Platelet Factor 4 (CXCL4) Seals Blood Clots by Altering the Structure of Fibrin*

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Platelet factor-4 (PF4/CXCL4) is an orphan chemokine released in large quantities in the vicinity of growing blood clots. Coagulation of plasma supplemented with matching amount of PF4 results in a translucent jellylike clot. Saturating amounts of PF4 reduce the porosity of the fibrin network 4.4-fold, and decreases the values of the elastic and loss moduli by 31- and 59-fold, respectively. PF4 alters neither the cleavage of fibrinogen by thrombin, nor the cross-linking of protofibrils by activated factor XIII, but binds to fibrin and dramatically transforms the structure of the ensuing network. Scanning electron microscopy showed that PF4 gives rise to a previously unreported pattern of polymerization where fibrin assembles to form a sealed network. The subunits constituting PF4 form a tetrahedron having at its corners a PRH motif that mimics (in reverse orientation) the HRP motif of peptides that co-crystallize with fibrin. Molecular modeling showed that PF4 could be docked to fibrin with remarkable complementarities and absence of steric clashes, allowing the assembly of irregular polymers. Consistent with this hypothesis, as little as 50 µM of the QVRPRHIT peptide derived from PF4 affects the polymerization of fibrin.

In addition to catalyzing the conversion of fibrinogen to fibrin, thrombin activates the G protein-coupled protease activated receptor 1, triggering platelet adhesion and activation (1, 2). Following activation, large quantities of platelet factor-4 (PF4/CXCL4), a major component of the α-granules (15-20 µg/10⁹ platelets), are released into the vicinity of growing blood clots, such that amounts in excess of 5 µg/ml are found in serum (3, 4). PF4 is an asymmetrically associated homotetrameric (70 residues/subunit) chemokine that interacts with a variant of CXCR3. The physiologic function of PF4 is not fully understood even if one of the first clues to the existence of endogenous angiogenesis inhibitors came with the observation that it inhibits endothelial cell proliferation (5). PF4 is otherwise mainly known for binding polysulphated glycosaminoglycans such as heparin and has been extensively studied for its role in heparin-induced thrombocytopenia (6). Generally speaking, PF4 binds to glycosaminoglycans, modulating their activity. Both pro- and anti-coagulant properties for PF4 have been reported. PF4 accelerates the activation of the anticoagulant protein C (7, 8); in contrast studies on transgenic mice demonstrate that, while PF4 contributes to thrombus formation, PF4-null mice had no overt bleeding diathesis (9).

Fibrinogen is a Mr 340,000 glycoprotein consisting of a pair of α, β and γ chains. The molecule comprises two outer D-nodules connected through coiled-coil domains to a central E-nodule (10, 11). The E-nodule includes all six NH₂ termini, and each D-nodule contains the COOH terminus of the β and γ chains folded into globular domains, namely βC and γC (12, 13). Fibrin formation is initiated by a thrombin-catalyzed removal of two peptides (fibrinopeptides A) from the NH₂ termini of the α-chains within the central E-nodule, thus exposing two new NH₂ termini that begin with GPRV (14, 15). These new NH₂ termini constitute A-knobs capable of binding to an appropriate site (the "a-hole") in the distal γC-domain of another fibrin molecule. The
ensuing non-covalent interaction assembles fibrin monomers into overlapping half-staggered structures called protofibrils (16). Protofibrils undergo a transverse association to form a three-dimensional insoluble, yet porous, network of thicker and branched fibers. A second or concurrent step is further cleavage by thrombin of fibrinopeptides B of the β-chains, again within the central E-nodule. Each new NH2 terminus exposes a GHRP sequence constituting a B-knob that potentially complements an additional site (the "b-hole") within the βC-domain of an outer D-nodule (14). While the role of the A-knob/a-hole interaction is well established, that of the B-knob/b-hole interaction is unclear, since it is not critical for network formation, although it presumably enhances lateral aggregation and assembly of protofibrils (17-19). Snake venom toxins such as reptilase (Ancrod) that remove only fibrinopeptide A from fibrinogen (leaving fibrinopeptide B intact), trigger fibrin network assembly resembling that induced by thrombin (18, 20). Accordingly, Gly-Pro-Arg-Pro-amide (GPRPam), which mimics the A-knob, impedes polymerization, whereas Gly-His-Arg-Pro-amide (GHRPam), which mimics the B-knob does not (21, 22). In a final step the fibrin network is strengthened by activated factor XIII (FXIIIa) through transglutamination of γC-domains, covalently cross-linking the network (23, 24).

Fibrin is eventually degraded by plasmin such that D-nodules originally belonging to different fibrinogen molecules are kept together by the covalent linkages formed by FXIIIa. Named D-dimers, these fragments include two γC-domains (with an a-hole), two βC-domains (with a b-hole) and part of the coiled-coil domains originally linking the D- to E-nodules. Most D-dimer crystal structures show an offset arrangement, in accord with the anticipated half-staggered overlapping structure of protofibrils and the banding pattern of mature fibrin fibers observed by transmission or scanning electron microscopy (16, 17). Two interfaces (namely, I and II) have been observed (13). Interface I, most commonly deduced from X-ray diffraction data seems to be the "preferred" interface (11). Unexpectedly, the γC-γC cross-linking takes place unambiguously across the alternate interface (II), whereas it had never been confirmed to occur through interface I. Nevertheless, while their geometry differs, both the interface I and II models are consistent with the organization of protofibrils into overlapping half-staggered fibrin.

