PURIFICATION AND PARTIAL PRIMARY SEQUENCE OF A CHEMOTACTIC PROTEIN FOR POLYMORPHONUCLEAR LEUKOCYTES DERIVED FROM HUMAN LUNG GIANT CELL CARCINOMA LU65C CELLS

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Polymorphonuclear leukocytes (PMN)[1] have been demonstrated to exhibit a cytotoxic activity against tumor cells in vitro (1), as well as other immune cells, including cytotoxic lymphocytes, NK cells, and macrophages (2, 3). For cellular immune reaction against tumor cells, these immune cells migrate to tumor-growing sites for attacking tumor cells, thus implying a possibility that tumor cells might produce a chemotactic factor for immune cells. In this regard, Bottazzi et al. (4) have reported that the neoplastic cells of mouse or human origin produce chemotactic factors for macrophages and there is a positive correlation between production of the factors by tumor cells and the amount of macrophage infiltration in tumor tissues. It may be assumed that PMN migrate to tumor-growing sites responding to tumor-producing chemotactic factors before the migration of any other immune cells, since PMN infiltration into inflammatory sites has been demonstrated to occur at the earliest stage of infection (5). In some surgical specimens, PMN infiltration has been observed in the primary tissue of tumors. Infiltration of PMN has also been demonstrated in lung giant cell carcinoma (6). This observation has suggested that the tumor cells might release extracellularly a PMN chemotactic factor.

This paper describes the purification of PMN chemotactic protein (lung carcinoma-derived chemotaxin [LUCT]) from the culture fluid of the human lung giant cell carcinoma cell line LU65C (6), and some characterization of LUCT, including NH_2-terminal partial amino acid sequence and amino acid composition. Comparison of amino acid sequences was made between LUCT and other reported chemotactic factors and protein deduced from the 3-10C cDNA.

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[1] *Abbreviations used in this paper:* CTU, chemotactic migration unit; LUCT, lung carcinoma-derived chemotaxin; pI, isoelectric point; PMN, polymorphonuclear leukocytes.

Materials and Methods

LU65C cells (clone C of LU65 cell line) were cultured in 50 ml of Iscove's modified Dulbecco's medium containing 0.1% BSA supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.3% sodium bicarbonate without FCS in a 75-cm^2 flask. After 4 d of cultivation, the culture fluid was harvested and centrifuged for 10 min at 1,500 *g* at 4°C. The cells were cultured another 4 d with 50 ml of the fresh medium and the culture fluid was again similarly harvested. Thus, 51.9 liters in a total volume of the culture fluid was frozen at −80°C in 200 packages. Buffer solutions were used as follows: buffer A, 50 mM sodium phosphate buffer (pH 7.4); buffer B, 0.005% BSA (81-028; Miles Scientific Div., Naperville, IL) was contained in buffer A; buffer C, 0.001% BSA was contained in buffer A; buffer D, 1.0 M NaCl was contained in buffer B; and buffer E, 20 mM sodium phosphate buffer (pH 7.4). All labware was rinsed with buffer B or C to avoid nonspecific adsorption of LUCT. After 2.5–3 liters of the culture fluid was thawed and dialized overnight against water in membrane tubings (mol wt 3,500) (Por3; Spectrum Medical, Los Angeles, CA), the fluid was mixed with 150 ml of DEAE-Sepharose CL6B equilibrated with the buffer A and gently stirred for 2 h at 4°C. Then the resin was filtered off on a Kiriyama Roto funnel. After CM-Sepharose CL6B equilibrated with buffer B as 50 ml slurry (10-ml gel volume) was added to the filtrate and stirred for 3 h at 4°C, the resin was collected by aspirating with a bottle top filter (7105; Falcon Labware, Oxnard, CA). After washing the gel with 75 ml of buffer B, protein adsorbed on the gel was eluted with 30 ml of buffer D. Thus, the 3 liters of culture filtrate was concentrated to 30 ml. The concentrated crude LUCT solution was frozen at −80°C until for further purification. These procedures were repeated 21 times for concentration of a total of 51.9 liters of culture fluid. A total of 750 ml of concentrated crude LUCT solution was centrifuged for 15 min at 3,000 rpm at 4°C to eliminate insoluble materials and was dialyzed against 15 liters of buffer A overnight. Successively, LUCT was purified by carboxyl-methylated-polyvinylalcohol resin, hydrophobic resin, and reversed-phase column on HPLC. SDS-PAGE was carried out by the method of Laemmli (7) using 1.3 μg of LUCT. Isoelectric point (pI) of LUCT was determined by chromatofocusing. LUCT solution containing 0.005% BSA dialyzed against 25 mM triethylamine HCl buffer (pH 11.0) was applied to a polybuffer exchanger PBE 118 column (1.0 ϕ × 15 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 25 mM triethylamine HCl buffer (pH 11.0); the column was eluted with 225 ml of diluted Pharmalyte (1/45, pH 8.0) at a flow rate of 45 ml/h. Amino acid analysis of LUCT (8.5 μg) was performed by an amino acid analyzer (6300E; Beckman Instruments, Inc., Fullerton, CA) after hydrolysis with 0.5 ml of 6N HCl in an evacuated sealed tube for 24 h at 110°C. NH_2-terminal sequence of LUCT (3.8 μg) was directly determined with a gasphase peptide sequencer (477A; Applied Biosystems, Inc., Foster City, CA). Migration activity of PMN was determined by the migration distance in a filter (3 μm in pore size) (Millipore Continental Water Systems, Bedford, MA) using a modified Boyden chamber method (8). Assay samples at each purification step were dialyzed against 5.0 liters of PBS(−) overnight before assay. All samples were diluted to one half with a chemotaxis medium, which consisted of 2% BSA, 1.25 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 0.1% glucose, and 0.6% $NaHCO_3$. The diluted sample was placed into the lower compartment of the chamber, and human PMN suspension (2×10^6 cells/ml) was placed in a mixture of PBS(−) and chemotaxis medium (1:1) into the upper compartment and then incubated for 35 min at 37°C under 5% CO_2 in air. The leading front distance was expressed as a mean of values of the final concentration of the sample determined in five microscopic fields. Chemotactic unit of LUCT was calculated from three parameters; FMLP (10^{-8} M)-stimulated maximal migration (FM), minimal migration without chemotactic factor, termed random migration (RM), and migration induced by LUCT sample (SM). Chemotactic migration unit (CTU) was defined as 100 × (SM − RM)/(FM − RM). Total CTU in each step was calculated by multiplying the dilution of a sample for 50 CTU in the dilution-activity curve.

