

PURIFICATION AND AMINO ACID ANALYSIS OF TWO HUMAN GLIOMA-DERIVED MONOCYTE CHEMOATTRACTANTS

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A central tenet of tumor immunology is that tumor-specific antigen may induce a host immune response, which results in a cellular immune reaction characterized by accumulation of cytotoxic macrophages in the tumor site. Our laboratory, among many others, has explored *in vivo* and *in vitro* aspects of this hypothesis. It was shown in experiments with transplantable tumors in inbred guinea pigs that at dermal sites of delayed hypersensitivity reactions to one tumor cell line, antigenically unrelated tumor cells were also destroyed (1). This suggested that the attack on the antigenically unrelated tumor cells was immunologically nonspecific, and was mediated by activated macrophages infiltrating the site. It was shown in many experimental models that macrophages were capable of destroying tumor cells *in vitro*, provided that they were activated (2, 3). The activated macrophage was thus assigned a critical role in host destruction of tumors. A minimal requirement for the reaction would include (a) local elaboration of a chemoattractant to mediate accumulation of macrophages at the site, and (b) factors that activate macrophages to become cytotoxic.

On the other hand, although macrophages are often found in human tumors, the degree of infiltration varies; and neither the role of tumor-associated macrophages nor the mechanism of infiltration has been clarified (4, 5). It has been suggested, for example, that tumor-associated macrophages may stimulate tumor growth or connective tissue development (4).

Our current interests center on the suggestion that chemoattractants mediate macrophage infiltration into tumor sites. There are at least two possible cellular sources for chemoattractants released at foci of growing tumors. One would be in the context of a cellular immune reaction to the tumor, since it is known that stimulation of sensitized lymphocytes with specific antigen causes elaboration of chemotactic factors (6, 7). An additional source of chemoattractant is the tumor itself. Meltzer et al. (8) reported macrophage chemotactic activity (MCA)¹ in culture fluid of murine tumor cell lines. Bottazzi et al. (9) found MCA in culture supernatants of mu-

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¹ *Abbreviations used in this paper:* CM-HPLC, carboxymethyl HPLC; GDGF, glioma-derived chemotactic factor; LDGF, lymphocyte-derived chemotactic factor; MCA, monocyte chemotactic activity.

rine and human tumor cells. There was a correlation between the amount of MCA in culture supernatants and the number of tumor-associated macrophages found when the cultured mouse tumor cells were transplanted into mice.

Reports of tumor cell-derived chemotactic activity raise several interesting questions. Is the chemoattractant structurally related to attractants released by normal cells? Is attractant production a property of most tumor cells? Is the attractant specific for macrophages? Can the attractant not only induce translational movement but also activate macrophages to become cytotoxic? Since unequivocal answers to these questions require a pure product, we attempted to isolate and purify a tumor-derived chemoattractant. Recently, we found MCA in culture supernatants of human glioma cell lines (9a). In this communication, we report purification to apparent homogeneity of glioma-derived chemotactic factor (GDGF) from the U-105MG cell line, which constitutively secretes high amounts of MCA.

Materials and Methods

Cell Culture. Human glioma cell line U-105MG, initiated by J. Pontane and B. Westermarck at the University of Uppsala, Uppsala, Sweden (10), was a generous gift from Dr. Y. Gillespie at the University of Alabama at Birmingham. Cells were cultured in 150-cm² tissue culture flasks (Costar, Cambridge, MA) in RPMI 1640 medium (Advanced Biotechnologies Inc., Silver Spring, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 20 mM L-glutamine, and 50 µg/ml gentamycin. When cells became confluent, medium was replaced with 100 ml of FCS-free RPMI 1640 medium, which was collected 4 d later and frozen at -20°C.

