MINIREVIEW

Platelet Factor 4: Production, Structure, and Physiologic and Immunologic Action (43309)

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Platelet factor 4 (PF4) is a protein found in megakaryocytes and platelet α-granules (1, 2). Immunocytochemical studies show that it is present as well in mast cell granules (3) and on the endothelium of human umbilical veins, but not arteries (4). Early work on PF4 has been reviewed elsewhere (5–7). Human platelets contain about 18 ± 4 μg of PF4/10^9 (8).

Thrombospondin, platelet-derived growth factor, and compounds derived from platelet basic protein such as β-thromboglobulin (β-TG) are found in platelet α-granules in addition to PF4. All are secreted when platelets are appropriately stimulated; for example, since thrombin is a strong stimulus, these compounds are present in much higher concentrations in serum than in plasma (e.g., 5334 vs 1.8 ng/ml for PF4 [9]). They are also secreted after contact of platelets with collagen in damaged blood vessels, for example.

Production and Structure

PF4 is synthesized by megakaryocytes (10), an ability that correlates with cytoplasmic maturity (2, 11). The PF4 is first packaged into vesicles and from there it is transferred to α-granules (2). Megakaryocytes express mRNA transcripts for PF4, but not for fibrinogen or albumin, which are taken up by megakaryocytes from plasma (12). Even blood platelets, which contain little mRNA, contain mRNA for PF4, whereas none is evident in human lymphocytes, cultured fibroblasts, and four types of malignant cells (13).

Human PF4 is a 7.8-kDa protein that contains 70 amino acids, with two disulfide bonds, no tryptophan or methionine, two histidines, and a single tyrosine (Fig. 1). The position of its two disulfide bridges has been inferred by homology with the related compound β-TG (14). Its isoelectric point is 7.6 (15) and its extinction coefficient (1%, 280 μm) is reported to be 2.9 (3) or 5.4 (9). PF4 has been quantified by a modification of the Lowry method, with lysozyme as the standard protein; PF4 concentrations were 1.47 times the equivalent concentration of lysozyme (16).

Rat PF4 cDNA was the first to be cloned and sequenced, and the gene was isolated as well (17). A λ-gt11 cDNA expression library was constructed from rat megakaryocyte poly (A)-selected RNA, and a rabbit anti-rat PF4 antibody was used to detect positive clones. The cDNA exhibited a single open reading frame encoding a hydrophobic leader sequence of 29 amino acids, as well as a mature polypeptide sequence of 76 amino acids. A genomic library prepared from rat DNA was used to isolate the gene. The coding region of the mature molecule was interrupted by two introns.

Human PF4 was expressed in *Escherichia coli* using the nucleotide sequence deduced from the reported amino acid sequence; one product with additional amino acids at both termini (18) and another that was identical to the native molecule (19) had the immunoregulatory properties of the native protein (see below).

PF4 was cloned using a cDNA library derived from a human erythroleukemia line that synthesizes a number of megakaryocyte proteins (20). The DNA insert includes the 3′-untranslated region, the entire amino acid coding region for the mature PF4 protein, and a 5′ region containing coding information for an additional 18 amino acids. Supplemental genomic DNA sequencing showed that the full-length leader sequence codes for a typical hydrophobic signal sequence with an initial methionine (20). This recombinant PF4 was expressed in *E. coli* and had the same amino acid composition and amino-terminal sequence as native

