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SDF-1α is a novel autocrine activator of platelets operating through its receptor CXCR4

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ABSTRACT

Platelets store and secrete the chemokine stromal cell-derived factor (SDF)-1α upon platelet activation, but the ability of platelet-derived SDF-1α to signal in an autocrine/paracrine manner mediating functional platelet responses relevant to thrombosis and haemostasis is unknown. We sought to explore the role of platelet-derived SDF-1α and its receptors, CXCR4 and CXCR7 in facilitating platelet activation and determine the mechanism facilitating SDF-1α-mediated regulation of platelet function. Using human washed platelets, CXCR4 inhibition, but not CXCR7 blockade significantly abrogated collagen-mediated platelet aggregation, dense granule secretion and thromboxane (Tx) A_2 production. Time-dependent release of SDF-1α from collagen-activated platelets supports a functional role for SDF-1α in this regard. Using an in vitro whole blood perfusion assay, collagen-induced thrombus formation was substantially reduced with CXCR4 inhibition. In washed platelets, recombinant SDF-1α in the range of 20–100 ng/mL^{-1} could significantly enhance platelet aggregation responses to a threshold concentration of collagen. These enhancements were completely dependent on CXCR4, but not CXCR7, which triggered TxA_2 production and dense granule secretion. Rises in cAMP were significantly blunted by SDF-1α, which could also enhance collagen-mediated Ca(2+) mobilisation, both of which were mediated by CXCR4. This potentiating effect of SDF-1α primarily required TxA_2 signalling acting upstream of dense granule secretion, whereas blockade of ADP signalling could only partially attenuate SDF-1α-induced platelet activation. Therefore, this study supports a potentially novel autocrine/paracrine role for platelet-derived SDF-1α during thrombosis and haemostasis, through a predominantly TxA_2-dependent and ADP-independent pathway.

1. Introduction

Platelet activation relevant to thrombosis and haemostasis is a finely coordinated process, involving a complex interplay of subendothelial matrix proteins (collagen, fibrinogen, vWF), localized thrombin and platelet-derived factors, in particular the secondary mediators, ADP and thromboxane (Tx) A_2, which culminate to regulate platelet signalling responses following vessel damage [1]. Moreover, numerous other bioactive molecules are present in the bloodstream or released from activated platelets, with the ability to potentiate or prime platelet activation and it's believed that these molecules pose a substantial risk to pathological thrombus formation without being significantly relevant to primary haemostasis, thus offering potential therapeutic avenues for targeting thrombotic disorders [2–4].

Despite being classically known for their chemotactic roles in leukocyte recruitment and inflammatory responses, chemokines represent an additional layer of complexity to the array of known platelet agonists. Platelets express a variety of CC and CXC chemokines and while the majority of these chemokines can support non-conventional aspects of platelet function (i.e. leukocyte recruitment, tissue regeneration) [5], certain chemokines were identified that could induce platelet activation. Macrophage-derived chemokine (MDC, CCL22) and thymus and activation-regulated chemokine (TARC, CCL17) are two CC chemokine receptor 4 (CCR4) ligands, which along with stromal cell-derived factor-1α (SDF-1α, CXCL12) were all shown to induce aggregation in platelet-rich plasma (PRP) and potentiate platelet responses to physiological agonists, ADP and thrombin [6–10]. More recently, the chemokine's CXCL16 and CX3CL1 (fractalkine) were also demonstrated to trigger platelet activation [11,12]. Notably, early evidence suggested that platelets did not express these specific chemokines and it was proposed that other vascular sources, including macrophages,

Abbreviations: SDF-1α, stromal cell-derived factor-1α; CXCR4, CXC chemokine receptor type 4; CXCR7, CXC chemokine receptor type 7; PRP, platelet-rich plasma; TxA_2, thromboxane A_2; GPCR, G-protein-coupled receptor; PGE_1, prostaglandin E_1; AR-C-66096, AR-C.

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endothelial and smooth muscle cells during vasculitis or atherosclerotic plaque rupture, could provide localized concentrations sufficient to potentiate platelet responses [6,9].

Importantly, evidence in recent years has conclusively shown that platelets express and release functional SDF-1α upon activation, whereas evidence supporting the release of platelet-derived MDC and TARC is less established [13–15]. Studies have demonstrated novel roles for platelet-derived SDF-1α in recruiting $CD34^+$ progenitor cells to arterial thrombi and supporting their differentiation to endothelial progenitor cells in vivo, to facilitate vascular remodelling and repair [16,17]. More recently, SDF-1α from activated platelets was demonstrated to be crucial for the transformation of circulating monocytes into multipotential cells with the capacity to differentiate into mesenchymal and endothelial lineages [18]. Despite these insights, the possibility of platelet-derived SDF-1α exerting autocrine/paracrine signalling effects regulating platelet activation relevant to thrombosis or haemostasis, in a manner similar to the classical secondary mediators ADP or TxA_2, has not been explored. Moreover, the mechanisms by which SDF-1α mediates enhancements of platelet function are poorly defined.

SDF-1α was originally believed to signal exclusively via the Gαi-coupled G-protein-coupled receptor (GPCR), CXC chemokine receptor type 4 (CXCR4), but more recent studies identified CXCR7 as a higher affinity receptor for SDF-1α [19–22]. Like CXCR4, CXCR7 possesses all the canonical components of GPCRs, but it is unable to activate heterotrimeric G proteins, so that the mechanisms mediating activation of intracellular signals remain controversial [23–25]. CXCR7 is believed to operate as a decoy receptor, which can internalize bound SDF-1α to regulate SDF-1α gradients necessary for optimal signals via CXCR4 [26]. Additionally, both receptors can heterodimerise to regulate SDF-1α-mediated functions [27]. Importantly, both receptors are expressed on the platelet surface and while CXCR4 appears functional, the relevance of CXCR7 to platelet physiology is only starting to be elucidated [6,28–30].