Dye-ligand Chromatography. 4 liters of culture fluid was concentrated to 50 ml on a 150-mm-diameter Diaflo membrane (Amicon Corp., Danvers, MA) (YM-5, molecular weight cutoff, 5,000), dialyzed against 20 mM Tris-HCl, pH 8.0, and applied on a column of Orange A-Sepharose (1 × 5 cm; Amicon Corp.) that was equilibrated with the same buffer. The column was eluted with a linear NaCl gradient (limit, 0.6 M) at a flow rate of 0.5 ml/min; 2-ml fractions were collected, and those with chemotactic activity were pooled.

Cation Exchange HPLC. The pool of active fractions eluted from Orange A-Sepharose was concentrated to 2 ml, dialyzed overnight at 4°C against starting buffer (20 mM MOPS, pH 6.5, in 0.1 M NaCl), and applied to a 0.75 × 7.5-cm CM 3SW column (Toyo Soda, Tokyo) at room temperature. The column was eluted with a series of linear NaCl gradients (limit, 20 mM MOPS, pH 6.5, in 0.4 M NaCl) at a flow rate of 1 ml/min. 1-ml fractions were collected and assayed for chemotactic activity. Two separate peaks were found.

Reverse Phase HPLC. Each of the active peaks from the cation exchange column was applied to a 0.5 × 25-cm Hi-Pore reverse phase column (Bio-Rad Laboratories, Richmond, CA), equilibrated with a starting solvent of 0.1% trifluoroacetic acid in water. A linear gradient was programmed, with a limit buffer of 70% (vol/vol) acetonitrile in water containing 0.1% TFA. Flow rate was 1 ml/min; 1.0-ml fractions were collected, and those in the region of A₂₈₀ peaks were assayed for chemotactic activity.

SDS-PAGE. Electrophoresis was carried out on a vertical slab gel of 15% acrylamide with a discontinuous tris glycine buffer system (11). Samples, as well as a solution of molecular weight standards, were mixed with equal volumes of double-strength sample buffer (20% glycerol, 6% SDS, with or without 10% 2-ME), boiled, and applied to the gel. After electrophoresis at 10 mA for 4 h, the gel was stained with a silver staining kit (ICN Biomedicals, Irvine, CA).

Amino Acid Composition and Sequence Analysis. After a 24-h hydrolysis in 6 M HCl in vacuo at 106°C, amino acid composition was determined on a Beckman System 6300 (Beckman Instruments, Inc., Fullerton, CA). NH₂-terminal sequence analysis was performed on protein sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA).

Chemotaxis Assay. Mononuclear cells from human venous blood were separated by centrifugation on metrizoate/Ficoll (Accurate Chemical & Scientific Corp., Westbury, NY) and

used for chemotaxis assay in multiwell chambers (12). Cell suspensions were added to upper wells of the chambers; they were separated from lower wells containing chemoattractant by a 10- μm -thick polycarbonate membrane with 5- μm -diameter holes (Neuro Probe, Inc., Cabin John, MD). The number of monocytes that migrated through the holes to the attractant side of the membrane during a 90-min incubation was counted with an image analyzer (13). Results were expressed as the percentage of the input number of monocytes that migrated per well (for duplicate wells). The reference chemoattractant, FMLP (Peninsula Laboratories, Inc., Belmont, CA), was dissolved in ethanol at a concentration of 1 mM and diluted for assay.

Results

Purification of GDCF. 4 liters of conditioned medium from U-105MG cells was concentrated to 50 ml, dialyzed against starting buffer and applied to an Orange A-Sepharose column. The column was eluted with a linear NaCl gradient. Fig. 1 shows that the bulk of the protein did not bind to the column, and emerged directly in the first 27 fractions. Chemotactic activity bound to the column and was eluted between 0.2 and 0.45 M NaCl. As shown in Table I, MCA was separated from $\sim 98\%$ of the conditioned medium protein, and recovery of chemotactic activity was 78%. Thus, this step was very efficient. Pooled active fractions were concentrated to 2 ml and applied to a carboxymethyl HPLC (CM-HPLC) column. Fig. 2 shows that chemotactic activity was recovered in two separate peaks that coeluted with two major A_{280} peaks. Sequential fractions corresponding to the two MCA peaks were analyzed by SDS-PAGE. The first MCA peak (GDCF-1), which had maximal chemotactic activity in fractions 36 and 37, showed a major band with maximal intensity in these fractions. There was also a narrower band immediately above the major band, which can be seen in the lanes of fractions 35 and 36 (Fig. 2). The second MCA peak (GDCF-2), with maximal chemotactic activity in fractions 45 and 46, showed a single