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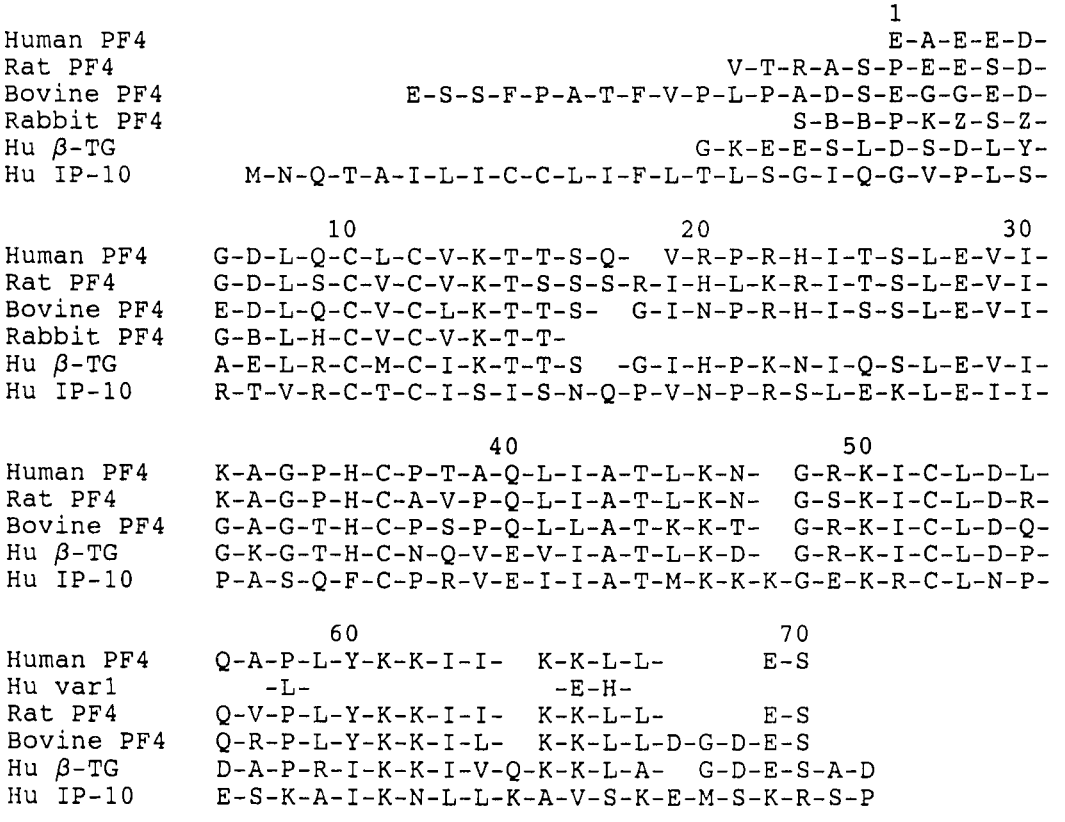

Figure 1. Amino acid composition of PF4 (human, human variant [only differences from PF4 shown; 23, 24], rat [17], bovine [26], and rabbit [N-terminal end only, 27]), human β-TG, and IP-10 (114). A recent publication (115) describes the composition of porcine and ovine PF4.

PF4, except for an N-terminal methionine. Since this compound exhibited chemotactic activity, this attribute of native PF4 is not due to a contaminant (21). The gene for PF4 is mapped to chromosome 4q12-q21 (22).

Two laboratories described a variant of human PF4 called PF4var1 (23) or $PF4_{alt}$ (24). This has a number of different amino acids in the leader sequence, and three amino acid differences in the C-terminal tridecapeptide (Fig. 1). RNA studies show that only the PF4 gene and not the gene for $PF4_{alt}$ is expressed in human erythroleukemia line cells (25).

Figure 1 shows the partial or complete amino acid sequence of PF4 of human, rat, bovine (26), and rabbit (27) origin. The four cysteine molecules are aligned. The nonhuman proteins have additional amino acids at the N terminus, whereas the C terminus is highly conserved. PF4 from the rhesus monkey is presumably very similar to human PF4, since the two show complete immunologic identity (28).

PF4 has considerable amino acid homology with derivatives of platelet basic protein such as β-TG and connective tissue-activating peptide (Ctap)-III (6, 7, 29), as well as with their relatives IP-10, interleukin (IL) 8, and others; all are produced by a family called small inducible genes (30) or "intercrine" cytokine genes (31). The immunologic and chemotactic functions of these proteins have been reviewed recently (31). Figure 1 shows the sequence of human β-TG and IP-10. All of these proteins have similar placement of the four cysteine molecules, as well as sequence homologies that are especially marked at the amino terminus. In another subgroup, the first two cysteines are in an adjacent position (31). Ctap-III and neutrophil-activating peptide (NAP)-2 are formed by enzymatic cleavage of amino acids from the N terminus of platelet basic protein by a serine protease (32), which may be derived from monocytes (33) or neutrophils (34). Platelet-derived growth factor is structurally unrelated to these compounds.

