In Vitro Interactions of PGE and cAMP With Murine and Human Erythroid Precursors


Addition of prostaglandins of the E series (PGE₁, PGE₂) in methylcellulose cultures of murine marrow results in a dose-dependent inhibition of the cloning efficiency of both BFU-E and CFU-C. However, CFU-E growth is unaffected. The inhibitory action of PGE is progressively overcome by increasing amounts of colony-stimulating factor (CSF), and with some limitations, also of erythropoietin (Epo). Addition of PGE₂₅₀, associated or not with indomethacin, does not exert any significant effect on these hematopoietic precursors. In an attempt to unveil the mechanism(s) underlying these phenomena, which mimicks that by PGEs, additionally, theophylline potentiates the inhibitory action of PGE₁. In all these studies, the inhibitory action of various concentrations. Both db-cAMP and theophylline induce an inhibitory influence on both BFU-E and CFU-C growth, which mimicks that by PGEs; additionally, theophylline potentiates the inhibitory action of PGE₁. In all these studies, the CFU-E number was not significantly modified. PGE action on BFU-E proliferation is clearly species-dependent, since PGE₁ addition to human marrow methylcellulose cultures induces a significant enhancement of the number of both BFU-E- and CFU-E-derived colonies. This action was abolished upon removal of adherent cells, thus suggesting that PGE₁ evokes a release of factor(s) enhancing human erythroid colony growth by adherent cells.

The model of erythropoiesis in mammals is characterized by an early erythroid precursor (erythroid burst-forming unit, BFU-E), which derives from the pluripotent stem cell (spleen colony-forming unit, CFU-S) and feeds the compartment of late erythroid progenitors (erythroid colony-forming unit, CFU-E).¹ The CFU-E finally differentiates into the erythroblastic compartment. A third pool of erythroid precursors, apparently intermediate between BFU-E and CFU-E, has been recently identified in both murine and human marrow.²,³ Recent studies by Kurland and Moore⁴ indicate that prostaglandins of the E series (PGE₁ and PGE₂) are potent inhibitors of CFU-E growth. Elevated numbers of peritoneal macrophages in soft-agar culture inhibit CFU-C growth: this phenomenon is apparently mediated via PGE release in the medium and accordingly suppressed by indomethacin. Low concentrations of peritoneal macrophages, instead, are associated with a stimulatory effect on CFU-C, via production of colony-stimulating factor (CSF). These observations have led to a tentative concept whereby granulopoiesis is modulated by macrophage release of either stimulatory (CSF) or inhibitory (PGE) agents, respectively, in face of low or high macrophage cellularity. It has been further suggested that the PGE action is mediated by activation of the adenilate cyclase system.

Interactions between the PGE-cAMP system and BFU-Es (i.e., the progenitor in the erythroid differentiation pathway corresponding to the CFU-C in the myelomonocytic line) have not been investigated. In regard to CFU-E and erythroblasts, the influence of these agents is apparently species-dependent. Thus, a stimulatory effect on CFU-E growth has been observed in humans⁵ and dogs,⁶ an inhibitory action on erythropoiesis in rats and guinea-pigs,⁷ and variable influences in mice.⁸,⁹,¹⁰

A role for the PGE-cAMP system in the in vitro modulation of murine hematopoietic precursors is supported by present studies, which provide evidence for an inhibitory effect of this system on expression of BFU-E and CFU-C, but not CFU-E. On the other hand, addition of PGE₁ to human marrow cultures exerts a significant enhancing effect on the clonogenic capacity of both CFU-E and BFU-E. The latter phenomenon, abolished via removal of adherent cells, is possibly mediated via PGE-induced release by adherent cells of factor(s) enhancing erythroid colony growth.

Materials and Methods

Reagents

PGE₁, PGE₂, PGE₂₅₀ were a kind gift of Dr. J. Pike (Upjohn Co., Kalamazoo, Mich.). The other reagents, of analytical grade, were obtained from commercial sources (dibutyryl cAMP and cGMP, Boehringer Co., Mannheim, West Germany; theophylline, Serva Co., Heidelberg, West Germany; indomethacin, Sigma Co., St. Louis, Mo.).

