Chemokines and thrombogenicity

Michele P. Lambert1,2, Bruce S. Sachais3, M. Anna Kowalska1,4
1Division of Hematology, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania, USA; 2Department of Pediatrics, and 3Department of Pathology and Laboratory Medicine University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA; 4Center for Medical Biology, Polish Academy of Science, Lodz, Poland

Summary
Thrombosis is an important clinical entity, and pathologic thrombosis, in the form of atherosclerosis, is a major cause of morbidity and mortality. Recent research points to the role of chemokines, normally key factors in inflammation, in thrombogenesis. Many recent studies in murine transgenic and knockout models show that chemokines and their receptors are important modulators of the process of thrombus formation, particularly in atherosclerosis. Platelet-released chemokines can potentiate or inhibit thrombosis and inflammation. This review focuses on the role of chemokines in platelet activation and thrombosis, particularly as it relates to atherosclerosis. Further studies to define this complex interaction are underway.

Keywords
Chemokines, thrombosis, atherosclerosis, chemokine receptors, platelets

Introduction
Thrombosis is a complex process requiring a delicate interplay between multiple cell types and involving release and/or activation of multiple proteins. The interaction of blood cells with the vascular endothelium is key in the process of thrombus formation, and the subsequent release of proteins either potentiates or inhibits this process. Accumulating literature points to the role of chemokines, normally involved primarily in inflammation, in thrombogenesis.

Chemokines are the family of small chemotactic cytokines that are structurally related and are mostly involved in leukocyte trafficking and activation (Table 1). Amino acid sequence homology between chemokines is low (15–50%) with the exception of the conserved Cys-, Pro- and Gly- residues. However, despite this low sequence homology, there is remarkable structural homology, so that each has a central β-sheet made of three anti-parallel β-strands as well as a C-terminal β-helix. There are two major subgroups of chemokines: “CXC” where the two cysteine residues nearest the N-terminal of the protein are separated by a single amino acid and “CC” with the two cysteines adjacent. While new chemokines are still being discovered, this family of proteins (1) consists of 15 members in the CXC subfamily (which generally play a role neutrophil chemotaxis), 28 members of the CC family (which act to induce migration of monocytes, basophils, lymphocytes and eosinophils) and two additional related proteins lymphotactin (a “C” chemokine) and fractalkine (a “CX3C” chemokine). An important structural property of chemokines is their quaternary structure; chemokines exist mainly as dimers or tetramers and rarely as monomers. In addition, the CXC chemokines can be grouped by whether or not they contain the glutamic acid-leucine-arginine (ELR) motif immediately before the first cysteine. ELR-containing chemokines function mainly as neutrophil chemoattractants and activators and promote angiogenesis (2). In contrast, the function of non-ELR chemokines is not uniformly described.

There are several sources of chemokines including monocytes, macrophages and endothelial cells, but most pertinent to this review are the platelets (Table 1). Platelet α-granules contain and release several chemokines upon activation. Most abundantly released are CXCL4 (platelet factor 4, PF4) and CXCL7 (and in particular, connective tissue-activating peptide III [CTAP-III], a product of cleavage of platelet basic protein, PBP [3]). CXCL4/PF4 was actually the first chemokine to be described (4) and sequenced (5). Subsequent studies have shown that platelets also release CCL3 (macrophage inflammatory peptide, MIP-1α), CCL5 (regulated upon activation, normal T-cell expressed and secreted, RANTES), CCL7 (monocyte chemottractant protein-3, MCP-3), CCL17 (thymus and activation-regulated chemokine, TARC), CXCL1 (Gro-α), CXCL5 (epithelial neutrophil-activating protein 78, ENA78), and CXCL8 (interleukin-8, IL-8) which can act as chemoattractants or to further
activate platelets (6). Other cells, such as some endothelial cells, secrete CXCL12 (stromal-cell derived factor-1, SDF-1), CCL2 (monocyte chemotactrant protein-1, MCP-1), CXCL8/IL-8 and monocytes and macrophages secrete CCL17/TARC and CCL22/MDC (Table 1). Chemokines that either originate or target blood platelets are marked bold in this table.