Results and Discussion

We purified 35 μg of LUCT as a single peak by a reversed-phase column on HPLC in the final step (Fig. 1). Table I summarizes the results of purification of LUCT.

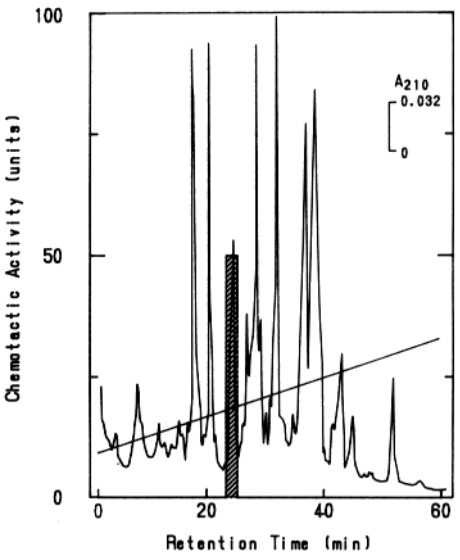

FIGURE 1. Reversed-phase column chromatography on HPLC. A typical elution profile of seven chromatographies. Dialyzed crude LUCT (200 ml) of CM-Sepharose was applied to an Asahipak ES 502C column (cation exchanger, 7.5 φ × 100 mm). LUCT was eluted with a linear gradient of NaCl 0–0.4 M in buffer B at a flow rate of 1.0 ml/min for 45 min, and then successively eluted with 0.4 and 1.0 M NaCl for 5 and 15 min. Combined fractions (48.2 ml) were supplemented with 2.0 M ammonium sulfate and applied to a TSK gel Phenyl 5PW column (7.5 φ × 75 mm) equilibrated with buffer E containing 2.0 M ammonium sulfate. LUCT activity was eluted with a linear gradient of 2.0–0 M of ammonium sulfate in buffer E at a flow rate of 0.5 ml/min. The eluate containing LUCT (4.2 ml) was applied to a reversed-phase column (Cosmosil 5 TMS-300, 4.5 ϕ × 250 mm) and eluted with CH_3CN from 22.5% to 60% containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. These procedures were repeated for 750 ml of the concentrated crude LUCT. ▨, chemotactic activity of a 310-fold diluted fractions with chemotactic medium; ———, absorbance at 210 nm; – – –, a linear gradient of CH_3CN (22.5–60%).

