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Research Article

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Human Recombinant Erythropoietin Promotes Differentiation of Murine Megakaryocytes In Vitro

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Abstract

To determine if erythropoietin affects megakaryocytopoiesis, we measured acetylcholinesterase (AchE) activity, a marker of the murine megakaryocytic lineage, after the addition of human recombinant erythropoietin to serumless murine bone marrow cultures. Erythropoietin increased AchE activity substantially. Moreover, when the hormone was added to serumless cultures of 426 isolated single megakaryocytes derived from megakaryocytic colonies, erythropoietin induced a significant increase in the diameters of these cells. From a Bayesian analysis of the likelihood that some megakaryocytes increased in DNA content during the culture period, we estimate that 61% of the cells increased in ploidy. These data indicate that the action of erythropoietin is not restricted to the erythroid lineage.

Introduction

Erythropoietin, known to be the primary humoral regulator of erythropoiesis, has been shown in some studies to promote megakaryocytopoiesis and increase the platelet count (1–8). These results have been controversial, however, because other laboratories have reported no influence of the hormone on the megakaryocytic lineage (9–11). The difficulties in evaluating the effect of erythropoietin on megakaryocytopoiesis include the impurities of the available preparations of the hormone, non-physiologic concentrations employed, and the possibility that any potential effects of erythropoietin are mediated by accessory cells contaminating the progenitor cells in marrow cultures. In this report, we show that human recombinant erythropoietin (rEpo)[1] directly influences some aspects of murine megakaryocytic differentiation in culture.

1. *Abbreviations used in this paper:* AchE, acetylcholinesterase; β-ME, β-mercaptoethanol; IMDM, Iscove's modified Dulbecco's medium; rEpo, human recombinant erythropoietin.

Methods

Mice. 6- to 8-wk-old specific pathogen-free C57Bl/6 males obtained from Jackson Laboratories (Bar Harbor, ME) were used for all experiments.

Liquid marrow cultures. Femoral marrow was flushed from the bones and made monodisperse by expulsion through an 18-gauge needle and then a 22-gauge needle. Marrow cells were treated with 0.5 mM diisopropylfluorophosphate in 100% ethanol for 20 min to inactive endogenous cholinesterase prior to culture (12). 1×10^5 cells per well were cultured in a final volume of 0.2 ml of Iscove's modified Dulbecco's medium (IMDM, Irvine Scientific, Santa Ana, CA) containing 1% Nutricyte (a serum-free medium supplement with albumin, transferrin, insulin, and lipids; J. Brooks Lab, San Diego, CA) and rEpo (Amgen Biologicals, Thousand Oaks, CA) over a four-log range of concentrations.

Quantitation of acetylcholinesterase (AchE) activity. AchE, a relatively specific marker of the megakaryocytic lineage in some rodents, was measured fluorometrically (12, 13). After 3 d in culture, each plate was centrifuged at 2,000 rpm for 10 min and the supernatants of the wells were discarded. 0.2 ml of a solution of 0.2% Triton X-100 in 1 mM EDTA, 0.12 M NaCl, and 50 mM Hepes, pH 7.5, was added to each well, followed by the addition of 20 μl acetylthiocholine iodide (final concentration 0.56 mM). After 4 h of incubation, 10 μl of 0.4 mM coumarinphenylmaleimide (Molecular Probes Inc., Junction City, OR) in acetonitrile was added to 10 μl of the reaction mixture in borosilicate glass tubes, followed by 2 ml of 5 mM sodium acetate, pH 5.0, containing 1 mM EDTA and 0.2% Triton X-100. The fluorescence emission was measured with a filter fluorometer with an excitation filter of 390 nm and an emission filter of 450 nm.

Single cell cultures. Bone marrow was enriched for progenitor cells on a 1.070/1.077 g/cm³ discontinuous Percoll gradient (13). 1–2 × 10⁴ cells were cultured in IMDM containing 1% methylcellulose, 15% horse serum, 5% pokeweed mitogen-stimulated spleen cell conditioned medium (14), 50 μM β-mercaptoethanol (β-ME), and 100 U/ml of penicillin-streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. After 5 d in culture, megakaryocytic colonies were identified in situ (15) and selectively removed under an inverted microscope with a micropipette in 2-μl volumes and dispersed into 2 ml of IMDM containing 50 μM β-ME. Fewer than five colonies were plucked at a time to maximize the dilution of the initial culture constituents. Individual cells were removed in 1-μl volumes and recultured for 48 h in microtiter plate covers in 35 μl of medium containing 3% crystalline bovine serum albumin, 50 μM β-ME, 1% Nutricyte, and 0.01 U/ml, 0.2 U/ml, or 1 U/ml of rEpo. The final dilution of the initial culture constituents was at least 1: 7,200. Cell diameter was measured by determining the geometric mean of two perpendicular cell diameters using an inverted microscope equipped with an ocular micrometer before and after cell culture.