Since the highest amount of PF4 is released in the vicinity of expanding fibrin clots, and sub-nanomolar amounts of monomeric (25) PF4 enhance fibrin fiber polymerization (26, 27), we investigated whether large amounts of PF4 could contribute to blood coagulation. We here provide direct biophysical evidence that tetrameric PF4 (25) in amounts released in the neighborhood of platelets incorporates into and structures a previously unidentified soft and transparent fibrin network having a low porosity.

Experimental Procedures

Proteins and Reagents - Blood was collected with informed consent from healthy volunteers by venipuncture in 1:9 (v/v) ACD. Platelet-rich plasma was prepared by centrifugation (170 g, 12 min, 20 °C) and used immediately. Platelet-poor plasma was obtained after further centrifugation (680 g, 12 min, 20 °C). Human prothrombin and fibrinogen were purified from outdated plasma, and prothrombin converted to thrombin and titrated as previously described (28, 29). Soluble fibrin (10 µM) was prepared from clots formed in the presence of 5 mM iodoacetamide. Clots were washed in kinetic buffer (Tris-HCl 50 mM pH 7.5 containing 0.15 M NaCl and 5 mM CaCl2) and solubilized by incubation (1 h, 20°C) with 50 mM CH3COOH. Mixtures of D-dimers and D-nodules were prepared according to Everse et al. (30) by affinity chromatography on silica gel linked to the C-terminal end of peptide GPRPAAAAA (Altergen, Schiltigheim, France). Briefly, plasmin (300 nM) was added to fibrin clots prepared without iodoacetamide, incubated (16 h, 20°C) and neutralized with aprotinin (10 µM). Bound material was eluted by NaH2PO4 50 mM pH 3.7, and precipitated with ammonium sulfate (70% saturation, 4°C) after correcting the pH to 7.5 with 2.0 M Tris-HCl. Recombinant PF4 and the chromogenic substrate H-D-Phe-Pip-Arg-pNA (S2238) were purchased from Biogenic (Maurin, France), reptilase (Ancrod), plasmin, FXIIIa, and sheep anti-human PF4 from Kordia (Leiden, the Netherlands), aprotinin (BPTI), GPRPam, poly-L-lysine (Mr 15,000-30,000), Polybrene, and iodoacetamide from Sigma-Aldrich (Saint-...
Quentin, Fallavier, France), and rabbit brain phospholipids (Platelet factor 3 reagent) from Organon Teknika (Durham, NC). The peptide QVRPRHIT derived from PF4 and the control peptide RQVHIRPT were prepared by Altergen. Buffers were made daily and all proteins were stored at -80°C in small aliquots until use. Experiments were reproduced a number of times in various conditions and several repeated independently by two of the authors. Data obtained in identical conditions were fully exchangeable. Figures illustrate representative experiments.

**Turbidimetry and enzyme kinetics** - Turbidity was recorded at 405 nm in pre-warmed (37 °C) microtiter plates after adding 170 µl pre-warmed plasma to a mixture of 10 µl platelet factor 3 reagent and 20 µl CaCl₂ (60 mM) with or without PF4. With purified fibrinogen (1-5 µM), A₄₀₅ was recorded in kinetic buffer after adding thrombin (0.2-5 nM) or reptilase (3-5 U/ml). Fibrin re-polymerization (3 µM) was triggered in the presence or absence of PF4 by raising the pH to 7.5 by adding (v/v) a mixture of 50 mM MOPS, 50 mM NaOH, 20 mM CaCl₂ and 0.15 M NaCl. In all experiments evaluating fibrin (re)polymerization, particular care was taken to maintain NaCl and CaCl₂ concentrations, total ionic strength, and pH identical in all samples as these influence dramatically the fibrin network structure (27, 31). The progress of fibrinopeptides A and B release was studied according to Higgins et al. (32), as previously described (28). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29). Briefly, reactions were stopped at timed intervals by adding phosphoric acid (10%) and fibrinopeptides separated and quantified by reverse phase chromatography on a C₁₈ column (218TP54, Vidac) developed with a linear gradient (0-50% CH₃-CN in 0.5%, v/v, phosphoric acid). Progress of γ-γ dimer formation by FXIIIa was also examined as previously described (29).

**Rheology** - The dynamic viscoelasticity of fibrin gels prepared with and without PF4 was studied at 25 °C using a strain controlled rheometer (ARES LS1, TA Instruments, New Castle, DE) essentially according to Janney et al. (33) and Ryan et al. (34). The reaction components were thoroughly mixed and 300 µl quickly transferred between the titanium cone and stainless plate of the apparatus (25 mm diameter, 0.04 rad angle, 45 µm gap). Elastic (G') and loss (G'') moduli (1% strain, 1 Hz) were recorded every 9 sec for 1 h. Following polymerization, the frequency dependence of G' and G'' between 1 and 100 Hz were characterized at 1% strain. Sequentially increasing strain (up to 1000%, 1 Hz) was then applied allowing detection of strain hardening when relevant and the minimum required for the gel to collapse. Sample evaporation was prevented by a homemade cover maintaining a 100% humidity atmosphere and preliminary short strain sweep scanning indicated that with all gels studied, a 1% strain deformation at 1 Hz was fully reversible.

