A 29 000 MOLECULAR WEIGHT FRACTION FROM FETAL RAT LIVER ADHERING CELLS THAT COOPERATES WITH ERYTHROPOIETIN IN STIMULATING THE GROWTH OF ERYTHROID PROGENITORS

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SUMMARY

The erythroid-potentiating effects of a protein fraction produced by 20-day rat fetal liver-adhering cells are studied. Partial purification by gel filtration gave an active fraction (apparent molecular weight = 29 × 10^3) that significantly increased the erythroid colony counts (CFUe and late BFUe) in cultures of liver cell fractions depleted of adhering cells at both limiting and saturating concentration of recombinant human erythropoietin. The sensitivity of CFUe and BFUe to erythropoietin was increased by the activator.

Key words: erythropoietin potentiating; adhering cells; fetal liver.

INTRODUCTION

Erythropoiesis is a multistep process involving the sequential differentiation of multipotential stem cells into at least two subpopulations of erythroid progenitors, the erythroid burst-forming unit (BFUe) and the erythroid colony-forming unit (CFUe). The BFUe gives rise to colonies of more than 100 erythroblasts, whereas the CFUe forms 8-50 erythroblasts in semi-solid culture (Clark and Housman, 1977; Ogawa et al., 1977; Eaves and Eaves, 1984). BFUe generally require erythropoietin (Epo), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Emerson et al., 1988), or interleukin 9 (IL-9) (Donahue et al., 1987). BFUe generally require erythropoietin (Epo), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Emerson et al., 1988), or interleukin 9 (IL-9) (Donahue et al., 1990) to develop erythroid colonies in vitro; CFUe colony formation, however, is dependent on Epo (Isocove, 1977; Gregory and Eaves, 1978). Rich and Kubanek (1980), Kanamaru et al. (1982), and Rich (1992) have found evidence for a change in the erythropoietin-sensitivity of CFUe during the ontogeny of erythropoiesis in the mouse. We found that the rat fetus spleen and liver CFUe of the same stage react differently to corticosteroids (Nagel and Nagel, 1987). We also isolated a factor from a granulocyte-rich splenic fraction that inhibited CFUe in cultures of fetal liver and spleen (Nagel and Nagel, 1992). These data suggest that CFUe are regulated by humoral factors released by other cells within the microenvironment. The present study uses a methyl-cellulose culture system to examine the role of adhering cells in 20-day fetal rat liver in determining the sensitivity of CFUe to Epo. A 29 000 molecular weight (Mr) protein fraction secreted by adhering cells was found to stimulate the proliferation and increase the sensitivity to recombinant human erythropoietin (rh Epo) at both early (BFUe) and late stage (CFUe) in vitro.

MATERIALS AND METHODS

Animals

All experiments were performed on Wistar rats (CF strain, from CNRS). Coitus was assessed by the presence of spermatozoa in the morning vaginal smears. Rat pups were generally born during the night between Days 21 and 22 postcoitus or on the morning of Day 22.

Chemicals

Bovine hemin (type I, Sigma Chemical Co., St. Louis, MO) was dissolved in 0.2 M KOH, diluted to 0.01 M in supplemented alpha medium (GIBCO, Grand Island, NY), neutralized with 1 N HCl (Ross and Sautner, 1976) and sterilized by filtration (0.45 μm, Millipore Corp., Bedford, MA).

Erythroid Colony Assays

Cell suspensions of total liver hematopoietic cells. Pregnant rats were killed by decapitation. Six to eight 20-day-old fetuses from two litters were used in each experiment. The fetal liver were removed aseptically, placed in sterile ice-cold alpha medium, and gently disrupted in a Potter-Elvehjem homogenizer with a loose-fitting plastic pestle. The resulting cell suspension was passed through a stainless steel screen (50-μm mesh) to remove particles, connective, and parenchyma cells. Aliquots of this cell suspension were diluted fivefold in 0.4 N acetic acid [to remove mature red blood cells (RBC)] and the hematopoietic cells were counted in a hemacytometer.

Liver cell fractions. Discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient components were prepared by diluting a stock solution of Percoll (Percoll 100) (9 vol of commercial solution plus 1 vol of 10 times phosphate buffered saline (PBS) concentrate, Bio-Merieux, Lyon, France), to 80, 60, 40, and 20% (Percoll 80, etc.) with alpha medium.