Chemokines generally bind to chemokine receptors, which are G-protein-coupled integral membrane proteins that are expressed on a variety of cell surfaces. It is likely that the expression pattern of the chemokine receptor determines the specificity of the chemokine. Platelets express a number of chemokine receptors and three chemokines in particular, CXCL12/SDF-1, CCL22/MDC and CCL17/TARC, act together to activate platelets through interaction with the CXCR4 and CCR4 receptors, respectively, leading subsequently to adhesion, aggregation and secretion of platelet granule contents. In addition to the CXCR4 and CCR4 receptors, platelets express also CXCR1, CCR1 and CCR3 and CX3CR1 receptors that facilitate the binding of a number of chemokines (7).

### The role of chemokines in platelet activation

Members of both the CC and CXC chemokine families have been described as platelet activators and/or platelet “primers” allowing for synergistic activation of platelets by multiple agonists. These activities of chemokines are important in thrombogenesis as platelets play such a critical role in thrombosis and hemostasis. Platelets, and their precursor, megakaryocytes (terminally differentiated hematopoietic cells that release mature platelets into the bloodstream) express CXCR4 on their surface (27, 28) and binding of CXCR4 ligand, CXCL12/SDF-1 (29) activates megakaryocyte precursors (27, 28, 30).

### Table 1: Chemokines known to affect thrombosis or atherosclerosis.

<table>
<thead>
<tr>
<th>CC chemokines</th>
<th>Name</th>
<th>Common name</th>
<th>Cell of origin</th>
<th>Target cell</th>
<th>Thrombosis effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>MCP-1</td>
<td>Monocytes, endothelial cells,</td>
<td>Monocytes, T cells, eosinophils, basophils, <strong>platelets (weak)</strong></td>
<td>Speeds resolution of DVT (8), recruits leukocytes and initiates inflammatory response</td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α</td>
<td>T cells</td>
<td>Granulocytes, SMC</td>
<td>Expressed in atherosclerotic plaques (9)</td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1α</td>
<td>T cells</td>
<td>SMC</td>
<td>Induces tissue factor expression on SMC (10)</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>T cells, <strong>platelets</strong></td>
<td>T cells, eosinophils, basophils, NK cells</td>
<td>Promotes recruitment of monocytes on inflamed endothelium (11)</td>
<td></td>
</tr>
<tr>
<td>CCL6</td>
<td>C10, MRP-2</td>
<td>Neutrophil/macrophage lineage</td>
<td>Myeloid cells</td>
<td>Stimulates MCP-1 and MIP-1α generation by monocytes (12)</td>
<td></td>
</tr>
<tr>
<td>CCL7</td>
<td>MARC, MCP-3</td>
<td><strong>Platelets</strong>, SMC</td>
<td>Monocytes, macrophages, SMC</td>
<td>Activates SMC (13)</td>
<td></td>
</tr>
<tr>
<td>CCL17</td>
<td>TARC, dendrokin, ABCD-2</td>
<td>Monocytes, macrophages, <strong>platelets</strong></td>
<td>T cells, <strong>platelets</strong>, dendritic cells</td>
<td>Induces platelet activation (14-16)</td>
<td></td>
</tr>
<tr>
<td>CCL18</td>
<td>PARC, DC-CX1, AMAC-1, MIP-4</td>
<td>Macrophages, SMC</td>
<td>T cells, other lymphocytes</td>
<td>Attract lymphocytes (17)</td>
<td></td>
</tr>
<tr>
<td>CCL19</td>
<td>ELC, Exodus-3, MIP-3B</td>
<td>Dendritic cell</td>
<td>Monocytes (?)</td>
<td>Found within atherosclerotic plaques (17, 18)</td>
<td></td>
</tr>
<tr>
<td>CCL22</td>
<td>MDC</td>
<td>Dendritic cells and macrophages</td>
<td>T cells, <strong>platelets</strong>, monocytes, dendritic cells, NK cells</td>
<td>Induces platelet activation (14-16, 19)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CXC chemokines</th>
<th>Name</th>
<th>Common name</th>
<th>Cell of origin</th>
<th>Target cell</th>
<th>Thrombosis effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>Gro-α, GRO1, NAP-3</td>
<td><strong>Platelets</strong>, endothelial cells</td>
<td>Neutrophils, monocytes</td>
