Chemotactic Factor for Polymorphonuclear Leukocytes Derived from Human Lung Giant Cell Carcinoma Lu65 Cells

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Summary A chemotactic factor for polymorphonuclear leukocytes was found in the culture fluid of human lung giant cell carcinoma cell line Lu65. This factor was defined as chemotactic one by checkerboard analysis. Partial purification of the chemotactic factor was performed by DEAE-Sepharose and gel filtration with Sephadex G-100, which showed it to be basic and low molecular weight. The factor was also found in the filtrate of culture fluid passed through a Centriflo CF25 filter, thus indicating a molecular weight of less than 25 kDa. Furthermore, three cell clones having higher activity of chemoattractant were selected from the original cell line.

Key Words: chemotactic factor, lung carcinoma, polymorphonuclear leukocytes (PMN), cell cloning

Polymorphonuclear leukocytes (PMN) show cytotoxicity *in vitro* and damage tumor cells [1-7]. It has been reported that myeloperoxidase, hydrogen peroxide, superoxide anion, and lysosomal enzymes are involved in this tumor cell killing by PMN [4, 7-11]. PMN *in vivo* are required to migrate toward the inflamed site from the peripheral blood in order to manifest this function in sites of tumor cell

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Abbreviations: HBSS, Hanks' balanced salt solution; fMet-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine; PBS, phosphate-buffered saline without calcium and magnesium.

growth. It has been observed that PMN infiltration into sites of inflammation occurs at the earliest stage, that is, PMN infiltrate into the site of tumor growth prior to infiltration of macrophages and lymphocytes [12, 13]. This indicates that PMN migrate rapidly to the inflamed site. As PMN migrate toward chemoattractants produced at this site [14, 15], it is assumed that PMN infiltration must be stimulated with a chemoattractant produced by tumor cells in this region. For substantiation of this hypothesis, many PMN must be observed in the primary tissue of the tumor, and, furthermore, the presence of chemoattractants in cell lines established from the tumor must be confirmed.

An infiltrative picture of PMN has been observed in the cancer tissues of a lung giant cell carcinoma [16], and histological examination suggested that a PMN chemotactic factor might be secreted from this carcinoma. A cell line, Lu65 has been established from a specimen of this carcinoma, and colony stimulating factor is secreted by it [16]. Following tumor transplantation, remarkable leukocytosis without evidence of infection has been observed in nude mice [16]. Furthermore, amplification of onco-genes *K-ras* and *myc* was also observed in this cell line [17].

The present report describes the finding of chemotactic activity in the culture fluid of the Lu65 cell line and the conditions for its optimal production. Moreover, partial purification of the chemotactic factor was performed.

MATERIALS AND METHODS

Preparation of chemotactic factor from culture fluid. Lu65 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (GIBCO, NY). The chemotactic factor was obtained from the culture fluid of Lu65 cells by centrifugation for 10 min at $1,500 \times g$ at 4°C , and the supernatant was used for the chemotactic assay.

Preparation of human PMN. Human PMN were obtained by the method described previously [18, 19]. Cells at a concentration of 2×10^6 cells/ml were suspended in Hanks' balanced salt solution (HBSS) containing 0.3% NaHCO_3 .

Migration assay. Migration activity of PMN was determined by the migration distance through a Millipore filter ($3 \mu\text{m}$) in a modified Boyden chamber [20]. Culture medium conditioned by the Lu65 cells was placed in the lower well, and PMN suspension, in the upper well. After incubation of the chamber for 45 min at 37°C under an atmosphere of 5% CO_2 in air, the filter was fixed with ethanol and dried and stained with Weigert's hematoxylin. Migration distance from the upper chamber side of the filter to the leading edge of cells was measured with a high power microscope. The values were expressed as means of values determined in five microscopic fields. fMet-Leu-Phe (10^{-8} M) was used as a positive control.

Checkerboard analysis of culture fluid of Lu65 cells. In order to determine whether the supernatants contained chemotactic activity as well as the capacity for

stimulation of locomotion, we placed different concentrations of supernatants in upper and lower compartments [21]. Migration distance for each concentration of the fluid was measured.

Filtration of culture fluid. The approximate molecular weight was determined by passage of culture medium through a Centriflo CF25 filter (Amicon Co., Denvers, MA). Lu65 cells were incubated for 6 days and the culture fluid was then taken and frozen at -20°C . After being thawed, 0.75 ml of this culture fluid was filtered with a Centriflo CF25 by centrifugation for 15 min at $1,500 \times g$ at 4°C . The non-filtered fraction was resuspended in HBSS.

Partial purification of the chemotactic factor from the culture fluid. Ten milliliters of the culture fluid was dialyzed against 50 mM sodium phosphate buffer (pH 7.4) overnight and applied to a DEAE-Sepharose column (2.5×10 cm) equilibrated previously with 50 mM sodium phosphate buffer (pH 7.4). The chemotactic activity appeared in the pass-through fraction and the major part of the protein adsorbed to the column. The pass-through fraction containing chemotactic factor (45 ml) was concentrated to 12 ml in a dialysis tubing using polyethylene glycol (m.w. 2,000) and dialyzed against phosphate-buffered saline without calcium and magnesium (PBS). Five milliliters of the concentrated sample was applied to Sephadex G-100 column (2.4×90 cm) equilibrated previously with PBS and eluted with the same.