PF4 is secreted from platelets in a complex composed of four molecules of PF4 and two of chondroitin-4-sulfate (5–7). In platelets, the glycosaminoglycan is present as part of a proteoglycan, which can also be secreted (35) and combined with PF4 (35, 36). When PF4 is separated from its carrier, chromatographic observations and, more recently, studies using sucrose gradient ultra-centrifugation showed that it forms tetramers at physiologic pH and ionic strength (37). Bovine PF4 is also tetrameric when examined by x-ray crystallography (38). The equilibria (among monomers, dimers, and tetramers were examined by ^1H nuclear magnetic resonance spectroscopy (39). The secondary structure of human (40, 41) and bovine (42) PF4 has been described.

Relationship to Heparin *In Vitro*

PF4 was originally recognized by its ability to neutralize the anticoagulant activity of heparin, and it can be readily isolated from the chondroitin sulfate complex in platelet releasates by making use of its ability to bind strongly to heparin-agarose (15). Several other secreted compounds (β-TG, thrombospondin) also bind to heparin, but are eluted at 0.5 *M* NaCl, whereas elution of PF4 requires approximately 1.2–1.4 *M* NaCl. The affinity of PF4 for glycosaminoglycans decreases in the following order: heparin, heparan sulfate, dermatan sulfate, chondroitin-6-sulfate, and chondroitin-4-sulfate (15, 43).

The C-terminal portion of PF4, which contains two double lysines and is thought to form an α helix (42, 44), is very important in promoting binding of heparin, since guanidination of the lysines (15) or digestion with carboxypeptidase (45) decreases binding. Thus, it is not surprising that rPF4var1, with three amino acid substitutions in the C-terminal end (46), as well as rPF4-241, in which the lysine pairs are replaced by glutamine-glutamate (47), bind less readily to heparin than does rPF4. Two heparin molecules of 8–10 disaccharide units bind to one PF4 tetramer (48, 49). As might be anticipated from the requirement for tetramer formation, the secondary structure of PF4 is also very important, so that reduced PF4 and the C-terminal 47–70 and 58–70 peptides of PF4 bind much less strongly to heparin-agarose than native PF4, requiring only 0.2–0.5 *M* NaCl for elution (50).

Physiologic Activities

Effects on Blood Coagulation. Heparin inhibits coagulation mainly by increasing the reaction between the plasma protein antithrombin III (AT III) and thrombin (51). Heparin molecules larger than 18 saccharide units effectively enhance the antithrombin activity of AT III, and this ability of heparin is neutralized by PF4. The antifactor Xa activity of AT III is also enhanced by these heparin molecules, and this action is less effectively neutralized by PF4. Heparin molecules as small as 8 saccharide units enhance the ability of AT III to inactivate factor Xa but not thrombin, and this activity is not counteracted by PF4 (52). Neutralization of the antifactor Xa activity of commercial heparin requires a 3- to 4-fold excess of PF4 (w/w); rPF4-241 is ineffective (47).

PF4 inhibits activation of purified Hageman factor (Factor XII) by the negatively charged substance ellagic acid. It also inhibits the shortening of the partial thromboplastin time by glass or kaolin that results from Hageman factor activation (53).

PF4 in the Circulation. PF4 binds to the endothelium of umbilical veins (4) and to the glomerular microvascular matrix (54), as well as to cultured endothelial cells from human umbilical veins (43) and bovine aorta (55, 56). Binding is saturable (55). It is abolished after incubation of the cells with heparinase from platelets (43) or Flavobacterium heparinase (55, 56), but not with chondroitin-ABCase (54, 55) or hyaluronidase (54, 56), suggesting that PF4 binds to the heparan sulfate on the cell surface. Endothelial heparan sulfate can act as a co-factor for the effect of plasma AT III on thrombin, and PF4 can antagonize this antithrombic action (57–59).

PF4 inhibits the spontaneous migration of cultured bovine aortic endothelial cells in a reversible, dose-dependent manner. Migration depends on endogenous basic fibroblast growth factor (bFGF), and 5 μg/ml of PF4 inhibits binding of ^{125}I-bFGF to the specific bFGF receptor of NIH 3T3 cells. Nonspecific binding of bFGF to the heparan sulfate on the cellular matrix is abolished by 2 *M* NaCl at neutral pH and is less readily inhibited by PF4 than is binding of bFGF to its specific receptor (60).

