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Synthesis and biological characterization of monocyte-derived neutrophil chemotactic factor

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MDNCF is a human monocyte-derived, 72-residue chemotactic peptide, which has sequence similarity with members of a family of pro-inflammatory cytokines. The peptide was synthesized by the solid-phase method, and is identical to the natural peptide in amino acid composition, sequence and chemotactic potency. MDNCF forms two loops via a neighboring pair of disulfide bridges, the probable locations of which are residues 7–34 and 9–50. Reduction and alkylation eliminated chemotactic activity. MDNCF fragments 7–37, 30–72 and 17–72 were all biologically inactive. The data suggest that the region of the clustered pair of disulfide bridges is important for biological activity.

Peptide synthesis; Neutrophil chemotaxis

1. INTRODUCTION

We recently described [1,2] the isolation and purification to homogeneity of a human monocyte-derived, neutrophil chemotactic factor (MDNCF). Reports on the isolation of the molecule appeared from two other laboratories at about the same time [3,4]. MDNCF was purified from culture fluid of LPS-stimulated human mononuclear cells in a series of steps that included ion-exchange chromatography, gel filtration and reverse-phase HPLC. Amino acid analysis and sequence data show that MDNCF is a unique molecule that differs completely from known cytokines [2]. The cDNA for MDNCF was recently cloned [5], and is identical to a cDNA derived from the mRNA of staphylococcal enterotoxin-stimulated human blood mononuclear cells [6]. The cDNA contains a coding sequence of 99 residues, the last 72 of which correspond to MDNCF. We undertook the solid-phase synthesis of MDNCF as well as portions of the molecule, in order to investigate structure-activity relationships.

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2. MATERIALS AND METHODS

2.1. *Synthesis of MDNCF and portions of MDNCF*

MDNCF was synthesized with a solid-phase synthesizer (model 430A, Applied Biosystems, Foster City, CA). Boc-Ser(Bzl)-PAM resin (0.28 mmol/g) or Boc-Thr(Bzl)-PAM resin (0.696 mmol/g) was placed in the reaction vessel. Boc-amino acid derivatives were coupled in DMF by the symmetric anhydride method, except for Boc-Asn, Boc-Arg(Tos), and Boc-Gln, which were coupled by the HOBT ester method. The following side chain protection groups were used: Asp(OcHEX), Glu(OcHEX), Ser(Bzl), Thr(Bzl), Lys(Cl-z), Arg(Tos), His(Z), Trp(CHO), and Tyr(Br-Z). Arg, Asn, Gln, His, Ile and Val were coupled twice. After coupling, remaining amino groups were capped by acetic anhydride. The low-high HF procedure was used for deprotection and cleavage from resin. 600 mg of resin was equilibrated first with 10 ml of dimethyl sulfide/*p*-cresol/*p*-thiocresol/HF (65:7.5:5:22.5) at −3°C for 2 h, and then with 10 ml of *p*-cresol/*p*-thiocresol/HF (7.5:5:87.5) for 1 h. After the resin was washed with ether containing 1% mercaptoethanol, the peptide was extracted with 5% acetic acid containing 20 mM mercaptoethanol.

2.2. *Purification, analysis and modification of synthetic peptides*

After reduction in volume of the peptide extracts by evaporation, they were partially purified by gel filtration on columns of

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Sephadex G-25 or G-50 equilibrated with 5% acetic acid containing 20 mM 2-mercaptoethanol. Peptides were further purified on a Vydac C-4 HPLC column with a gradient from a starting composition of 0.05% TFA in water to a limit of 0.05% TFA in acetonitrile. After reduction of peptides in DTT, air oxidation was induced by dialysis for 2 days at 4°C against 0.05 M Tris-HCl buffer, pH 9.0, containing 1 M urea. Amino acid composition was determined with a Waters Picotag system (Waters Corp., Milford, MA) on samples hydrolyzed 24 h in the gas phase above 6 M HCl in vacuo at 110°C. N-terminal sequence was determined by Edman degradation on an automated Applied Biosystems 470A sequencer. For studies on reduced MDNCF, samples were treated with 10 mM DTT in 7 M guanidine, 0.3 M Tris, pH 8.5, alkylated with 22 mM iodoacetic acid or iodoacetamide, and dialyzed against 50 mM Tris, pH 8.3.

