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Purification and properties of a 155 kDa bone marrow-derived glycoprotein enhancing the activity of granulocyte-macrophage colony stimulating factor (GM-CSF)

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A protein has been purified from pig bone marrow which enhances the activity of exogenously added granulocyte-macrophage colony stimulating factor (GM-CSF) on mouse myelopoietic colony formation in vitro. The substance alone did not exhibit any colony stimulating activity. The active fraction was isolated from the medium of pig bone marrow suspension cultures during 20–24 h. Purification to homogeneity was then performed by subsequent HPLC and SDS-PAGE. The GM-CSF enhancing protein was identified as a 155 kDa glycoprotein.

GM-CSF; Bone marrow; Colony formation enhancement

1. INTRODUCTION

In the present work we have investigated conditioned medium from bone marrow culture in order to identify high molecular weight substances which may be involved in the stimulation of colony formation induced by GM-CSF. It appears that bone marrow cells produce a regulatory protein ('enhancing protein'; EnP), which may be a cofactor in the regulation of myelopoiesis.

2. MATERIALS AND METHODS

2.1. *Production of serum-free conditioned medium*

Bone marrow cells were obtained from the ribs of freshly killed adult pigs by crushing followed by washing in standard Eagle's minimum essential medium (Flow Lab, Glasgow, UK). About 100 ribs (~0.035 kg each) were used in order to obtain approximately 30 l of the conditioned medium. The bone crumb was mixed with 500 ml of medium (ratio 4:1 v/w) in a round bottle tissue culture flask and placed on a heated shaker (Hereaus GmbH, Germany) for 1 h at 37°C, followed by separation of the bone fragments by filtration through 0.5 mm pore-size multilayered nylon mesh. The filtrate (consisting approximately 50% of fatty substances) was centrifuged at $200 \times g$ for 10 min and the deposited cells washed twice: first in standard Eagle's medium, followed by medium 199 (Flow Lab, Glasgow, UK). The cells were counted, tested for viability by the Trypan blue exclusion test, and then seeded into sterile glass culture flasks at a concentration of 2×10^7 cell/ml in serum-free medium 199 contained 20 mM of glutamine, HEPES 0.01 M, penicillin 100 U/ml. The cell suspension was incubated at 37°C and 5% CO_2 in humidified air. Cell viability during the period of incubation was more than 95%.

After 20–24 h of incubation, the conditioned medium was collected by centrifugation at $10,000 \times g$ for 30 min and then concentrated 10 times by ultrafiltration using an Amicon LP-1A pump fitted with HIP3 hollow fibre filters (cut off 3 kDa) at 10 ml/min flow rate. The subsequent purification steps (except HPLC) were performed in a cold room below 4°C.

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2.2. *Gel filtration*

Ten ml of the conditioned medium concentrate was applied to a column (2.6 × 100 cm, LKB, Sweden) fitted with Toyopearl HW-55 (Toyo Soda Manufacturing Co., Japan) equilibrated with double-distilled water. A Multiperpex 2115 peristaltic pump, Uvicord SII monitor, equipped with a 214 nm absorbance filter was used together with an Ultrarac 7000 automatic fraction collector (all from LKB Pharmacia, Uppsala, Sweden). The flow rate was 100 ml/h. The samples were washed through the column with approximately 600 ml of the eluent and the active fraction was eluted in the void volume. Thereafter the active fractions were pooled and freeze dried. This purification protocol resulted in ~0.1% yield of the active fraction from the content of UV-absorbing material loaded onto a column.

2.3. *High performance liquid chromatography (HPLC)*

Using an appropriate column, HPLC was performed at room temperature using a Spectra-Physics model SP8000B liquid chromatograph fitted with a 1 ml sample loop. The samples emerging from the column were monitored with a LDC Spectromonitor III at 226 nm wavelength, and the protein content was assessed by comparing the absorption of a known amount of IgG Fab' fragment at the same wavelength. A lyophilized sample of the active fraction from the Toyopearl column was dissolved in normal saline (1 mg in 0.2 ml) and then chromatographed on a TSK 3000SW size exclusion column (60 × 0.75 cm internal diameter, Toyo Soda Manufacturing Co., Japan). The mobile phase consisted of aqueous sodium chloride (0.9%, w/v), pH 5.6, at a flow rate of 1 ml/min at ambient temperature, and the proteins eluted from the column were detected by UV absorption. One ml fractions were collected automatically into propylene tubes containing 100 μl of 0.2% (w/v) Tween solution (Fluka AG, Switzerland). Aliquots of 10 μl were tested for biological activity.

