Effect of Burst-Promoting Activity (BPA) and Erythropoietin on Hemoglobin Biosynthesis in Culture

By Takashi Terasawa, Makio Ogawa, Pamela N. Porter, David W. Golde, and Eugene Goldwasser

The influence of erythropoietin (Ep) and burst-promoting activity (BPA) on the relative rates of synthesis of fetal and adult hemoglobins (HbF and HbA) were investigated in clonal cell culture. In the case of crude Ep, a positive dose-response relationship between the concentration and the relative rate of HbF biosynthesis by circulating erythropoietic precursors was clearly observed. In contrast, increasing the concentration of purified Ep enhanced the synthesis of HbF and HbA equally. BPA from bone marrow conditioned media and T-cell conditioned media enhanced the relative rate of HbF biosynthesis and burst number at limiting serum concentrations (10%-15%). We, therefore, conclude that BPA is important in the modulation of HbF biosynthesis in culture.

**Materials and Methods**

**Erythropoietic Cell Culture**

Peripheral blood mononuclear cells obtained from healthy volunteers were cultured using a modification of the methylcellulose technique described by Iscove et al. Unless otherwise specified, 3 x 10^5 mononuclear cells were plated in 35 mm Lux standard non-tissue culture dishes (Flow Laboratories, Inc., Rockville, Md.) in a 1 ml mixture containing α-medium (Flow Laboratories, Inc.), 0.8% methylcellulose (Fisher Scientific Co., Norcross, Ga.), 30% fetal calf serum (FCS) (Flow Laboratories, Inc.), 1% bovine serum albumin (Calbiochem, San Diego, Calif.), and 1.0 U/ml Ep. Modifications of the culture conditions in specific experiments are described in Results. The dishes were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. On day 14 of incubation, the bursts were scored on an inverted microscope and Hb biosynthesis was analyzed.

**Erythropoietin and Burst-Promoting Activity**

Homogenously-pure Ep with specific activity of 70,400 U/mg was prepared from pooled urine from anemic patients. Crude human urinary Ep preparation with specific activity of 40 U/mg protein was supplied by the Blood Diseases and Resources Branch, NHLBI. Step III preparation of sheep plasma Ep with specific activity of 14 U/mg protein was purchased from Connaught Labs, Ltd., Willowdale, Ontario, Canada. Human marrow BPA was prepared by suspension culture of human marrowuffy-coat cells in glass flasks for 1 wk. Human T-lymphocyte BPA was obtained from a permanent T-cell line that had been established from the spleen of a patient (M.O.) with T-cell variant of hairy-cell leukemia.

**Analysis of Hb Synthesis in Culture**

Details of the measurements of the rates of Hb synthesis in erythropoietic bursts in culture have been described previously. Briefly, 2 μCi of [³⁵S]labeled amino acid mixture (NEC-445, New England Nuclear, Boston, Mass.) was added to cultures on day 12 of incubation and the bursts were harvested 2 days later. Hemolysates were subjected to isoelectric focusing on an LKB Multiphor apparatus under the conditions described by Allen et al. The gels were fixed in 15% trichloroacetic acid, stained with Coomassie Brilliant blue R-250 (Eastman Kodak Co., Rochester, N.Y.) and exposed to Kodak NS-2T x-ray film for about 3 days. The densitometric tracing was carried out as previously described.

When the separation of HbA and HbF was not clear, HbF was estimated to be 15% of HbF₆₅, since this ratio is constant in cultures of adult peripheral blood cells. HbF is expressed as the sum of F₁ and F₂, and the results are expressed as mean ± SD of quadruplicate tracings.
Iron Incorporation

Duplicate cultures were labeled for 24 hr prior to harvesting with 0.2 ml of α-medium containing 50% FCS and 0.5 μCi of 59Fe-citrate (13 mCi/mg, Amersham, Arlington Heights, Ill.). Cells were collected as described previously and heme was extracted by cyclohexanone.12 Samples were counted in an automatic gamma counter (Nuclear Chicago, Model #1185).

RESULTS

Peripheral blood mononuclear cells from three normal subjects were cultured in the presence of varying concentrations of pure Ep, and the plating efficiency of BFU-E and the relative rate of HbF synthesis were determined. The results of the experiments in which cells were simultaneously cultured in the presence of crude human urinary or sheep plasma Ep are presented in Table 1. In cultures with pure Ep, the percentage of HbF biosynthesis remained constant between Ep concentrations of 0.25 and 4.0 U/ml. The autoradiogram derived from cultures of donor 1 containing 3 different concentrations of pure Ep is shown in Fig. 1. With crude Ep preparations, there was a significant correlation between HbF biosynthesis and Ep concentration (Table 1). These results strongly suggested that the positive correlation between HbF biosynthesis and Ep concentration that we previously observed13 and confirmed here, may be due to non-Ep factors present in the crude Ep preparations.

We then examined the effects of human bone marrow derived BPA (marrow BPA) and BPA derived from a human T-lymphocyte cell line (T-cell BPA). The results of a total of eight experiments are presented in Table 2. Both BPA preparations increased the number of bursts observed in culture. In some preparations, T-cell conditioned media inhibited the growth of bursts in culture. We found that the inhibitory activity could be inactivated by boiling for 10 min.3 Only those experiments in which inhibitors had been inactivated are included. The enhancement of burst formation by BPA was more pronounced in cultures with 10% and 15% FCS than in cultures with 30% FCS, presumably because FCS contains some BPA. The percent enhancement of burst formation by BPA was 45 ± 23% (mean ± SD) and 25 ± 11% for the low (10% and 15%) and high (30%) FCS groups, respectively. BPA from both sources augmented the relative rate of HbF biosynthesis when cultures contained limiting levels of FCS. The mean and SD of the percent enhancement of HbF biosynthesis by BPA was 52% ± 21% for the low FCS groups. The addition of BPA to cultures containing 30% FCS did not enhance relative HbF biosynthesis, even in experiments in which there was an increase in burst number.

