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THE CARBOXYL-TERMINAL TRIDECAPEPTIDE OF PLATELET FACTOR 4 IS A POTENT CHEMOTACTIC AGENT FOR MONOCYTES

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Summary: The carboxyl-terminal tridecapeptide of platelet factor 4, Pro-Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser, was synthesized and found to have chemotactic activity towards monocytes at concentrations 1/30 that of the parent molecule, while eliciting the migration of cells comparable in number to that seen using the parent and the control C5a. In an α-helical conformation the tridecapeptide would be amphiphilic, making it a good candidate for binding to membrane surfaces. Indeed, addition of sodium dodecyl sulfate, an amphiphile, induces an increase in the peptide's α-helicity, as shown by the circular dichroism spectrum. Pro-Leu-Tyr methyl ester was found to be inactive, indicating that the N-terminal region is not the recognition site of the tridecapeptide. These results suggest that the tridecapeptide incorporates the active site of human platelet factor 4 for monocyte chemotaxis.

Cells are thought to migrate to sites of inflammation by the process of chemotaxis, the directed movement of cells in response to a chemical gradient. Stimuli identified *in vitro* as positive chemotactic agents include small hydrophobic N-formylated peptides (1), oxygenated arachidonic acid derivatives (2), and the complement fragment C5a (3).* Inflammatory cells are found *in vivo* surrounding aggregated platelets (4), suggesting platelets may contain substances which serve as chemoattractants.

Recently, we demonstrated that the potent heparin binding protein platelet factor 4 (PF4) is a strong chemoattractant for monocytes and polymorphonuclear leucocytes, using *in vitro* assay systems (5). PF4 is a 70-residue polypeptide which is released from α-granules during platelet activation such as occurs in blood coagulation or when

*Abbreviations: PF4, human platelet factor 4; SDS, sodium dodecyl sulfate; C5a, human complement factor 5a; HPG, high power grid; tlc, thin layer chromatography.

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platelets are exposed to injured endothelium (6). More recently, we have found that another platelet α-granule protein, the human platelet derived growth factor, also is a strong chemoattractant protein (7). Apart from releasing chemotactic factors, platelets also effect generation of chemotactic activity by releasing protease activity that can cleave complement (8).

While PF4 is active in vitro, its cellular site of recognition and its structure-activity relationships have yet to be elucidated. For two reasons the carboxyl-terminal tridecapeptide of PF4 (6) (Pro-Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser-COOH) seemed to be a reasonable structural determinant for the chemotactic activity. First, it possesses an unusual alternation of paired basic and hydrophobic residues, and second, it forms an amphiphilic structure if arranged in an α-helical conformation. We have demonstrated the structural importance of amphiphilic helical regions for a number of peptides which bind to lipid surfaces and membranes. In particular, we constructed a model amphiphilic α-helical docosapeptide having minimum homology to apolipoprotein A-I (apo A-I), which retains the fundamental properties of apo A-I, including binding to phospholipid surfaces, surface activity at the air-water interface, and activation of lecithin:cholesterol acyltransferase (9). We now demonstrate that the chemically synthesized carboxyl terminal tridecapeptide of PF4 (58-60) retains the full chemotactic potential for human monocytes of the 70 residue intact PF4, suggesting that this peptide contains the active site of PF4 for monocyte chemotaxis.

Methods

Synthesis of tridecapeptide. The peptide was prepared by the solid phase method employing a 1% crosslinked styrene-divinylbenzene support. The deprotection and coupling program, similar to that described by Yamashiro and Li (10), utilized the symmetric anhydrides (11) of the appropriate Boc amino acids. The side-chain functionalities were protected by benzyl type protecting groups. The peptide was cleaved from the resin and deprotected by treatment with liquid HF-anisole at approximately 0°C.