2.4. *Rechromatography on the PHPLC column*

The fractions containing enriched material were collected manually and rechromatographed in order to avoid impurities. The total volume applied was not more than 0.5 ml. SDS-PAGE under reducing conditions (in presence of 2-mercaptoethanol) was used to assess the purity of the fractions.

2.5. *SDS-PAGE and protein blotting*

SDS-PAGE (12.5% acrylamide, w/v) was performed as described previously [1]. Each sample was boiled for 5 min in the starting buffer containing 2-mercaptoethanol and 3–5% SDS before use. Apparent molecular weight was estimated by comparison with protein standards. The gels were stained with Coomassie blue R-250 or with Schiff's reagent [2,3]. The protein separated by SDS-PAGE were subsequently blotted onto nitrocellulose paper using a Trans Blot unit (Bio-Rad Laboratories, CA, USA) under conditions described previously [4] and stained with Amido black dye [5].

2.6. *Biological testing*

Assay was performed in semisolid agar culture using 75,000 C57Bl mouse bone marrow cells per 35 mm plate [6]. The proteins to be tested were added with or without mouse recombinant GM-CSF (Amgen Corp., USA), to the petri dishes and mixed with the cell suspension. All samples were sterilized by filtration (0.22 μm Acrodisc, Gelman Sci., USA). Colony formation (>50 cells) was determined after 7 days of incubation using an inverted microscope.

3. RESULTS

The conditioned medium (CM) obtained from bone marrow culture was filtered through a 0.45 μm sterile filter unit (Millipore Corp., USA) and then concentrated 100 times by ultrafiltration. This procedure was performed in two steps. First, a 10-fold concentration was achieved by desalting with double-distilled water in the same equipment overnight, with the procedure being repeated in the following day. No aggregation or lipid-like formation was found in the concentrate. During this procedure, a major part of the constituents with molecular weights lower than 3000 Da was discarded. About 30 l of the primary CM were processed in this manner to obtain enough material for further purification.

3.1. *Gel chromatography*

In the chromatographic profile five peaks were registered in CM concentrate by two different absorption wavelengths (Fig. 1). Medium which had not been in contact with the cells was analyzed for control purposes and this control profile is also presented in Fig. 1. A difference between the CM control sample was found only in the first fraction (void volume). Since double-distilled water was used as an eluent on the Toyopearl HW-55 column the separation pattern was not a result of a size exclusion effect only. This is because Toyopearl-type matrix gels show a slight anion exchange effect which appears at low ionic strength. In our experiments, the anion exchange effect was utilized to eliminate unwanted components from the incubation medium. Fraction 1 was pooled from about 20 chromatographic runs, frozen and lyophilized. The presence of an amino group- as well as carbohydrate-containing substances was shown by staining tests with ninhydrin and diphenylamine reactions [7].

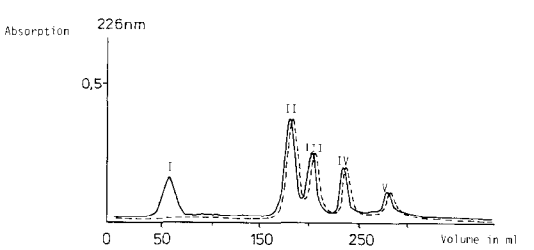

Fig. 1. Gel filtration chromatography of the conditioned medium on Toyopearl HW-55 column. Constant line: conditioned medium profile. Dashed line: culture medium (control sample) profile.

3.2. *Biological activity of CM fraction*

The CM fraction obtained by gel chromatography on Toyopearl HW-55 was assayed for possible colony stimulating activity (CSA). No CSA in semisolid agar culture of mouse bone marrow cells was observed. When optimal (10 ng) or suboptimal (1 ng) doses of GM-CSF were added together with the CM fraction, enhancement of the GM-CSF activity was observed (Fig. 2). The CM fraction did not affect colony morphology and the ratio of granulocyte to macrophage colonies was not changed (data not presented).

