PURIFICATION OF A FACTOR FROM HUMAN BLOOD MONOCYTE-MACROPHAGES WHICH STIMULATES THE PRODUCTION OF COLLAGENASE AND PROSTAGLANDIN E_2 BY CELLS CULTURED FROM RHEUMATOID SYNOVIAL TISSUES

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1. Introduction

The complex interactions involved in the degradation of joint structure in inflammatory synovitis such as rheumatoid arthritis have been studied in this laboratory. We have shown that monocyte-macrophages isolated from human blood and incubated for one or more days in culture release a soluble factor into the medium. This factor (MCF) stimulates the production of collagenase and prostaglandin E_2 in a population of cultured rheumatoid synovial cells which adhere to the culture dish (ASC) [1,2]. Highly purified preparations of the monokine interleukin I, also known as lymphocyte activating factor (LAF), derived from a murine macrophage cell line have MCF activity and MCF and interleukin I copurify [3]. Here, the purification and further characterization of human MCF employing a combination of chromatographic techniques are described. The sequential use of ion-exchange, gel filtration, hydrophobic interaction and high performance liquid chromatography (HPLC) with a protein separation column has enabled us to achieve a high degree of purity of MCF.

2. Materials and methods

2.1. *Production of MCF by human blood monocyte-macrophage cultures*

Primary cultures of human monocyte-macrophages were prepared as in [2,4]. After 4–5 days of culture, cell-free supernates were obtained by centrifugation and stored at −20°C for further purification of MCF. Just before chromatography, batches were pooled, millipore-filtered (0.22 μm, Millipore Corp., Bedford MA), dialyzed extensively against distilled water using 3500 M_r cut-off membranes (A. H. Thomas, no. 3787–H45, Philadelphia PA) and freeze-dried.

2.2. *Preparation of synovial cell cultures*

Adherent cells (ASC) were prepared from samples of synovium, obtained at synovectomy from patients with clinically defined rheumatoid arthritis as in [5,6]. For bioassays, ASC were plated at a second or third passage in 16 mm diam. wells (Costar, Cambridge MA) at ~1×10^5 cells/well. At this stage the basal production rates of collagenase and PGE_2 were usually lower than in primary culture, which permitted measurement of a dose-related response to added fractions containing MCF. The results (collagenase or prostaglandin) are corrected for synovial cell number at the end of the incubation. Since the synovial cell cultures were near confluency, no effect of monoclear cell media on cell proliferation was detected; the cell numbers under all incubations were within ± 5% of the mean.

2.3. *Bioassay of mononuclear cell factor (MCF) in target synovial cells (ASC)*

Aliquots of dialyzed, freeze-dried column fractions to be assayed were taken up in DMEM, 10% FCS (Grand Island Biological Co., Grand Island NY) (Microbiological Associates, Walkersville MD), millipore-filtered, and added to the synovial cell cultures. After 3 days incubation the cell-free supernates were assayed for collagenase activity and PGE_2 concentration

Abbreviations: MCF, macrophage cell factor; ASC, adherent synovial cells; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

[5,6]. A 'standard' MCF fraction, lyophilized from a pooled peak from an AcA 54 column, was added to each bioassay to assess the activities of the different target cell preparations.

2.4. *Collagenase and prostaglandin E_2 assays*

Collagenase activity was measured by solubilization of ^{14}C-labeled reconstituted purified guinea pig skin collagen fibrils. Trypsin activation followed by excess soybean trypsin inhibitor was used to convert latent to active enzyme. One unit represents solubilization of 1 μg native collagen/min [5,6]. The specific activity of the [^{14}C]collagen was ~15 000 cpm/mg.

PGE_2 was measured using a radioimmunoassay [7]. The results reported are in terms of PGE_2 equivalents based on prior observations that rheumatoid synovial organ cultures produce PGE_2 and no detectable PGE_1 [8].

2.5. *Labeling semipurified MCF with ^{125}I*

The entire peak of biological activity eluted from the Water's protein column was labeled with ^{125}I by a modification [9] of the chloramine T method [10]. Material containing ~6×10^6 cpm was isolated. This sample had only a trace of absorbance at 254 nm prior to labeling. This material was added to uniodinated MCF in order to correlate biological activity with ^{125}I following chromatography. One column was used to locate biological activity and the same column was re-run to locate ^{125}I radioactivity.