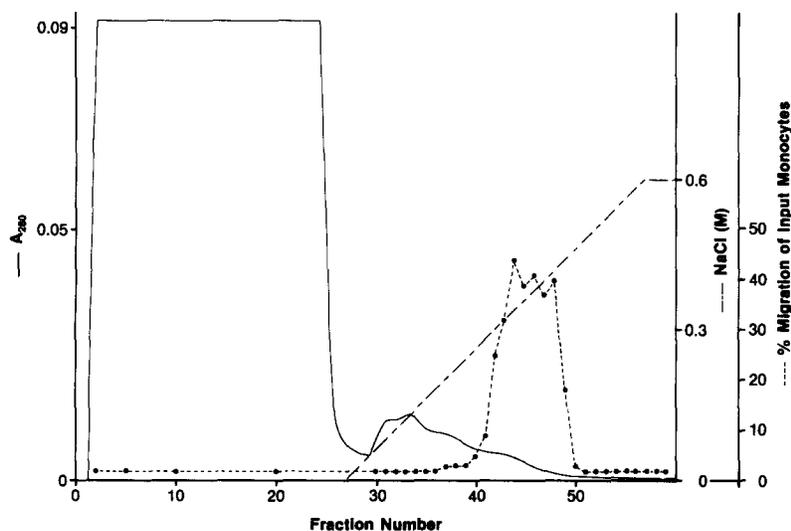


FIGURE 1. Monocyte chemotactic activity eluted from Orange A-Sepharose column. (—) A_{280} ; (- - -) monocyte chemotactic activity; (- - -) NaCl gradient.

TABLE I
Purification Schema for Human GDCF

Purification step	Total protein <i>mg</i>	Total MCA* <i>U</i>	Specific activity <i>U/mg</i>
Crude supernatant	29 [‡]	200,000	6,900
Concentrated and dialyzed supernatant	29 [‡]	190,000	6,600
Orange A-Sepharose	0.52 [‡]	148,000	288,000
CM-HPLC			
P-I (fractions 36 + 37)	0.03 [‡]	21,600	720,000
P-II (fractions 45 + 46)	0.03 [‡]	18,200	607,000
Reverse phase PHLC			
GDCF-1	0.005 [§]	5,700	1,140,000
GDCF-2	0.019 [§]	20,000	1,053,000

* MCA concentration of 1 U/ml was defined as the reciprocal of the dilution at which 50% of the maximal chemotactic response was obtained.

[‡] Protein concentration was determined by dye protein assay with BSA as standard.

[§] Protein concentration was calculated from amino acid composition.