2.3. *Chemotaxis assay*

The chemotactic activity of peptides for human neutrophils was measured in multiwell chemotaxis chambers [7]. In this assay, cells migrate from upper wells through 3 μm diameter holes in a polycarbonate membrane toward attractant in lower wells. Since the migrated cells adhere to the under side of the membrane, they can be stained and counted for quantification of their response.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of MDNCF (1-72) and partial sequences of MDNCF*

MDNCF (1-72) and partial sequences (17-72, 30-72 and 7[Ala substituted for Cys]-37) were synthesized as described. Amino acid composition of oxidized and purified peptides was in good agreement with predicted values. The oxidized form of synthetic MDNCF was eluted at the same position as native MDNCF on reverse-phase HPLC. A single, high yield N-terminal sequence of synthetic MDNCF (1-72) was obtained, which was in excellent agreement with native MDNCF (table 1).

3.2. *Chemotactic activity of synthetic MDNCF*

Potency and efficacy of native and synthetic MDNCF as chemoattractants for human neutrophils were compared. Fig.1 shows that the concentration required for a maximal response (potency) and the percentage of neutrophils migrating at the optimal concentration (efficacy) were virtually identical for the two peptides. The decline in response at concentrations above the optimal is typical for pure chemoattractants.

3.3. *Structure-activity correlations*

MDNCF has 4 cysteines, located in positions 7, 9, 34 and 50. In purified native MDNCF these cysteines are apparently oxidized, since addition of radioactive iodoacetic acid without reduction resulted in no detectable incorporation. In the case of β-thromboglobulin, a peptide with sequence similarity to MDNCF [2] and which has 4 cysteines in the same locations as MDNCF, the peptide is in the form of 2 loops, created by disulfide bridges at residues 7-34 and 9-50 [8]. It is likely, therefore, that the MDNCF configuration is similar. To determine if the oxidized form is required for biological activity, we added dithiothreitol to a solution of native MDNCF, which was unfolded in 7 M guanidine hydrochloride; an aliquot of the reduced peptide was then alkylated with iodoacetamide. Chemotactic activity of native MDNCF was compared with the reduced or the reduced and alkylated preparations. Fig.2 shows that the potency and the efficacy of reduced MDNCF were comparable to the untreated (oxidized) peptide, probably because of re-oxidation; whereas the reduced and alkylated preparation was practically devoid of activity.

Two different peptide fragments were synthesized and oxidized to form loops via disulfide bridging. One fragment comprised residues 7-37, in which the position 7 cysteine was replaced by alanine, so that the oxidized peptide had a disulfide bond between residues 9 and 34. This forms a loop comparable to the loop created by a disulfide bond between residues 7 and 34 of the native molecule (fig.3, loop 1). The other was fragment 30-72, with a disulfide bond between residues 34 and 50. Since the proximity of cysteines 7 and 9 in the native peptide brings the 2 disulfide bridges close together, the 34-50 disulfide bond creates a loop containing almost the same residues as the native 9-50 loop (fig.3, loop 2). These two peptide fragments were completely devoid of chemotactic activity. Fragment 17-72, which cannot assume the 2-loop configuration since it is devoid of cysteines 7 and 9, also had no chemotactic activity. In another test for ligand-neutrophil interaction, peptide fragments added to neutrophil suspensions in the cell wells of the chemotactic chamber did not prevent migration toward native MDNCF in the attractant wells. In contrast, 10-7 M native MDNCF in the neutrophil suspension inhibited the chemotactic response to 10-8 M MDNCF in the attractant wells.