When serum is injected intravenously in man, much of its PF4 (measured by radioimmunoassay) disappears extremely rapidly from the circulation. This is in contrast to β-TG, which disappears much more slowly (61). The half-life of injected human PF4 has been measured more precisely in animals, either by radioimmunoassay or by use of radiolabeled PF4. In rhesus monkeys (28), rats (62), and rabbits (63, 64), PF4 disappearance has a fast component with a half-life of a few minutes and a slow component with a half-life of over half an hour. Injection of 1000-fold excess of cold PF4 does not alter the disappearance rate of about 1 μg of ^{125}I-PF4, indicating that the binding sites were still not saturated (64). Clearance is not affected by nephrectomy (65). Over 40% of the injected, radiolabeled PF4 is found in the liver within 5 min, but since only about 50% of the injected radioactivity is found in the major organs, the PF4 may also bind to capillary endothelium in the skin and muscles (64). Calculations from the extrapolated value of the fast component give a value for the amount of PF4 injected that is considerably smaller than the injected amount, suggesting that some of the PF4 disappears on its first passage through the circulation (28, 62–64). The rapid disappearance of the PF4 in injected serum (61) indicates that the responsible binding sites have a higher affinity for PF4 than does chondroitin-4-sulfate.

PF4 and β-TG occur in only trace amounts in plasma collected with precautions to avoid platelet stimulation (e.g., 13.9 ± 6.1 (SD) ng of PF4/ml and 30.7 ± 13.7 (SD) ng of β-TG/ml [61]). An increase in the amount of PF4 and β-TG in carefully collected plasma is noted in many clinical conditions, and is thought to indicate that platelet stimulation has occurred *in vivo* (see Ref. 9, for example). Actually, the ratio between the amounts of these proteins in the patients' plasma compared with the ratio in plasma

from normal individuals may be a better measure of *in vivo* release than measurement of either protein alone, since the PF4 released *in vivo* will disappear faster than the released β-TG (66).

Washed platelets that have not been intentionally stimulated bind ^{125}I-labeled PF4 in a specific, saturable manner, and binding is inhibited by unlabeled PF4 and by heparin (37). More radiolabeled antibody to PF4 (67) or its Fab fragments (37) binds to thrombin-stimulated platelets than to control platelets, suggesting that some of the secreted PF4 binds to the platelet surface. No significant binding of radiolabeled β-TG or antibody to β-TG was noted (37).

Concentrations of PF4 up to about 4 μg/ml do not cause aggregation of washed platelets, or secretion of serotonin. However, at concentrations between 50 and 1000 ng/ml, PF4 often potentiates submaximal platelet aggregation and secretion caused by other agonists. Fab fragments of anti-PF4 antibody, but not of anti β-TG antibody, inhibit platelet aggregation (37).

Response to Intravenous Heparin. In man, intravenous injection of heparin (usually >5000 units, or about 33 mg) results in an immediate 15- to 30-fold increase in the plasma concentration of PF4 measured by radioimmunoassay (4, 61, 62, 68, 69), without altering intraplatelet PF4 (69). The increase is about 5% of the amount potentially available from the platelets (4). Doubling the heparin dose from 60 to 120 units/kg does not increase the amount of heparin released (69). The released material is indistinguishable from PF4 prepared from platelets on the basis of its molecular weight as determined by sodium dodecyl sulfate polyacrylamide electrophoresis, affinity for heparin-agarose, and reactivity with antibody (62). No β-TG is released (61, 62). The postheparin increase in PF4 is normal in patients with arterial disease or diabetes (68–71), and is greater in patients with thrombocytosis than in normal subjects or patients with normal platelet counts (68).

Although the plasma concentration of PF4 returns to its normal low level within 4 hr after heparin injection, a second heparin injection given at that time barely increases PF4. The ability of heparin to release PF4 is progressively restored, reaching 50% of the original value in 24 hr and the original value in 48 hr or more (4, 69).