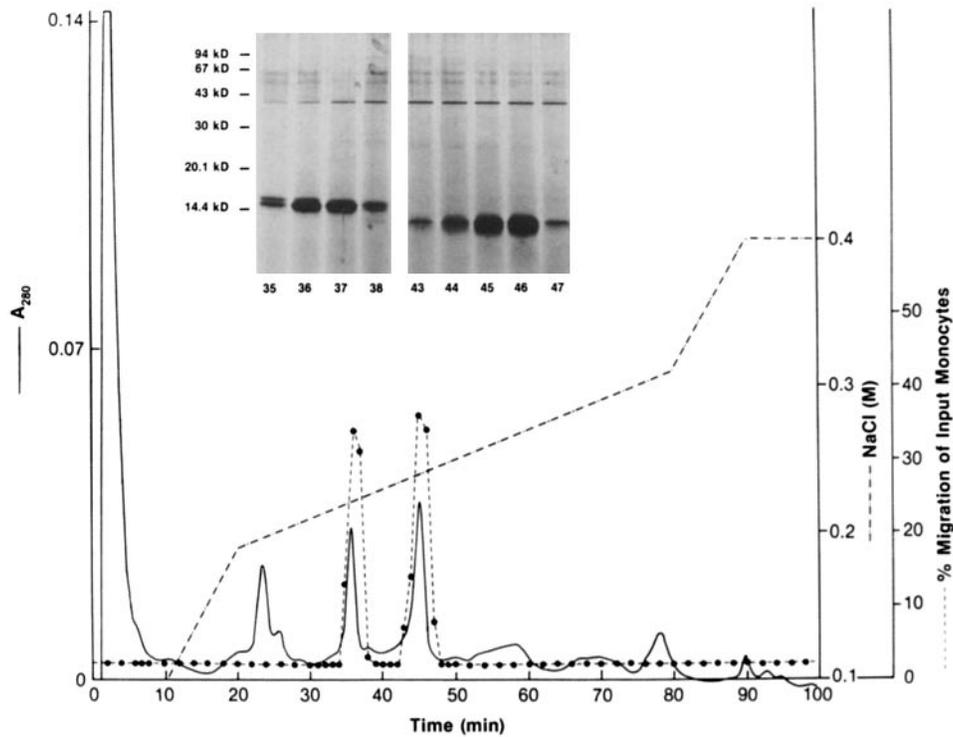


FIGURE 2. Monocyte chemotactic activity eluted from cation exchange HPLC column. (—) A₂₈₀; (---) monocyte chemotactic activity; (· · ·) NaCl gradient.

major band with peak intensity in these fractions. By reference to the mobility of protein standards, estimates of the molecular masses of GDCF-1 and -2 were 15 and 13 kD. For further purification, GDCF-1 (fraction 37) and GDCF-2 (fractions 45 and 46) were applied to reverse phase HPLC columns and eluted with a linear acetonitrile gradient. Fig. 3 *a*, and *b* show that each MCA peak coeluted with a single, sharp A_{226} peak. The presence in these chromatograms of absorbance peaks without chemotactic activity shows that the reverse phase column removed residual extraneous protein. This is also shown in Table I by the increased specific activity of the RP-HPLC products. When RP-HPLC GDCF-1 and GDCF-2 were analyzed by SDS-PAGE, single bands were found, with estimated molecular masses of 15 and 13 kD,

