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Fractionation of CSF Activities from Human Placental Conditioned Medium

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A. Introduction

Colony stimulating factors (CSFs) are required for the survival, proliferation and differentiation of hemopoietic progenitor cells in vitro and possibly in vivo. Recently, several human CSFs have been purified and molecularly cloned from human tumor cell lines. These include granulocyte colony stimulating factor (G-CSF), from the bladder carcinoma cell line 5637 [1, 2] and the squamous cell carcinoma cell line CHU-2 [3], and granulocyte-macrophage colony stimulating factor (GM-CSF), from the Mo T-lymphoblast cell line [4, 5] and the monocytic cell line U937 (personal communication, Dr. J. DeLamarter). In addition, human erythroid potentiating activity (EPA) has been purified and molecularly cloned from the Mo T-lymphoblast cell line [6, 7]. The relationship between these CSFs and those contained in media conditioned by normal human tissues has not been established. We have therefore fractionated human placental conditioned medium (HPCM) to determine the number of CSFs produced by this tissue able to stimulate human progenitors in vitro. Furthermore, the biological relationship of these factors to the known CSFs has been determined by comparing their ability to stimulate human and murine colony formation with the proliferation of murine factor-dependent cell lines in vitro.

B. Results

Cultures of low-density non-adherent bone marrow (3×10^4 cells/ml) or peripheral blood cells (2×10^5 cells/ml) were established in 35-mm Petri dishes containing Iscove's Modified Dulbecco's Medium, 25% fetal calf serum (FCS) and 0.3% agar and were scored after 14 days' incubation (5% CO_2, 37 °C). Murine (CBA) bone marrow cultures (5×10^4 cells/ml) in 35-mm Petri dishes containing Dulbecco's Modified Eagle's Medium with 20% FCS and 0.3% agar were incubated for 7 days. Cultures were scored under a dissecting microscope and then stained with Luxol-Fast-Blue and hematoxylin. Cellular proliferation of the murine cell lines 32D Cl.3 (IL-3-responsive) and FDC-P1 (IL-3 and GM-CSF-responsive) was assessed in 15-µl suspension cultures containing 200 FDC-P1 or 32D Cl.3 cells. Viable cells were counted after 2 days of incubation.

Initial fractionation of HPCM by phenyl-Sepharose chromatography [8] allowed separation of CSFα from CSFβ. While CSFβ, the human analogue of murine G-CSF [9], only stimulated neutrophil and some neutrophil-macrophage or macrophage colony formation by human progenitor cells in vitro, CSFα stimulated neutrophil, neutrophil-macrophage, macrophage and eosinophil colony formation and, in the presence of erythropoietin (Epo), also stimulated multi-

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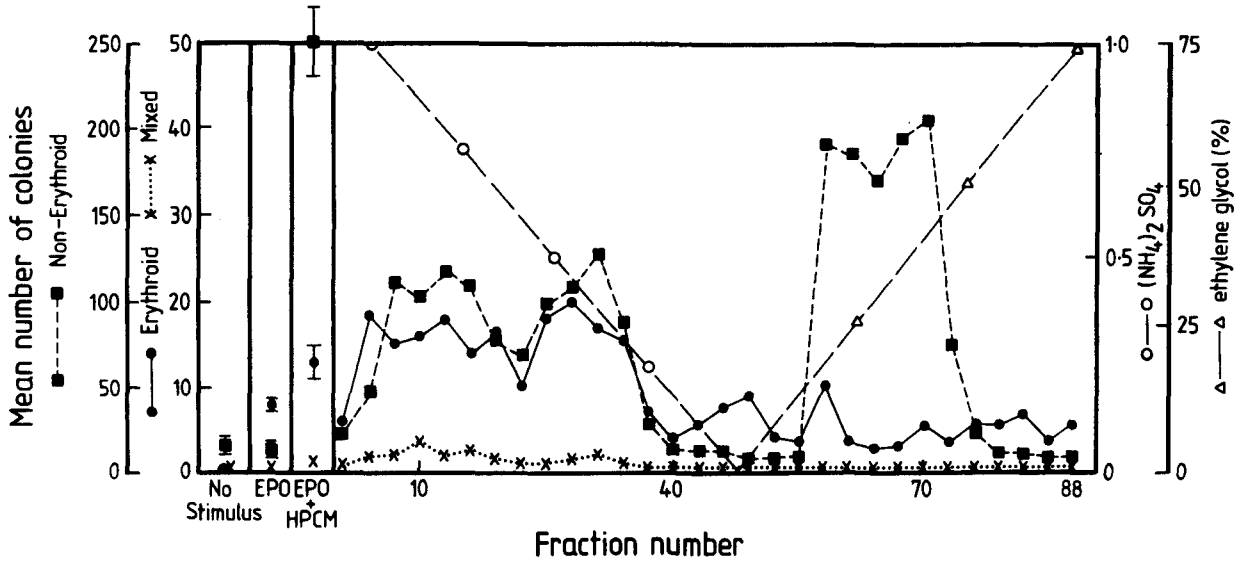