<td>Monocyte arrest/attenuation (20)</td>
<td></td>
</tr>
<tr>
<td>CXCL4</td>
<td>PF4</td>
<td><strong>Platelets</strong>, monocytes</td>
<td><strong>Platelets</strong>, endothelial cells</td>
<td>Stimulates APC generation (21), activate neutrophils in presence of TNF-α (22)</td>
<td></td>
</tr>
<tr>
<td>CXCL5</td>
<td>ENA-78</td>
<td><strong>Platelet</strong>, eosinophils</td>
<td>Neutrophils, endothelial cells</td>
<td>Monocyte arrest on vascular endothelium (23)</td>
<td></td>
</tr>
<tr>
<td>CXCL7</td>
<td>NAP-2, CTAPIII, β-TG, PBP</td>
<td><strong>Platelets</strong>, monocytes</td>
<td>Neutrophils</td>
<td>Recruits neutrophils, mobilizes calcium (24, 25)</td>
<td></td>
</tr>
<tr>
<td>CXCL8</td>
<td>IL-8, NAP-1, MDNCF, GCP-1</td>
<td>Endothelial cells, monocytes, <strong>platelets</strong></td>
<td>Macrophages</td>
<td>Speeds resolution of DVT (26)</td>
<td></td>
</tr>
<tr>
<td>CXCL12</td>
<td>SDF-1, PBSF</td>
<td>Bone marrow stromal and some endothelial cells</td>
<td><strong>Platelets</strong>, lymphocytes, megakaryocytes</td>
<td>Activates megakaryocyte precursors, co-stimulate platelet activation (15, 16, 19, 27)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CX,C chemokines</th>
<th>Name</th>
<th>Cell of origin</th>
<th>Target cell</th>
<th>Thrombosis effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX3CL1</td>
<td>Fractalkine</td>
<td>Endothelial cells</td>
<td>Endothelial cells, <strong>platelets</strong>, monocytes</td>
<td>Induces platelet activation, found within atherosclerotic lesions (19)</td>
</tr>
</tbody>
</table>
shown that CXCL12/SDF-1 alone did not have any effect on aggregation of platelets (27). However, CXCL12/SDF-1 binding to the CXCR4 receptor stimulated the G_α_i pathway and inhibited adenylyl cyclase activity (19) (Fig. 1). Importantly, CCR4, the receptor for CCL22/MDC (31) and CCL17/TARC (32) is also present on platelet surfaces (7, 19) but couples to a different G protein, perhaps G_α_q, and stimulates calcium mobilization (19) (Fig. 1). In our studies, both CCL22/MDC and CCL17/TARC synergized with subthreshold doses of CXCL12/SDF-1 to enhance platelet activation, acting through these two different activation pathways (Fig. 1). Low concentrations of ADP can potentiate this effect. Subsequent studies have shown that CCL22/MDC and CCL17/TARC can activate platelet aggregation under arterial flow conditions (16) and that this process depends strongly on the presence of low levels of primary agonists such as ADP or thrombin (14–16). Additionally, CXCL12/SDF-1 has been shown to have similar effects (33), and CCL5/RANTES may modulate the response of platelet to CXCL12/SDF-1 (34). CXCL12/SDF-1 and CCL22/MDC can also stimulate platelet adhesion to immobilized collagen or fibrinogen (16). CX3CL1/fractalkine (35) has also been reported to activate platelets in concert with other weak agonists, such as ADP (36) or perhaps other chemokines such as CXCL12/SDF-1, CCL22/MDC or CCL17/TARC. CX3CL1/fractalkine mediated activation occurs through a G-protein-coupled intracellular signaling cascade, most probable to Gi protein, as pertussis toxin completely prevented its action (36). Presented in Figure 1 is a chemokine-platelet activation model. The activation pathways include binding of chemokines to seven-transmembrane domain receptors and activation of G-proteins that generate second messengers, which lead to activation of platelet responses. Aggregation of platelets in turn leads to release of platelet α-granule proteins, which are rich in other chemokines. Released chemokines, including CCL5/RANTES, CCL17/TARC, CXCL8/IL-8 and CCL22/MDC, can feedback on platelets to cause further aggregation. These platelet chemokines may also recruit additional leukocytes to the region. Thus, it is clear that chemokines can potentiate and expand the platelet response to increase thrombus formation.