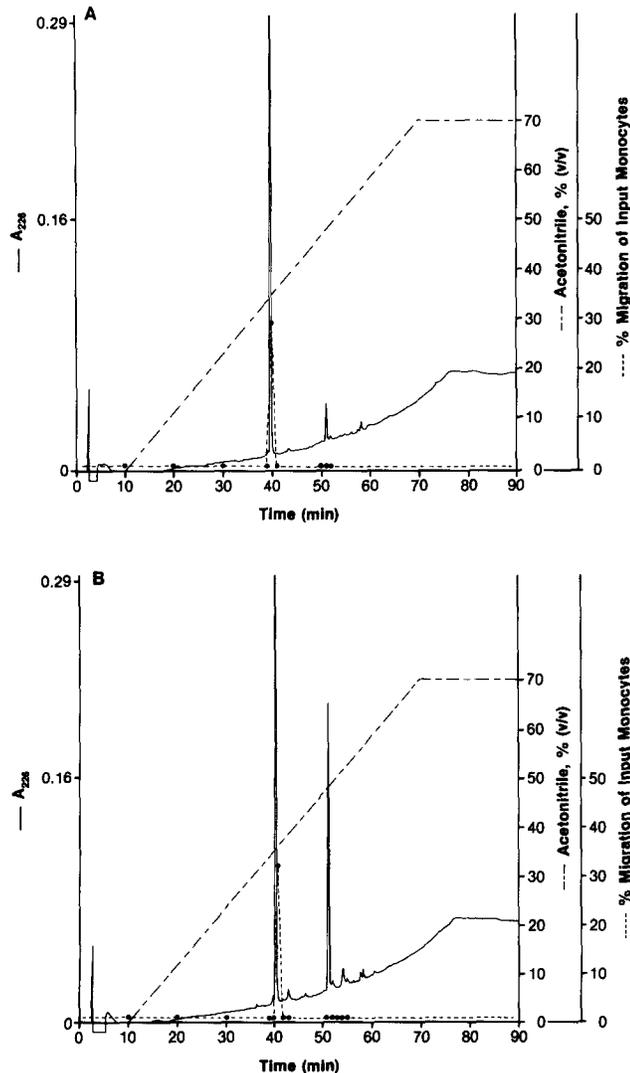


FIGURE 3. Monocyte chemotactic activity eluted from reverse phase HPLC column. (—) A_{226} ; (---) monocyte chemotactic activity; (· · ·) acetonitrile gradient. (A) CM-HPLC fraction 37 applied to column. (B) CM-HPLC fractions 45 and 46 applied to column.

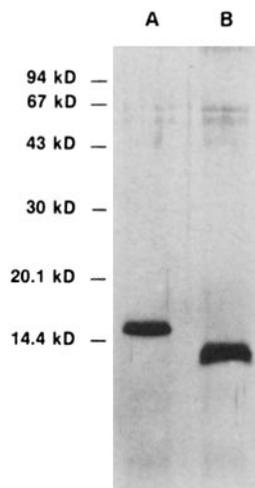


FIGURE 4. SDS-PAGE of purified GDCF. Approximately 25 ng of GDCF from the reverse phase HPLC column was applied to a 15% polyacrylamide gel under reducing conditions. (A) GDCF-1; (B) GDCF-2. Positions of molecular mass markers on this gel are indicated at the left.

respectively (Fig. 4). As summarized in Table I, from 4 liters of conditioned medium, $\sim 5 \mu\text{g}$ of GDCF-1 and $19 \mu\text{g}$ of GDCF-2 were purified to apparent homogeneity. Specific activity was 165 times that of the starting material for GDCF-1, and 150 times for GDCF-2. Total recovery was $\sim 13\%$.

Amino Acid Analysis of GDCF-1 and GDCF-2. Table II shows the amino acid composition of purified GDCF-1 and -2, based on two separate analyses of each peptide. Within the limits of error of the method, the amino acid composition of the peptides

TABLE II
Amino Acid Composition of Human GDCF

Amino acid	Residues per molecule*	
	GDCF-1	GDCF-2†
Asp + Asn	7.6	8.0
Thr	6.8	6.8
Ser	4.6	4.6
Glu + Gln	8.4	8.0
Pro	5.1	4.5
Gly	2.0	0.3
Ala	5.7	6.1
Val	4.7	4.5
Met	0.9	0.7
Ile	5.3	5.0
Leu	2.3	2.3
Tyr	1.8	1.8
Phe	2.1	2.0
His	1.2	0.9
Lys	8.6	9.1
Arg	4.0	3.6
Cys	ND	3.5‡
Trp	ND	ND

* The data were calculated on the basis of a total of 74 residues/molecule.

† GDCF-2 was reduced and [^3H]carboxymethylated for composition analysis.

‡ [^3H]Carboxymethylcysteine.