After extraction from the resin-peptide mixture with 25% aqueous acetic acid, the deprotected peptide was purified by gel permeation chromatography on Sephadex G-25 (Superfine) in 0.2 N acetic acid. Purification to homogeneity was achieved by reverse phase HPLC on a Waters C_{18} μ-Bondapak semi-preparative column, using 0.1% H_3PO_4 as the

aqueous phase and acetonitrile as the organic phase. A gradient from 20% to 30% acetonitrile over 30 min was used with the peptide eluting at 26% acetonitrile. After desalting and lyophilization, the peptide gave the expected amino acid composition within experimental error and showed one spot upon thin layer chromatography in three solvent systems. The yield of purified peptide based on the amount of material recovered after the HF cleavage was 60%.

Circular Dichroism. Circular dichroism spectra of 1.0×10^{-5} M solutions of the tridecapeptide in 0.02 M sodium phosphate, pH 7.4, or in the same solution containing 0.25% (w/v) sodium dodecyl sulfate were recorded from 200 nm to 250 nm on a Cary 60 spectropolarimeter.

Synthesis of Pro-Leu-Tyr methyl ester. This tridecapeptide was synthesized by the solid phase benzophenone oxime method of DeGrado and Kaiser (12). Boc-Leu was coupled to the resin using direct DCC coupling in CH_2Cl_2. Deprotection and coupling of Boc-Pro utilized the program described by DeGrado and Kaiser. Tyrosine methyl ester was employed to cleave the dipeptide from the resin with acetic acid catalysis, yielding the protected tripeptide Boc-Pro-Leu-Tyr methyl ester. The excess tyrosine methyl ester was extracted into 5% aqueous citric acid. The protected tripeptide was crystallized from $CHCl_3$, and the Boc group removed by treatment with 50% trifluoroacetic acid in CH_2Cl_2. The deprotected peptide was homogeneous on tlc in three solvent systems, and was characterized by its 270 MHz NMR spectrum in D_2O/DCl. ^1H NMR: δ 0.86 (d, 6H, Leu C_δ), 1.55 (m, 3H, Leu C_β and C_γ), 1.88 (m, 4H, Pro C_β and C_γ), 3.00 (d, 2H, Tyr C_β), 3.38 (m, 1H, Pro C_δ), 3.64 (m, 1H, Pro, C_δ), 4.08 (m, 1H, C_α), 4.24 (m, 1H, C_α), 4.35 (m, 1H, C_α), 6.68 (d, 2H, Tyr C 2',6'), 6.91 (d, 2H, Tyr C 3',5').

Preparation of PF4 and C5a: PF4 was purified as previously described (6). A partially purified preparation of C5a (the chemotactic activity derived from the fifth component of complement) was made from human serum by incubation with zymosan and ϵ-aminocaproic acid (1 M) at 37°C, followed by gel filtration over Sephadex G-100 (13).

Chemotaxis assays: Highly purified preparations of mononuclear cells were obtained from venous blood by Ficoll/Hypaque gradients. The cells were suspended in modified Eagles medium supplemented with 2% human serum albumin (v/v) at 2.5×10^6 cells/ml. Chemotaxis was quantified in triplicate using modified Boyden chambers with 5 µM filters

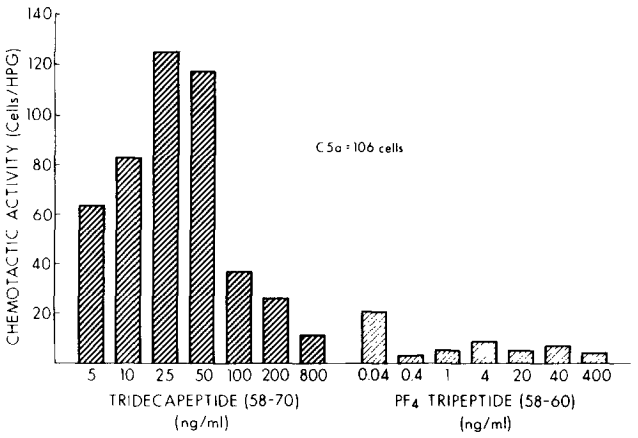

Figure 1 - Chemotactic activity of the human platelet factor 4 carboxyl terminal tridecapeptide 58-70 and tripeptide 58-60 for human monocytes. The peptides were tested in the lower compartment of modified Boyden chambers at the concentrations indicated. Chemotactic activity is expressed as cells/high power grid. The response of monocytes to C5a is included for comparison.