2.6. *Column chromatography*

Details of the chromatography are given in the figure legends. DEAE-cellulose, Ultrogel AcA 54, Phenyl-Sepharose CL-4B and the Water's protein column I-125 were used. In all cases when samples containing MCF were dialyzed, 3500 M_r cut off tubing was used.

3. Results and discussion

In [1] MCF from cell-free medium from cultured human macrophages was partially purified using columns of Ultrogel AcA 54. Biological activity assayed in the rheumatoid synovial cell system (ASC) as stimulation of collagenase or prostaglandin E_2 production, was eluted in the same fractions. Although we have shown that lectins such as concanavalin A, phytohemagglutinin and pokeweed mitogen, Fc fragments of immunoglobulin G, and collagens stimulate the production of MCF [2,4,11], we chose to perform the initial purification under non-stimulated conditions.

MCF was eluted from the anion exchange DEAE-cellulose columns at ~0.2 M NaCl, using linear gradients as indicated in fig.1. This step resulted in ~100-fold increase in specific activity. Because of the relatively large amount of albumin present in the fetal calf serum, calculations of the initial specific activities have little significance.

The use of the Ultrogel sizing column AcA 54 as shown in fig.2 was helpful because of its high capacity and ability to separate biological activity from most of the albumin as indicated by comparison of the absorbance with the peak of biological activity. MCF was eluted in the 14 000–25 000 M_r range. Lyophilized MCF from this column stored at −20°C retained activity for months. Several column batches were pooled at this stage to obtain sufficient material for further purification. Material in the column buffers in the freeze-dried samples accounted for most of the weight in samples before dialysis.

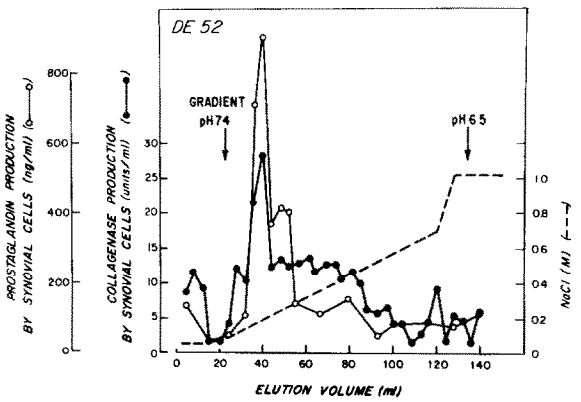

Fig.1. Chromatography of MCF on DEAE-cellulose. Pooled lyophilized material with biological activity from Ultrogel AcA 54 columns dialyzed against 0.05 M Tris–HCl (pH 7.4) was applied in 4.5 ml to a 0.9 × 2.8 cm column of DE-52 (Whatman, Clifton NJ) and eluted at 0.5 ml/min, collecting fractions of 1.0 ml. Initially, 20 ml equilibration buffer, 0.05 M Tris–HCl (pH 7.4) were added followed by a linear gradient of 0–1.0 M NaCl in 0.05 M Tris–HCl (pH 7.4) in 100 ml. At ~0.7 M NaCl (120 ml elution vol.), 60 ml 1.0 M NaCl, 0.05 M Tris–HCl at pH 6.5 were applied. The molarity of NaCl in the gradient was determined by conductivity. For biological assays on synovial cells, fractions were dialyzed against DMEM, Millipore-filtered, and applied in DMEM, 10% FCS at a 1:5 dilution to the adherent synovial cells.

