inflammatory protein [MIP]-1α; MIP-1β; and I-309). Chemokines were tested at 25, 40, and 100 nmol/L (each chemokine concentration was used in a different experiment). Of the chemokines tested, only SDF-1 induced platelet aggregation (Figure 1A). At all concentrations tested (25 to 100 nmol/L), the other chemokines did not induce platelet aggregation measured by the lumi-aggregometer.

SDF-1α and SDF-1β are CXC chemokines derived from a single SDF-1 gene by alternative splicing with SDF-1β containing an additional 3’ exon encoding 4 C-terminal amino acids. The effect on platelets of SDF-1α and SDF-1β was concentration dependent (Figure 1B and 1C). The concentration of SDF-1α and β necessary to induce a maximum aggregatory response varied between experiments and was between 10 and 100 nmol/L.

Platelets have several levels of response to stimuli. The first level consists of platelet shape change, seen as a minor change in aggregometer traces. Primary aggregation is the second level of response, defined as aggregation without secretion, and is at least partially reversible. Secondary aggregation, the third level of activation, is associated with...
maximal irreversible aggregation, platelet granule secretion, and prostanoid synthesis. Low concentrations of SDF-1 only induced the primary phase of aggregation (6.2 and 2.5 nmol/L for SDF-1\(_{\alpha}\) and SDF-1\(_{\beta}\), respectively, Figure 1B and 1C). However, increasing amounts of either SDF-1\(_{\alpha}\) or SDF-1\(_{\beta}\) caused both primary and secondary response (open and closed arrow, respectively, Figure 1C). Of 12 healthy donors tested, 10 had a full response to SDF-1 (primary and secondary phase of aggregation), whereas 2 had only a primary aggregatory phase. We also observed that SDF-1 induced a more robust aggregation when PRP was kept at 4°C before the aggregation studies compared with room temperature or 37°C. These data suggest that other factors may regulate platelet responsiveness to SDF-1.

**SDF-1 Induces Ca\(^{2+}\) Flux in Platelets**

Because chemokines characteristically induce elevation in cytosolic Ca\(^{2+}\) concentration in leukocytes, we tested the ability of SDF-1 to induce a similar response in platelets. Ca\(^{2+}\) elevation in washed human platelets was reproducibly induced by 100 nmol/L SDF-1 (Figure 2). SDF-1 at 50 nmol/L had a minimal effect on Ca\(^{2+}\) flux in platelets. ADP, a known platelet agonist, was used as a positive control for comparison. I-309, one of the other chemokines tested that did not induce platelet aggregation, was used as a negative control and had no effect on Ca\(^{2+}\) flux in platelets.

**CXCR4 Mediates the Effect of SDF-1 on Platelets**

SDF-1 signals cells through the chemokine receptor CXCR4,\(^{29,30}\) a 7-transmembrane–spanning G protein–coupled cell-surface glycoprotein. We found that human peripheral blood platelets expressed CXCR4 by flow cytometry using 2 different mAbs specific for CXCR4 (data not shown), which confirmed 3 recent reports.\(^{31-33}\) A mAb to CXCR4 inhibited SDF-1–induced platelet aggregation by 84.4 ± 2.4%, demonstrating that SDF-1 activates platelets through CXCR4 (Figure 3A). An isotype-matched control Ab had no effect on SDF-1–induced platelet aggregation. Also, pertussis toxin inhibited SDF-1–induced platelet aggregation by 83.9 ± 6% (Figure 3B), confirming that this effect was at least in part mediated by a pertussis toxin–sensitive G protein, such as G\(_{\alpha}\).

**Anti–SDF-1 Ab Inhibits the Effect of SDF-1 on Platelet Aggregation**

To confirm that SDF-1 was directly responsible for the observed effects on platelets, SDF-1 was incubated for 15 to 30 minutes with a neutralizing goat anti–SDF-1 polyclonal Ab before addition to the PRP. Preadsorption with the SDF-1...
polyclonal Ab inhibited the SDF-1 effect on platelets by 92.9 ± 2.7% (Figure 4).