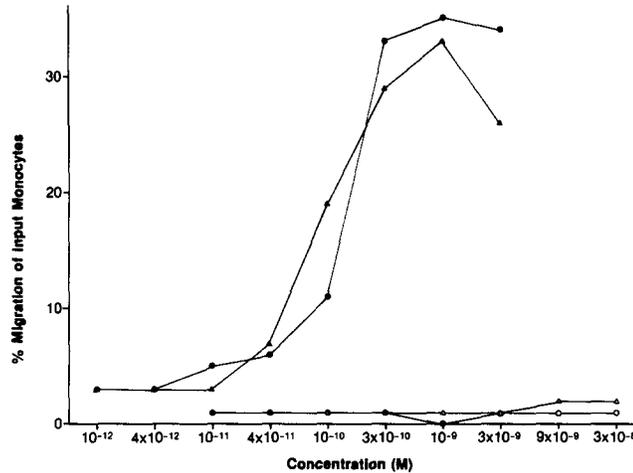


FIGURE 5. Chemotactic activity of GDCF for human monocytes and neutrophils. (●) GDCF-1 for monocytes; (○) GDCF-1 for neutrophils; (▲) GDCF-2 for monocytes; (△) GDCF-2 for neutrophils. Pure proteins obtained by reverse phase HPLC were used for this assay.

is identical. A minimal molecular mass, calculated from the amino acid composition, is $\sim 8,400$ daltons. The amino acid composition of GDCF is different from IL-1, TNF, granulocyte-macrophage CSF, macrophage CSF, and transforming growth factor, cytokines that have been reported to be chemotactic for monocytes (14–18).

When NH_2 -terminal amino acid analysis was attempted, no degradation of either peptide occurred, suggesting that the NH_2 terminus was blocked.

Assay of GDCF Chemotactic Activity for Monocytes and Neutrophils. Fig. 5 shows potency and efficacy of pure GDCF-1 and -2 for monocytes. For both peptides, $\sim 35\%$ of monocytes added to assay wells migrated at the optimal concentration of 1 nM. Migration to the optimal concentration of FMLP in the same assay was also 35%. No significant neutrophil migration was observed over a GDCF concentration range of 0.01–30 nM in that experiment. Thus, GDCF attracts monocytes but not neutrophils.

Assay to Distinguish Chemotaxis from Chemokinesis. Purified GDCF was added in different concentrations to top and bottom wells of multiwell chambers, as outlined in Table III. Dose-dependent monocyte migration was observed only when GDCF

TABLE III
Assay to Distinguish Chemotactic from Chemokinetic Activity

Concentration in top wells (M)	Monocyte migration* at concentration in bottom wells of (M):			
	0	4×10^{-11}	2×10^{-10}	10^{-9}
GDCF-1				
0	1 ± 0.2	5 ± 0.9	22 ± 2.4	35 ± 0.7
4×10^{-11}	1 ± 0.2	4 ± 0.5	15 ± 1.3	34 ± 4.6
2×10^{-10}	2 ± 0.4	2 ± 0.3	3 ± 1.2	21 ± 4.2
10^{-9}	1 ± 0.2	1 ± 0.1	1 ± 0.1	3 ± 0.2
GDCF-2				
0	2 ± 0.2	12 ± 1.8	25 ± 6.2	27 ± 3.9
4×10^{-11}	1 ± 0.1	5 ± 0.5	18 ± 0.6	26 ± 5.0
2×10^{-10}	3 ± 0.5	2 ± 0.2	5 ± 0.6	24 ± 1.5
10^{-9}	1 ± 0.1	2 ± 0.1	2 ± 0.1	4 ± 0.1

* Percent of input cell number \pm SEM.

was in bottom wells. No significant migration occurred when top and bottom wells contained equal concentrations of GDCF, showing that migration was due primarily to chemotaxis, not chemokinesis.

Discussion

Two chemotactic peptides for human monocytes, GDCF-1 and GDCF-2, were purified to apparent homogeneity from culture fluid of a human glioma cell line. Although these two peptides were separated into two completely distinct peaks by CM-HPLC chromatography, their elution patterns from a reverse phase HPLC column were identical; and their amino acid compositions were indistinguishable. Chemotactic potency and efficacy of both peptides were very similar (Table II, Fig. 5), and both were chemotactic for monocytes but not neutrophils. It is possible that the two peptides differ only by post-translational modifications, such as phosphorylation, glycosylation, or degradation. Based on the amino acid composition, our estimate of the molecular mass of GDCF is 8,400 daltons, which is considerably less than the 15- and 13-kD values determined by SDS-PAGE for GDCF-1 and -2. Discrepancies between molecular mass estimates obtained by these different methods of biologically active peptides have been reported by others (19).