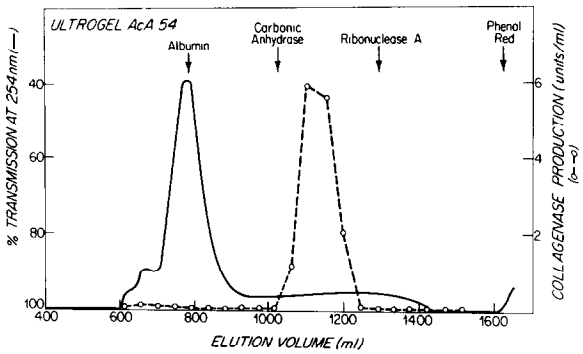

Fig.2. Chromatography of MCF on Ultrogel AcA 54. Partially purified MCF was applied to an 80 × 5 cm column containing Ultrogel AcA 54 (LKB Instruments Inc., Rockville MD). Pooled freeze-dried MCF from DEAE-columns was dialyzed extensively against distilled water containing 0.04% sodium azide followed by water alone. After freeze-drying to reduce the volume, the samples were taken up in 15 ml 10 mM Tris–HCl (pH 7.5), 165 mM NaCl and 5 mM $CaCl_2$, millipore-filtered and applied to the column pre-equilibrated with the buffer. The flow rate was 1.2 ml/min and 15 ml fractions were collected. Aliquots of 70 μl from each of 3 tubes were pooled, diluted 1:10 with DMEM, 10% FCS and assayed for biological activity (collagenase production) on ASC. The column had been standardized with 20 mg each of blue dextran, carbonic anhydrase, human serum albumin, ribonuclease A and phenol red. Pooled fractions from 10 such columns were used for most further purifications. Samples in this large pool had all been through DEAE-cellulose and hydroxylapatite columns prior to the Ultrogel AcA 54, although the order of use of the initial two columns was varied.

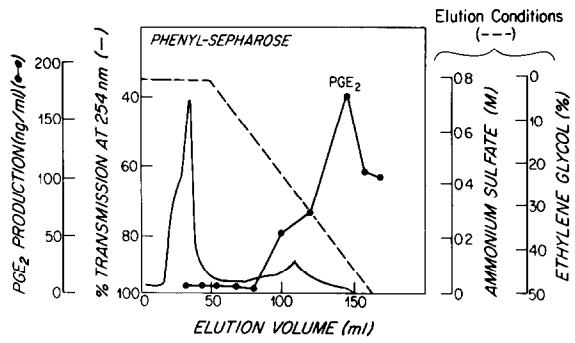

Fig.3. Chromatography of MCF on phenyl–Sepharose. Pooled lyophilized fractions containing MCF eluted from AcA 54 columns were dialyzed for 2 days against water and freeze-dried. The sample was then dissolved in 3.5 ml 0.01 M Na-phosphate, 0.8 M $(NH_4)_2SO_4$ at pH 6.8, and applied to a 25 × 1.5 cm column of phenyl–Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala) eluted at 1.5 ml/min collecting 4.5 ml fractions. Elution was initiated by the addition of 50 ml 0.8 M $(NH_4)_2SO_4$, 0.01 M Na-phosphate (pH 6.8) followed by a linear gradient of 60 ml 50% ethylene glycol, 0.01 M Na-phosphate (pH 6.8) into a reservoir of 60 ml of the initial buffer, giving a decreasing concentration of $(NH_4)_2SO_4$ and an increasing concentration of ethylene glycol. Samples from 4 consecutive tubes were pooled, dialyzed against water and stored freeze dried until bioassay (PGE_2 production).

Larger batches of pooled material were purified to this stage and applied to phenyl–Sepharose columns where the bulk of the absorbance at 260 nm was eluted in earlier fractions than those containing MCF activity, thus achieving a significant further purification (fig.3). The hydrophobic phenyl groups linked to the uncharged agarose gel Sepharose CL-4B, separate proteins on the basis of their hydrophobic sites [12]. Since MCF was eluted at relatively high concentrations of the organic eluant, MCF could be considered to be fairly hydrophobic. We did not apply the usual protein concentration assays to any pooled fractions beyond this column because of the small amounts of MCF available for further purification.

The semi-purified preparations from either AcA 54 or phenyl–Sepharose columns were placed on the Water's protein column using high performance liquid chromatography (HPLC) which resulted in further purification as measured by the absorbance at 254 nm (fig.4). Recovery of MCF activity was ~50% of that added to the column. The MCF activity emerged in an area where there was minimal absorbance at 254 nm, barely detectable with a full-scale deflection of 0.5 units. Elution characteristics on the protein column were reproducible, judging from observations that the peak of biological activity emerged in the same tube in 6 separate expt. When freshly iodinated samples of MCF were added to a separate column, also shown in fig.4, a peak of ^{125}I radioactivity was eluted which coincided with the peak of both prostaglandin- and collagenase-stimulating activity. An estimate of the M_r of MCF using the AcA 54 and Water's protein columns indicates a size between 14 000–24 000. It is not possible to establish purity by the usual means until more material is obtained.