The bursts cultured in the presence of BPA were larger than those grown in the absence of additional BPA; the differences were particularly striking when low concentrations of FCS were used. This observation agrees with our previous finding that marrow BPA increased the incorporation of 59Fe into heme to a greater extent than the burst number.8 In order to gain further insight into the mechanisms of the BPA augmentation of HbF biosynthesis, we carried out simultaneous quantitation of relative HbF biosynthesis and incorporation of 59Fe by the erythropoietic bursts. Results of serial examinations of cultures of normal adult peripheral blood BFU-E are presented in Fig. 2. Relative HbF biosynthesis on day 18 was significantly (p < 0.001) lower than that on day 14.
and supported the data by Papayannopoulou et al. At all days of culture examined, both the relative HbF biosynthesis and the 59Fe incorporation were augmented by BPA. Since HbF synthesis was less than 15% of total Hb synthesis, BPA augmented biosynthesis of not only HbF but also HbA.

DISCUSSION

Studies of the effects of varying doses of Ep on the Hb biosynthetic capabilities of peripheral blood BFU-E have yielded conflicting results. Whereas we observed a positive correlation between the concentration of crude Ep and relative HbF biosynthesis, Clarke and Housman observed no such dose response. Papayannopoulou et al. observed an inverse relationship between Ep concentrations and HbF biosynthesis in studies of patients with polycythemia vera. Ep preparations used in these experiments were crude and possibly contained BPA. Our reinvestigation of this controversy using pure Ep clearly showed that there is no dose relationship between Ep concentrations and HbF biosynthesis. Concurrent with this investigation, however, Papayannopoulou et al. observed a dose-related inhibition of relative HbF biosynthesis by pure Ep. Since they also found a higher HbF/HbA ratio in immature bursts, they postulated that Ep enhances erythroid cell maturity and thereby decreases the relative synthesis of HbF. Our results agree with theirs and those of Dover and Boyer in that the percent HbF biosynthesis decreases during erythroblast maturation. In our experiments, however, the response to BPA was not dependent on maturation since a significant effect was observed as late as day 18, a time at which essentially all of the bursts are mature in our culture system. At the present time, we cannot provide an adequate explanation for

<table>
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<tr>
<th>Experiment</th>
<th>BPA (%)</th>
<th>FCS (%)</th>
<th>HbF (%)</th>
<th>BPA (%)</th>
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<tbody>
<tr>
<td>Marrow BPA</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>10</td>
<td>7 ± 2</td>
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<td>2</td>
<td>10</td>
<td>12 ± 1</td>
<td>13.4</td>
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<td>3</td>
<td>10</td>
<td>17 ± 3</td>
<td>24.6</td>
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<td>4</td>
<td>10</td>
<td>32 ± 4</td>
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<tr>
<td>5</td>
<td>10</td>
<td>45 ± 5</td>
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<td>7</td>
<td>10</td>
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<td>8</td>
<td>10</td>
<td>32 ± 7</td>
<td>23.1</td>
<td>2.8</td>
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*Cells were cultured with and without 10% (v/v) BPA.
†(Culture with BPA - Culture without BPA)/Culture without BPA x 100%.
‡Levels of significance (NS = not significant). Experimental groups of cultures containing BPA were compared to corresponding cultures without BPA by t test. The data are expressed as mean ± SD.
HbF BIOSYNTHESIS IN CULTURE

Fig. 2. Time course of the effects of marrow BPA on relative HbF biosynthesis and $^{59}$Fe incorporation (net heme cpm) by normal adult blood erythropoietic bursts. The circles and bars represent mean ± SD of triplicate analyses. (● + BPA; ○ – BPA).

HbF biosynthesis by bursts. More recently, they reported that PHA-LCM greatly increases HbF biosynthesis by mixed colonies (CFU-GEMM). One interpretation of our data is that the addition of BPA resulted in a shift in the population toward earlier precursors. Since the reported incidence of mixed colonies in culture is low, augmentation of HbF biosynthesis by BPA may be due to recruitment of committed but very early BFU-E. High HbF biosynthesis by this population would thereby be responsible for the observed increase in HbF synthesis. An alternative explanation is that the increase in HbF synthesis is due to qualitative changes of the same population. In contrast to Fauser and Messner who observed that a significant portion of the BFU-E population is incapable of HbF synthesis, we have shown using biochemical and F-cell analyses, that all peripheral blood BFU-E are capable of HbF biosynthesis in culture. At the present time, we are unable to choose between the two possible hypotheses.

Since bursts and mixed colonies respond to BPA and are able to reactivate HbF biosynthesis whereas the more mature progenitors, CFU-E, are incapable of HbF synthesis, augmentation of HbF synthesis in culture appears to be closely related to cell proliferation during the early phase of burst formation. Our results suggest that BPA may be the primary modulator of HbF biosynthesis in culture. Although the precise physiologic role of BPA has not been determined, the presence of high levels of BPA in the sera of patients with aplastic anemia and in the urine from anemic subjects may suggest a physiologic role. Significant synthesis of HbF in vivo has been observed in patients with severe anemia. It is of interest that BFU-E proliferation is augmented in the regenerating marrow of mice following radiation injuries and marked augmentation of HbF synthesis in vivo is seen in patients who have successful bone marrow transplantation. Therefore, it is conceivable that BPA augmented reactivation of HbF synthesis in these adult subjects.

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