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Inhibition of Angiogenesis by Recombinant Human Platelet Factor–4 and Related Peptides

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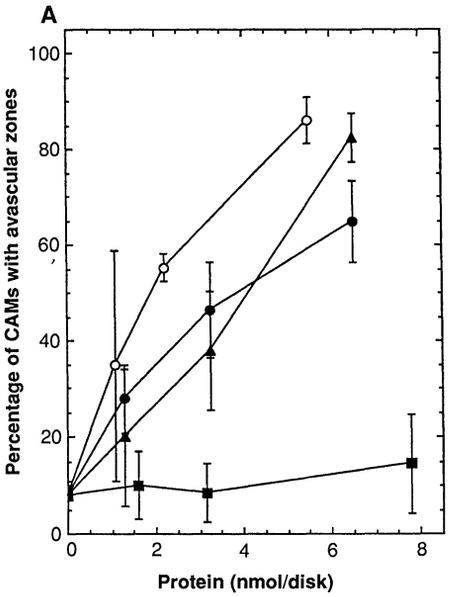
Recombinant human platelet factor–4 (rhPF4), purified from *Escherichia coli*, inhibited blood vessel proliferation in the chicken chorioallantoic membrane in a dose-dependent manner. Treatment of several cell types with rhPF4 in vitro suggested that the angiostatic effect was due to specific inhibition of growth factor–stimulated endothelial cell proliferation. The inhibitory activities were associated with the carboxyl-terminal, heparin-binding region of the molecule and could be abrogated by including heparin in the test samples, an indication that sulfated polysaccharides might modulate the angiostatic activity of platelet factor–4 in vivo. Understanding of the mechanisms of control of angiogenesis by endogenous proteins should facilitate the development of effective treatments for diseases of pathogenic neovascularization such as Kaposi's sarcoma, diabetic retinopathy, and malignant tumor growth.

PLATELET FACTOR–4 (PF4) IS A platelet α-granule protein originally characterized by its high affinity for heparin (*1, 2*). The protein is released from platelets during aggregation as a high molecular weight complex of a tetramer of the PF4 polypeptide and chondroitin sulfate (*3, 4*), which dissociates at high ionic strength. The complete primary structure of PF4 was determined by amino acid sequencing of proteolytic peptides (*5*) and later by isolation of the human gene (*6*). Although PF4 has several biological activities, including prevention of immunosuppression (*7*), chemotactic activity for neutrophils and monocytes (*8*) as well as for fibroblasts (*9*), inhibition of bone resorption (*10*), and inhibition of angiogenesis (*11*), its physiological significance is obscure. Of these activities, only the immunomodulatory effects have been confirmed with recombinant PF4 (*12*).

Angiogenesis inhibitors might be useful agents for treatment of solid tumors that require neovascularization for growth (*13*), and interest in this therapeutic approach has increased with the recognition that diseases



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Peptide	Sequence
	58 60 65 70
C-13	P L Y K K I I K K L L E S
C-12	L Y K K I I K K L L E S
C-11	Y K K I I K K L L E S
C-10	K K I I K K L L E S
58-68	P L Y K K I I K K L L

Fig. 1. Inhibition of angiogenesis by recombinant PF4 and related peptides. Angiostatic activity was measured by blind assessment of the presence or absence of avascular zones in the chicken CAM as described (*14*), with minor modifications. Three-day-old fertilized eggs were carefully removed from their shells and placed into petri dishes in an incubator with 3% CO_2, test substances were implanted on day 6, and vessel growth was assessed on day 8. Implant disks were made by air-drying 10-μl aliquots of a protein solution containing 0.5% (w/v) methyl cellulose. (**A**) Inhibition of angiogenesis by rhPF4 (●), C-41 (○), C-13 (▲), and N-29 (■). Data represent the average inhibition from several assays; at least ten sample disks were implanted per assay. Error bars represent SEM. (**B**) Heparin at 50 μg/disk (Upjohn) was either included (+) or omitted (−) from test samples during implant preparation. Peptides were present at 6.5 nmol/disk. Methyl cellulose alone or with heparin was inactive in these assays. (**C**) Inhibition of CAM angiogenesis by short carboxyl-terminal peptides of human PF4. Each peptide was tested at 6.5 nmol/disk in a minimum of three assays (30 implants). Error bars represent SEM. (**D**) Sequences of short carboxyl-terminal peptides used in these studies.