**The role of platelets and platelet-specific chemokines in thrombosis**

Concentrations of chemokines, stored in platelet α-granules, vary widely for different chemokines. Two closely related chemokines, CXCL4/PF4 and CXCL7/PBP/CTAP-III/NAP-2/-β-thromboglobulin are abundant platelet α-granule chemokines released during the process of platelet activation (37–39). The biosynthesis of CXCL4/PF4 and CXCL7 proteins is almost exclusively limited to megakaryocytes. In platelets, these chemokines account for >5% of the total α-granular content on a molar basis. Interestingly, very low levels of CXCL4/PF4 and CXCL7 have recently been reported in activated human monocytes (40, 41). Concentrations of CXCL4/PF4 and CXCL7 in serum are 0.4–2 μM and 1.6–4.8 μM, respectively (42), while concentrations of these chemokines in plasma are 1,000 times lower, suggesting that the concentration in platelets exceeds 100 μM. The concentrations of CXCL4/PF4 tetramers in the vicinity of platelet activation have been estimated to be greater than 280 μM (43), which is over 100-fold greater than levels present in serum.

The CXCL4/PF4 and PBP genes are only ~5 kb apart in both the human and murine genome (44) and they have ~40 amino acid (aa) homology (45). Mature human CXCL4/PF4 is a 7.8 kDa protein that contains 70 aa residues and exists at physiologic pH and tonicity as a tetramer with high affinity for heparin (38). The PF4 gene is duplicated and the expression of the second gene product, PF4_α, is about 1–10% of normal PF4. This variant PF4 has three amino acid substitutions in its C-terminus, a region critical for PF4-heparin interaction (46). Expressed PF4_α has lower affinity to heparin in comparison to PF4 and

**Figure 1**: Activation of platelets by chemokines. Four main chemokines that activate platelets are shown. CCR22/MDC and CCL17/TARC interact with CCR4 receptor that may bind to G_α_q protein and thus stimulate intracellular calcium mobilization. CXCL12/SDF-1 on the other hand acts through Gi proteins as it inhibits adenylyl cyclase. ADP stimulates two G-protein coupled receptors bound to either G_α_q or Gi, and additionally ATP secretion can contribute to activation of platelets through ligand-gated ion channel, thus stimulation by either chemokine can be potentiated by ADP. In addition, CX3CL1 (the receptor for CX3CL1/factalkine) is most probably linked to Gi protein as its action is inhibited by pertussis toxin.
was found to be more effective than PF4 in blocking angiogenesis in vivo (47).

Human PBP is a 10.3 kDa protein that contains 93 aa residues with a lower affinity to heparin as compared to CXCL4/PF4. PBP is converted by limited N-terminal proteolysis to connective tissue activating peptide-III (CTAP-III) (48), and further N-terminal cleavage of CTAP-III results in formation of β-thromboglobulin (48) and neutrophil-activating peptide (NAP)-2 (24, 25, 49). CXCL7/NAP-2 can be generated from CTAP-III by several tissue proteases such as chymotrypsin, cathepsin G, and trypsin (50). Cathepsin G is likely to be involved in this process in vivo, since it is present in primary granules of neutrophils (51) and monocytes (52). Neither PBP nor β-thromboglobulin is a strong activator of neutrophils. However, human CXCL7/NAP-2, in contrast to its precursors, is a potent activator of neutrophils, and stimulates neutrophil chemotaxis, Ca²⁺ mobilization and exocytosis (24, 25). Full-length PBP lacks potent heparin-neutralizing activity, but it has been shown to down-modulate increases in the secretion of the lipid prostaglandin I₂ (54), suggesting that it may have a pro-coagulant activity (53).