The effect of heparin on the disappearance of exogenous PF4 has also been studied. The rapid component of PF4 disappearance is lost when heparin and human PF4 are injected together into rabbits, which results in a single compartment disappearance curve with a half-life of about 30 min (50, 63, 64). Similar results are obtained in rats (62). The slow clearance of β-TG is not affected by heparin (4, 61, 64).

It seems likely that injected heparin releases PF4 that was bound to heparan sulfate on endothelial cells (4). Over 40% of radiolabeled PF4 injected into rabbits is found in the liver within 5 min, from which it can be partially removed by injection of heparin (64), suggesting that at least part of the PF4 binds to heparan sulfate in the liver vasculature. However, cultured hepatocytes also bind PF4 and then internalize and degrade it (72). Injected β-TG accumulates in the kidneys rather than in the liver (64).

After injection, radiolabeled PF4 appears in the urine in relatively small amounts after a lag of 15 min. In contrast, when heparin is injected simultaneously with the PF4, 100 times more radioactivity appears in the urine with a much shorter lag time (64). Thus, it is difficult to interpret changes in PF4 excretion in heparinized patients (9).

PF4 may be a clinically useful substitute for protamine, since it counteracts the anticoagulant action of injected heparin without causing the adverse pulmonary effects of protamine (73).

Megakaryopoiesis. Human PF4 inhibits megakaryocytopoiesis *in vitro* (74–76). According to one group, inhibition requires 25 μg of PF4/ml. The 24 amino acid C-terminal peptide of PF4 is active, but the C-terminal tridecapeptide (amino acids 58–70), or β-TG, is not. The 24-amino acid peptide inhibits megakaryocyte maturation, as measured by production of Factor V rather than megakaryocyte number (74). Other workers, however, reported that both PF4 and β-TG, at concentrations of only 1–2.5 μg/ml, inhibit development of megakaryocytes in marrow cultures and have an additive effect (75, 76). At the concentrations that affect megakaryopoiesis, PF4 has no effect on erythroid or granulocyte-macrophage colony formation (74, 75).

Angiogenesis. Recombinant human PF4 inhibits angiogenesis in the chicken chorioallantoic membrane in a dose-dependent manner (19). The effect is marked at about 20 μg per implant disk and is due to specific inhibition of endothelial cell proliferation. The C-terminal 12 amino acids are also extremely inhibitory at about 8.5 μg/disk. Although heparin at 50 μg/disk (approximately 8 units) inhibits the effect of PF4 (19), rPF4-241, which does not bind to heparin, also inhibits angiogenesis (47). The growth in mice of melanoma and human colon carcinoma is inhibited when rPF4 is injected into the lesion at 50 μg/day (77). This antitumor activity may result from inhibition of angiogenesis, since rPF4 has no effect on the proliferation of the tumor cells in culture.

Miscellaneous Activities. Human PF4 stimulates the activity of human leukocyte elastase (78). It also inhibits collagenases derived from both cultured human skin and granulocytes; this activity is not inhibited by heparin (79). Since a cartilage-derived substance unrelated to PF4 also inhibits both neovascularization and collagenase activity, these properties may be related (80). PF4 inhibits bone resorption in parathyroid

hormone-stimulated cultures (81). PF4 and its C-terminal amino acids 59–70 stimulate histamine release from basophils (82).

PF4 induces intercellular adhesion molecule (ICAM)-1 in cultured endothelium; the C-terminal 41-amino acid peptide, but not the N-terminal 29-amino acid peptide, is also active (83). Treatment of neutrophils with PF4 causes them to adhere to endothelial cells (84), but treatment of endothelial cells is not effective.

PF4 can be used as a substrate for cell attachment. However, fibroblasts form tight focal contacts (85) or microfilaments (86) and neuroblastoma cells form neurite extensions (87) much less readily on PF4-coated surfaces than they do on surfaces coated with fibronectin or its cell-binding fragment.

At a concentration of only 7.6 ng/ml, PF4 increases the mass to length ratio of fibrin fibers, resulting in larger, thicker fibers (88).