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such as Kaposi's sarcoma, diabetic retinopathy, and neovascular glaucoma are conditions of dysregulated endothelial cell proliferation and inappropriate capillary growth. Steroid derivatives in combination with sulfated polysaccharides effectively inhibit corneal and embryonic neovascularization (*14*) and represent one class of promising therapeutic candidates. A number of human proteins (*15*) as well as animal tissue extracts (*16*) have angiostatic activity, but no therapeutic drugs based on these activities have been developed because of excessive toxicity, limited efficacy, and difficulty in obtaining or purifying active material. In contrast to the relatively well-defined involvement of growth factors in promoting vascular development (*17*), roles for these inhibitory molecules as endogenous regulators of angiogenesis have not been established. The recent characterization of a 140-kD glycoprotein angiogenesis inhibitor present in normal but absent from transformed hamster kidney cell culture medium (*18*) may help to define the physiological importance of natural angiostatic molecules in controlling normal and neoplastic vascularization.

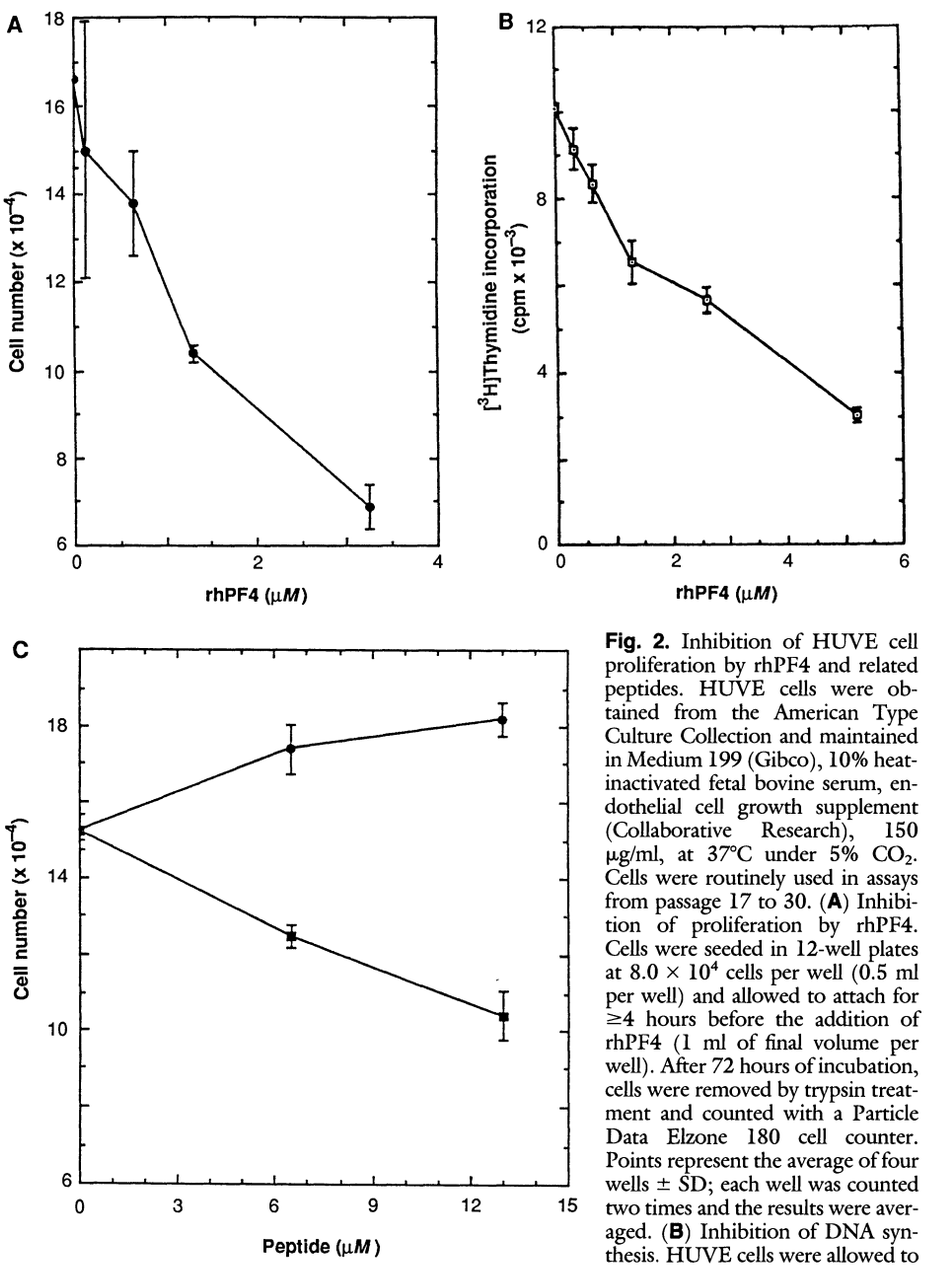

Fig. 2. Inhibition of HUVE cell proliferation by rhPF4 and related peptides. HUVE cells were obtained from the American Type Culture Collection and maintained in Medium 199 (Gibco), 10% heat-inactivated fetal bovine serum, endothelial cell growth supplement (Collaborative Research), 150 μg/ml, at 37°C under 5% CO_2. Cells were routinely used in assays from passage 17 to 30. (**A**) Inhibition of proliferation by rhPF4. Cells were seeded in 12-well plates at 8.0×10^4 cells per well (0.5 ml per well) and allowed to attach for ≥4 hours before the addition of rhPF4 (1 ml of final volume per well). After 72 hours of incubation, cells were removed by trypsin treatment and counted with a Particle Data Elzone 180 cell counter. Points represent the average of four wells ± SD; each well was counted two times and the results were averaged. (**B**) Inhibition of DNA synthesis. HUVE cells were allowed to attach for ≥4 hours before the addition of rhPF4 (100 μl of final volume), incubated with the protein for 24 hours, then treated with [^{3}H]thymidine at 1 μCi per well. The plates were incubated for an additional 6 hours then frozen at −70°C. The plates were subjected to two freeze-thaw cycles before harvest to assure complete removal of cells and matrix. Well contents were aspirated onto a fiber filter, washed with distilled H_2O and fixed with methyl alcohol. Each sample point represents the average of three wells ± SEM. (**C**) Proliferative response of HUVE cells to PF4-based peptides. HUVE cells were treated with N-29 (●) and C-41 (■) and counted. There was no response in this assay to either the C-13 peptide (Fig. 1C) or polylysine (molecular weight 3300).