CXCL4/PF4 has been reported to have a number of biologic activities in vitro, including potential roles in thrombosis and inflammation. There have been a number of in-vitro studies of the role of CXCL4/PF4 in coagulation, many of which suggested opposing effects. For example, CXCL4/PF4 has been suggested to strongly influence thrombus formation. However, studies conflict as whether this influence results in increased fibrin fiber mass-length ratio and therefore increased turbidity of clots and thicker fibers (54, 55), or decreased turbidity of clots and thinner fibers (56). As another example, CXCL4/PF4 inhibited the heparin-dependent acceleration of thrombin inactivation by antithrombin (57) and potentiated platelet aggregation in the presence of suboptimal concentrations of agonists (58), suggesting a procoagulant function. In contrast, CXCL4/PF4 plays a role in activation of protein C (PC). CXCL4/PF4, unlike other cationic proteins that impair thrombomodulin (TM) anticoagulant function, stimulates TM-mediated generation of activated PC (APC) by as much as 25-fold (21). CXCL4/PF4 binds in a calcium-independent manner to the extracellular domain of TM that expresses glycosaminoglycans (GAG+TM) but not to glycosaminoglycans without TM (GAG-TM). Further, CXCL4/PF4 binding to PC requires its anionic γ-carboxyglutamic acid (Gla) domain. This stimulation of TM function was specific for CXCL4/PF4 because it did not occur in the presence of the two other major platelet α-granule heparin-binding proteins, thrombospondin and β-thromboglobulin. As was the case with eosinophilic cationic proteins, the CXCL4/PF4 interaction with TM appeared to be electrostatic because it was also reversible by polyanionic GAGs, such as heparin (59). CXCL4/PF4 potently stimulates generation of APC in vivo after infusion of recombinant human CXCL4/PF4 into primates (60). In vivo studies by our group showed, by using knockout PF4 mice (mPF4⁻) and human overexpressor mice (hPF4⁺) that overexpress five to six times more human PF4 than concurrently studied human controls, that endogenously released CXCL4/PF4 increased the generation of APC that may lead to impaired coagulation and increased thrombolysis. (Kowalska et al., unpublished data).

CXCL4/PF4 does not possess significant chemotactic activity for neutrophils in comparison with the other chemokines, such as CXCL7/NAP-2 or CXCL8/IL-8 (61). However, in the presence of a co-stimulus such as tumor necrosis factor (TNF)-α, CXCL4/PF4 can activate neutrophils, cause exocytosis and promote neutrophil adhesion to protein matrices or endothelium (22). CXCL4/PF4 was also found to facilitate macrophage differentiation during the inflammatory process (62, 63).

CXCL4/PF4 has high affinity to heparin and to cell surface glycosaminoglycans (GAGs) (64). For this reason, the half-life of CXCL4/PF4 after infusion is only 17 minutes, but increases to 30 minutes if it is injected simultaneously with heparin (65). Unlike other chemokines, for which specific, high-affinity G-protein-coupled receptors have been characterized, there is only a single report suggesting that, in some endothelial cell lines, an alternatively spliced form of CXCR3B binds CXCL4/PF4 with high affinity (K_D = 4nM) (66). CXCL4/PF4 is released at sites of injury at concentrations >10⁻⁵-fold higher than that of the K_D for the putative CXCR3B receptor, and the in-vitro effects ascribed to CXCL4/PF4 have been shown at that higher concentration. Therefore, a biological role for this high-affinity receptor has yet to be determined. Recent reports have also shown that CXCL4/PF4 may activate endothelial cell E-selectin expression in an nuclear factor (NF)-κB dependent fashion by signaling through the LRP-1 receptor on endothelial cells (67). In addition, we have reported high affinity binding of CXCL4/PF4 to the LDL receptor, localizing binding to the LDL ligand binding domain region of the receptor (68). PF4 binds to low-density lipoprotein receptors and disrupts the endocytic itinerary, resulting in retention of low-density lipoprotein on the cell surface (68).

Further exploration of the in-vivo relevance of these findings is underway.