With this study, we sought to determine if SDF-1α plays a significant autocrine/paracrine signalling role regulating human platelet function through its two cognate receptors and to address the mechanism of SDF-1α-mediated regulation of platelet activation with relevance to ADP and TxA_2 signalling. Since the annucleate platelet is not amenable to genetic manipulation, we used a pharmacological inhibitor and blocking antibody approach to delineate novel signalling roles for target proteins. Our findings demonstrate that blocking CXCR4, but not CXCR7, significantly reduced collagen-mediated platelet aggregation, dense granule secretion and TxA_2 production. Consistently, in vitro collagen-induced thrombus formation required CXCR4-dependent signalling. To support a synergistic signalling mechanism between collagen and SDF-1α, co-stimulation experiments revealed that SDF-1α, via CXCR4, could substantially enhance platelet activation to threshold concentrations of collagen, through an amplification pathway which is primarily TxA_2-dependent, but ADP-independent.

2. Materials and methods

2.1. Materials

Platelet agonists used were fibrillar HORM® Collagen (Type I) of equine tendon (Nycomed, Munich, Germany) and human recombinant SDF-1α (R&D Systems Europe, Abingdon, UK). Pharmacological inhibitors; AMD3100 octahydrochloride (CXCR4), MRS-2279 ($P2Y_1$), and AR-C66096 ($P2Y_{12}$ — abbreviated AR-C) were from R&D Systems and indomethacin (cyclooxygenase) was from Sigma-Aldrich (Poole, UK). All inhibitors used were dissolved in modified HEPES-Tyrodes, with the exception of indomethacin, which was dissolved in DMSO (vehicle conc. was 0.1%). Anti-CXCR7 blocking antibody (Clone 11G8) and isotype control (Mouse IgG_1) were from R&D Systems and anti-CD32/FcγRIIA (Clone IV.3) was from StemCell Technologies (Grenoble, France). D-Phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem (Merck Chemicals, Nottingham, UK) and heparin was from Sigma-Aldrich. Unless stated, all other reagents used were from Sigma-Aldrich.

2.2. Human platelet preparation

Venous human blood from healthy, drug-free volunteers was drawn in 0.4% trisodium citrate (v/v) in accordance with the local ethics committee and informed consent. In brief, citrated blood was acidified with 12.5% (v/v) acid citrate dextrose (85 mM trisodium citrate, 71 mM citric acid, 111 mM glucose) and centrifuged at 190 ×*g* for 17 min. PRP was removed and centrifuged at 650 ×*g* for 10 min in the presence of 140 nM prostaglandin (PG) E_1 and 0.02 U mL^{-1} apyrase (Grade VII, Sigma). The resulting platelet pellet was resuspended at the required density in modified HEPES-Tyrode's buffer (10 mM HEPES, 145 mM NaCL, 1 mM $MgCl_2$, 3 mM KCL, 5 mM Glucose, pH 7.3) containing 0.02 U mL^{-1} apyrase.

2.3. Lumi-aggregometry — platelet aggregation and ATP secretion

Simultaneous monitoring of platelet aggregation and ATP secretion was performed at 37 °C with constant stirring (1000 rpm) in a Chronolog 700 aggregometer (Chronolog, Havertown, PA, USA). For all experiments involving pharmacological inhibitors, platelets (2×10^8 mL^{-1}) were pre-treated with vehicle control or inhibitors for 10 min. Also, for experiments involving functional blockade of platelet CXCR7, platelets were pre-incubated for 10 min with 10 μg/mL^{-1} IV.3 prior to a 10 min incubation with 10 μg/mL^{-1} anti-CXCR7 (11G8) or IgG_1 control to prevent non-specific platelet activation via the FcγRIIA receptor. Before agonist stimulation, 5 μL of luciferin-luciferase (Chronolog) was added. Post aggregation, 1 nM ATP standard was added as a reference value for quantification of secreted ATP. Data analysis was performed with Aggro/Link5 software (Chronolog).

2.4. Measurement of TxA_2 production

To assess TxA_2 levels, the stable metabolite TxB_2 was analysed using a commercial ELISA kit (Enzo Life Sciences, Exeter, UK). Briefly, activated platelet samples (from aggregation reactions) were quenched at 5 min with 5 mM EDTA and 200 μM indomethacin to inhibit further TxA_2 formation. Samples were centrifuged for 4 min at 12,000 ×*g*, with the supernatant removed and stored at −80 °C for subsequent TxB_2 analysis according to manufacturer's instructions.

2.5. SDF-1α ELISA

To monitor soluble SDF-1α release, washed platelets (4×10^8 mL^{-1}) were stimulated under aggregating conditions for indicated times. Samples were immediately transferred to an eppendorf containing 280 nM PGE_1 (final conc.) and subjected to 2 × pulse centrifugation steps (15 s at 12,000 ×*g*), with the final releasate stored at −80 °C for subsequent analysis by SDF-1α ELISA as per manufacturer's instructions (R&D Systems).

2.6. cAMP ELISA

For quantification of cyclic adenosine monophosphate (cAMP), washed platelets (4×10^8 mL^{-1}) were pre-treated with inhibitors and stimulated with 1 μM PGE_1 in the absence or presence of SDF-1α for 5 min at 37 °C. cAMP accumulation was terminated by the addition lysis buffer (0.5% TritonX-100) containing 0.1 M HCL to stop endogenous phosphodiesterase activity for 30 min at room temperature. Samples were clarified by centrifugation at 12,000 × *g* for 5 min with the supernatant stored for subsequent analysis by cAMP ELISA according to manufacturer's instructions (Enzo Life Sciences).