Intracellular Signaling Pathways Involved in SDF-1–Induced Platelet Aggregation

In an attempt to identify the mechanism of SDF-1–induced platelet activation, we explored the signaling pathways involved in this process using a variety of known inhibitors. Aspirin is known to inhibit platelet cyclooxygenase and the second wave of ADP-induced platelet aggregation characterized by maximal irreversible aggregation, platelet granule secretion, and prostanoid synthesis. Aspirin inhibited the SDF-1 effect on platelets by 70.1 ± 3.9%. Aspirin inhibited the second but not the first wave of SDF-1–induced aggregation, suggesting a requirement for prostanoid synthesis in SDF-1–induced platelet aggregation (Figure 5A). Genistein, a tyrosine kinase inhibitor, decreased by 72.3 ± 4.4% SDF-1–induced platelet aggregation. Genistein also completely inhibited the SDF-1–induced secondary phase of aggregation but not the primary phase (even at a 10 mmol/L concentration), indicating that the secondary phase of SDF-1–induced platelet aggregation appears to require tyrosine kinase activation. The maximal inhibitory effect of genistein was seen at a concentration of 200 to 800 μmol/L (Figure 5B). Wortmannin and LY294002, 2 structurally unrelated PI 3-kinase inhibitors, completely inhibited SDF-1–induced platelet aggregation. The inhibitory effect of wortmannin and LY29004 on SDF-1–induced platelet aggregation was concentration dependent (Figure 5C). Under our experimental conditions, a wortmannin concentration of 400 to 500 nmol/L was required to induce complete (100%) inhibition of SDF-1 effect on platelets. Because in the nanomolar range wortmannin can inhibit at least 2 other enzymes, namely, phosphati-
dylinositol 4-kinase and phospholipase A, the effect of another PI-3 kinase inhibitor, LY29004, was also tested. LY92004 inhibited SDF-1–induced platelet aggregation in a dose-dependent manner, reaching complete inhibition of platelet aggregation at 50 μmol/L (Figure 5D). Taken together, these data suggest that SDF-1–induced platelet aggregation likely involves PI-3 kinase and depends, at least in part, on both prostanoid synthesis and tyrosine kinases.

Atherosclerotic Plaques Express SDF-1 Protein

In view of the role of platelet activation in atherosclerosis and thrombosis, we investigated the expression of SDF-1 protein in normal human arteries and atherosclerotic plaques. Western blot analysis revealed a striking increase in SDF-1 immunoreactivity in atherosclerotic plaques isolated from 4 different carotid atheromas compared with nonatherosclerotic arteries (Figure 6). Immunohistochemical staining using 2 different anti–SDF-1–specific Abs showed abundant expression of SDF-1 protein in atheromatous arteries but not in normal arteries (Figure 7). Double immunofluorescence localized SDF-1 expression in plaques to endothelial cells (CD31+), smooth muscle cells (α-actin+), and macrophages (CD68+) (Figure 7).
Discussion

It is becoming increasingly clear that chemokines have other activities in addition to leukocyte chemotaxis. Mice deficient in SDF-1 and CXCR4 have defects in B-cell lymphopoiesis and bone marrow myelopoiesis and die perinatally with defects in cerebellar, cardiac, and vascular morphogenesis. SDF-1 and CXCR4 appear to be a monogamous receptor ligand pair, which is unusual for the chemokine system. SDF-1 is constitutively expressed in bone marrow stromal cells and to date has not been shown to be upregulated in inflammatory diseases. Recently, SDF-1 was shown to increase megakaryocyte production in the presence of thrombopoietin and to induce megakaryocyte chemotaxis and adhesion to endothelial cells. Our finding that SDF-1 is a potent activator of platelets has identified a novel activity for the chemokine family involving an important cell type not previously known to respond to chemokines.

SDF-1–induced platelet activation was measured by 2 different methods, platelet aggregation and Ca²⁺ flux. Moreover, a mAb to CXCR4 reduced the SDF-1 effect on platelets by ~84%, which is similar to previous inhibition studies reported with this anti-CXCR4 mAb. Pertussis toxin also inhibited SDF-1–induced aggregation, suggesting that its effect on platelets is mediated via a pertussis toxin–sensitive G protein such as Gαi. The complete inhibition of the effect of SDF-1 on platelets by wortmannin and LY29004 strongly suggests the involvement of PI-3 kinase in the initial primary phase of aggregation. In contrast, the inhibition of only the secondary wave of SDF-1–induced platelet aggregation by genistein suggests the need for tyrosine kinases to achieve maximal irreversible aggregation and platelet granule secretion after CXCR4 activation. These data are consistent with the previous reports suggesting that PI-3 kinase acts upstream of tyrosine kinases in the SDF-1 signal transduction pathway. The SDF-1–induced secondary wave of platelet aggregation was inhibited by aspirin, suggesting the involve-

Figure 5. Intracellular signal transduction pathways involved in SDF-1–induced platelet aggregation. A through D, Representative tracings of aspirin (ASA) inhibition of SDF-1–induced platelet aggregation (1 mmol/L; 2 donors, n=2) (A); genistein inhibition (100 and 200 μmol/L; 2 donors, n=2) (B); wortmannin inhibition (50, 100, and 400 nmol/L; 2 donors, n=2) (C); and LY29004 inhibition (10, 25, and 50 μmol/L; 2 donors, n=2) (D). Wortmannin at a concentration of 200 nmol/L gave the same tracing as the 100 nmol/L concentration and therefore is not shown in panel C. Vertical spikes represent the addition of aspirin or vehicle in panel A, genistein or vehicle in panel B, wortmannin or vehicle in panel C, and LY29004 or vehicle in panel D.

