A potent inhibitor of endothelial cell proliferation is generated by proteolytic cleavage of the chemokine platelet factor 4

SHALLEY K. GUPTA*, TOM HASSEL, AND JAI PAL SINGH†
Cardiovascular Research, Lilly Research Laboratories, Indianapolis, IN 46285

Communicated by Henry Lardy, University of Wisconsin, Madison, WI, May 11, 1995

ABSTRACT Platelet factor 4 (PF-4) is an archetypal of the "chemokine" family of low molecular weight proteins that play an important role in injury responses and inflammation. From activated human leukocyte culture supernatants, we have isolated a form of PF-4 that acts as a potent inhibitor of endothelial cell proliferation. The PF-4 derivative is generated by peptide bond cleavage between Thr-16 and Ser-17, a site located downstream from the highly conserved and structurally important CXC motif. The unique cleavage leads to a loss of one of the structurally important large loops in the PF-4 molecule and generation of an N terminus with basic residues that have the potential to interact with the acidic extracellular domain of the G-protein-coupled chemokine receptor. The N-terminal processed PF-4 exhibited a 30- to 50-fold greater growth inhibitory activity on endothelial cells than PF-4. Since endothelial cell growth inhibition is the only known cellular activity of the cleaved PF-4, we have designated this chemokine endothelial cell growth inhibitor. The N-terminal processing of PF-4 may represent an important mechanism for modulating PF-4 activity on endothelial cells during tissue injury, inflammation, and neoplasia.

Platelet factor 4 (PF-4) is a member of a recently discovered multigene family of low molecular weight proteins known as "chemokines" (1-5). All members of the chemokine supergene family share four highly conserved cysteine residues (1-3). The members are classified as α or β based on the nature of the cysteine motif present at the N terminus. The α gene family characterized by a CXC motif (cysteines separated by another amino acid) includes PF-4, interleukin 8 (IL-8), human protooncogene Gro/melanocyte growth-stimulating activity, β-thromboglobulin, neutrophil-activating protein 2, neutrophil-activating protein from epithelial cells (ENA-78), and interferon-inducible protein 10 (1-3). The β gene family characterized by a CC motif (adjacent cysteines) includes monocyte chemotactic protein 1 (MCP-1), RANTES, and macrophage inflammatory proteins 1α and 1β (1-3). The genomic exon organization of >20 of the chemokines identified to date is nearly identical, suggesting a divergence from a common ancestral gene (3, 4). The members of the superf gene family play an important role in modulating a variety of biological activities during inflammation and tissue repair including cell adhesion, migration, activation, proliferation, hematopoiesis, and angiogenesis (1-6).

Human PF-4 is a tetramer of four identical polypeptides each containing 70 amino acid residues (7). Primary and three-dimensional structural studies have shown that PF-4 contains three large loops that participate in joining the three strands of β-sheet and the C-terminal α-helix (8). The highly conserved CXC motif at the N terminus plays an important role in conferring the tertiary structure to native PF-4 by forming intramolecular disulfide bonds with Cys-36 and -52. Recent studies have revealed that PF-4 is an important modulator of endothelial cell proliferation (5, 9) and angiogenesis (5, 10). Although a selective inhibition of endothelial cells by PF-4 has been an important finding, the unusually high concentration required for endothelial cell growth inhibition (micromolar range as compared to nanomolar concentrations for other chemokines) has been puzzling. We report here the identification of a cleavage product of PF-4 that acts as a potent inhibitor of endothelial cell proliferation. The unique cleavage site located downstream from the N-terminal CXC motif is such that cleavage leads to important structural and functional changes. Our studies also show that the highly conserved CXC motif thought to be important for structure and function of PF-4 is not essential for endothelial cell growth inhibition. Furthermore, these results suggest that N-terminal processing of PF-4 may represent an important biological mechanism regulating its action on endothelial cells and homeostasis in the vasculature.

MATERIALS AND METHODS

Cell Culture. Fetal bovine heart endothelial cells were obtained from the American Type Culture Collection. Cell cultures were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), and basic fibroblast growth factor (FGF) (20 ng/ml). Cultures were passaged once a week by trypsin treatment. Human aortic endothelial cells were obtained from Clonetics (San Diego). The cells were maintained in endothelial cell growth medium supplemented by Clonetics.