**Scanning electron microscopy** - Clots (10 µl) were formed in kinetic buffer on a glass lens by mixing (v/v) thrombin (2.5 nM) with fibrinogen (5 µM) and varying concentrations of PF4. Immediately following the initiation of clotting, the solution was sprayed and polymerization allowed to proceed in a humid chamber (1 h, 20 °C). Clots were washed in kinetic buffer, fixed in buffered glutaraldehyde (1 h, 2%, v/v), dehydrated by stepwise ethanol gradients and critical-point dried in hexamethyldisilizane (HMDS, Sigma-Aldrich). Samples were mounted on specimen stubs, sputter coated with gold using a JEOL JFC100, and examined at 16 KV using a JEOL ISM 35CF (Croissy sur Seine, France).

**Modeling studies** - Swiss-Pdb viewer (http://www.expasy.org/spdb/) was used to generate models of complexes between PF4 tetramer (1PFN) and human D-dimers (1FZF) or lamprey D-dimers (1N73). Docking was achieved by superimposing the Arg²² Cα of the PRH motif in PF4 with the Arg³ Cα of GHRPam co-crystallized with D-dimer (the Cα’s were separated by 0.1 Å for double docking of PF4 to a-holes). A unique docking satisfying both overlapping of the tripeptides (running antiparallel) and absence of steric hindrance was achieved by simple rotation around the overlapping points.

**RESULTS**

*Physiologic amount of PF4 alter the rate*
of formation of fibrin clots - The progress of clot formation may be monitored through it's absorbance, since protofibril association increases light scattering. Typically, the shorter the clotting time, the thinner (thus less turbid) is the bunch of protofibrils constituting the fibrin network. Platelet rich plasma has a higher intrinsic turbidity than platelet-poor plasma due to light scattering by platelets. We nevertheless observed that the difference between final and initial turbidities was less in platelet-rich plasma than in platelet-poor plasma (Fig. 1A). This observation was puzzling because the time to gelation (i.e. to reach half maximum turbidity) differed by less than 4% (10 seconds), whereas the relative turbidity almost doubled. To investigate the possible contribution of PF4 to clot formation, we compared the effect of various amounts of PF4 on the turbidity of clots formed in platelet-poor plasma (Fig. 1B). As little as 25 nM PF4 had a discernible effect on final turbidity and increasing amounts of PF4 decreased final turbidity further. In contrast to clots triggered in platelet-rich plasma, however, the time to gelation was also affected. At a concentration occurring in the vicinity of blood clot extension in vivo (300-600 nM) PF4 not only caused a dramatic decrease of the final turbidity, it also considerably increased the lag phase (from 218 seconds without PF4 to 552 seconds with 375 nM PF4). Platelets alter the fibrin network structure, through thrombospondin release (35-37). Similar to PF4, thrombospondin decreases the final turbidity of clots, but contrary to PF4, it decreases the lag phase (36). Thus the low increase of turbidity in platelet rich plasma required PF4 possibly in synergy with thrombospondin, whereas the unaltered lag phase perhaps originated from their opposing effects.

Thrombin catalysis is unaffected by PF4 - Since PF4 is heavily charged we investigated whether the observed effects could simply reflect an electrostatic steering interference with thrombin catalysis. Polybrene and poly-L-lysine are polycationic compounds having an average Mr of 7500 comparable to that of PF4 monomers and of 22,500 comparable to that of PF4 tetramers, respectively. If the effect of PF4 were non-specific and resulted solely from charge repulsion, Polybrene and/or poly-L-lysine would mimic PF4. We therefore compared the effect of PF4, Polybrene, and poly-L-lysine on thrombin-mediated clotting of purified fibrinogen. Increasing amounts of PF4 resulted in a similar effect as observed in platelet-poor plasma: as concentration increased, the final turbidity decreased and the lag phase lengthened, yet gelation ultimately occurred (Fig. 1C). Polybrene had little or no effect on fibrinogen clotting (Fig. 1E). As reported by Carr et al. (38), poly-L-lysine had an effect opposite to PF4, i.e. increasing concentrations enhanced the final turbidity, whereas the lag phase decreased (Fig. 1F). Thus the effects of PF4 clearly differ from that of two other positively charged compounds and were therefore unlikely to result from a general unspecific charge effect on fibrin assembly.

When fibrinogen clotting was triggered by reptilase instead of thrombin, PF4 still affected the outcome (Fig. 1D). The final turbidity decreased and the lag phase lengthened with increasing PF4 concentration, yet gelation ultimately occurred. Similar effects were thus obtained with both reptilase and thrombin, supporting the hypothesis that PF4 interacted with fibrinogen and/or fibrin; alternately, PF4 would have to affect both proteases through discrete allosteric alterations. The rates of H-D-Phe-Pro-Arg-pNA hydrolysis by either thrombin or reptilase were unaffected by large amounts of PF4 or poly-L-lysine, and only marginally by Polybrene (data not shown), rendering it unlikely that the impact of PF4 on clot formation was mediated through allosteric alteration (39). That the effect of PF4 resulted from a specific interaction, and not from an incompletely controlled parameter, was also attested by the observation that stochiometric amount of an anti-PF4 antibody attenuated the effect of PF4 by 75% (Fig. 2A).

Fibrinogen cleavage is little affected, if at all, by PF4 - Fibrinogen hydrolysis is a partially ordered reaction with release of fibrinopeptide A preceding that of fibrinopeptide B. We considered that, through binding to fibrinogen, PF4 could shield it from thrombin and/or reptilase, thus preventing cleavage. However, the release of fibrinopeptide A by thrombin or reptilase was little affected, if at all, by PF4 (Fig. 2B). In contrast, hydrolysis of fibrinopeptide B by thrombin was delayed in the presence of PF4 (Fig. 2C). Thus, binding of PF4 to partially hydrolyzed fibrinogen (lacking fibrinopeptide A only) could not be ruled out. Nevertheless, since the cleavage of
fibrinopeptide B is not required for fibrin to polymerize, we concluded that the dramatic effect of PF4 on clot formation must occur at a later stage during assembly of the network.