From www.bloodjournal.org by guest on November 9, 2017. For personal use only.
INTERACTIONS OF PGE WITH ERYTHROID PRECURSORS

Mice

Random-bred, CD1 female animals (derived from HAm/ICR strain, i.e., Hauschka-Mirand, Roswell Park Memorial Institute, Swiss, provided by Charles River Italy, Calco, Italy) weighing 20–25 g were maintained on a standard diet of lab-pellets (diet 4RF2, Italiana Mangimi, Settimo Milanese, Italy) and tap water ad libitum. In a limited number of experiments DBA/2 mice (same source, sex, and weight) were employed.

Erythropoietin (Ep) Purification

The urine from a pure red cell aplasia patient, collected at –20°C and concentrated against glycopolyethylene at +4°C, was lyophilized (specific activity, 20–27 IU/mg of protein). This crude Ep preparation was further purified by means of a 3-step chromatography method12† final specific activity, ~50–300 IU/mg of protein. This semipurified Ep, virtually free of both activity-stimulating myeloid-macrophage colonies and inhibitor(s) of erythroid colony formation, was employed as the stimulus for BFU-E and CFU-E growth. It must be further emphasized that endotoxin was virtually absent in this Ep preparation, i.e., the Limulus assay showed the presence of as little as 0.02 ng/2.4 IU of Ep.12° This amount does not significantly modify expression of CFU-E and BFU-E in culture.14

Assay of the Number of BFU-E, CFU-E, or CFU-C Colonies

Stock solutions of PGs in ethanol (10 M), kept frozen at –20°C, were diluted in Dulbecco’s Modified Eagle’s Medium (DEM).* Plates given 10 M PGs contained therefore 1 μl of stock solution/ml; those with 10 M or less, 0.1 μl of ethanol ml. Corresponding vehicle plates contained the appropriate volume of ethanol. Stock solutions of indomethacin (10 M) were 10% ethanol in DEM (1 μl of ethanol in 10 μl of stock solution/dish when plating 10 M indomethacin). Stock solutions of the other reagents were 10 M in DEM, and accordingly diluted.

The assay of murine BFU-E, CFU-E, and CFU-C colonies was performed by means of methylocellulose cultures, according to a slight modification of a previously reported method.15,16 The animals were killed by cervical dislocation under light ether anesthesia. The number of nucleated cells in tibial marrow, flushed out in DEM, was evaluated by means of a ZBI Coulter Counter. Each 1-ml plate (2 plates/group) contained the following components in DEM: methylocellulose (0.8%, final concentration), α-thioglycerol (10 M), fetal calf serum (FCS; 30%), 2 x 10 nucleated cells, and either purified Ep (3 IU, unless otherwise indicated) or CSF, i.e., lung-conditioned medium16 (0.1 ml, unless otherwise indicated). Preliminary experiments indicated that these amounts of Ep or lung-conditioned medium induced maximal growth of BFU-E and CFU-E or CFU-C colonies. In order to strengthen further this conclusion, 8 IU Ep/plate were randomly plated in the vehicle groups in the various studies performed here. No shift of dose–response curves was observed, i.e., the total number of erythroid progenitors was comparable when calculated from plates with 8 or 3 IU of Ep.

The plates were incubated in a humidified 7.5% CO2 in air atmosphere at 37°C. CFU-C colonies containing a minimum of 50 cells were scored on days 7–8. CFU-E and BFU-E colonies, containing a minimum of either 8 or 200 cells, were scored at 36–48 hr or 8–10 days, respectively. The identification was performed in situ on the basis of standard morphological criteria: the validity of these criteria had been previously demonstrated by control studies involving benzidine staining of colonies smeared on glass slides.12,13

The assay of human marrow CFU-E and BFU-E was performed in methylocellulose cultures by means of a slight modification of previously reported procedures.13 “Normal” bone marrow was aspirated from young hematology-oncology patients without marrow involvement and prior to initiation of treatment. Fully informed consent had been obtained from each individual. Briefly, cell separation from marrow aspirates was carried out as described by Eaves and Eaves,1 with an additional step involving passage of the cell-rich plasma, obtained from the centrifuged marrow, through progressively smaller needles (down to no. 25). Adherent cells were removed as reported by Mesner et al.17 Methylcellulose cultures were set up as described above. Ep (1–2 IU/dish) was plated at concentrations inducing plateau expression of CFU-E and either suboptimal (1 IU) or optimal (2 IU) expression of BFU-E. Single clusters of erythroblasts, characterized by their orange-red color and containing a minimum of 20 cells, were scored on day 8 as CFU-E colonies. Bursts, i.e., erythroid colonies arranged in clusters and containing a minimum of 300–600 cells, were scored on day 13.