Determination of DNA Synthesis and Cell Proliferation. For determination of DNA synthesis, endothelial cells were cultured in 96-well plates at a starting density of 4 × 10^5 cells per well in growth medium. The cultures reached confluence after 5 days at which time the medium was replaced with fresh DMEM containing 10% FBS, FGF (20 ng/ml), [3H]thymidine (1 μCi/ml; 1 Ci = 37 GBq), and various concentrations of PF-4 or fractions containing cleaved PF-4. After 24 hr, cells were fixed in methanol and radioactivity was determined by scintillation counting. For determination of cell proliferation, 1 × 10^4 endothelial cells were plated in 24-well plates in DMEM containing 10% FBS, basic FGF (20 ng/ml), and the indicated concentration of PF-4 or cleaved PF-4. Cells were counted with a ZM Coulter Counter after 4 days.

Human Leukocyte Conditioned Medium. Medium conditioned by human peripheral blood leukocytes was prepared as described (11). Cells from the buffy coat preparations were washed in Hanks' balanced salt solution and plated at a density of 3 × 10^6 cells per ml in serum-free minimal essential medium

Abbreviations: PF-4, platelet factor 4; FGF, fibroblast growth factor; FBS, fetal bovine serum; IL-8, interleukin 8; TFA, trifluoroacetic acid.

*Present address: SmithKline Beecham, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406.
†To whom reprint requests should be addressed at: Cardiovascular Research, DC 0520, Lilly Research Laboratories, Indianapolis, IN 46285.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
supplemented with 2 mM l-glutamine, nonessential amino acids, 0.8 mM d-glucose, penicillin (100 units/ml), streptomycin (100 μg/ml), and lipopolysaccharide (20 μg/ml). The cells were incubated for 30 hr at 37°C in 5% CO₂/95% air. The conditioned medium was harvested by filtration through a 0.2-μm filter.

Isolation and Characterization of Endothelial Cell Growth Inhibitor. Four liters of cell-free conditioned medium was acidified by addition of 0.1% trifluoroacetic acid (TFA) and then loaded directly onto a semipreparative reversed-phase column (Bio-Rad C4-RP-304; 250 × 21.5 mm). The column was eluted with a linear gradient of 0–50% acetonitrile in 0.1% TFA over 40 min and then for 10 min at 50% acetonitrile and 0.1% TFA. The flow rate was 15 ml/min. One-minute fractions were collected. The elution profile was monitored at 210 nm. Fractions eluted with 33–39% acetonitrile were pooled. The pool was further fractionated by heparin affinity chromatography using a Bio-Rad Econo-Pac 5-ml heparin-Sepharose cartridge. The bound proteins were eluted with a linear gradient of 0–2.0 M NaCl in phosphate-buffered saline at a flow rate of 2.0 ml/min. Proteins eluted from the column were pooled into three fractions as non-heparin binding (flow-through), low-affinity heparin binding (0–0.7 M NaCl), and high-affinity heparin binding (0.7–2.0 M NaCl) fractions. The high-affinity heparin binding fractions containing endothelial cell growth inhibitory activity were pooled and applied to an analytical C-4 column (Vydac; 0.46 × 10 cm; Hesperia, CA), previously equilibrated with 27% acetonitrile/0.1% TFA. The column was eluted with a linear gradient of 32% acetonitrile to 42% acetonitrile in 0.1% TFA, over 10 min. Absorbance was monitored at 210 nm. Fractions were analyzed for inhibition of DNA synthesis in endothelial cells.