In contrast, human CXCL7/NAP-2 has a well-defined chemokine receptor, CXCR2 (69). This receptor can be stimulated as well by CXCL8/IL-8, which binds to both CXCR1 and CXCR2 in humans (69, 70). Thus, while CXCL7/NAP-2 activates neutrophils, it cannot desensitize these cells to CXCL8/IL-8, but CXCL8/IL-8 can desensitize cells to CXCL7/NAP-2 (70, 71). Human CXCR1 and CXCR2 are closely related (72), but in the mouse there is only one functional receptor that is the likely homologue of CXCR2, but not CXCR1 (73). Also, CXCL8/IL-8 has not been identified in mice, but the murine chemokine KC (N51) is accepted as a homologue of human CXCL1/GRO-α, which has 67% identity with CXCL8/IL-8. KC contains an N-terminal ELR sequence like CXCL1/GROα and CXCL7/NAP-2, and binds to the human CXCR2 receptor (74). A recent report demonstrates that there may be a mCXCR1, which can functionally bind hCXCL8/IL-8 and human and mouse CXCL6/GPC-2 but not KC (75).

The role of CXCL4/PF4 in thrombus formation – animal studies

The role of CXCL4/PF4 in thrombus formation was studied in our laboratory using the FeCl₃ injury model (76). This model, which depends on the development of a platelet-rich thrombus, may provide a more sensitive and relevant measure of the effects of CXCL4/PF4 during thrombosis. Our data show that thrombus formation and stability is maximal at physiological levels of
CXCL4/PF4; fewer occlusive thrombi and impaired thrombus stability were observed when platelet CXCL4/PF4 levels fell by even 50% or when they exceeded the normal level by less than four-fold. This conclusion was confirmed by studies in which infusion of recombinant hPF4 into mPF4−/− mice maximized thrombus formation and stability only over a very narrow range of concentrations (76).

We propose a simple model to explain the role of CXCL4/PF4 in thrombosis, accounting for our observations after infusions of unfractionated high-molecular-weight heparin, low-molecular-weight heparin (LMWH) and protamine sulfate (76). This model is based on the known high affinity of CXCL4/PF4 for negatively charged molecules such as heparin and heparan-sulfate glycoproteins that are expressed on cell surfaces. We propose that the release of CXCL4/PF4 in large molar amounts neutralizes the negatively charged surfaces of platelets and endothelial cells, allowing closer approximation of platelets to each other and to the endothelial lining, thereby enhancing thrombus formation. The existence of electric field differences between the endothelial lining and flowing blood has been previously recognized (77). When insufficient CXCL4/PF4 is released, cell surfaces may retain sufficient negative charges to prevent optimal approximation of platelets and endothelial cells. Conversely, release of excessive amounts of CXCL4/PF4 may cause cell surfaces to become too positively charged for optimal cell-cell approximation (Fig. 2). Our studies with infusions of heparin and protamine sulfate in the setting of FeCl3-induced carotid arterial injury are consistent with this model (76). The development of thrombi in both mPF4−/− and mPF4+/− mice was especially sensitive to heparin infusion, i.e. thrombus development was prevented at lower doses of heparin than was required in wild-type animals. We postulate that less heparin is needed to neutralize the CXCL4/PF4 released in these PF4-deficient animals. More telling, infusion of comparable amounts of heparin enhanced thrombus formation in the hPF4−/+ over-expressors by neutralizing excess CXCL4/PF4. The converse was observed with protamine sulfate infusions, a positively-charged molecule that, similar to CXCL4/PF4, neutralizes large negatively charged molecules such as heparin (78). Protamine sulfate appears to be able to substitute for CXCL4/PF4 to enhance thrombus formation in the mPF4−/− mice. Consistent with the proposed model, hPF4+ mice are particularly sensitive to the anti-thrombotic effect of protamine sulfate. The absence of a comparable paradoxical effect of LMWH infusion in hPF4 mice is consistent with the proposed model as LMWH has a decreased binding affinity for CXCL4/PF4 (79).