Introduction of a synthetic gene for human PF4 into *Escherichia coli* allowed expression and routine purification of 20- to 50-mg batches of recombinant human PF4 (rhPF4) of authentic sequence. The recombinant gene was first expressed as a fusion protein bearing 35 amino acids of *E. coli* β-glucuronidase at the amino terminus linked to rhPF4 by the only internal methionine. The insoluble fusion protein was cleaved with CNBr, and the soluble authentic rhPF4 was purified to >95% by heparin-agarose affinity chromatography. This high affinity for heparin suggested that the recombinant protein had spontaneously recovered a substantial amount of the native structure during the purification process.

Purified rhPF4 inhibited angiogenesis in the chicken chorioallantoic membrane (CAM) system (Fig. 1A). The measured inhibition was dose-dependent and quantitatively similar to that previously reported for protamine and platelet-derived PF4 (*11*). Although heparin alone had no effect in this system, the inclusion of heparin in experimental implants abrogated the effects of rhPF4 (Fig. 1B). To investigate the role of specific portions of the molecule in producing its biological effects, we synthesized a series of polypeptides representing the entire 70–amino acid sequence of PF4, including two carboxyl-terminal fragments containing 41 and 13 amino acids, respectively (C-41 and C-13) containing the heparin-binding domain (*5*) and an amino-terminal peptide of 29 amino acids (N-29). With these peptides the angiostatic activity was shown to be associated with the heparin-binding domain (Fig. 1A); the C-41 and C-13 peptides retained full activity in the CAM assay, whereas the N-29 peptide was completely inactive. The inhibitory effects of the carboxyl-terminal peptides could also be abrogated by the addition of heparin (Fig. 1B), a result that was particularly surprising in that the C-13 peptide failed to bind immobilized heparin or to neutralize the anticoagulant activity of heparin. Smaller peptides (C-12, C-11, C-10, and 58–68) (Fig. 1D) were progressively less inhibitory (Fig. 1C), suggesting a direct role for the deleted amino acids in producing inhibition or an indirect involvement through the support of peptide structures essential for angiostatic activity.

Angiogenesis is a multistep process dependent on the proliferation and migration of vascular endothelial cells (*17*). Our findings show that rhPF4 completely suppresses the growth factor–dependent proliferation of human umbilical vein endothelial (HUVE) cells in culture with only one

addition of reagent during a 72-hour time course. After rhPF4 treatment, cell proliferation could be restored by the addition of fresh medium containing growth factors and heparin, indicating that rhPF4 suppressed the HUVE cell response to growth factor stimulation without cytotoxicity. Growth factor–stimulated human dermal fibroblast or keratinocyte proliferation were not inhibited by rhPF4 at concentrations that completely suppress HUVE cell growth. The observation that rhPF4 did not prevent the growth of these cell lines after they were stimulated with basic fibroblast growth factor (bFGF) indicated that rhPF4 inhibition of HUVE cell proliferation was not due to binding and sequestering of soluble bFGF. In experiments we conducted, rhPF4 also failed to inhibit proliferation of several human and murine tumor cell lines even at concentrations two to five times those that inhibited endothelial cell growth.