Liver cell fraction (L1). Hematopoietic cells (6.4 × 10^9 in 0.6 ml alpha medium) were layered onto a gradient of (from bottom to top) 0.2 ml Percoll 100, 0.6 ml Percoll 80, 0.6 ml Percoll 60, 0.3 ml Percoll 40, and 0.5 ml Percoll 20. The tubes were centrifuged at 500 g for 20 min at 4 °C and the cells at the Percoll 60-Percoll 40 interface from 10 separate centrifuged tubes were pooled and then washed 3 times with alpha medium. Cell viability was over 95% by trypan blue exclusion.

Adhering and non-adhering cell fractions. Liver total hematopoietic suspensions were prepared as described above and 15 ml containing 45 × 10^6 cells were incubated in 100-mm plastic culture dishes (Falcon) for 2 h at 37 °C in alpha medium plus 10% fetal bovine serum (FBS). The supernatant was harvested, and the cells adhering to the culture dish were gently washed twice with alpha medium. The washings and supernatant
were pooled to give the non-adhering cell fraction. The cells adhering to the culture dish constituted the adhering cell fraction.

Preparation of Conditioned Medium from Adhering Cells

Adhering cells were incubated overnight at 37 °C in 10 ml RPMI 1640 (GIBCO) without FBS buffered to pH 7.4 with 5 mM HEPES. The conditioned medium was removed, filtered through a 0.45-µm Millipore membrane, and stored at -20 °C. Control medium was the same volume of cell-free RPMI 1640 buffered to pH 7.4 and incubated under the same conditions. Conditioned media were either added to cultures (0.25 ml) or pooled for gel filtration chromatography.

Gel Filtration Chromatography

Conditioned media were pooled and 2-ml samples were fractionated on a 60 × 0.9-cm column of Sephadex G100 superfine (Pharmacia). The gel was equilibrated in and fractions eluted with PBS, pH 7.4. The flow rate was 5 ml/h and 1-ml fractions were collected. Protein standards, Blue Dextran 2000 (gel filtration calibration kit, Pharmacia) and glucose were all dissolved in PBS. The eluted fractions were pooled to give five fractions. These were passed through a 0.45-µm Millipore membrane for CFUe activation. Stimulation activity was found only in 29 000 Mr fraction. The stimulation activity corresponded to 1.3% of total protein placed on the column. Fractions eluted after the glucose marker (postglucose) were used as controls.

Protein was estimated by the Lowry procedure (Lowry et al., 1951).

Erythroid Colony Cultures

Erythroid progenitors were cultured on methylcellulose by the technique of Iscove et al., (1974), as modified by Urabe and Murphy (1978). The culture medium was 1.25 ml 2% methylcellulose (AAM premium, Dow Chemical Corp., Colorcon Ltd, England) in alpha medium containing rh Epo (Ampen, 100 IU/µm protein), 0.25 ml 10% bovine serum albumin (BSA, grade V, Sigma) in alpha medium, 25 µl 200 mM L-glutamine in water, 25 µl kanamycin (kanamycin solution, ×100, GIBCO), 25 µl penicillin and fungus (antibiotic antinymotic solution, ×100, GIBCO), 0.75 ml heparinized PBS (batch 01107, Lab. J. Boy, Reims, France, or batch 40G96141 GIBCO-BRL), 50 µl 5% sodium bicarbonate in water, 25 µl β-mercaptoethanol, and 50 µl hemin solution (2 × 10⁻⁴ M). The BSA was prepared and deionized according to Murphy and Sullivan (1978). The final concentration of cells was 0.5 × 10⁷/ml. The volume of adhering cell-conditioned medium or active fraction was 0.25 ml. Controls were 0.25 ml RPMI 1640 incubated as for the conditioned medium or 0.25 ml postgeluclose elastate.

Erythroid Colonies

Two-day erythroid colonies. The erythroid colonies containing eight or more cells after 2 days in culture in each well were counted without staining, as described by Iscove and Sieber (1975). These colonies were considered to be CFUe.

Seven-day erythroid colonies. In hematopoietic liver cell cultures of 20-day-old fetuses, some colonies developed from Day 4 to Day 7. They did not give bursts as in younger ages, but medium-to-large well-hemoglobinized intermediate colonies between BFUe and CFUe. Colonies of over 64 cells were therefore counted and designated mature or late BFUe.

