Interaction of PF4 (CXCL4) with the vasculature:
A role in atherosclerosis and angiogenesis

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Summary
Platelet factor-4 (PF4), a platelet-derived chemokine, has two important functions in the vasculature. It has a pro-atherogenic role while also having anti-angiogenic effects. The activity of platelet factor-4 (PF4), unlike other chemokines that bind to specific receptors, depends on its unusually high affinity for proteoglycans and other negatively charged molecules. High affinity for heparan sulfates was thought to be central to all of PF4’s biological functions. However, other mechanisms have been described such as direct growth factor binding, activation of the CXCR3B chemokine receptor isoform that is present in some vascular cells or binding to lipoprotein-related protein-1 (LRP1). Furthermore, PF4 also binds to integrins with affinities similar to matrix molecules. These interactions may explain the effects of PF4 in healthy and pathological tissues. However, the mechanisms involved in PF4’s activity are complex and may depend on a given tissue or localisation. Overall, while much is already known about PF4, its specific role in atherosclerosis and angiogenesis remains still to be clarified.

Keywords
PF4 (CXCL4), CXC chemokine, integrins, angiogenesis, atherosclerosis

Introduction
Platelet factor 4 (PF4 or CXCL4) is a CXC-chemokine that is predominantly synthesised in megakaryocytes, sequestered in platelet-granules, and released upon platelet activation (1). PF4 is released in high concentration at injury sites, where it can reach 25 μM (2). PF4 binds with unusually high affinity to heparin and other glycosaminoglycans (GAG), which led to its early isolation and sequencing (3, 4). It plays an important role in the direct regulation of several cellular functions such as thrombosis (5, 6) megakaryocytopoiesis (7–9), immune modulation (10), angiogenesis (11), and atherosclerosis (12). The majority of these functions involves interactions with heparan sulfates; however, some of them involve GAG-independent mechanisms. For example, as we will discuss in this review, the effect of PF4 on atherosclerotic plaque development is at least partially dependent on GAG, whereas the anti-angiogenic effects involve, besides GAGs, direct growth factor binding, activation of chemokine receptors or integrin binding.

Another form of PF4, named PF4 alt or PF4V1 (CXCL4L1) was described in 1989, but only recently became the focus of research (13, 14, 15, 16). PF4V1 (CXCL4L1) is a variant that has been isolated from thrombin-stimulated human platelets and exhibits a potent effect in blocking angiogenesis in vitro and in vivo (14–17). PF4V1 shows only 4.3% amino acid divergence in the mature protein which contains three amino acid substitutions in the COOH-terminus, a region known to be critical for PF4-heparin interactions (14). In addition, PF4 and PF4V1 exhibit a 38% amino acid divergence in the signal peptide region and present distinct subcellular localisations. In contrast to PF4, which is stored and released upon activation, PF4V1 appears to be secreted through a constitutive pathway (15).

In this review, we will focus exclusively on PF4 due to the significant existing body of data on this chemokine. In the first part of this review, we will summarise the role of PF4 in atherosclerosis. We will then focus on the potential role of PF4 in angiogenesis and describe some recent findings on the mechanisms involved in angiogenesis inhibition.

PF4 and atherosclerosis
It has been shown for many years that platelets contribute to the progression of atherosclerosis. Evidence for the particular importance of platelets in human atherosclerosis has been obtained from a conclusive prospective study that found an association between
platelet concentrations, ability to aggregate and long-term incidence of fatal coronary heart disease in a population of apparently healthy middle-aged men (18). A direct role for platelets in the initiation of atherosclerotic lesions has been demonstrated in several experimental models. For example, in apolipoprotein E-deficient (ApoE-/-) mice fed with a Western diet, platelet adhesion to regions of the carotid artery prone to atherosclerosis precedes both leukocyte adhesion and the formation of visible lesions (19). Blockade of platelet adhesion using either glycoprotein (GP)IIb-IIIa or GPIb decreased platelet adhesion, leukocyte recruitment and lesion size. Furthermore, activated platelets and platelet-leukocyte aggregates interact with atherosclerotic vessels in ApoE-deficient mice (20). In both ApoE-deficient mice and in isolated cell systems, platelet-derived chemokines (PF4 and RANTES) are delivered to endothelial cells and macrophages by activated platelets (20). These effects require platelet activation and are dependent on P-selectin. Overall, these studies provide direct evidence that platelet adhesion to the vasculature is important for early lesion formation and help to explain how PF4 and other platelet-derived chemokines contribute to atherosclerotic lesions (21, 22).