Chemotaxis. PF4 and its C-terminal peptides have chemoattractant activity. The necessary concentrations of PF4 for human polymorphonuclear leukocytes and monocytes have been reported as 1–5 μg/ml, with a decrease in efficacy at higher concentrations (89), whereas others reported that its activity on neutrophils increased from about 8 μg/ml to about 80 μg/ml (84) and a third group reported that neutrophils of only three of nine subjects showed significant chemotaxis, with peak activity at about 30 μg/ml (90). The discrepancies may be related to the method used for studying chemotaxis (90). Recombinant PF4 with an N-terminal methionine was active at 0.3 μg/ml, with a maximum effect at 5 μg/ml and no decrease at higher concentrations (20). In the presence of cytochalasin B, PF4 causes the release of neutrophil enzymes (84, 89). Prior incubation of neutrophils with PF4 specifically inhibits receptor-mediated internalization of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine without altering binding to its receptors, but does not deactivate the effect of other chemotactic factors (91).

The C-terminal tridecapeptide is active as a chemoattractant at 5–50 ng/ml, with a decrease at higher concentrations (92). The dodecapeptide was reported to be increasingly active at up to 1500 ng/ml (91); although it caused high chemotactic responses, its EC_{50} was 1000-fold greater on a molar basis than that of NAP-1 or C5a (90).

Five daily injections of 25 μg of PF4 recruits granulocytes and mononuclear cells and causes marked fibrosis. The effect is also produced by the 41-amino acid C-terminal peptide, but not by an N-terminal peptide (93).

PF4 also causes directed fibroblast migration and, thus, together with β-TG and platelet-derived growth factor, may play a role in wound repair (94).

Immunoregulation

The immunoregulatory effects of mouse PF4, native and recombinant human PF4 (rPF4), and synthetic C-terminal peptides of human PF4 have been described by our group (18, 95–102) and others (46, 103).

***In Vivo* Effects.** We showed initially that intravenous injection of mouse or human serum enhanced the murine immune response to sheep red blood cells (SRBC) or TNP-Ficoll when the response had been suppressed by injection of syngeneic mouse lymphoma cells (95) or concanavalin A (Con A) (96). The response measured was the number of plaque-forming cells (PFC) in the spleen (104, 105). A suppressive agent and serum were injected on Day −1, and SRBC was injected on Day 0. Spleens were removed on Day 5, dissociated into single cell suspensions, washed, and plated on duplicate slides in agar containing 8% SRBC as indicator. The slides were incubated at 37°C for 1.5 hr, then flooded with complement and incubated for an additional 1.5 hr. The secreted antibodies plus complement cause lysis of the SRBC surrounding the secreting cells, resulting in clear areas (plaques) on the slides. The plaques are counted and the number per spleen is calculated.

The serum factor that alleviated immunosuppression was derived from platelet α-granules, since activity was absent from plasma, from serum prepared in the absence of platelets, and from serum from a patient whose platelets lacked α-granules (95). Incubation of mouse or human platelets with thrombin (95) or the calcium ionophore A23187 (96) released a factor with similar properties, i.e., injection of the releasate restored PFC responses to the unsuppressed value. As little as 50 μl of serum or 20 μl of releasate from 10^9 platelets/ml completely alleviated suppression (95). Activity was attributed to PF4, since it was absorbed by heparin-agarose, neutralized in serum by goat anti-human PF4, and present in purified human PF4 (97) as well as rPF4 (18). Platelet-derived growth factor was inactive (97) and β-TG had less activity than PF4 (97, 98). Il-8, which shares about 30% structural homology with PF4 at the amino terminus, was also inactive (99).

The ability of rPF4 to reverse Con A-induced immunosuppression was confirmed by Johnson *et al.* (46).

To determine whether the whole PF4 molecule is necessary for immunoregulatory activity, we tested enzymatic cleavage products of PF4, as well as synthetic peptides. Peptides from the C-terminal end of PF4 (amino acids 55–69 and 58–70) reversed Con A-induced immunosuppression, whereas the peptides comprising amino acids in the middle and N-terminal portions of PF4 or three irrelevant peptides did not do so (98). However, the amino acid requirement for immunoregulatory activity was surprisingly nonspecific. Thus, the C-terminal 18-amino acid peptide of β-TG

and Ctap-III, which is similar but not identical to the C-terminal 13 amino acids of PF4 (Fig. 1), was active. Although the lysine groups are critical for heparin binding (see above), they do not seem important for immunoregulatory activity. Thus, an analog of the C-terminal tridecapeptide in which lysine was replaced with arginine and the last two amino acids were omitted (106) was active, as was rPF4 in which the free amino groups had been reacted with fluorescamine (98). Furthermore, PF4var1, in which three of the amino acids in the C-terminal tridecapeptide were altered (Fig. 1) and which binds poorly to heparin, had greater immunoregulatory activity than the native molecule (46). Finally, we found that three substituent peptides of PF4's C-terminal tridecapeptide were active; they consisted of amino acids 58–62, 58–66, and 63–70 (98).