Cloning of chemoattractant-producing cell from Lu65 cell line. Cells of the original line were plated at a density of 1×10^4 cells in $500 \mu\text{l}$ into each well of a 24-well plate and cultured for 7 days. Cell populations possessing high chemotactic activity were transferred into new 24-well plates at a 1/10 dilution with RPMI-1640 medium containing 10% fetal bovine serum and cultured for 12 days. Furthermore, each of the populations having higher activity in the second selection was plated into 96-well plate as one cell per well and cultured from 14 to 23 days. After growth the cell colony in the well was picked up and used as a clonal cell line.

RESULTS AND DISCUSSION

Optimal culture period to produce chemotactic factor

As shown in Fig. 1, PMN migration was stimulated by the culture fluid of Lu65 cells but not by culture medium only which had been incubated under the same conditions as the cell culture. The highest stimulatory activity was observed in the fluid harvested at for 6 days. Whether this stimulation factor for PMN migration is a chemotactic factor was determined by checkerboard analysis. Table 1 shows checkerboard analysis of the chemotactic factor. PMN migrated to the culture fluid in the lower compartment in a dose-dependent manner and responded to that in the upper with inhibition. This indicates that culture fluid of Lu65 cells contained a chemotactic factor for PMN.

Partial purification and approximate molecular weight of the chemotactic factor

When the culture fluid was applied to Centriflo CF25 filter and centrifuged, the chemotactic activity was detected in the filtrate (Table 2). These results suggest that the chemotactic factor secreted in the culture fluid by the Lu65 cells had a relatively low molecular weight one of less than 25 kDa. On the other hand, the

Fig. 1. Chemotactic activity in culture fluid of lung giant cell carcinoma LU65 cells. After preculture of Lu65 cells for 4 days, cells non-adherent and adherent to the plastic flask were removed by pipetting, and plated again in RPMI-1640 containing 10% heat-treated fetal bovine serum. The cells were incubated for 4 to 7 days at 37°C in 5% CO₂. The culture fluid (6–7 ml) was harvested after a lapse of time and centrifuged for 10 min at 1,500×g at 4°C. The supernatant was used as an assay sample. , Lu65-conditioned culture fluid; , medium culture without cells.

Table 1. Checkerboard analysis of culture fluid from Lu65 cells.

% Fluid in lower compartment	Migration distance (μm/45 min)			
	% Fluid in upper compartment			
	0	25	50	100
0	27.1	32.0	28.4	27.6
25	52.0	34.9	39.5	29.4
50	60.1	48.8	31.6	28.8
100	82.5	57.6	42.5	31.1

Table 2. Distribution of chemotactic factor in filtrate and non-filterable fraction of centriflo CF25 filtration.

Sample fraction	Migration distance (μm/45 min)	
	Conditioned culture fluid	Non-conditioned culture fluid
Original fluid	45.5	26.5
Filtrate	50.5	21.9
Non-filterable fraction	38.0	25.4
Control fMet-Leu-Phe (10 ⁻⁸ M)		75.8

Fig. 2. Sephadex G-100 column chromatography. ●—●, Chemotactic activity; ----, absorbance at 280 nm. Molecular markers used were bovine serum albumin, 66 kDa; chymotrypsin, 25 kDa; cytochrome *c*, 12.5 kDa.

Table 3. Clones of chemoattractant-producing cells from the original cell line Lu65.

Cell clone	Migration distance ($\mu\text{m}/45 \text{ min}$)
Lu65-A	51.6
B	55.3
C	59.5
Control	
Random migration	37.9
fMet-Leu-Phe (10^{-8} M)	72.3

factor was eluted in the pass-through fraction of DEAE-Sepharose column chromatography and it was eluted in fraction of less than 12.5 kDa by Sephadex-G100 column chromatography (Fig. 2). These results strongly suggest that the factor may be a basic protein. Moreover, the molecular weight of this factor is relatively similar to that of complement C5a [22]. However, as the fetal bovine serum used in the present study was treated for 30 min at 56°C, it is likely that C5a does not exist in the medium. In addition, medium cultured in the absence of cells did not show chemotactic activity (Fig. 1). These results strongly suggest that the chemotactic factor in culture of Lu65 is different from C5a.

Cloning of chemoattractant-producing cell from Lu65 cell line

Cells of the original line were plated into 24-well plates and cultured for 7 days. Of the 24 populations obtained, 5 possessed high chemotactic activity and were transferred to a new 24-well plate at a 1/10 dilution with RPMI-1640 medium containing 10% fetal bovine serum and cultured for 12 days. Furthermore, each of

two populations having the highest activity in the second selection was plated into a 96-well plate at one cell per well and cultured from 14 to 23 days. The cell colony that appeared in a given well was picked up and cultured to establish a clonal cell line. Finally, three clones of chemoattractant-producing cells from the Lu65 cell line were selected (Table 3).

Bottazzi *et al.* [23] have demonstrated a chemotactic factor for macrophages in the supernatant of tumor cell cultures. The preliminary evidence for a PMN chemoattractant in the present study agrees with their observation. Their report and the present study suggest that macrophages and PMN participate in immunosurveillance of growth of tumor cells. Recently, it was reported that PMN are the first cells to infiltrate into the intraperitoneal site after injection of a stimulant such as casein or glycogen [12, 24]. Furthermore, PMN have tumor-killing activity [1-7]. These findings suggest that PMN migrate via a tumor cell-secreted chemoattractant to sites of growing tumor cells to kill the tumor cells there. Hence, secretion of chemoattractant for PMN by tumor cells is important at the first step of immunosurveillance by PMN in their host-defense function.

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