The fact that PF4 is one of the most abundant proteins released and rapidly mobilized after platelet activation, places this chemokine as a first-line mediator in the control of early events in thrombosis or plaque formation (5, 6). PF4 is deposited early in the development of atherosclerotic lesions and continues to accumulate in foam cells as lesions advance (22). Presence of PF4 within atherosclerotic lesions was associated with clinical parameters including lesion grade and presence of symptoms (22). This suggests an important role of this chemokine in the pathogenesis of atherosclerosis.

Different mechanisms by which PF4 may enhance the development of atherosclerosis are described in the literature. PF4 can promote the recruitment of peripheral blood monocytes toward lesions facilitating their differentiation into macrophages (23, 24). Indeed, PF4 may induce the differentiation of monocytes into macrophages by preventing monocyte apoptosis and by mediating up-regulation of differentiation markers (23). Recent data showed that sphingosine kinase 1 is involved in the survival of PF4-induced monocytes and suggest that this kinase plays a key role in the pro-inflammatory responses triggered by PF4 in human monocytes (25). Furthermore, it was shown that PF4 stimulates the differentiation of monocytes into a specific subtype of macrophages lacking HLA-DR on their surface (23). Gleissner et al. also showed that PF4-induced pro-inflammatory macrophages, with a phenotype that lack CD163 expression, are found in atherosclerosis lesions in vivo (26). CD163 down-regulation by PF4 requires binding to GAG present on the cell surface of monocytes and macrophages (26, 27). Later, the same group found that PF4 induces not only changes in the macrophage transcriptome that shares similarities with the classical pro-inflammatory macrophage phenotypes (M1 and M2 macrophages) but also induces changes that reflect a unique macrophage phenotype named M4 (28). These authors postulated that M4 macrophage phenotypes are present in human atherosclerotic lesions. The role of the M4 macrophage phenotype, however, is not clear and may be pro- or anti-atherogenic (28). PF4 can also cooperate with RANTES to trigger monocyte arrest on inflamed endothelium (20, 29). The amplification of monocyte arrest requires structural motifs important in RANTES oligomerisation and the presence of chondroitin sulphate (30, 31). The structural requirements of RANTES-oligomerisation and GAG binding are similar to those that determine interaction with PF4. While a tetrameric mutant of RANTES with impaired PF4 binding caused monocyte arrest, no synergy with PF4 was observed. This indicates that PF4 enhances monocyte arrest induced by RANTES through the formation of heteromers (30, 31). In light of the above research, chemokine heteromerisation has emerged as an additional regulatory mechanism (30–34). In line with this idea, the selective disruption of RANTES-PF4 interactions has been shown to attenuate monocyte recruitment and inhibit atherosclerosis in hyperlipidemic mice (35). On the other hand, PF4 may enhance the development of atherosclerosis by binding to the LDL-R, thus inhibiting the degradation of LDL-R and facilitating LDL oxidation (36). Indeed, PF4 inhibits binding of native LDL to its receptor and subsequent internalisation, thereby, potentially promoting LDL oxidation (37). The ability of PF4 to inhibit binding and degradation of LDL is dependent on the cell surface GAG (36, 37). Furthermore, PF4 can directly bind to oxidised LDL and mediates its binding to vascular cells and macrophages (37), which again depends on the presence of proteoglycans such as chondroitin sulphate, and enhances the esterification of oxidised LDL in macrophages (37). Finally, deletion of the PF4 gene encoding PF4 results in reduced lesion size in atherosclerotic ApoE-/- mice, thus showing that PF4 has a net proatherogenic effect (12).

In light of the above research, the following schema for the role of PF4 in atherosclerosis can be put forward: PF4 is first released by activated platelets or vascular cells in the vicinity of the atherosclerotic lesion, then deposited on the endothelial cell (EC) surface and retained by endothelial GAG and/or additional binding molecules. It promotes atherosclerosis by recruiting and arresting peripheral monocytes at the lesion site facilitating their differentiation into macrophages, inhibiting the degradation of LDL-R and increasing the uptake and esterification of ox-LDL in macrophages (Fig. 1). Overall, a growing body of evidence indicates that PF4 plays an important role for the development of atherosclerosis and suggests that PF4 can be a target for atheroprotective therapeutic strategies.