As shown in the last column of Table II, purification of GDCF to homogeneity was associated with only a 150-fold increase in specific activity, which reflects the relatively high concentration of GDCF in U-105MG glioma cell culture fluid. This is due to the absence of FCS in the medium, and also indicates that GDCF represents a significant percentage of the proteins secreted by the U-105MG cell line.

The amino acid composition of GDCF is different from other cytokines that have been reported to be chemotactic for monocytes (14–18). This includes IL-1, TNF, granulocyte-macrophage CSF, macrophage CSF, and transforming growth factor β . GDCF is also distinct from other cytokines produced by glioma cells, including IL-1 and platelet-derived growth factor (20, 21). In contrast to these chemically defined cytokines, there are reports in the literature of incompletely characterized monocyte chemoattractants, the molecular masses of which are similar to that of GDCF (8, 9, 22–27). Of particular interest in the light of the basic pI of GDCF (9a) are two reports. Valente et al. (26) recently found that baboon aortic medial smooth muscle cells produce monocyte chemotactic factor with a molecular mass of 10–12 kD and pI above 10.5. This factor may be involved in the generation of atherosclerotic lesions.

The other report, by Altman et al. (27), is on monocyte chemotactic factors produced by mitogen-stimulated lymphocytes (lymphocyte-derived chemotactic factor [LDCF]). These factors had a molecular mass of 13 kD, with isoelectric points of 5.6 and 10.1. Despite the probable importance of LDCFs as mediators of cellular immune reactions, they have never been completely characterized. Therefore, we applied our procedure for purification of GDCF to LDCF, and obtained a product the amino acid composition of which is indistinguishable from that of GDCF (28). Thus, monocyte chemotactic factors produced by different normal tissues or by tumors derived from different organs may be similar or identical. These attractants may account for not only monocyte infiltration into tumors but also accumulation of mononuclear phagocytes at sites of delayed hypersensitivity reactions.

Pure GDCF provides an opportunity to answer the questions posed in the in-

roduction to this communication. In addition, development of specific antibody to GDCF should provide immunochemical means to determine the incidence and localization of tumor-derived attractants at sites of human tumors.

Summary

Two chemoattractants for human monocytes were purified to apparent homogeneity from the culture supernatant of a glioma cell line (U-105MG) by sequential chromatography on Orange A-Sepharose, an HPLC cation exchanger, and a reverse phase HPLC column. On SDS-PAGE gels under reducing or nonreducing conditions, the molecular masses of the two peptides glioma-derived chemotactic factor 1 and 2 were 15 and 13 kD, respectively. Amino acid composition of these molecules was almost identical, and differed from other cytokines that have been reported. The NH₂ terminus of each peptide was apparently blocked. When tested for chemotactic efficacy, the peptides attracted ~30% of the monocytes added to chemotaxis chambers, at the optimal concentration of 10⁻⁹ M. Potency and efficacy were comparable with that of FMLP, which is often used as a reference attractant. The activity was chemotactic rather than chemokinetic. In contrast to their interaction with human monocytes, the pure peptides did not attract neutrophils. These pure tumor-derived chemoattractants can now be compared with attractants produced by normal cells and evaluated for their biological significance in human neoplastic disease.

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Note added in proof: The amino acid sequence of GDCF-2 has now been completely analyzed (29). In addition, we recently prepared a cDNA from the U-105MG glioma cell line (30). By Northern blot analysis, this cDNA hybridized with RNA from both glioma cells and PHA-stimulated human blood mononuclear cells. The result provides additional evidence that the monocyte attractant can be produced by different cell types. In future articles, the attractant will be referred to by the generic term monocyte chemoattractant protein 1 (MCP-1). Greek letter suffixes will indicate different forms of the protein.

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