In addition to counteracting nonspecific immunosuppression, native PF4, as well as rPF4, alleviates antigen-specific suppression of the antibody response to pneumococcal polysaccharides (100). PF4 interferes with induction of antigen-specific low dose tolerance when injected at the same time as a low dose (0.2 μg) of Type 14 pneumococcal polysaccharide, 3 days before an optimal immunizing dose of 25 μg. Furthermore, injection of platelet releasate together with an optimal primary immunizing dose of pneumococcal polysaccharide Type 14 enhances the secondary response to killed bacteria injected 2 weeks later, but has no effect on the primary response. Both of these effects may be due to interference with antigen-specific suppressor cell induction during primary immunization.

Gregg *et al.* (103) showed that PF4 also affects the delayed-type hypersensitivity response to SRBC, a model of specific cell-mediated immunity. Fifty micrograms of PF4 injected intraperitoneally or subcutaneously 1 hr before sensitization with SRBC significantly enhanced the delayed-type hypersensitivity reaction to rechallenge with SRBC. The study suggested that PF4 may be an inhibitor of suppressor cell function. On the other hand, intravenous injection of up to 450 μg of PF4/m^2 into patients with malignant melanoma and elevated numbers of circulating suppressor cells does not reduce the suppressive activity of these cells (P. O. Livingston, S. Cunningham-Rundles, and M. K. Hoffman, unpublished manuscript), although cyclophosphamide does so (107).

Inhibitor of PF4 Action. Platelet releasates prepared by stimulating once-washed human platelets with the calcium ionophore A23187 in the presence of the serine protease inhibitor *p*-amidinophenylmethanesulfonyl fluoride (APMSF), or boiled within 2 min after secretion, have much less immunoregulatory activity than releasates prepared without the protease inhibitor, although they contain the same amounts of PF4 (97). This led us to propose that an enzyme acted on the amino terminus of PF4 (97), as it does when platelet basic protein is converted to its physiologically active subunits (32–34). However, this hypothesis proved to be incorrect when it was found that the whole rPF4 molecule is active (18). Subsequently, we found that reduction of the APMSF releasates or absorption with Con A-agarose restores immunoregulatory activity, whereas boiling or exposure to pH 1.5 for 5 min does not. Moreover, adding an eluate of the Con A-agarose used to absorb PF4 prepared from releasates from once-washed platelets markedly decreases the immunoregulatory activity of rPF4. Thus, APMSF-treated releasates of once-washed platelets contain an inhibitor of the PF4 effect. Like PF4, this inhibitor is derived from platelets, because releasates from thrice-washed platelets fail to alleviate immunosuppression in spite of their high PF4 content, even without APMSF treatment. These results suggest that a serine protease from plasma that contaminates releasates made from once-washed platelets may be required to destroy a platelet-derived inhibitor before the contrasuppressive effect of the PF4 in platelet releasates can be demonstrated. The inhibitor appears to be a carbohydrate-containing, heat- and acid-stable peptide, susceptible to disulfide reduction and to digestion by a serine protease (101).

Neutralization of Activity by Incubation with Spleen Cells. Cells from the mouse spleen and lymph node, but not from the thymus or from spleens of athymic mice, neutralize the immunoregulatory activity of serum after 1 hr of incubation at 4°C (95). Phenotype studies show that these cells belong to an activated Ly2$^+$ subpopulation of T cells (96). Immunostimulatory activity of rPF4 (18), as well as that of the C-terminal tridecapeptide amino acids 58–70 (98), is also eliminated by incubation with spleen cells from normal, but not from athymic, mice. Despite loss of activity, we have been unable to demonstrate loss of more than half of the PF4 in the supernatant when it is measured by enzyme-linked immunoabsorbent assay or radiommunoassay. Further studies are in progress.