Fig. 1. Phenyl-Sepharose chromatography. Ten-fold concentrated HPCM equilibrated in 1 *M* ammonium sulfate was loaded on a Pharmacia phenyl-Sepharose CL4B column (2.6 × 20 cm). First CSFα was eluted with a linear gradient from 1 *M* ammonium sulfate to water; this was followed by CSFβ, which eluted during a second linear gradient from water to 75% ethylene glycol. Cultures of low-density non-adherent human peripheral blood cells (2×10^5/ml) were scored after 14 days

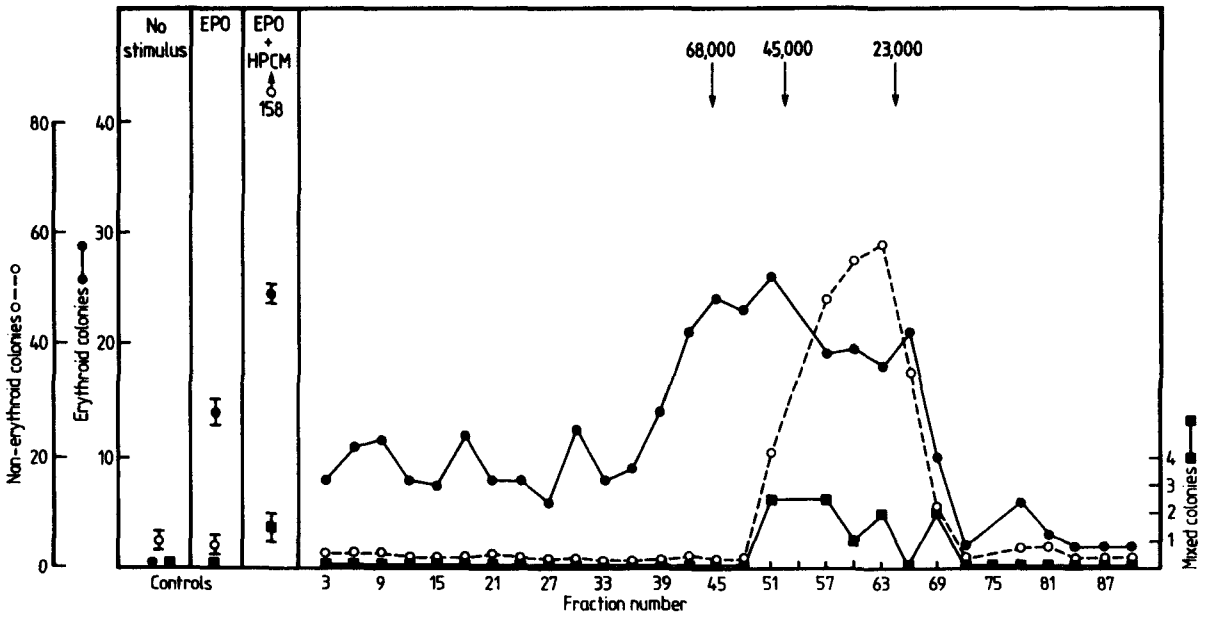

Fig. 2. Gel filtration chromatography. CSFα was loaded on an Ultrogel AcA44 column (2.6 × 100 cm) and eluted with phosphate buffered saline, 0.04 *M*, pH 7.4. *Arrows* denote the elution points of bovine serum albumin, ovalbumin and chymotrypsinogen. Colony number per 2×10^5 low-density non-adherent human peripheral blood cells was determined after 14 days

potential colony formation and potentiated erythroid colony formation (Fig. 1).