Figure 6. SDF-1 protein is expressed in human atherosclerotic plaques. Western blot analysis of 4 carotid artery plaques and 3 normal arteries all isolated from different individuals showing increased expression of SDF-1 in atherosclerotic plaques compared with normal vessels. rSDF-1 indicates recombinant human SDF-1 α; arrow indicates position of SDF-1. Molecular mass in kDa is indicated on the left of the blot.
Figure 7. SDF-1 protein is expressed in human atherosclerotic plaques. A, Immunoperoxidase staining of SDF-1 in a normal carotid artery and in an atherosclerotic plaque using a goat anti–SDF-1 polyclonal Ab. As a control, an adjacent section of the atherosclerotic plaque was stained with a nonimmune goat IgG. SDF-1 was not detected in the normal vessel but was detected in the plaque, whereas the control IgG did not stain the plaque. Magnification, ×100. B, Colocalization of SDF-1 in CD31+ endothelial cells (EC), α-actin+ smooth muscle cells (SMC), and CD68+ macrophages (Mø) in a representative plaque. Green or red immunofluorescence is obtained with different light polarization. Arrow indicates endothelial cells stained for SDF-1. Magnification, ×400.
ment of prostanoids in the pathway leading to irreversible aggregation. This was in contrast to the SDF-1–induced Ca\(^{2+}\) flux, which was seen even when washed platelet preparations were pretreated with aspirin.

In contrast to our findings, Kowalska et al.\(^{33}\) recently reported that \(^{125}\)I-labeled SDF-1 specifically bound to CXCR4 on platelets but failed to induce aggregation or Ca\(^{2+}\) flux. The reason for the discrepancy between our findings and those of Kowalska et al.\(^{13}\) are not readily apparent, but they may have to do with methods of platelet collection and handling. Our finding that SDF-1 induces platelet activation was seen in \(>24\) independent experiments using 13 different blood donors. We have also used 3 different recombinant SDF-1 preparations from 2 different manufacturers and have consistently observed SDF-1–induced platelet aggregation. Furthermore, we observed \(\approx 93\%\) inhibition of SDF-1 effect on platelets by preadsorption with a neutralizing SDF-1 Ab. Therefore, we believe that it is exceedingly unlikely that a contaminant in our SDF-1 preparations accounts for the activity we have observed. In addition, in our studies SDF-1 induced Ca\(^{2+}\) fluxes in washed platelets, making it unlikely that another plasma-derived factor interacting with SDF-1 was responsible for activating the platelets. Thus, we have established that SDF-1 can activate platelets.

Platelets play a critical role in hemostasis and participate in the pathophysiology of important thrombo-occlusive diseases, such as myocardial infarction, a leading cause of death, which is increasing in worldwide prevalence.\(^1\) Coronary thrombosis, the immediate cause of acute coronary syndromes, usually results from atherosclerotic plaque disruption and in situ platelet aggregation.\(^1\)–\(^3,5\) Plaque rupture or erosion is associated with vascular endothelium damage, which changes the normally antithrombotic vessel into a prothrombotic surface partly through the exposure of subendothelial structures and perhaps also as a result of a local decrease in the production of platelet antagonists, such as endothelial cell–derived nitric oxide and prostacyclin.\(^42\) Our findings that SDF-1 localizes within atherosclerotic plaques and induces platelet aggregation suggest that SDF-1 may play a role in the formation of a platelet-rich thrombus after plaque disruption. The activated platelet may also contribute to the local inflammatory response at their site of activation and may therefore contribute to the development of atherosclerosis.\(^18\)

Activated platelets release their own proinflammatory cytokines, chemokines, and lipid metabolites.\(^4,17,21\) In addition, activated platelets express the CD40 ligand and P-selectin, which induce the secretion of chemokines from endothelial cells and monocytes, respectively.\(^43,44\) These pathways serve to amplify the inflammatory response at vessel sites where platelets become activated, such as sites of vascular endothelial damage or plaque rupture. Atherosclerotic plaques also contain another chemokine, MCP-1, a potent monocyte chemotactant.\(^12,15\) Recent studies using mice deficient in MCP-1 and its receptor, CCR2, have revealed an important role for MCP-1 in the early recruitment of monocytes into the vessel wall and subsequent lipid deposition and lesion formation in murine models of atherosclerosis.\(^6,7\) However, in both mutant mouse strains, the recruitment of monocytes into nascent atherosclerotic lesions was only partially reduced, suggesting that other factors contribute to monocyte recruitment and lesion formation. In addition to the activity we have described for SDF-1 on platelets, SDF-1 is a potent chemotactic factor for T cells and monocytes\(^23\) and can arrest circulating lymphocytes.\(^24\) Because monocytes, lymphocytes, and platelets are involved in the pathogenesis of atherosclerosis, plaque rupture, and acute thrombus formation, inhibiting SDF-1-CXCR4 signaling could prove beneficial for the treatment of atherosclerosis.

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