Table 1

Sequence analysis of synthetic MDNCF (320 pmol)

Cycle	Residue	Yield (pmol)	Cycle	Residue	Yield (pmol)
1	S	234	21	F	16
2	A	167	22	I	16
3	K	149	23	K	9
4	E	162	24	E	7
5	L	132	25	L	16
6	R	95	26	R	11
7	C	ND[a]	27	V	9
8	Q	110	28	I	11
9	C	ND	29	E	7
10	I	87	30	S	6
11	K	85	31	G	5
12	I	46	32	P	6
13	Y	56	33	H	ND
14	S	32	34	C	ND
15	K	27	35	A	3
16	P	41	36	N	0.8
17	F	37	37	T	0.5
18	H	10	38	E	1.3
19	P	25	39	I	1.3
20	K	13			

[a] Not detected

The deduced complete sequence of MDNCF [5] is as follows:

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          10        20        30        40        50        60        70
SAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS
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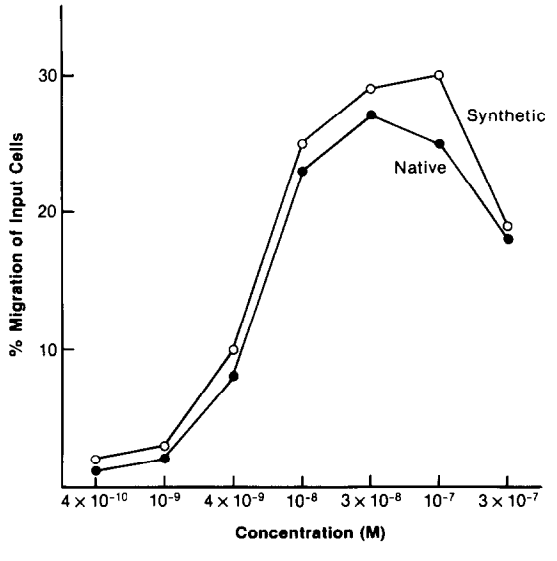

Fig.1. Neutrophil chemotactic response to native and synthetic MDNCF.

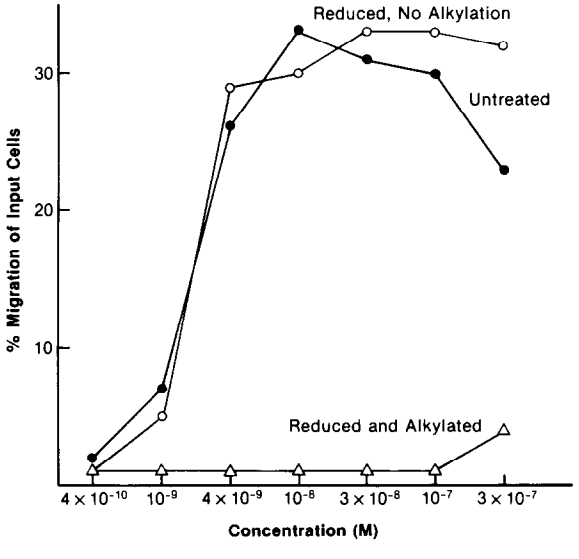

Fig.2. Effect of reduction and alkylation on MDNCF chemotactic activity.

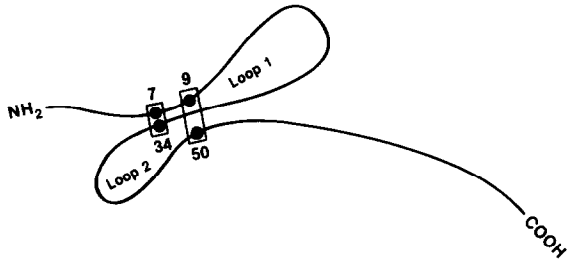

Fig.3. Loop configuration, based on disulfide locations of β-thromboglobulin.

Studies of the oxidized peptide fragments lead to the following conclusions and speculations. Neither the carboxyl-terminus nor either of the synthetic loops formed by disulfides 7-34 or 34-50 are sufficient for biological activity. It is unlikely that the amino-terminus is the active site, since activity is unaltered by extensions of the amino-terminus (Yoshimura et al., submitted). Of added interest is the fact that neither of the loops inhibits activity of intact MDNCF. This indicates that they cannot inhibit the interaction of MDNCF with neutrophil-saturable MDNCF binding sites, which have recently been demonstrated (Leonard et al., unpublished). When these observations are considered in the light of the inactivity of reduced and alkylated MDNCF, it appears likely that the region of the clustered pair of disulfide bridges is important for biological activity.

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