**PF4 and angiogenesis**

Angiogenesis plays an important role in physiologic processes such as wound healing and in disease progression such as in cancer, diabetic retinopathy and various inflammatory disorders (38). The expansion of solid tumours and other cancers critically depends on angiogenesis (39) making anti-angiogenesis strategies relevant for cancer therapy (40) and PF4 was one of the first agents revealed to inhibit angiogenesis in vitro and in ex vivo systems (41). At the molecular level, angiogenesis is governed by pro-angiogenic factors, such as VEGF or FGF, cell adhesion molecules and integrins.
An angiogenic stimulus by growth factors induces increased integrin expression in endothelial cells undergoing angiogenesis, thereby leading cells to spread, to migrate and to ultimately form new vessels (42–44). It was shown that angiogenesis inhibitors could interfere with these different events to exert their anti-angiogenic effect (40, 45). PF4 and a peptide derived from its carboxyl-terminal domain (PF4(47–70)) display potent anti-angiogenic activity in vitro (41, 46–47) and in vivo (41, 47–48). They suppress growth of various tumours (49–51) and metastasis (52) in vivo. This effect is related to their anti-angiogenic action and not to tumour cell proliferation (47, 48–50). In contrast to the anti-tumour effects described for recombinant molecules or fragments, the role of endogenous PF4 in tumour development has not yet been demonstrated using PF4 -/- mice (53).

Although PF4 is one of the first agents discovered to have an anti-angiogenic action in ex vivo systems (41), the specific receptor mechanisms that transduce the anti-angiogenic signal of PF4 are still not completely understood. Different mechanisms for the anti-angiogenic effect of PF4 have been proposed. These include: i) direct interaction with angiogenesis factors (54), ii) competition of the cooperative effect of heparan sulfate on growth factor binding (55–57), iii) activation of CXCR3-B receptors (58), iv) interaction with LRP1 (59) or v) integrin binding (60).

Interaction with growth factors

We have previously shown that PF4 is able to directly interact with pro-angiogenic factors such as FGFs and to inhibit their dimerisation required for receptor activation (54–57). We have also established the binding domain by which PF4 interacts with FGF-2. We used nuclear magnetic resonance (NMR) spectroscopy to demonstrate direct interactions of the C-terminal sequence of PF4 (47–70) with FGF-2 and characterised the residues mainly involved in the contact area. Altogether, the NMR data describing PF4/FGF-2 interactions point to a major role of the hydrophobic contributions of the C-terminal region of PF4, mediated by Y60 and leucines and/or isoleucine residues, in addition to specific contacts established by the N-terminal region through the cysteine side chain.

The question remains whether this interaction could represent the basis of the inhibition of FGF-2 biological activity evidenced in different assays (61). Binding studies with 125I-FGF-2 in bovine capillary endothelial cells showed a PF4(47–70) concentration-dependent inhibition of FGF-2 binding to the receptor. This is in line with the inhibition of FGF-2-induced endothelial cell proliferation, migration and tube formation in vitro or ex vivo in the rat aortic ring assay. Furthermore, PF4(47–70) inhibited FGF-2-induced signalling and strongly inhibited the rate of 125I-FGF-2 internalisation in bovine endothelial cells in a time-dependent manner (61). Overall these data reinforce the view that PF4 in-
Interactions with proteoglycans

PF4 is a strong heparin-binding molecule that interacts with high affinity with heparin, heparan sulfate and chondroitin sulfate (3, 63). PF4, together with fibroblast growth factors (FGFs), belongs to the proteins in the organism with the highest heparin-binding affinity. Heparan sulfates are important co-receptors for several growth factors such as fibroblast growth factors or transforming growth factor-β (64). Growth factor binding to heparan sulfates has two consequences. It may serve to concentrate growth factors at the cell surface or be important for receptor activation by promoting ligand/receptor dimerisation (65). This mechanism has been elegantly explained for FGF signalling (65). PF4 is able to inhibit the binding of fibroblast growth factor-2 to cell surface heparan sulfates (55). Thus, this will lead to lower surface concentration of FGF-2 and may impair signalling. The involvement of chondroitin sulfates in growth factor activity is not well established to date.