The inhibitory effect of recombinant PF4 on HUVE cells is correlated with the inhibition of DNA synthesis (Fig. 2B). The concentration of rhPF4 found to be highly inhibitory in both assays (~25 μg/ml), although far above that normally found in human plasma (10 ng/ml), is theoretically attainable at sites of platelet aggregation and α-granule release (*19*). The C-41 peptide extensively inhibited cell proliferation, although substantially higher concentrations of this peptide were required to produce inhibition comparable to that produced by full-length rhPF4 (Fig. 2C). The amino-terminal peptide (N-29) was not inhibitory even at very high doses.

The suppression of growth of vascular endothelial cells without inhibition of other cell types closely associated with capillaries suggests a possible physiological role for PF4 in the regulation of vessel development. In contrast, a highly specific platelet-derived endothelial cell mitogen has recently been cloned and characterized (*20*), suggesting that a balance of platelet proteins may be important for the control of angiogenesis. Regardless of its physiological function, the inhibitory specificity of rhPF4 suggests that it might be useful for the treatment of pathological vascularization. Although angiostatic in vitro and in vivo, protamine is not used for the clinical control of neovascularization because of unacceptably high toxicity (*21*). Because rhPF4 is identical in sequence to the natural protein, it would be expected to be less immunogenic and perhaps better tolerated than protamine. Further investigation of the conditions and growth factors that regulate the vascularization process should facilitate the development of agonists and antagonists for treatment of angiogenic diseases.

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A Peptide Sequence Confers Retention and Rapid Degradation in the Endoplasmic Reticulum

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A nonlysosomal pathway exists for the degradation of newly synthesized proteins retained within the endoplasmic reticulum (ER). This pathway is extremely selective: whereas some proteins are rapidly degraded, others survive for long periods in the ER. The question of whether this selectivity is due to the presence within the sensitive proteins of definable peptide sequences that are sufficient to target them for degradation has been addressed. Deletion of a carboxyl-terminal sequence, comprising the transmembrane domain and short cytoplasmic tail of the α chain of the T cell antigen receptor (TCR-α), prevented the rapid degradation of this polypeptide. Fusion of this carboxyl-terminal sequence to the extracelluar domain of the Tac antigen, a protein that is normally transported to the cell surface where it survives long-term, resulted in the retention and rapid degradation of the chimeric protein in the ER. Additional mutagenesis revealed that the transmembrane domain of TCR-α alone was sufficient to cause degradation within the ER. This degradation was not a direct consequence of retention in the ER, as blocking transport of newly synthesized proteins out of the ER with brefeldin A did not lead to degradation of the normal Tac antigen. It is proposed that a 23–amino acid sequence, comprising the transmembrane domain of TCR-α, contains information that determines targeting for degradation within the ER system.

THE IDENTITY AND FUNCTIONS OF intracellular organelles in eukaryotic cells are established by the specific targeting of appropriate proteins among these membrane compartments. The idenfification within proteins of signals that determine their fates within the cell has facilitated an understanding of this targeting (*1*). These peptide signals contain the information for import of cytosolic proteins (or nascent proteins) into the endoplasmic reticulum (ER), mitochondria, chloroplasts, peroxisomes, and nuclei. Within the secretory system, proteins have many fates, including retention within specific organelles or transport to the plasma membrane, secretory granules, and lysosomes. Proteins are targeted to the latter two organelles by information that is localized to peptide sequences or structural motifs (*2*). We have described one potential fate of proteins that are inserted into the ER but fail to be transported into the Golgi system. We observed that some of these proteins are rapidly degraded by a nonlysosomal process, which we refer to as ER degradation (*3–5*). One characteristic of this degradative system is its selectivity. This selectivity became apparent in studies of the fate of newly synthesized chains of the multisubunit T cell antigen receptor (TCR). Normally, this seven-chain complex ($\alpha\beta\gamma\text{-}\delta\epsilon\zeta_2$) assembles in the ER soon after synthesis. If these chains do not reach the Golgi system, either because of incomplete assembly or pharmacologic manipulation [for example, treatment of cells with the fungal

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