Ploidy analysis of single cells. After measurement of their diameters, single cells were placed on glass slides, fixed with 70% ethanol for 10 min and stained with 1.7 × 10⁻⁵ M chromomycin A3 (Calbiochem-Behring Corp., La Jolla, CA). DNA content was measured by fluorescence cytophotometry as previously described (16). Granulocytes were used as diploid standards. A Bayesian classification rule was utilized to classify 49 cells into initial preculture ploidy classes, given their postculture ploidies and preculture diameters, as well as estimated frequency distributions
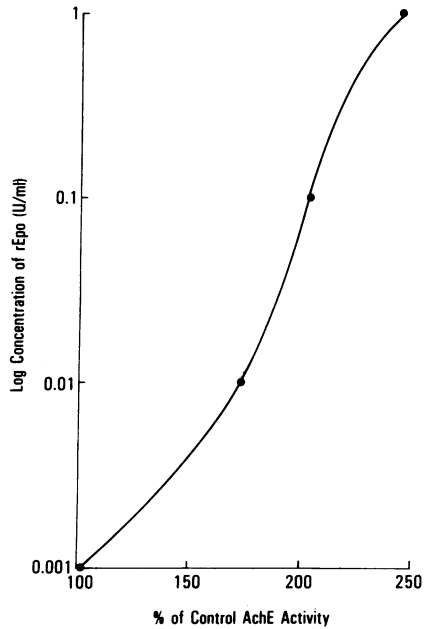
Figure 1. The promotion of AchE production in murine marrow cultures stimulated by human rEpo. At concentrations of 0.01 U/ml, a significant increment in AchE production was observed ($P < .05$) with further increases at higher concentrations.

of preculture diameters within each ploidy class (17). This analysis entails the calculation via Bayes' theorem of the posterior probabilities of membership in initial preculture ploidy classes for each of the 49 cells, and hence the posterior likelihood of ploidy shift.

Results

AchE activity was measured in serumless liquid cultures of murine marrow 3 d after the addition of rEpo. Fig. 1 shows a significant increase in AchE at a concentration of 0.01 U/ml, with further increments noted at higher concentrations. Visual inspection of the cultures showed an increase both in the size and number of megakaryocytes when compared to control cultures without rEpo.

Table I. Effect of rEpo on the Size of Single Megakaryocytes

Initial cell diameter	Number of cells increasing in diameter (%)			
	0 U/ml	0.01 U/ml	0.2 U/ml	1 U/ml
μm				
12–20	4/34 (12)	10/31 (32)	25/41 (61)	10/17 (59)
20–25	3/30 (10)	8/28 (29)	29/46 (63)	5/12 (42)
25–30	1/19 (5)	1/16 (6)	9/42 (21)	5/17 (29)
>30	0/14 (0)	1/20 (5)	3/36 (8)	2/23 (9)

Cell diameter was measured on the same cells before and after culture with rEpo. An increase in diameter was defined as an increment of ≥0.5 μm. The range of increments was 0.5–10 μm.

Table II. Classification of 49 Cells into Preculture Ploidy Classes

Predicted preculture ploidy class (n)	Observed postculture ploidy class (n)			
	4	8	16	32
2	0	1	0	0
4	1	4	1	0
8		10	15	3
16			6	6
32				2
Total cells	1	15	22	11

The increase in AchE activity in the cultures may have been related to an increase in megakaryocytic proliferation and/or differentiation; moreover, the role of rEpo in this augmentation of AchE activity may have been mediated via accessory marrow cells. To determine if erythropoietin acted directly on megakaryocytes, rEpo at concentrations of 0.01–1 U/ml was added to single megakaryocytes isolated from megakaryocytic colonies. Cell diameter was measured on 426 megakaryocytes at the outset of culture and 48 h later. As shown in Table I, rEpo promoted an increment in diameter, the response being dependent both on the concentration of the hormone and the initial cell diameter. Isolated granulocytes ($n = 30$) cultured in the same manner did not change in diameter.

A Bayesian rule was used to classify cells into ploidy classes at the outset of culture, given their preculture cell diameters and postculture ploidies (17). A summary of the classification results is given in Table II. On the basis of observed postculture ploidy class and our estimated prior densities of cell diameters, we would estimate a substantial number of cells (30 of 49 or 61%) to have shifted ploidy class while growing in culture. Indeed, as shown in Table III, a monotone trend in probability of shift may be discerned; we would predict 0%, 33%, 73%, and 82% of cells in ploidy classes 4, 8, 16, and 32 n, respectively, to have shifted ploidy class while growing in culture. Fig. 2 illustrates the appearance of a typical megakaryocyte after 48 h in culture with 0.2 U/ml of rEpo.