**PF4 alters the structure of the fibrin network** - Given that PF4 appeared to act neither through binding to the enzyme nor to fibrinogen, we hypothesized that it interacted with fibrin. Fibrin monomers associate both through non-covalent interactions and through covalent \( \gamma \)-\( \gamma \) cross-linking. If \( \gamma \)-\( \gamma \) cross-linking has been prevented, the fibrin network will dissolve at low pH, whereas spontaneous re-polymerization occurs when the pH is raised to 6.5. We reasoned that if PF4 chaperoned fibrin network assembly, it should also affect the re-polymerization of soluble fibrin. Conclusively, when re-polymerization of fibrin monomers was triggered in the presence of increasing amounts of PF4, final turbidity decreased and gelation was delayed albeit it still occurred (Fig. 2D). In addition, the macroscopic aspect of the clot was evidently affected by PF4 as the fibrin gel gradually turned jellylike, even though supra-physiologic amount of PF4 did not prevent gelation.

A-knob to a-hole interactions are essential for the formation of fibrin polymers, we therefore examined if PF4 prevented binding. GPRPam (which mimics the A-knob) binds to the a-hole and thus prevents self-assembly of protofibrils and network formation (20, 22). In accord with the concept that size of the fibrils augments when polymerization is retarded, the final turbidity and the lag phase increased with the amount of GPRPam added (Fig. 2E). Above 500 \( \mu \)M, however, GPRPam extended the lag phase to the point that turbidity (and gelation) no longer developed within two hours. Thus the effect of GPRPam on fibrin polymerization was clearly different from that of PF4. In particular, fibrin polymerization in the presence of PF4 always resulted in a discernible jellylike mass even when clots were near transparent. Binding of synthetic B knobs to fibrinogen also increases the turbidity of fibrin clots (40), suggesting that PF4 did not act through simple blockage of either the a- or b-holes.

**PF4 considerably alters the porosity and viscoelasticity of the fibrin network** - Turbidity reflects only in part the structure of a fibrin network; porosity and viscosity are also essential features (34, 41). The porosity of a clot can be evaluated by measuring flow-through. With stiff structures, clots can be formed in a small plugged syringe. Once the fibrin has polymerized, the syringe is unplugged, a given volume of buffer is added, and flow-through recorded. Clots formed in the presence of saturating amounts of PF4 were too soft to stand alone in a syringe. Thus, to evaluate the effect of PF4 on porosity, fibrinogen containing various amount of PF4 was polymerized on top of a "normal" fibrin network serving as a supporting grid. PF4 dramatically changed the porosity. Permeability decreased rapidly with increasing PF4 concentrations, and leveled-off at about 25 \( \% \) that of the control. The flow rates for PF4-free and clots containing 300 nM PF4 were 1.1±0.16 \( \mu \)l min\(^{-1}\) and 0.25±0.01 \( \mu \)l min\(^{-1}\), respectively (Fig. 2F). Thus the maximum effect of PF4 on porosity occurred when the molar ratio of fibrin to PF4 was four (two fibrin molecules for each PF4 monomer). Assuming that viscosity of the buffer was 10\(^{-3}\) poise (dyne s cm\(^{-2}\)), the permeation coefficient (Darcy constant, which reflects the structure of the pore within the network) would drop from 1.054±0.168 \( 10^{-9}\) cm\(^2\) to 0.239±0.01 \( 10^{-9}\) cm\(^2\) with saturating amounts of PF4 (42-44).

PF4 evidently modified the viscoelastic properties of the fibrin network: it induced the formation of jellylike masses instead of the stiff clot that formed in its absence. The values of \( G' \) and \( G'' \) decreased simultaneously when the amount of PF4 increased: from 559±6 to 18.1±1.5 and from 26.3±1.9 to 0.44±0.16, respectively (Fig. 3A and 3B). Thus, irrespective of the amount of PF4 added, the elastic resistances to deformation (the shear modulus \( G''/G' \)) were relatively comparable for all gels (Fig. 3C). Interestingly, gel prepared in the presence of increasing amount of PF4 encountered gradual stress hardening with the result that all fibrin gels collapsed at about 200\% strain (Fig. 3D). In accordance with the apparent stoichiometry deduced from the porosity experiments, the rheological data also suggested that the effect of PF4 reached a maximum with two fibrin molecules per PF4 monomer.

**PF4 does not prevent \( \gamma \)-\( \gamma \) cross-linking of fibrin** - FXIIIa stabilizes fibrin clots by cross-linking fibrin monomers. Its zymogen (factor XIII) is activated by thrombin. FXIIIa strengthens the internal architecture of fibers such that the

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stiffness of the network increases (24). The extent of the reaction depends in part upon the rate of protofibril assembly. Thus, a delay in γ-γ dimer formation could reflect impaired factor XIII activation and/or retarded protofibril formation. Factor XIII is a minor contaminant that co-purifies with fibrinogen with the result that, in the absence of an inhibitor such as iodoacetamine, γ-γ dimers form during fibrin network assembly without the addition of extra factor XIII (29). Progress of transglutamination is conveniently monitored by SDS-PAGE under reducing conditions. As γ-chain cross-linking progresses, the γ-chain band disappears and a new band having about twice its molecular mass becomes visible. Adding 300 nM PF4 had no detectable influence on the cross-linking of γ-chain (Fig. 4A), suggesting that PF4 had little impact, if any, on the activation of factor XIIIa by thrombin or on fibrin cross-linking.