3.3. *Purification of CM fraction*

The active fraction was subjected to SDS-PAGE on 12.5% gels and stained by Coomassie blue R-250 (Fig. 3). The SDS-PAGE showed that the proteins in the CM fraction were well resolved according to molecular weight. High performance size-exclusion liquid chromatography was chosen as the purification method to maintain the conditions favourable to flexible molecules, and proved to be effective. The fraction from the Toyopearl HW-55 column containing stimulating activ-

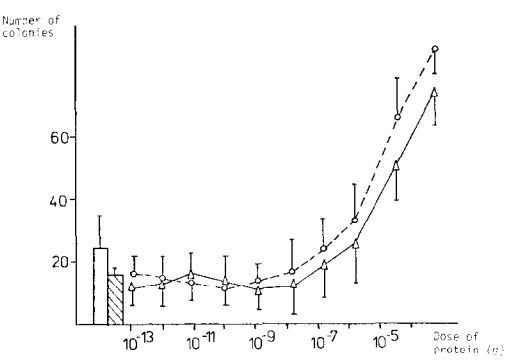

Fig. 2. Stimulation of the colony formation in GM-CSF activated mouse bone marrow cells by CM fraction. Matched bar: 1 ng of GM-CSF alone. Open bar: 10 ng of GM-CSF alone. ○: CM fraction + 10 ng of GM-CSF. △: CM fraction + 1 ng of GM-CSF.

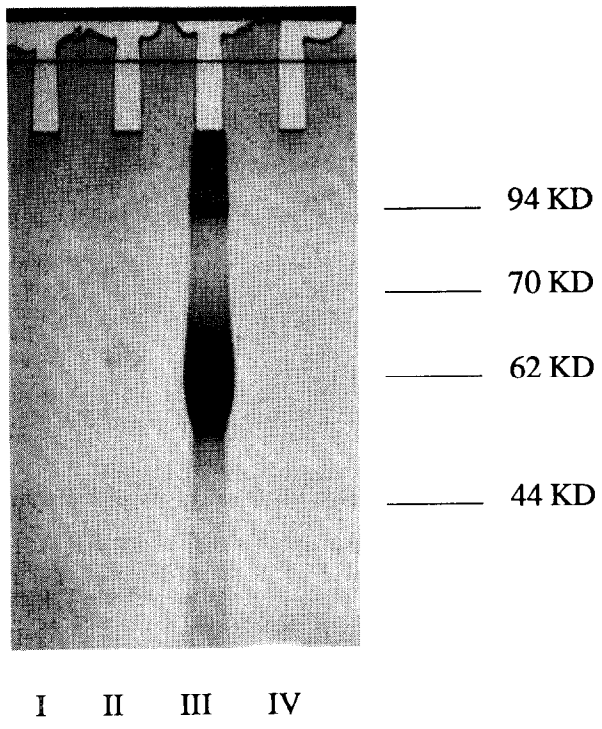

Fig. 3. SDS-PAGE analysis of the different fractions. Track I, F2 fraction; Track II, F3 fraction; Track III, CM fraction; Track IV, FI fraction.

ity was lyophilized and chromatographed on a TSK 3000SW column. Each 1 ml fraction was collected, filtered through a sterile 0.22 μm filter and tested for biological activity. The active substances were eluted between 14 and 17 min (F2) as shown on Fig. 4. No other fractions were found to stimulate the response to GM-CSF so dramatically. Each of the first four fractions was collected separately and analyzed by SDS-PAGE (Fig. 3), and no proteins were detected in F1 even after concentration of this fraction. The molecular weight of F2 was found to be 155 kDa.

3.4. *Rechromatography of F2*

Before rechromatography F2 was concentrated 10 times by lyophilization and dialyzed against normal saline, pH 5.6. Rechromatography of F2 revealed five fractions. One of them (about 80% of the subjected material according to UV absorbance) eluted between 15 and 17 min and showed biological activity (Fig. 5). The optimal dose of active fraction was 0.1–1.0 μg of protein per 35 mm plate, i.e. 1 ml culture medium.