Molecular mass of LUCT was determined as a single band at ~10 kD by SDS-PAGE (Fig. 2), and its pI was determined as 10.7 by chromatofocusing. Comparing the chemotactic activity of LUCT with that of FMLP, the chemotactic activity of LUCT was 0.75×10^{-9} M for ED_{50}, a higher specific activity than that of the bacterial chemotactic peptide FMLP (5×10^{-9} M) (8), and comparable with that of human C5a (10^{-9} M) (9).

We determined the amino acid sequence of NH_2-terminal 32 residues of LUCT and compared it with amino acid sequences of several chemotactic factors (Fig. 3). The NH_2-terminal sequence of LUCT was quite different from those of human C5a and C3b (10). Recently, PMN chemotactic factors (MDNCF and monokine) from human peripheral leukocytes stimulated with LPS or Con A were isolated (11, 12). NH_2-terminal 42- and 20-amino acid sequences of MDNCF and the monokine have been determined, respectively. The NH_2-terminal amino acid sequence from

TABLE I

Summary of Purification for LUCT

	Total volume	Total activity	Total protein	Specific activity	Fold	Yield
	ml	*CTU**	*mg*	*CTU/mg*		%
Culture fluid	51,900	21,200,000	44,600	475	1	100
DEAE-Sepharose	58,600	12,700,000	2,340	5,430	11.4	59.9
CM-Sepharose	750	5,250,000	26.3	200,000	421	24.8
CM-PV	48.2	1,990,000	6.0	332,000	699	9.4
Hydrophobic	4.2	714,000	0.571	1,250,000	2,630	3.4
Reversed phase	7.35	570,000	0.035	16,300,000	34,300	2.7

CM-PV, carboxymethylated polyvinylalcohol.

* CTU, units in 100-μl sample = 100 × (SM − RM)/(FM − RM).

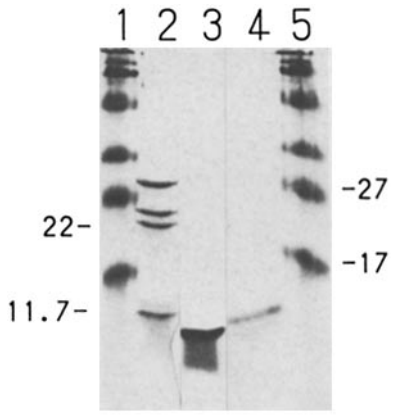

FIGURE 2. SDS-PAGE of purified LUCT. LUCT (1.3 μg) was applied to SDS-PAGE. Lanes *1* and *5*, M_r markers; lane *2*, pertussis toxin subunits (28, 23, 22, 11.7 kD); lane *3*, Kazal's trypsin inhibitor (6.3 kD); lane *4*, LUCT.

the sixth amino acid of LUCT was completely identical to the NH_2-terminal sequence of either chemotactic factor, except for the 11th, 12th, and 31st amino acids. Considering the homology of the NH_2-terminal amino acid sequences, the leukocyte-derived chemotactic factors could be hydrolyzed by a trypsin-like protease, resulting in des(1-5) of LUCT. Moreover, the NH_2-terminal amino acid of LUCT was Ala, and the NH_2-terminal 32-amino acid sequence was extensively homologous to the 32-amino acid sequence of the protein consisting of 77 amino acids encoded by 3-10C cDNA derived from mRNA isolated from human leukocytes (13). LUCT consisted of 75 (Trp was not tested) amino acids and this amino acid composition was similar to the reported amino acid composition deduced from the 3-10C cDNA sequence (Table II). Schmid and Weissmann (13) have suggested that the NH_2-terminal 22 amino acids are the sequence for signal peptide, and therefore, the 23rd amino acid Ala is the NH_2 terminal of the mature protein consisting of 77 amino acids, strongly suggesting that the LUCT-coding gene structurally resembles the 3-10C cDNA in the carcinoma cells and that LUCT may be initially synthesized as a 99-amino acid protein and then the first 22-amino acid signal peptide may be removed upon extracellular secretion. The NH_2-terminal sequence of five amino acids