**PF4 binds to fibrin degradation products** - The effect of PF4 on fibrin network formation supported a model in which its influence occurred after fibrin had assembled into protofibrils. In addition, the apparent stoichiometry was consistent with an incorporation of PF4 into the fibrin network itself. Equilibrium binding studies between PF4 and fibrin were precluded because PF4 did not prevent gelation of fibrin. Compounds such as GPRPam that bind fibrin also bind soluble fibrin fragments. Therefore, we searched for evidence of PF4 binding to fibrin degradation products. PF4 was not captured by GPRPAAAAA linked to a silica gel via its C-terminal end (30), but if the column was pre-loaded with fibrin degradation products, PF4 was bound and co-eluted with the D-dimers (Fig. 4B). In fibrinogen, the α-hole within the γC-domain is accessible such that GPRPAAAAA-coupled resins also retain fibrinogen. Surprisingly, PF4 was also captured by fibrinogen bound to the GPRPAAAAA-column and co-eluted at low pH.

**PF4 dramatically alters the morphology of fibrin networks** - Biochemical data suggested that PF4 impaired the lateral aggregation of protofibrils. To investigate the resulting structures, fibrin clots were prepared in the presence of various amount of PF4, and observed by scanning electron microscopy. The typical network obtained in the absence of PF4 (Fig. 5A) gradually disappeared with increasing amounts of PF4. One hundred nM PF4 was sufficient to profoundly transform the appearance of the network (Fig. 5B). Fibrils were still discernible, but PF4 apparently triggered the formation of layers that tended to form a contiguous mesh. Adding 300 nM PF4 was sufficient to induce formation of a two-dimensional coat in which only few holes were visible (Fig. 5C). Above 500 nM PF4, the fibrin network was completely sealed, and a continuous sheet was formed that was underlaid with a framework of barely apparent fibrils (Fig. 5D and 5E). We concluded that PF4 not only affected the lateral polymerization of fibrin: it structured the network.

**PF4 has a considerable potential to shape fibrin molecular edifices** - Fibrin(ogen) structure(s) are known from X-ray diffraction studies (12, 13, 45-49) and that of PF4 has been deduced through both X-ray diffraction and NMR spectroscopy (50, 51). Three intriguing features of these structures attracted our attention. First, the D-nodule of fibrinogen has a strong electrostatic potential that is opposite to that of PF4 (Fig. 6). Second, PF4 is an asymmetric tetramer with PRH motifs exposed at its corners that are reminiscent of the B-knobs uncovered by fibrinopeptide B cleavage (NH2-GHRP). Third, the distance between Arg22 Cα of the PRH motifs in PF4 is either 26.7 or 30.5 Å (depending on which edge of the tetrahedron is considered) and the lower figure matches the distance separating the Arg3 Cα of the GHRPam peptides (26.9 Å) that were co-crystallized in the α-holes of human D-dimers.

Docking PF4 to the b-hole of a D-dimer co-crystallized with GHRPam was especially informative. Using the Arg22 Cα of a PRH motif in PF4 and the Arg3 Cα of GHRPam as an anchoring point, we found a unique orientation that allowed side chains of each motif to overlap without creating steric hindrance between PF4 and D-dimer (Fig. 7A). A model of four D-dimers bound to a single PF4 was constructed in which no conflict occurred (Fig. 7B). According to this model, PF4 would not impede cross-linking by FXIIIa, as found experimentally. Also consistent with our data, while PF4 binding would not prevent protofibril formation it would impede their lateral association. In this b-hole model, each D-dimer could connect two PF4 and each PF4 connect four D-dimers, opening an avenue to the formation of complex molecular edifices. Following the long edge of PF4, D-dimers would...
shape an elongated helix making a complete turn every seventh PF4 (Fig. 7C and 7D), whereas a complex loop would form following the short edge (not shown). The above PF4/b-hole model was based upon the classical association between γC-domains (interface I in reference 13). A complex network would also result from the association of PF4 with D-dimers arranged through interface II, albeit quite different from that constructed according to interface I (not shown).

In structures of D-dimers obtained through X-ray diffraction studies, GHRPam peptides mimicking the B-knob occupy the b-holes (as expected) but also occupy the a-holes. Accordingly, PF4 displays PRH motifs separated by a distance equal to that separating GHRPam peptides inserted in the a-holes of a D-dimer. Double docking of PF4 to a single D-dimer not only proved feasible, it also revealed surprising complementarities (Fig. 7E), allowing construction of a model in which one PF4 coupled two D-dimers offset by 51° with respect to each other (Fig. 7F). Interestingly, the distance between ArgCα of GHRPam peptides occupying the a-holes in interface II of lamprey D-dimers is 21.3 Å, thus too short to bind simultaneously two of the PRH motifs in PF4.

The NH2-termini of synthetic A- and B-knobs are essential for binding to the a- and b-holes of fibrin (47), raising the prospect that internal PF4 sequences would not interact with fibrin. We therefore examined if the QVRPRHIT peptide (derived from the PF4 sequence) bound fibrin. A scrambled peptide (RQVHIRPT) was used as a control. Unexpectedly the QVRPRHIT peptide, while affecting fibrin polymerization, had an opposite effect to that of PF4; as little as 50 µM had a detectable effect, 500 µM almost doubled the final turbidity (Fig. 8). Nevertheless the peptide clearly affected fibrin polymerization suggesting that, in the context of the PF4-derived peptide (and likely of PF4), an α-amino group is not strictly required for binding. Accordingly, monomeric GHRPam (40) and the QVRPRHIT peptide enhanced fibrin polymerization, whereas tetrameric PF4 dramatically altered the structure of the network.