Interactions with cell surface receptors

It has been reported that PF4 is able to bind a variant of CXCR3 called CXCR3B (58). This interaction induces signalling events including cAMP-dependent signalling that may be responsible for the phenotypic modifications of endothelial cells expressing CXCR3B. Recently the signalling mechanisms of CXCR3B activation by CXCL4 have been studied in more detail (66). In HEK-293 cells expressing CXCR3B, PF4 induced the activation of the p38 (MAPK), MKK3/6 and MAPKAPK-2. Only a modest induction of ERK or JNK was observed upon CXCR3-B activation. Activation of p38 (MAPK) was also observed when human microvascular endothelial cells were stimulated with PF4. Thus, p38 (MAPK) seems to be a downstream effector of PF4-induced activation of CXCR3-B. However, CXCR3-B is not found in bovine or mouse endothelial cells, albeit a similar inhibitory effect of PF4 is observed. Furthermore, these data are in contradiction to former studies where no CXCR-3B activation could be evidenced in human endothelial cells (62). Thus, it has not yet been clearly established whether or not CXCR3-B mediates the anti-angiogenic effects of PF4.

Another possible binding molecule of PF4 is the lipoprotein-related protein-1 (LRP1). LRP1 has been described to mediate PF4 effects on megakaryocytopoiesis (67). Blocking LRP1 with either a receptor-associated protein (RAP), an antagonist of LDL family member receptors, or specific anti-LRP1 antibodies reversed the inhibition of megakaryocyte colony growth by PF4. In addition, using shRNA to reduce LRP1 expression was able to restore megakaryocyte colony formation in bone marrow isolated from PF4-overexpressing mice. Further, shRNA knockdown of LRP1 expression was able to limit the effects of PF4 on megakaryocytopoiesis. Low-density lipoprotein (LDL) binding to receptor-related protein-1 (LRP1) has also been shown to mediate endothelial cell responses to PF4 (59). However, a direct interaction of LRP1 and PF4 has not been demonstrated and thus there is no proof that LRP1 is a receptor for PF4. Thus, LRP1 may also be a co-receptor or located downstream of the PF4 signalling pathway.

Interaction of PF4 with integrins

Integrin receptors are major regulators of angiogenic processes. Integrins are the main adhesion receptors used by endothelial cells undergoing angiogenesis to interact with their extracellular microenvironment (ECM). We recently proposed a novel mechanism for the anti-angiogenic effects of PF4, which implicates the direct targeting of vessels through integrins (60). Our data showed that PF4 and a derived anti-angiogenic peptide from the C-terminus of PF4 (PF4(47–70)) interact with αvβ3 integrins and to some extent with αvβ5 and αvβ1 integrins on the surface of endothelial cells. This interaction is of functional significance, since PF4 modulates endothelial cell adhesion and migration, in an integrin-dependent and-specific manner (60). Furthermore, soluble PF4 or PF4(47–70) inhibits endothelial cell adhesion and migration to immobilised fibronectin and vitronectin, the specific αvβ3 and αvβ1 ligands (60).

Our data suggest that PF4 and PF4(47–70) act as antagonists of integrins to their ligands, a mechanism by which PF4 may exert its anti-angiogenic activity. Thus, PF4 may compete with fibronectin or vitronectin for binding to the binding sites of its receptor, αvβ3. It is also possible that αvβ3 integrins may serve as functional receptors for PF4 to transduce the anti-angiogenic signal of PF4. In this context, PF4 may act as an agonist of negative signals rather than as an antagonist of positive signals. For example, tumstatin and endostatin, two endogenous angiogenesis inhibitors, were shown to interact with αvβ3 and αvβ1 and inhibit angiogenesis via inhibition of PI3 kinase and mTOR activity (68) and inhibition of actin cytokeraton turnover (69). Interestingly, modulation of integrin function and signalling is emerging as a common functional theme among various angiogenesis inhibitors (68–72). In addition to the
anti-angiogenic inhibitors that directly interact with integrins (see above) it was shown that the tumour necrosis factor α and the γ interferon disrupt tumour vasculature in vivo by reducing activation of the αvβ3 integrin. (73). Taken together with the above research, our data suggest that PF4-integrin interaction could have an effect on neovascularisation in vivo, e.g. by modulating integrin signalling involved in processes such as cell adhesion and cell migration. Since integrins are overexpressed in endothelial cells undergoing angiogenesis, this may explain why, in vivo, PF4 tends to preferentially target angiogenic blood vessels (74, 75).

Another issue to consider is the possibility of crosstalk among integrins or between integrins and growth factor receptors (76, 77) that may play a role in PF4 action. Indeed, activation of αvβ3 and/or their downstream integrin effectors stimulates VEGF expression (43) and may promote activation of the receptor for VEGF by direct physical interaction between β3 integrin and VEGFR2 (78). In turn, VEGF activates αvβ3 and therefore increases adhesion of endothelial cells to a variety of ligands (79). Thus, these different interactions may be impaired by PF4.