(Nucleopore, Pleasant, CA), overlying 0.45 μM pore filters (Millipore) (5). C5a at a concentration twice that required for 50% maximum chemotaxis served as a positive control. Five high power (x 400) grids (HPG) were counted per filter. Correction for cell migration was done by subtracting blank values in which the lower compartment contained medium alone. Blank values averaged 47 $\pm$ 2/HPG. The identity of individual filters was withheld from the observer until the analyses were complete.

Results and Discussion

Figure 1 demonstrates the migration of human monocytes to increased concentrations of the synthetic tridecapeptide. The ED_{50} for the tridecapeptide (2×10^{-8} M) is 1/30 that observed for intact PF4 (6×10^{-7} M). The maximal number ($\sim$ 120) of cells migrating toward the tridecapeptide is identical or greater than that found with PF4 in repeat experiments (data not shown) and compares to the number responding to C5a at a concentration equal to twice its ED_{50} (105 cells), as shown in control experiments. The data in Table 1 establish that the effect is truly chemotaxis and not chemokinesis. Addition of tridecapeptide to the upper chamber eliminates the migration of cells toward the peptide in the lower chamber. This can be partially overcome by increasing the concentration of tridecapeptide in the lower chamber. Addition of C5a to the lower chamber results in full mobility of the monocytes, overcoming the activity of the tridecapeptide

TABLE I

Monocyte Migration in Response to Active Agents in the Lower vs Upper Chambers

Tridecapeptide in Lower Chamber	Tridecapeptide in Upper Chamber 50 ng/ml	200 ng/ml
	Cells/HPF	Cells/HPF
0	0	0
50 ng/ml	0	0
200 ng/ml	10	8
C5a	100	90

in the upper chamber. These data indicate that the tridecapeptide is a potent chemotactic agent for human monocytes. Its greatly increased potency over the parent molecule coupled with the similarity of magnitude of the cellular migration, indicates that this peptide incorporates the chemotactic active site of PF4 for monocytes.

The cellular site of recognition for PF4 is not known yet, but it is likely to be different from the recognition site for C5a, since the response to a C5a concentration gradient is not significantly inhibited by the tridecapeptide, as shown in Table 1. The formyl-peptide receptor (14) which avidly binds peptides with sequences such as f-Met-Leu-Phe could be involved in the activity of the tridecapeptide. However, the tripeptide, Pro-Leu-Tyr methyl ester, corresponding to the amino terminal region was inactive, suggesting that these residues are not the active site of the tridecapeptide.

The finding that the tridecapeptide is highly active in chemotaxis assays will permit an investigation of the structural requirements for activity which would be extremely difficult to undertake for the intact PF4 molecule. The possibility that the biologically active form of the peptide has an amphiphilic helical conformation is supported by the finding that addition of sodium dodecyl sulfate, an amphiphile, induces an increase in helicity. In 0.25% SDS, $[\theta]_{222}$ changes from -1100 to -5200 deg $cm^2/dmol$. From these data, an increase in α-helicity from 10% to 20% can be calculated (15). An α-helical type of conformation could be induced at the cell-water interface, much as the amphiphilic α-helical character of model peptides based on apolipoprotein (A-I) (9) and melittin (16) appears to be induced when they interact with lipid or membrane surfaces. The importance of having the potential to assume an amphiphilic α-helical con-

formation as well as the need for paired positively charged and hydrophobic residues seen in the sequence of the tridecapeptide will be assessed by the synthesis of additiona model peptides and by appropriate chemical modification experiments.

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