**DISCUSSION**

We demonstrated that amounts of PF4 released in the vicinity of activated platelets have a remarkable impact on fibrin polymerization, especially on the porosity and viscoelastic properties of the network formed. Our data suggest that PF4 impeded the lateral association of the protofibrils and incorporated into the fibrin network with an apparent stoichiometry of two fibrins per PF4 monomer. In support of this hypothesis, tentative docking through superimposition of the PRH motif in PF4 and peptide GHRPam that co-crystallizes with fibrin fragments proved realistic and showed that PF4 might associate with fibrin to impede the lateral association of protofibrils without perturbing the γ-γ cross-linking catalyzed by FXIIIa.

The opacity of fibrin clots increases with lateral aggregation, thickening of fibers mainly accounting for turbidity (17, 52). Near transparent gels are constituted of thin filaments forming fine meshes, whereas in opaque (or turbid) gels, thick polymer strands surround wide open spaces. The final structure reflects the outcome of a competition between linear and lateral growth of the fibers (14, 27). This competition is influenced by the rate of monomer production and depends upon a number of factors including rate of polymerization, ionic strength, pH, and presence of ions such as calcium (15, 19, 31, 34). The structure of the fibrin network formed is also influenced by the release of thrombospondin, along with PF4, from the α-granules of platelets (35, 36). Generally speaking, faster protofibril formation and/or impaired lateral association results in lower turbidity and porosity. We observed that PF4 had a dual effect, decreasing the final turbidity while increasing the lag phase. We verified that PF4 was not inhibiting thrombin or reptilase and that fibrin production was not impeded. We also ruled out potential electrostatic steering effects of PF4. The strong positive field characterizing PF4 actually renders it unlikely that it tethered thrombin. In fact, while inhibition of thrombin or of fibrin production would increase the lag-phase (as observed), it would also increase turbidity (contrary to our observations). We further established that PF4 decreased the porosity as well as the G′ and G″ values of the fibrin network formed. The implication was that PF4 caused structural differences in the fibrin network itself concurrently with or subsequently to protofibril assembly.
The release of fibrinopeptide B was delayed in the presence of PF4. A number of studies suggest that assembly of protofibrils promotes fibrinopeptide B release, which in turn facilitates their lateral aggregation (17, 53, 54). Thus if polymerization enhances cleavage of fibrinopeptide B, the delay apparently induced by PF4 could reflect an impaired lateral association of protofibrils. However, PF4 had no detectable effect on the $\gamma$-$\gamma$ cross-linking by FXIIIa favoring the hypothesis that PF4 did not interfere with protofibrils assembly. Fibrin polymerization in the presence of large amounts of FXIIIa decreases both the turbidity and permeability, due to the formation of a finer fibrin mesh (23). Conversely, inhibiting cross-linking by FXIIIa results in a softer than normal network (24). Therefore, while PF4 mimicked in part the effects of FXIIIa on fibrin polymerization (causing lower turbidity and porosity), its effects were also opposite: far from strengthening the fibrin network, PF4 considerably softened it as attested by measurement of its viscoelastic properties.

The intimate structure of fibrin networks is known from light scattering and rheological studies, as well as from electron scanning and confocal microscopy (15, 17, 34, 52). Most fibrin networks described are fundamentally analogous: while their properties vary widely, they are all constituted of fibrils having various sizes linked by a variable number of branching points. Enhancing lateral aggregation of the protofibrils increases the size and length of the fibrils between branched points; conversely, impeding association results in thinner highly-branched fiber bundles (27, 55). To some extent, our data (network less turbid, stiff, and porous) are in accord with the concept that PF4 impaired the lateral association of protofibrils. A number of fibrinogen variants exhibiting impaired lateral association have been characterized (42, 44, 53, 56, 57). The networks formed are thinner than normal, but their viscoelastic properties are not dramatically affected: $G'$ and/or $G''$ values decrease 2-fold at the most. Surprisingly, their permeability either decreases or dramatically increases due to formation of large pores and perhaps to the intrinsic fragility of the gel. Micrographs of gels formed with fibrinogen Caracas II, where the fibers form secondary networks with many free ends (42), somewhat resemble the network observed in the presence of PF4. On the other hand, fibrinogen Caracas II forms fibrin gels that are highly porous and have viscoelastic properties near normal, and are thus quite different from the networks that formed in the presence of PF4.

Fibrin networks formed in the presence of PF4 were atypical in that they were simultaneously little turbid, permeable, and soft (i.e. had low $G'$ and $G''$ values). In addition, micrographs showed that the networks were not constituted of fine meshes of thin and branched fibers. At least two studies report micrographs of strikingly unusual gels that, to some extent, are reminiscent of those we observed in the presence of PF4. In the first (58), the clot was formed in the presence of an antibody directed against the fibrinogen $\gamma$-chain, leading to a network constituted of very short and thin fibers arranged in unusual bundles surrounding large pores. Compared with normal fibrin gels, the $G'$ value was decreased only slightly, whereas the permeability increased 25-fold; this antibody also impaired cross-linking by FXIIIa. Thus the network formed in the presence of the antibody was quite different from the one we report in the present study. The second study (59), concerns a fibrinogen variant having a C-terminal extension of its $\beta$-chain (end-linked to another molecule). Following an increased lag phase, this fibrinogen variant forms a network having a lower turbidity than normal and characterized by highly branched, fragile, lace-like structures. While the rheology of this fibrin gel was not reported, perfusion data suggest that the network also greatly differs from the one we report herein. In support of our proposal that PF4 mediates a specific pattern of polymerization, confocal micrographs illustrate that the network surrounding platelets differs from that of a “typical” fibrin clot (37) and, providing that retraction had been prevented, has a low permeability (60).