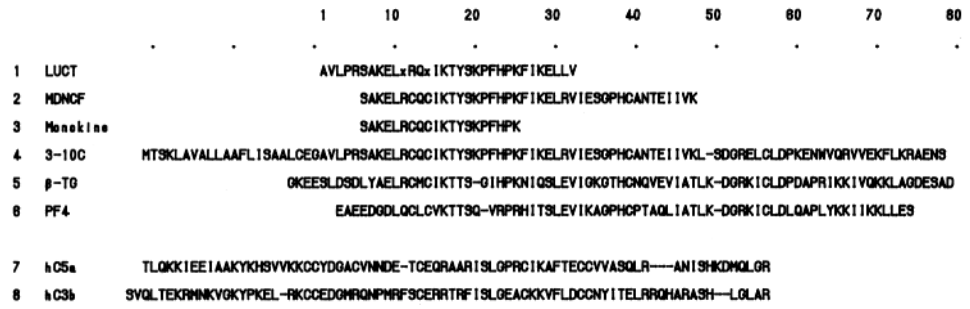

FIGURE 3. A comparison of NH_2-terminal amino acid sequence of various chemotactic proteins. *1*, LUCT; *2*, MDNCF; *3*, monokine; *4*, 3-10C-encoded protein; *5*, β-thromboglobulin; *6*, platelet factor 4; *7*, human C5a; *8*, human C3b.

TABLE II
Amino Acid Composition of LUCT

Amino acid	(mol)	LUCT	3-10C*
	%		
Lys	12.49	9	9
His	2.64	2	2
Arg	8.05	6	6
Asp	9.31	7	5
Thr	3.92	3	2
Ser	7.36	6	5
Glu	16.62	12	10
Pro	6.35	5	5
Gly	5.60	4	2
Ala	6.98	5	4
1/2Cys	2.15	2	4
Val	4.30	3	6
Met	0.28	0	0
Ile	2.40	2	5
Leu	7.47	6	7
Tyr	1.33	1	1
Phe	2.75	2	3
Trp	ND	ND	1
Total	100	75	77

* Cited from reference 12.

of LUCT was completely different from the corresponding amino acid sequences of both β-thromboglobulin and platelet factor 4 (Fig. 3), although both have been suggested to be homologous to the sequence of 3-10C-encoded protein (13). It should also be noted that the gene for LUCT is constitutively expressed in the human lung carcinoma cells without any added stimulant. On the other hand, the synthesis of the chemotactic factors in human leukocytes has been induced only by stimulation with LPS or Con A (11, 12).

It has been observed that PMN first infiltrate into inflammatory sites when mice are intraperitoneally injected with stimulants such as glycogen and casein (5). The infiltration of PMN into tumor-growing sites before infiltration of macrophages and lymphocytes has been also observed (1). Furthermore, Bottazzi et al. (4) have demonstrated from using tumor cell lines of mouse and human origin, that there is a correlation between chemotactic activity for mononuclear phagocytes in supernatants of cultured cells and amount of tumor-associated macrophages in vivo. In addition, PMN have tumor killing activity in vitro (1). Hence, tumor-secreting LUCT might be involved in the first step of immunosurveillance by PMN in their host-defense function. Further study is needed to clarify the physiological role of LUCT, especially in terms of tumor-associated PMN.

Summary

A chemotactic protein for polymorphonuclear leukocytes (lung carcinoma-derived chemotaxin [LUCT]) was purified from culture fluid of the human lung giant cell carcinoma LU65C cells to electrophoretically homogeneous form through five se-

quential purification steps: DEAE-Sepharose, CM-Sepharose, HPLC on carboxylmethylated-polyvinylalcohol resin, hydrophobic, and reversed-phase. The molecular mass was determined as ~10 kD by SDS-PAGE and isoelectric point was 10.7. The chemotactic activity (ED_{50} 0.75×10^{-9} M) was sevenfold more potent than that of FMLP (5×10^{-9} M) and comparable with that of C5a (10^{-9} M). NH_2-terminal amino acid sequence and amino acid composition of LUCT strongly suggest that it may be closely related to the putative protein encoded by the cDNA clone (3-10C) and almost identical with a part of sequence of the chemotactic factor derived from stimulated human leukocytes in the 6th to 32nd, but not the NH_2-terminal 5 amino acids. These results indicate that the carcinoma cells produce LUCT without any added stimulant and suggest that the previously isolated chemotactic monokines may correspond to des(1-5) of LUCT in the NH_2-terminal region.

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