Although the results of our studies are consistent with the proposed model, they may also be consistent with other models. CXCL4/PF4 has been reported not only to inhibit anti-thrombin III activation by heparin (prothrombotic) (80), but also to enhance the activation of protein C (antithrombotic) (21, 59). If these and perhaps other undefined mechanisms are of biological relevance, then these competing functions of CXCL4/PF4 may help explain why CXCL4/PF4 promotes clot formation only over a narrow range of concentrations and why both higher or lower CXCL4/PF4 concentrations have similar biological outcomes.

**Chemokines and thrombus resolution**

Interesting recent data have suggested that chemokines not only potentiate platelet activation and act as chemoattractants to increase thrombus formation, but that they also play a key regulatory role in thrombus resolution. In a mouse model, deletion of the chemokine receptor CCR2 impairs the animals’ ability to resolve thrombosis (81). In addition, administration of exogenous CXCL8/IL-8 (26) and CCL2/MCP-1 (8) can speed resolution of deep venous thrombosis in a rat model. CCR2 receptor deletion may represent a similar mechanism to CCL2/MCP-1 infusion as CCR2 is the receptor for CCL2/MCP-1, but CXCL8/IL-8 binds CXCR1 and CXCR2, suggesting this improvement in clot resolution occurs by a different mechanism. Disruption of CCR2 (82), CXCRR2 (83) and CXCR3 (84) have all been shown to decrease atherogenesis in mouse knockout models. A recent study also showed that CCR5 knockout mice were relatively protected from atherosclerosis when crossed with ApoE knockout mice and fed a high fat diet. In contrast, mice that were double knockouts for CCR1 and ApoE showed increased propensity to atherosclerosis (85). These findings emphasize the complex interactions of multiple chemokines in thrombosis and athero-

![Figure 2: Charge neutralization model of CXCL4/PF4 in thrombosis. Model of two cells with normal negatively charged surfaces and with less than optimal approximation and thrombus formation (left). Following CXCL4/PF4 release, the surface charge is partially neutralized, allowing better cell approximation and thrombus development (center). However, if there is excess CXCL4/PF4 release, too much of the surface charge is neutralized and the cells are relatively positively charged and again do not approximate to allow optimal thrombosis (right).]
sclerosis.

Platelets, platelet-specific chemokines, and atherosclerosis

Atherosclerosis is one of the most studied and clinically relevant vascular disorders in medicine and science. Consequences of atherosclerosis, typically caused by thrombotic complications of the disease, include heart disease and stroke and account for significant morbidity and mortality. The presence of platelet antigens in atherosclerotic lesions (86–88) raises the possibility that persistent platelet activation occurring over the course of years to decades may contribute to the progression of atherosclerosis itself. It has been known for many years that activated platelets release mediators that promote smooth muscle cell proliferation (87, 89–92) and the accumulation of lipoproteins in vascular cells and macrophages (87, 91, 93, 94). CD40 ligand (CD154) is present on multiple cell types including activated T-lymphocytes, macrophages, vascular smooth muscle cells and endothelial cell in atherosclerotic plaques and appears to be important in progression of atherosclerotic plaques (95). Platelet CD40 ligand on activated platelets facilitates endothelial cell activation that leads to secretion of the chemokines CCL2/MCP-1 and CXCL8/IL-8 (96) to recruit leukocytes and initiate inflammatory responses. On the other hand, platelet delivered CCL5/RANTES promotes recruitment of monocytes on inflamed endothelium and interaction of CCL5/RANTES with CXCL4/PF4 is important in this process (11, 97).

It has been demonstrated that the absence of P-selectin (which is important for platelet-endothelial interactions [98]) protects against development of atherosclerotic lesions in both LDL receptor (99) and apoE (100–102) knockout mice, especially early in lesion development (99). Importantly, studies in apoE-deficient mice fed a Western diet demonstrated that platelet adhesion to atherosclerosis prone regions of the carotid artery precedes both leukocyte adhesion and visible lesion formation, and blockade of platelet adhesion receptors with either α-glycoprotein (GP) IIb-IIIa or α-GPib decreased adhesion and leukocyte recruitment to these regions and decreased lesion size (103). Such studies provide direct evidence that platelet adhesion to the vasculature is important for early lesion formation.