The sequence motif of PF4 that integrins recognise is not yet determined. PF4 or PF4(47–70) interacts directly with purified αvβ3 integrin in a specific manner (60), with affinities similar to matrix molecules (unpublished results). Despite the fact that neither PF4 nor PF4(47–70) contains an RGD sequence, our data showed that the interaction of PF4 with integrins is RGD-dependent (60). Similarly, some other endogenous angiogenesis inhibitors such as endostatin (70), arresten (71) and tumstatin (68, 72) also bind directly to integrins, despite the fact that they lack the RGD-binding site. The fact that the interaction of PF4 with integrins is blocked by the RGD peptide (60) indicates that PF4 interacts with the same or an overlapping RGD-binding site in integrins. Importantly, PF4 and PF4(47–70) both contain the natural ligands of integrins, as fibronectin or by inducing an intracellular negative signal. d. PF4 interacts with cell surface molecules (CXCR3B, LRP1 etc...) and may inhibit angiogenesis by activating specific signalling pathways. EC, endothelial cell; HSPGs, heparan sulfate proteoglycans; GFRs, growth factor receptors.

**General mechanism of PF4’s anti-angiogenic activity**

A general picture of PF4’s anti-angiogenic mechanisms is emerging from the results described above (Fig. 2). PF4 inhibits neovascularisation in vivo by several distinct, although not necessarily exclusive, mechanisms including direct interaction with angiogenic molecules (54), binding to proteoheparan sulfates (55, 56, 63) interaction with LRP1 (59), activation of CXCR3-B (58), or direct binding to integrins (60). These different mechanisms may operate in parallel or in cooperation depending on the site and/or the type of vessel that is undergoing angiogenesis.

During angiogenesis, PF4 may be released from the α-granules of activated platelets in the vicinity of vessel wall injury (2) or from other vascular cells. It interacts with the different binding molecules on the surface of endothelial cells and induces inhibition of angiogenesis by competing with or activating specific signalling pathways (Fig. 2). The specific mechanisms involved are certainly context-dependent and may vary between different angiogenesis-dependent pathologies or organ localisations.
Concluding remarks

PF4 has two important functions in the vasculature. It has a pro-atherogenic role while, at the same time, it has anti-angiogenic effects. Re-endothelialisation after balloon angioplasty has been shown to reduce neointima formation (82). Thus, delaying re-endothelialisation by angiogenesis inhibitors could contribute to the progression of atherosclerotic lesions. On the contrary, it has been reported that angiogenesis within the vessel wall contributes to plaque formation while the inhibition of angiogenesis reduces it (83–86). These two effects are, in appearance, difficult to reconcile. PF4's function may depend on the physiopathological context and/or on the cell types present in the vicinity of vessel wall injury. PF4 may play a pro-atherogenic role by interacting with the vascular endothelium and with monocytes (23, 24), facilitating monocyte differentiation to inflammatory macrophages (23–26, 28–29), or by increasing the uptake and esterification of ox-LDL in lesional macrophages (36, 37). However, PF4 may also slow-down re-endothelialisation on the vessel surface and intravascular neoangiogenesis may also be inhibited, thus counteracting these effects to some extent. On the other hand, in other pathologies, such as cancer or retinopathy, PF4 is able to interact with "neo-angiogenic" endothelial cells and induce their growth arrest, thus exhibiting an anti-angiogenic function (Fig. 3).

The heterogeneity of PF4's functions on various cell types may also be explained by its binding to different receptors or surface proteins on the vessel wall. PF4, unlike other chemokines that bind to specific receptors, depends for its activity on various different mechanisms (heparan sulfates interaction, growth factor binding, chemokine receptor activation, integrin binding) (54–58, 60). All of these interactions may contribute to the effects of PF4 in healthy and pathological tissues. In vivo models to test biological relevance of each of these proposed mechanisms by which PF4 interacts with the vasculature may provide the answers to these remaining questions.

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References


Note added in proof

We (A.B.) have recently demonstrated that single amino-acid substitutions are responsible for the functional differences between CXCL4L1 (PF4V1) and CXCL4 (PF4). This leads to higher diffusibility, lower matrix binding and increase in biological activity for CXCL4L1 (PF4V1) and CXCL4 (PF4). This leads to higher diffusibility, lower matrix binding and increase in biological activity for CXCL4L1 (PF4V1) and CXCL4 (PF4).