In spite of a low protein concentration fraction 2a was the most pure and contained only a 155 kDa component and lacked the lower molecular weight impurities presented in F2.

4. DISCUSSION

Although the existence of the colony stimulating factors and other cytokines as potent stimulators of both hemopoiesis and leukocyte functions has been well established for several years (see e.g. [8–10]) the more complex underlying regulatory mechanisms have recently been documented. Firstly, rather complex interactions between these factors and the relative expression of their target cell receptors have been found. Secondly, it has been realized that hemopoiesis is a balance between stimulatory and inhibitory factors both of high [11] and low molecular weight [12,13].

It is also known that bone marrow cells in long term culture, and specifically macrophages secrete a factor which enhances the stimulatory effect of GM-CSF [14,15]. Recently, this factor has been identified as a glycoprotein with molecular weight 74 kDa which seems to be a unique cytokine different from other hematopoietic enhancing factors [16]. Using the PTH-derivative technique and an automatic protein sequencer, we have identified the first ten amino acids at the N-terminal end of our 155 kDa protein as a unique se-

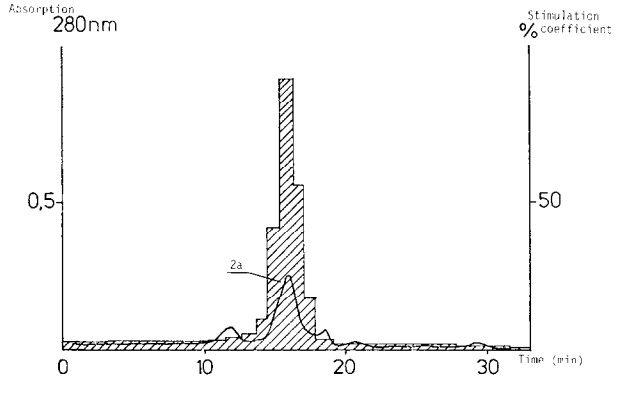

Fig. 4. Size exclusion chromatography of CM fraction on TSK 3000SW column. Shaded area: biological effect of the corresponding fractions.

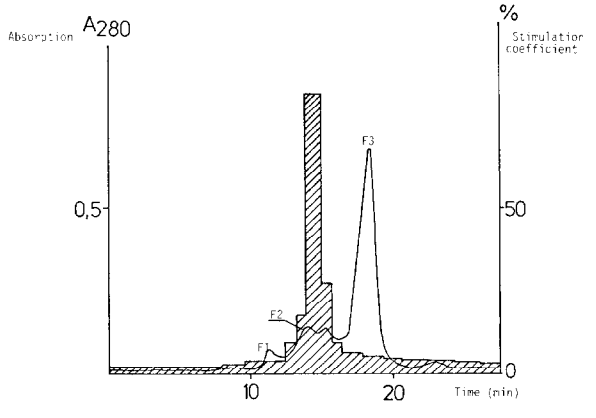

Fig. 5. Rechromatography of F2 fractions on TSK 3000SW column. Shaded area: biological effect of the corresponding fractions.

quence (the authors, manuscript in preparation). In addition to this, the differing molecular weight from the other described protein suggests that these are two different proteins with a similar enhancing activity. To what extent our protein is specific for hemopoiesis as appears to be the case for the other one [16], is so far not known.

It is earlier known that serum albumin may bind growth factors. In addition, BSA contains an activity which is necessary for the stimulatory effect of IL-3 [17]. However, this mechanism cannot explain our described enhancing activity. Since the EnP was found in pig bone marrow and acted on mouse cells, the factor does not seem to be species specific.

In short-term bone marrow suspension culture, both immunostimulating and immunosuppressing molecules [18–20] as well as hemoregulatory peptides of low molecular weight [21] may be present. In line with this, also the hemoregulatory peptide dimer may act as a GM-CSF enhancer [21]. Thus, both oligopeptides and proteins may strongly modify myelopoiesis.

We conclude that EnP is a novel glycoprotein which may have a modifying effect on myelopoietic activity in concert with hemopoietic growth factors and other regulatory molecules.

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