Further fractionation of CSFα by gel filtration chromatography (LKB Ultrogel AcA44, 2.6 × 100 cm column; Fig. 2) separated an erythroid potentiating activity (EPA) with an apparent mol.wt. of 40–45 kD which stimulated no human colony formation alone but potentiated erythroid colony formation in the presence of Epo. This activity could be separated from a broad CSF peak [CSFα (ii)] with an apparent mol.wt. of 30 kD which had all the biological properties of CSFα. Depending on

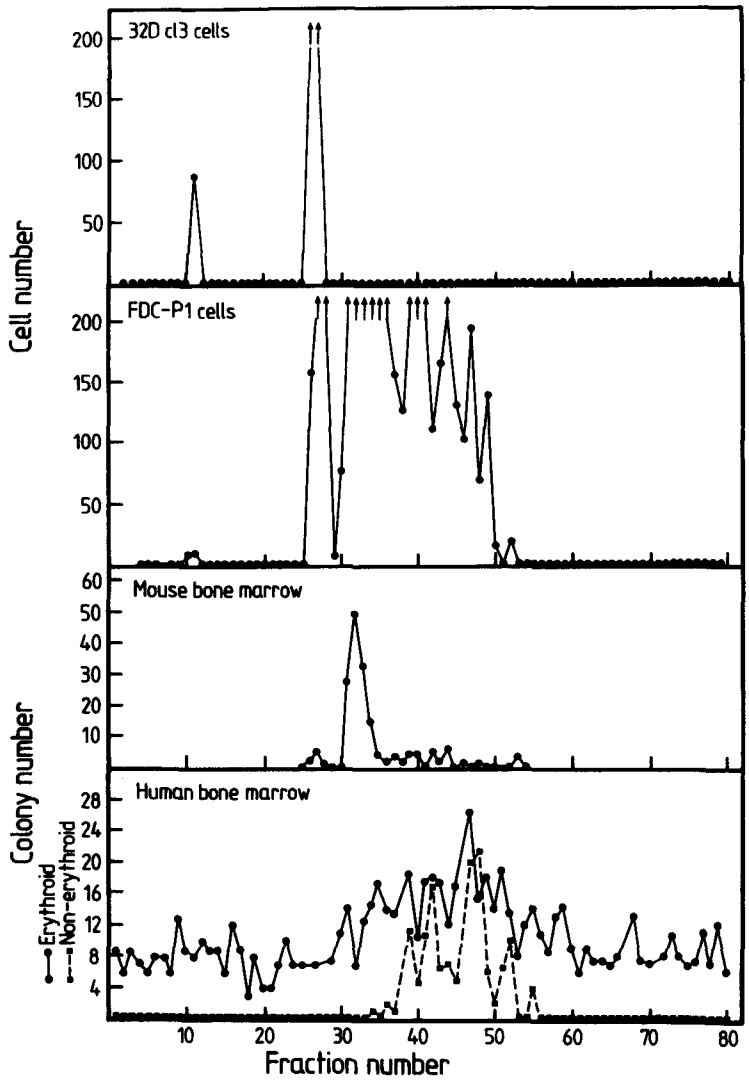

Fig. 3. Reverse-phase HPLC. Pooled fractions of CSFα (ii) were loaded onto a phenyl column (Waters). Bound proteins were eluted with two linear gradients of acetonitrile in 0.05% TFA. *Upper two panels:* fractions were assayed for the ability to stimulate the proliferation of 32D Cl.3 and FDC-P1 cells; *lower two panels:* the CSF activity of fractions was assayed on mouse bone marrow (5×10^4 cells/1 ml culture) and low-density non-adherent human bone marrow (3×10^4 cells/1 ml culture)

the specific activity of CSFα (ii), material was bound to lentil-lectin Sepharose and eluted with 0.2 *M* α-methyl-D-glucoside prior to HPLC fractionation. This intermediate step increased the specific activity of CSFα (ii) approximately 50-fold, although a fivefold loss of total activity was observed.