Discussion

Although studies in experimental animals and some clinical data suggest that erythropoietin may influence megakaryocytopoiesis,

Table III. Estimated Numbers of Shifts in Ploidy Class among 49 Cells during Culture

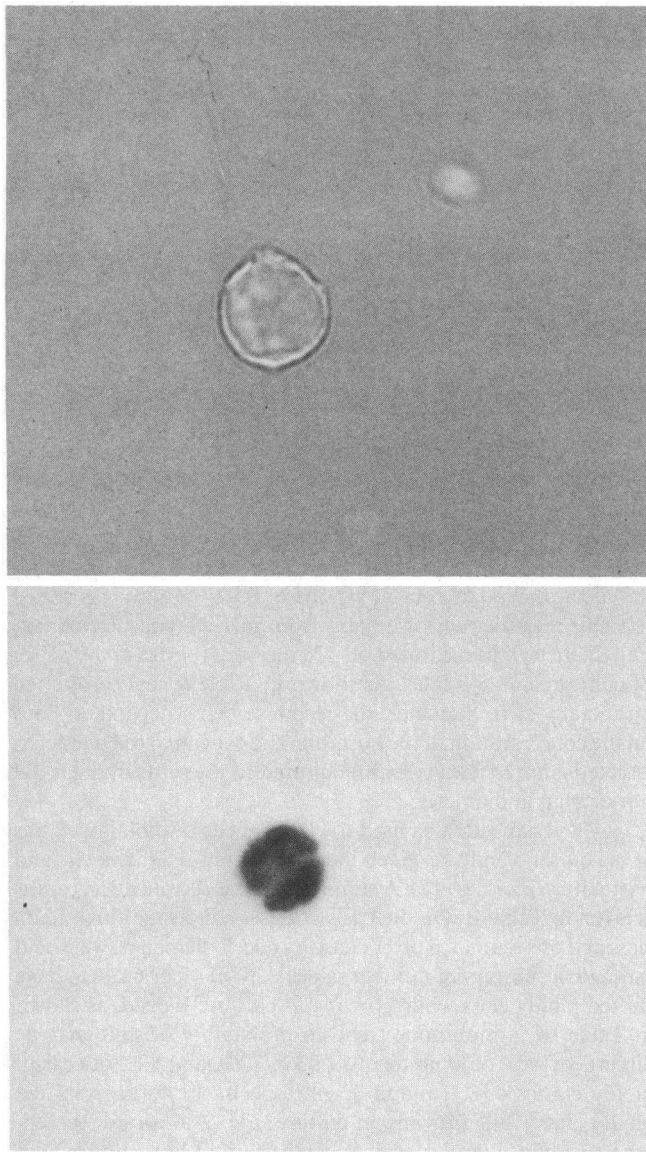|	Observed postculture ploidy class (n)			
	4	8	16	32
Predicted shifts	0	5	16	9
Predicted nonshifts	1	10	6	2
Percentage shifts	0	33	73	82

Figure 2. (*A*) A single megakaryocyte after culture in the presence of 0.2 U/ml rEpo. Initial diameter was 20.8 μm. The final diameter before fixation was 26.1 μm ($\times$500). (*B*) The identical cell observed under fluorescein optics. The measured ploidy was 32 N ($\times$500).

the multitude of effects of the experimental manipulations and the complexity of the clinical situations have precluded a definitive conclusion (4, 5, 18–21). More recent studies employing in vitro marrow culture have not resolved the question (1–3, 9–11). However, the data of Williams et al. (6), Dukes et al. (7), and Mizoguchi et al. (8) suggest that purified or recombinant erythropoietin may stimulate megakaryocytic colony formation in cultures of whole marrow. The production of rEpo in purified form has allowed for a more precise reexamination of the role of erythropoietin in megakaryocytopoiesis. In serumless liquid marrow cultures, the hormone promoted AchE production, a marker of megakaryocytes in mice and some other species (12). To determine if rEpo promoted some aspects of megakaryocytic differentiation, we measured the size of individual megakaryo-

cytes derived from colonies before and after culture with rEpo. In the megakaryocytic lineage size is one generally accepted marker of differentiation (22). At concentrations of erythropoietin achievable in vivo (~0.01 U/ml), a significant increment in size was observed. Polyploidy is another well-known characteristic of differentiated megakaryocytes. Although we were unable to preselect single megakaryocytes of a particular ploidy class prior to culture with rEpo, we would infer from a Bayesian analysis of our data that some megakaryocytes have increased in ploidy.

The studies reported herein demonstrate that rEpo is sufficient to promote some aspects of murine megakaryocytic differentiation in vitro, indicating that the influence of erythropoietin is not restricted to the erythroid lineage. The role of erythropoietin in the regulation of thrombocytopoiesis in vivo remains to be ascertained.

Acknowledgments

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