RESULTS

Isolation and Characterization of PF-4 Cleavage and Endothelial Cell Growth Inhibitor. Our initial studies suggested the presence of a low molecular weight endothelial cell growth inhibitory activity in the culture supernatant of activated human leukocytes that appeared unrelated to the previously described inhibitors such as transforming growth factor β (12), SPARC (13), or interferon (14). Further characterization of this activity has revealed a unique peptide sequence derived from PF-4 that acts as a potent inhibitor of endothelial cell proliferation. Serum-free culture medium from human peripheral blood leukocyte conditioned medium on a semipreparative reversed-phase C-4 column. The proteins were eluted with a linear gradient of 0–50% acetonitrile in 0.1% TFA. Active fractions were pooled, lyophilized, and then purified on a heparin-Sepharose column. Bound proteins were eluted with a linear gradient of 0–2.0 M NaCl in phosphate-buffered saline. High-affinity heparin binding fractions were pooled and then purified on an analytical C-4 column (0.46 × 10 cm). The activity peak was recycled through the C-4 column to obtain a homogenous preparation. (A) Protein (absorbance 210 nm) and activity profile (solid bars) from an analytical C-4 column are shown. Activity was determined by inhibition of DNA synthesis in fetal bovine heart endothelial cells stimulated with FGF (9). The activity peak was analyzed by gel electrophoresis in SDS/18% polyacrylamide (15) before and after reduction with 1 mM 2-mercaptoethanol. Lanes: 1, molecular weight standards; 2, purified human PF-4 from Sigma; 3, reduced PF-4; 4, activity peak; 5, activity peak after reduction. (B) Activity peak was analyzed for amino acid sequence and molecular mass. Sequence II was based on the 20 N-terminal residues of the 7.8- and 6.0-kDa bands obtained before or after reduction.

and 17. The latter is consistent with a shift in protein band migration in SDS/polyacrylamide gels from 7.8 to 6 kDa upon reduction. The identity of the cleaved PF-4 was further confirmed by mass spectrometry. As shown in Fig. 2, the molecular weights of PF-4 and cleaved PF-4 were 7765 and 7783, respectively. The latter corresponds to a molecular mass generated by addition of one H₂O molecule to PF-4. After reduction, the peptide exhibited a mass of 6033, a mass corresponding to PF-4 lacking the first 16 amino acids (Fig. 1B, sequence II). Thus, the amino acid sequence determination, SDS gel electrophoresis, and mass spectrometric analysis data clearly established the identity of the endothelial cell growth inhibitory activity as a derivative of PF-4 generated by a single cleavage between Ser-16 and Thr-17.

Biological Activity of Cleaved PF-4. Previous studies have shown that PF-4 inhibits endothelial cell proliferation and angiogenesis (5, 9). Therefore, we examined the effect of the HPLC-purified cleaved PF-4 on endothelial cell growth. These results have revealed an unexpectedly high specific activity of cleaved PF-4 compared to the native PF-4. As shown in Fig. 3A and B, the native PF-4 and the cleaved PF-4 produced a
N-terminal cleavage of PF-4 leads to a significant increase in endothelial cell growth inhibitory activity.

**Activation of PF-4 Is Cleavage Dependent.** Two important structural consequences of the N-terminal cleavage of PF-4 are the loss of a structurally important large loop joining the N terminus to the first strand of the β-sheet and the generation of a new N terminus. The highly conserved N-terminal CXC motif is thought to play a critical role in conferring the structure and functional activity to PF-4 (1, 8). The large loop is stabilized by the intramolecular disulfide bonds and hydrogen bond between Thr-16 and Cys-52 in the β-sheet (Fig. 4). A cleavage at this critical site is expected to induce significant changes in PF-4 structure. These structural changes have apparently led to the acquisition of a greater specific activity in the resulting molecule. To investigate whether the changes in PF-4 structure and biological activity are due to specific cleavage and not nonspecific unfolding of PF-4, we subjected PF-4 to unfolding conditions and reversed-phase chromatography. PF-4 purified from platelets was subjected to unfolding by reduction with 2-mercaptoethanol or reduction and denaturation and then fractionated on a reversed-phase C-4 column as in the purification of cleaved PF-4. Each preparation was then tested for inhibition of endothelial cell growth. Unfolding of PF-4 by reduction or denaturation produced structural changes resulting in increased hydrophobicity as revealed by a greater retention on the reversed-phase column (Fig. 5A). However, unfolding of the PF-4 molecule did not enhance its endothelial cell growth inhibitory activity. To the contrary, reduction of PF-4 led to a loss of activity. These results suggest that the increase in PF-4 activity could not simply be due to a nonspecific unfolding of the protein. Furthermore, reduction and dissociation of the N-terminal peptide from cleaved PF-4 did not result in a complete loss of endothelial cell growth inhibitory activity of PF-4 (Fig. 5B). A slight loss of activity of cleaved PF-4 upon reduction shown here may have been due to the experimental conditions used for achieving complete reduction. As before, the completion of reduction of cleaved PF-4 and separation from the short N-terminal peptide were confirmed by a shift in elution profile (Fig. 5A), by amino acid sequence determination, and by mass spectrometric analysis. The amino acid sequence of the reduced protein eluting at 41% acetonitrile was identical to that of sequence II (Fig. 1F). These results suggest that a 30- to 50-fold potentiation of PF-4 activity is due to a specific, single cleavage and not nonspecific protein changes during purification. Furthermore, our results suggest that the N-terminal CXC motif and the intramolecular disulfide bonds are not essential for endothelial cell growth inhibitory activity.