Activated platelets and platelet-leukocyte aggregates interact with atherosclerotic lesions in apoE-deficient mice (104). In both apoE-deficient mice and in isolated cell systems, platelet-derived chemokines (CCL5/RANTES and CXCL4/PF4) are delivered to endothelial cells and macrophages by activated platelets in a P-selectin dependent manner (104–106). These data help to explain how CXCL4/PF4 and other platelet derived chemokines enter atherosclerotic lesions (11, 86, 87, 97), and suggest that the contribution of platelets in the atherogenic process may begin at an earlier stage than previously appreciated.

Our laboratory has demonstrated that CXCL4/PF4 is localized to fatty streaks and atherosclerotic lesions in humans (86). Further, lesional CXCL4/PF4 correlated with histological and clinical severity of disease. We have also reported on several potentially proatherogenic properties of CXCL4/PF4, including effects on lipoprotein trafficking (107, 108) and activation of NF-κB (67). In order to directly assess the role of CXCL4/PF4 in the formation of atherosclerotic lesions, we have recently examined the effects of knocking out CXCL4/PF4 expression in two established murine models of atherosclerosis. Results from these studies demonstrate a significant decrease in atherosclerotic lesion formation in the absence of CXCL4/PF4, providing direct evidence that this platelet specific chemokine is proatherogenic (Sachais et al., unpublished data). Data from these knockout mice support our in-vitro findings and provide a model in which to examine the in-vivo mechanisms of CXCL4/PF4 atherogenicity.

In addition, many other chemokines have been localized within atherosclerotic plaques including CCL2/MCP-1, CCL3/MIP-1α and CCL4/MIP-1α, CCL5/RANTES, CCL17/TARC, CCL18/PARC, CCL22/MDC, CCL19/ELC, CCL1/309, CXCL8/IL-8, CXCL1/GRO-α, CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, CXCL12/SDF-1 and CXCL3/Fractalkine (see Zerneck and Weber [18] for review). CCL2/MCP-1 is found within atherosclerotic plaques in humans in macrophage-rich areas and in organized thrombi (109) and was one of the first chemokines described in human carotid endarterectomy specimens. The expression of CCL2/MCP-1 by monocytes within atherosclerotic plaques appears to be related to the process of atherogenesis as macrophages from normal arteries do not express CCL2/MCP-1 (110). In mice, chemokine KC (homologue of human CXCL1/GRO-α) triggers arrest of monocytes in early atherosclerotic endothelium (111). CCL3/MIP-1α and CCL4/MIP-1α and CCL5/RANTES are expressed by activated T-cells within advanced atherosclerotic lesions (112) and CCL5/RANTES interacting with CXCL4/PF4 facilitates monocyte arrest on endothelium (97). CCL4/MIP-1α was also shown to bind to CCR5 on SMC (smooth muscle cells) and induce tissue factor expression (10). CCL18/PARC is highly expressed by CD68+ macrophages and ELC is expressed by both macrophages and SMC and may play a role in attracting T lymphocytes into atherosclerotic lesions (17). These are just a few examples, and what has become clearer with the passage of time is that chemokines play an important role not only in recruitment of cells to atherosclerotic plaques, but also potentiate the inflammatory and procoagulant response in that region.

Conclusions

Recent advances in our understanding of the roles of chemokines have significantly expanded our knowledge of the molecular biology of thrombotic vascular diseases, including the specific example of atherosclerosis. Many transgenic and gene knockout studies in murine models show that chemokines and their receptors are important in this processes. Genetic evidence for the role of chemokines and their receptors in the human population is under investigation (113). Identifying chemokine polymorphisms (for example, in CX3CR1 [114]) could also help to determine pathways that are important in the pathology of atherosclerosis. Continuing research focusing on targeted disruption of one or more aspects of this very complex interaction will also allow the development of targeted therapies to help prevent sig-
Chemokines in vascular biology

References


26. Marcum JA, McKeeney JB, Rosenberg RD. Acceleration of thrombin-antithrombin complex formation