Subsequent reverse-phase HPLC fractionation of CSFα (ii) was carried out using a Waters phenyl column and a two-stage linear gradient with a constant flow rate of 1 ml/min, first from 0% to 30% acetonitrile in 0.05% trifluoroacetic acid (TFA) over 10 min collecting 5-ml fractions, followed by a second linear gradient from 30% to 60% acetonitrile in 0.05% TFA over 60 min collecting 1-ml fractions. Multiple human-active peaks were detected eluting from the column between 38% and 46% acetonitrile (fractions 39 to 52), and all were able to stimulate neutrophil, macrophage, and eosinophil colonies as well as multipotential colonies (not shown in figure) and to potentiate erythroid colonies in the presence of Epo (Fig. 3, lower panel). When assayed on mouse bone marrow, the human-active fractions also stimulated murine colony formation, although the majority of murine activity (which stimulated neutrophil, macrophage and eosinophil colonies) eluted from the HPLC column prior to the human CSF peaks. Proliferation of FDC-P1 cells was supported by all fractions active on murine and human progenitors, while 32D Cl.3 cells responded only to a narrow band of fractions which also stimulated normal murine cells and FDC-P1 cells but not human cells.

To reduce carbohydrate heterogeneity, the same batch of CSFα (ii) was treated with neuraminidase [from *Clostridium perfringens* (Sigma) pH 5.0, 37 °C for 60 min] before HPLC fractionation. This treatment completely separated a murine CSF activity which also stimulated 32D Cl.3 cell prolifer-

ation from a human CSF activity which also stimulated FDC-P1 cells. However, neuraminidase treatment did not resolve the multiple peaks of activity observed in the human and FDC-P1 assays. Other deglycosylation agents may be useful in determining whether these peaks are related to a single CSF protein or are a result of multiple CSF activities not yet characterized.

C. Conclusion

Human recombinant GM-CSF (rHGM-CSF) has recently been tested on a variety of human target cells and murine cell lines [10], and, although possessing the same human repertoire as material fractionated from HPCM, rHGM-CSF at comparable doses does not stimulate proliferation of FDC-P1 cells. It is possible that native GM-CSF from a non-tumor source is heterogeneous and has a greater ability to interact with murine GM-CSF receptors present on FDC-P1 cells. Binding studies are now in progress to determine whether HPCM-derived fractions can compete with ^{125}I murine recombinant GM-CSF binding sites on FDC-P1 cells. The ability of monoclonal antibodies directed against rHGM-CSF to block the response of human progenitor cells to HPCM-derived fractions is also under investigation.

The nature of the activity which stimulated both 32D Cl.3 proliferation and murine colony formation is not clear. Based on purification and biological studies, it appears to be unrelated to G-CSF or M-CSF, since the molecule exhibited low hydrophobicity, had an apparent mol.wt. of 30 kD, and could stimulate murine granulocyte, macrophage and eosinophil colony formation. The role of this activity in human hemopoiesis is not yet clear, since alone or in combination with erythropoietin no stimulation of human progenitor cells could be demonstrated. However, the possibility still exists that it has a synergistic effect with other known CSFs in vitro. A recent report that IL-2 could stimulate 32D-clone 23 cells [11] suggested the possibility that the HPCM-derived 32D Cl.3 activity might be due to IL-2, although we were not able to stimulate the murine IL-2-dependent cell line CTLL with these fractions (less than 0.7 units IL-2/ml), and recombinant human IL-2 did not stimulate 32D Cl.3 cells (personal communication, Dr. A. Kelso).

These studies show that a normal human tissue, the placenta, contains hemopoiesis-stimulating activities similar to those recently purified from tumor cell lines and molecularly cloned. These include G-CSF, GM-CSF and EPA. In addition, the studies suggest that there may be multiple forms of activity with similarities to GM-CSF but also some activities with apparent specificity only for normal murine cells and murine factor-dependent cell lines.

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