In a recent collaborative study we showed that human MCF has properties in common with mouse interleukin I [3]. MCF activity co-purified with interleukin I activity derived from the murine macrophage cell line $P388D_1$. Both activities had app. M_r of 14 000–25 000 with peak M_r 16 000 on Sephacryl S200, and both biological activities were present in the same fractions after electrophoresis on 10% Tris–

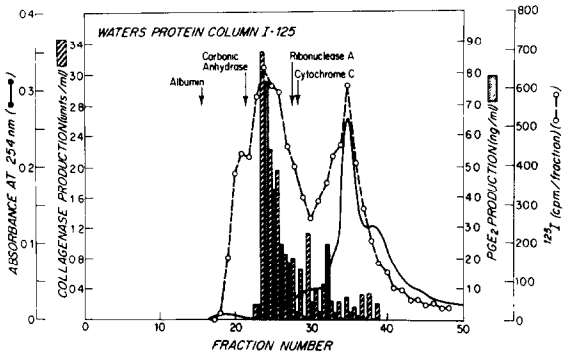

Fig.4. Chromatography of MCF on the Water's I-125 protein column using HPLC. The Water's ALC/GPC 204 liquid chromatograph system with a 254 nm detector was used. The column is a sizing column having a useful range of 2000–80 000 M_r. A sample with MCF activity eluted from AcA 54 columns was dissolved in 0.1 M triethylammonium acetate (pH 7.0) since trialkylammonium salts have been used successfully in this type of chromatography [13]. This was prepared by neutralizing freshly distilled triethylamine with acetic acid. The sample was centrifuged for 1 min at 10 000 rev./min to remove bits of undissolved material prior to application to the column. The protein column was washed with acetonitrile and equilibrated with 0.02 M triethylammonium acetate (pH 7.0) the eluting buffer. A 125 μl sample was applied at a flow rate of 0.3 ml/min, collecting 0.3 ml fractions which were freeze-dried immediately to remove the buffer prior to bioassay (collagenase production and PGE_2 production). The ^{125}I radioactivity curve was from a previous protein column run using pooled iodinated biologically-active material. Standard proteins (100 μg each) run individually under identical conditions indicated fairly broad peaks with large overlaps of one to another. The column separates mainly by size; however, it has a few functional groups which could affect the elution pattern of some basic proteins.

glycinate discontinuous polyacrylamide gels [3]. Since the synovial cells do not possess morphological or functional markers of macrophages or lymphocytes [4], these observations suggest that interleukin I has effects on human non-lymphoid cells. Now that we are able to obtain relatively pure material we are in a position to examine physical and chemical properties of MCF and to conduct immunological and biochemical experiments which may help in our understanding of the biological activities of this factor.

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References

[1] Dayer, J.-M., Robinson, D. R. and Krane, S. M. (1977) J. Exp. Med. 145, 1399–1404.
[2] Dayer, J.-M., Bréard, J., Chess, L. and Krane, S. M. (1979) J. Clin. Invest. 64, 1386–1392.
[3] Mizel, S. B., Dayer, J.-M., Krane, S. M. and Mergenhagen, S. E. (1981) Proc. Natl. Acad. Sci. USA in press.
[4] Dayer, J.-M., Passwell, J. H., Schneeberger, E. E and Krane, S. M. (1980) J. Immunol. 124, 1712–1720.
[5] Dayer, J.-M., Krane, S. M., Russell, R. G. G. and Robinson, D. R. (1976) Proc. Natl. Acad. Sci. USA 73, 945–949.
[6] Dayer, J.-M., Goldring, S. R., Robinson, D. R. and Krane, S. M. (1980) in: Collagenase in Normal and Pathological Connective Tissues (Woolley, D. E. and Evanson, J. M. eds) pp. 83–104, Wiley, New York.
[7] Levine, L., Gutierrez Cernosek, R. M. and Van Vunakis, H. (1971) J. Biol. Chem. 246, 6782–6785.
[8] Robinson, D. R., Smith, H., McGuire, M. B. and Levine, L. (1975) Prostaglandins 10, 67–85.
[9] Marx, S. J., Woodward, C., Aurbach, G. D., Glossmann, H. and Keutmann, H. T. (1973) J. Biol. Chem. 248, 4797–4802.
[10] Hunter, W. M. and Greenwood, F. C. (1962) Nature 194, 495–496.
[11] Dayer, J.-M., Trentham, D. E., David, J. R. and Krane, S. M. (1981) Trans. Assoc. Am. Phys. in press.
[12] Porath, J., Sundberg, L., Fornstedt, N. and Olsson, I. (1973) Nature 245, 465–466.
[13] Rivier, J. E. (1978) J. Liquid Chromatog. 1, 343–366.