Statistical Evaluation

The results were evaluated by Student’s t test or a one-way analysis of variance (ANOVA). Differences between the groups were considered significant if the P value was less than 0.05.

RESULTS

Erythropoietin-Dependent Erythroid Colony Formation in Cell Fractions from 20-Day Fetal Rat Liver Cultures

The number of CFUe colonies scored in total liver hematopoietic cell cultures increased between 0 and 2 U/ml of rh Epo and then remained unchanged (Fig. 1 a). In the cell fraction enriched in CFUe (L1) the number of colonies increased between 0 and 3 U/ml of rh Epo (Fig. 1 b) and was, at each dose of Epo, higher than in Fig. 1 a P < 0.001). Removal of adhering cells to give cultures of non-adhering cells (Fig. 1 c) resulted in fewer CFUe colonies at each dose of rh Epo assayed than was obtained with cultures of total liver hematopoietic cells (P < 0.001 or P < 0.005). The maximum number of colonies was formed only when the rh Epo concentration was 4 or more U/ml. Nevertheless, in the presence of adhering cells (0.2 ml/well of 3 × 10⁶ total hematopoietic liver cells/ml incubated 2 h at 37 °C in alpha medium and then removed as described in Materials and Methods), cultures of non-adhering cells (Fig. 1 d) showed a significantly higher number of CFUe (P < 0.001) than did non-adhering cell cultures alone (Fig. 1 e). The maximum number of colonies was produced at 3 U/ml of rh Epo. Results of each culture of Fig. 1 with 2, 3, and 4 U/ml of rh Epo were compared. ANOVA gave P > 0.1 a, P < 0.05 b, P < 0.001 c, and P < 0.001 d. Culture a was therefore significantly more sensitive to Epo than b,c, and d. Moreover, the analysis of d and e with 3, 4, and 5 U/ml of rh Epo gave P > 0.05 and < 0.01, respectively. The results indicate that the sensitivity of CFUe to rh Epo is reduced in the absence of adhering cells. Adhering cells in cultures of non-adhering cells significantly enhanced the number of CFUe colonies (P < 0.001). Control cultures of adhering cells alone gave only 7 ± 1.1 CFUe/2000 hematopoietic cells in the presence of 2 U of rh Epo/ml.

Influence of Conditioned Medium from 2-Day Liver Adhering Cells on the Effect of rh Epo on the Number of CFUe Colonies Formed by Liver Cell Fractions

Conditioned medium was prepared by incubating adhering liver cells (3 × 10⁶ cells/ml) in alpha medium without FBS for 16 h in plastic culture dishes. Liver cell fractions were then cultured in 10% of conditioned medium plus various concentrations of rh Epo, and the number of CFUe assayed. The concentration of rh Epo stimulating maximum numbers of colonies by control L1 cultures was 3 U/ml (Fig. 2 a). L1 fraction cells cultured in 10% conditioned medium produced maximum numbers of colonies at 2 U/ml rh Epo. In cultures with 2, 3, and 4 U/ml of rh Epo, the results analyzed by ANOVA gave P > 0.25 when conditioned medium was added and P < 0.001 in controls. Cell cultures in conditioned medium produced more colonies (P < 0.005) than control cultures: a 25.3% increase in CFUe at 1 U/ml rh Epo, and a 35.1% increase in CFUe at 2 U/ml rh Epo. The number of CFUe colonies scored in non-adhering liver cell cultures (Fig. 2 b) was maximal at lower Epo concentrations when the medium contained 10% conditioned medium. The concentration of rh Epo needed for optimal response was 3 U/ml vs. 4 U/ml in controls (ANOVA gave P < 0.001 and > 0.1, respectively, with 3, 4, and 5 U/ml of rh Epo), and the number of colonies produced was greater than for the controls: 50.9% more CFUe at 1 U/ml rh Epo, 43.7% more CFUe at 2 U/ml rh Epo, and 60.5% more CFUe at 3 U/ml rh Epo. All the differences were significant (P < 0.005, P < 0.001).

Effect of 10% Conditioned Medium from 20-Day Adhering Liver Cells on the Number of Late BFUe in L1 Cultures

The rh Epo concentration needed by control cultures to produce the optimal number of late BFUe was higher (3 U/ml) than that required by treated cultures (2 U/ml) (Fig. 3). In cultures with 2, 3, and 4 U/ml of rh Epo, the results were significantly different by