Our data suggest that PF4 bound fibrin monomers and/or protofibrils and molecular modeling permits a number of arrangements. Through binding to the b-holes of a D-dimer, we found that each PF4 could participate both in an elongated helix and a complex loop. Moreover, while we modeled PF4 binding to D-dimers, in a true PF4-fibrin network, each D-dimer would be replaced by two fibrin molecules, possibly engaged in a half-staggered protofibril. The
resulting network would be tightly packed and has a tremendous potential for complexity. Electrostatic potentials favored binding of PF4 to the a-holes rather than the b-holes, and we found striking topological complementarities. A major difference was that PF4 binding to a-hole would disrupt protofibril formation, and consequently their lateral association. Nevertheless, in both models PF4 binding would not impede cross-linking by FXIIIa, as found experimentally. From these multiple binding combinations, a number of arrangements were achievable with the result that association of PF4 to fibrin would form irregular edifices. Steric hindrance would limit the number of sites simultaneously accessible for a given PF4 tetramer, perhaps explaining the non-saturating stoichiometry observed. Undoubtedly, such complexity would account for the transparency of the network formed in the presence of PF4, because steric hindrance would rapidly limit regularity, rendering the structure amorphous in nature. The density of the network would account for its low porosity.

An overall dissociation constant of 0.7 µM (in 0.2 M NaCl, at 30 °C, and pH 7.0) had been extrapolated by H NMR for tetrameric PF4 (25). Depending upon its concentration, PF4 induces opposing effects on the fibrin network structure: at sub-nomolar concentrations PF4 is essentially monomeric and enhances polymerization (26, 27); we observed a similar phenomenon using the QVRPRHIT peptide derived from PF4. In contrast, our data suggest that in amounts released in the vicinity of platelet plug expansion, tetrameric PF4 incorporates to and profoundly transforms the structure of the network. While puzzling at first, the paradox is only apparent and may provide a clue to the true function of PF4 in hemostasis. The initial platelet plug must rapidly and efficiently seal the vascular injury. Thus in close proximity of platelets, PF4 in large quantities insures the formation of layers that seal the clot. Such a clot is however fragile, and needs to be stabilized by thick fibrin fibers. As PF4 diffuses from the site of vascular injury, its concentration gradually decreases and tetramers dissociate into monomers that tend to enhance fibrin polymerization, forming a stiffer (but porous) network. The implications of this study are manifold and prompt a reconsideration of the structure of blood clots surrounding platelets in vivo. A practical implication could be in the preparation of emergency fibrin glue (61) since PF4 dramatically decreases the porosity of the fibrin network.

REFERENCES


FOOTNOTES

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The abbreviations used are: PF4, platelet factor 4; GPRPam, Gly-Pro-Arg-Pro-amide; GHRPam, Gly-His-Arg-Pro-amide; FXIIIa, activated coagulation factor XIII; G', elastic modulus; G'', loss modulus.

FIGURE LEGENDS

**Fig. 1.** PF4 alters fibrinogen clotting. *A,* Progress of turbidity when clotting is triggered by adding calcium to either platelet-rich (PRP) or platelet-poor plasma (PPP): clotting times differ by less than 10 seconds but the relative turbidity doubles. *B,* Adding increasing amount of PF4 (between 24 and 1500 nM, as indicated) to PPP also decreases final turbidity while increasing clotting time. *C,* The same is true when clot formation is triggered by adding 1.25 nM thrombin to 2 µM fibrinogen and increasing amount of PF4 (as indicated). *D,* Same as in *C* except that clot formation was triggered by adding 3.5 U/ml reptilase. *E,* Same as in *C* with increasing Polybrene (up to 14 µM, lower curve) instead of PF4. *F,* In contrast to PF4, adding poly-L-lysine (between 1 and 32 µM, as indicated) decreased the clotting time while increasing the final turbidity.

**Fig. 2.** PF4 alters fibrin polymerization, but not its production. *A,* Progress of turbidity when clotting was triggered by adding 1.25 nM thrombin to 2 µM fibrinogen containing 600 nM PF4 (PF4 only), 600 nM PF4 and 600 nM anti-PF4 polyclonal antibody (PF4 + anti-PF4), or 600 nM anti-PF4 only. *B,* Progress of fibrinopeptide A (FpA) release from 2.5 µM fibrinogen hydrolyzed by 1.25 nM thrombin (squares) or 3.5 U/ml reptilase (circles) in the presence (open symbols) or not (closed symbols) of 600 nM PF4; solid lines were obtained by non-linear regression analysis according to the equations derived by Higgins et al. (32). *C,* In contrast, release of fibrinopeptide B (FpB) by thrombin is delayed in the presence of PF4. *D,* Progress of fibrin re-polymerization in the presence of increasing amounts of PF4, as indicated. PF4 delayed re-polymerization and lowered final turbidity. *E,* same as in *D* in the presence of increasing amount of GPRPam, as indicated. GPRPam delayed re-polymerization but increased the final turbidity. *F,*
Flow through (µl min⁻¹) as a function of added PF4 for clots (100 µl) formed by incubation (60 min, 20 °C) of 2.5 µM fibrinogen with 1.25 nM thrombin. The gel was 0.8 cm high for a cross-sectional area of 0.126 cm² and the differential pressure applied was 18 Pa (180 dynes/cm²). Error bars represent the mean±standard deviation for three measurements with each clot.