***In Vitro* Studies.** To avoid the complex *in vivo* environment and gain insight into the mechanisms of immunosuppression and its alleviation by PF4, we used as our assay system the anti-SRBC PFC responses of spleen cells *in vitro* (99, 102). The fetal calf serum used in the cultures had been passed over heparin-agarose to decrease its PF4. Spleen cells from mice immunized *in vivo* with SRBC were co-cultured with Con A blasts, which contributed an enriched source of suppressor cells. Con A blasts were generated in a 48-hr culture of whole spleen cells with Con A, a mitogen for T cells which causes them to transform into blasts. At the end of the 2-day culture, Con A was removed by washing the blasts twice with α-methylmannoside. This Con A blast-induced suppression is not due to absorption of IL-2 by activated blast cells, since addition of exogenous IL-2 to the cultures does not overcome suppression.

Suppression is mediated by $CD4^-$, $CD8^+$ cells, since it is abrogated when the Con A blasts are treated with anti-CD8 and complement.

As shown in *in vivo* studies, both rPF4 and its C-terminal fragment amino acids 58–70 significantly reduce *in vitro*-induced suppression when added during the first 20 hr of a 4-day culture. The concentrations of rPF4 used *in vitro* are very similar to those used *in vivo*; rPF4 (0.2 μg) is active when injected into a mouse with a plasma volume of perhaps 1 ml, and 0.02–0.2 μg/ml of rPF4 alleviates suppression of the anti-SRBC response *in vitro*. IL-8, a monocyte-derived, neutrophil-activating factor which, like PF4, belongs to the family of molecules known as small inducible proteins (30), does not alleviate Con-A-induced suppression (99), probably due to a lack of structural homology with PF4 at the C-terminal end (31).

Since elimination of prostaglandin production by the addition of indomethacin to cultures not only increases immunosuppression, but sometimes decreases the effect of PF4, PF4 may need the production of prostaglandins for its effect (99). Enhanced IL-1 production is not the mechanism of PF4 action, since addition of IL-1 to cultures has no effect on suppression or its alleviation by PF4.

We and others have found that blocking the histamine H2 receptor with cimetidine prevents Con A blast-induced suppression, and that stimulation of the receptor by the H-2 agonist dimaprit induces suppression. We find that dimaprit-induced suppression is abolished by rPF4 (99).

Others also demonstrated an immunoregulatory role of PF4 *in vitro*, but they measured the proliferative response of mouse spleen cells to Con A and its inhibition by Con A-induced irradiated suppressor cells, rather than the PFC response of spleen cells to SRBC (104). PF4, in contrast to IL-8, prevented the generation of Con A-induced suppressor cells at concentrations above 8 μg/ml, but had no effect on the proliferative response to a number of stimuli, as other cytokines do, or on its suppression.

A recent abstract reports that rPF4 and its non-heparin-binding analog PF4-241 inhibit replication of cultured Kaposi sarcoma cell lines (108).

Conclusion

Numerous *in vivo* and *in vitro* effects of PF4 have been described. The effects of PF4 on angiogenesis (19), tumor growth (77), and perhaps megakaryocyte maturation (74) require high concentrations of PF4. In contrast, its immunoregulatory activity (18, 46, 96–103) requires concentrations that could occur *in vivo* near secreting or disintegrating platelets. Thus 0.2 μg, the amount of PF4 that is active when injected into a mouse or added to a 1-ml culture, is present in about 10^7 platelets, or the number of platelets present in about 40 μl of human blood. Blood platelets, the main repository of PF4, may play a part in inflammatory processes (109–111), and PF4 has been demonstrated where platelets are localized at a site of injury (112). Proteoglycans, such as serglycin, which binds PF4, are gaining attention as modulators of growth factor activity (113).

Little attention has been paid to the PF4 found in mast cell granules. The PF4 presumably bound to heparan sulfate on the vascular endothelium may be important in modulating activity of antithrombin III (58, 59) and possibly in other ways as well. The relationship between the many effects of PF4 in different assays and its actual physiologic and pathologic roles needs further study.

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