**DISCUSSION**

The results presented here document an endogenous N-terminal processed form of PF-4. The cleavage site and the
identity of the resulting molecules were demonstrated by SDS/polyacrylamide gel electrophoresis, amino acid sequence determination, and mass spectrometry. The cleavage site is uniquely located downstream from the CXC motif and the hydrogen bond between the residues Thr-16 and Cys-52.

Accordingly, the cleavage leads to a loss of the first large loop in the PF-4 molecule. Our results show that cleavage at this site leads to a 30- to 50-fold potentiation of the endothelial cell growth inhibitory activity of PF-4. Since a significant level of endothelial cell growth inhibitory activity of the truncated PF-4 (produced after dissociation of peptide residues 1-16 by reduction) was maintained, these results suggest that the CXC motif is not essential for biological activity. The new N terminus generated by cleavage of PF-4 may have an important functional significance. The chemokine receptors are seven transmembrane G-protein-coupled receptors containing an acidic extracellular ligand binding domain. Cleavage of PF-4 at the Thr-16–Ser-17 bond leads to generation of an N terminus containing a stretch of basic amino acids -Arg-Pro-Arg-His. Conceivably, the newly formed N terminus with basic residues plays a role in facilitating binding of the cleaved PF-4 to its receptor. The identification of PF-4 receptor in the future would allow determination of the role of the newly formed N terminus in receptor–ligand interaction.

The proteolytic modification of PF-4 activity and perhaps chemokines in general could be an important physiological mechanism regulating injury and inflammatory responses. This may be particularly relevant for high-affinity heparin binding chemokines whose actions are highly localized at the injury site. In our studies, the cleaved PF-4 was isolated from cultures undergoing mixed lymphocyte reaction, a condition analogous to a localized inflammatory response. At an injury or inflammatory site, PF-4 produced from platelets during early phases may be activated by cleavage during a subsequent phase by the infiltrating leukocytes. Evidence for proteolytic modification of another chemokine, IL-8, at an N-terminal site different from that reported here for PF-4, has been presented (16, 17). The activity of a 77-residue IL-8 isolated from lipopolysaccharide-stimulated peripheral blood mononuclear cells has been shown to be different from an N-terminal truncated 72-residue IL-8 (16, 17). Recent studies on structure–function relationships of chemokines have indicated that the N-terminal sequences in IL-8 (18) and monocyte chemotactic protein 1 (19) play an important role in determining the
biological activity and receptor binding of chemokines. Mutational substitution or deletion in the N-terminal segment proximal to the CXC or CC motif has been shown to produce significant changes in the biological activities and receptor binding properties of IL-8 and monocyte chemotactic protein 1 (18, 19). Thus, the N-terminal processing of chemokines may represent a potential mechanism regulating their biological activity and needs careful investigation for the other members of the chemokine supergene family.

These findings may have implications for the role of PF-4 in vascular development, tissue repair, and angiogenic diseases. Endothelial cell proliferation and angiogenesis play an important role in the pathophysiology of atherosclerosis (20), restenosis following angioplasty (21), wound healing, and neoplasia (22). Endothelial cell–platelet interaction is a common event during normal and pathological vascular responses. It has been shown that extensive denudation of arteries leads to a prolonged proliferation of smooth muscle cells as compared to arteries that become rapidly covered with endothelial cells. Thus, impairment of reendothelialization by persistent generation of PF-4 from platelets and its activation to cleaved PF-4 by protease from inflammatory cells could contribute to retardation of endothelial regrowth and restenosis following angioplasty (21). Furthermore, since PF-4 is a selective inhibitor of endothelial cells in vitro and angiogenesis in vivo (5, 9), the highly potent derivative of PF-4 described here may have direct therapeutic potential for treatment of angiogenic diseases such as neoplasia, diabetic retinopathy, neovascular glaucoma, and endothelial tumors (22–25).