Fig. 3. Dynamic viscoelasticity of fibrin gels prepared with and without PF4. A, Progress of the elastic modulus (G’) in Pa (1 Pa = 10 dynes/cm²) when clotting was triggered by adding 2 nM thrombin to 5 µM fibrinogen containing or not PF4, as indicated. B, Corresponding progress of the loss modulus (G’’) in Pa. C, Final values in Pa of G’ (open squares) and relative G’’ values (closed circles) as a function of PF4 (for ease of comparison, G’’ values have been multiplied by 10). Inset represents the shear modulus (G’’/G’) as a function of PF4. D, G’ values (in Pa) as a function of the strain (in %) demonstrating strain hardening and the minimum strain required for the gel to collapse (PF4 as indicated). Similar plots were obtained when G’’ values were plotted as a function of the strain applied.

Fig. 4. PF4 allows γ-γ cross-linking and binds to plasmin-digested fibrin. A, Analysis by SDS–PAGE followed by Coomassie Blue staining of the progress of 2.5 µM fibrinogen clotting by 1.25 nM thrombin at 20 °C in the presence or absence of 300 nM PF4, as indicated. B, Analysis by SDS–PAGE followed by Coomassie Blue staining (upper panel) and Western blot analysis (lower panel) using an anti PF4 antibody (PF4 is poorly stained by Coomassie blue). Lane 1, plasmid digested fibrin; lane 2, material eluted from silica gel linked to peptide GPRPAAAAA loaded with a mixture of plasmin-digested fibrin and PF4; lane 3, PF4 alone; lane 4, molecular weight markers, size given in kDa.

Fig. 5. Scanning electron microscopy of fibrin clots. Fibrin network were formed by adding 1.25 nM thrombin to 2.5 µM fibrinogen without PF4 (A) or with PF4 added to a final concentration of 300 nM (B), 600 nM (C), or 1200 nM (D and E). Micrographs were taken at the same magnification and are representative of random observations (except E, which corresponds to the edge of the fibrin clot in D). Bar, 1 µm.

Fig. 6. Electrostatic potential. Potentials of human D-dimer (1FZF), thrombin (1PPB) and PF4 (1PFM) were obtained using coulomb computation of partial atomic charges for a solvent dielectric constant of 100. The electrostatic potential of PF4 was minimized, as it would otherwise overlap those of thrombin and D-dimer. Binding of PF4 to thrombin is unlikely, but interaction between PF4 and the fibrin γC-domain is highly favorable.

Fig. 7. Tentative docking of PF4 to D-dimers. A, The PRH motif (red) of PF4 (blue) was overlapped with HRP (dark green) of peptide GHRPam bound to a b-hole of fibrin D-dimer (yellow). B, In a docking of four D-dimers (yellow and red) to a single PF4 tetramer (blue) no conflict occurs. C and D. View along the axis (C) or from an offset angle (D) of extended PF4-D-dimer complexes. PF4 tetramers are depicted by lines drawn between Arg<sup>22</sup> Cα of each monomer and D-dimers by lines between Arg<sup>3</sup> Cα of the four bound GHRPam. The relative orientations of PF4 and D-dimer are as in B. Alternate D-dimers (yellow and black) bound to each end of the long edge of PF4 tetramers (blue) would form the backbone of an elongated helix. D-dimers bound to the short edge of PF4 form lateral projections (red and green). E, The PRH motif (red) of PF4 (blue) was overlapped with HRP (dark green) of peptide GHRPam bound to an a-hole of fibrin D-dimer (yellow). F, In a docking of the four a-holes of two D-dimers to a single PF4 no conflict occurs.

Fig. 8. Binding of the QVRPRHIT peptide to fibrin. Progress of turbidity when clotting was triggered by adding 1.25 nM thrombin to 2 µM fibrinogen containing increasing amount of QVRPRHIT peptide, as indicated. One nM of the scrambled peptide RQVHIRPT (open circles) had little influence on the turbidity progress curve.
Fig. 1.
Fig. 2.

A) Turbidity (A<sub>550</sub>) vs. time (T (min))

B) Fibrinogen (Fg (%)) vs. time (T (min))

C) Fibrin (Fb (%)) vs. time (T (min))

D) Turbidity (A<sub>550</sub>) vs. time (T (min)) with different concentrations of PF4

E) Activity (nmol/min) vs. PF4 (nM)

F) Activity (nmol/min) vs. PF4 (nM) with different concentrations of rep

Legend:
- Anti-PF4 only
- PF4 + anti-PF4
- PF4 only
- Rep + PF4
- Anti-PF4
- Ila + PF4
- Ila = PF4
- Thrombin + PF4
Fig. 4.

(A) Western blots showing a y-y Dimer, α, β, and γ proteins. Time points are shown in minutes (T). There are two panels, 1 and 2, indicating the presence or absence of PF4. The y-y Dimer band is present in both panels, with slight variations in intensity over time.

(B) Western blots showing molecular weight markers at 148, 98, 64, 50, 36, 22, 16, 12, and 4 kDa. These markers help in the identification and quantification of the protein bands.
Fig. 6.

D-dimer
B-holes
A-holes

PF4

Thrombin
Fig. 8.
Platelet factor 4 (CXCL4) seals blood clots by altering the structure of fibrin
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