\*DEM always contained 25 mM of Hepes buffer (1M), GIBCO.
†DEM was modified here to contain t-alanine (25 μg/ml), t-asparagine H2O (50 μg/ml), t-aspartic acid (30 μg/ml), t-cysteine (70 μg/ml), t-glutamic acid (75 μg/ml), t-proline (40 μg/ml), sodium pyruvate (110 μg/ml), vitamin B12 (0.025 μg/ml), and biotin (0.030 μg/ml).

Fig. 1. Effect of PGE, on the number of colonies derived from BFU-E (upper panel), BFU-E (central panel) and CFU-C (lower panel) in murine bone marrow. Each point represents the mean ± SEM of average values from a minimum of 3 separate experiments (2 plates/experiment). *p < 0.05 when compared with vehicle values. **p < 0.001 when compared with vehicle values.
RESULTS
Effects of PG of the E Series on Erythroid and Myelomonocytic Precursors

As indicated in Figs. 1 and 2, BFU-E and CFU-C growth is dampened by addition of PGE, in the presence of both plateau (Figs. 1 and 2, 3–6 IU of Ep, 0.1 ml of CSF/plate) or suboptimal (Fig. 2, 1 IU of Ep, 0.01–0.05 ml of CSF/dish) levels of, respectively, Ep or CSF. This inhibitory phenomenon is clearly dose-dependent, i.e., is directly correlated with PGE dosage comprised between $10^{-5}$ and $10^{-8}$M (Fig. 1). In addition, it can be partially overcome by increasing amounts of CSF and, with some limitations, also of Ep (Fig. 2). On the other hand, it is noteworthy that the cloning efficiency of CFU-E is apparently unaffected by addition of corresponding amounts of PGE, (Figs. 1 and 2). Similar phenomena were observed for the clonogenic capacity of BFU-E, CFU-E, and CFU-C when plating comparable amounts of PGE, (results not shown). Finally, expression of BFU-E, but not CFU-E, from DBA/2 mouse marrow was similarly inhibited by PGE, (between $10^{-5}$ and $10^{-8}$M) in a dose-dependent fashion (unpublished observations).

Effects of Dibutyryl-cAMP (db-cAMP) and Theophylline on Erythroid and Myelomonocytic Precursors

As shown in Fig. 3, addition of db-cAMP ($10^{-4}$ to $10^{-5}$M) induces a significant decline of the number of BFU-E and CFU-C colonies as compared to that in control plates. Once again, the inhibitory pattern is directly correlated with the db-cAMP dosage. Also of interest is the lack of any significant effect on CFU-E

Fig. 2. Effect of PGE, ($10^{-7}$ or $10^{-9}$M) on the number of colonies derived from BFU-E (upper panel), CFU-E (central panel) and CFU-C (lower panel) in murine bone marrow incubated with graded concentrations of Ep (upper and central panels) and CSF (lower panel). The colony-forming capacity of BFU-E, CFU-E, and CFU-C is expressed as a percentage of the number of colonies in vehicle plates. Each point represents the mean ± SEM from 2 separate experiments (2 plates/experiment).

Fig. 3. Effect of db-cAMP on the number of colonies derived from BFU-E (upper panel), CFU-E (central panel), and CFU-C (lower panel) in murine bone marrow. Each point represents the mean ± SEM of average values from a minimum of 3 separate experiments (2 plates/experiment). *p < 0.05 when compared to vehicle values. **p < 0.001 when compared with vehicle values.
growth. Control studies indicated that both butyrate and AMP (10^{-4} to 10^{-5} M) did not exert any significant influence on the in vitro expression of these precursors (results not shown).

Theophylline addition (10^{-4} M) induces an inhibitory action on the cloning efficiency of BFU-E and CFU-C, which is similar to that induced by db-cAMP; this agent, too, does not modify the number of CFU-E colonies (Fig. 4). Moreover, combined treatment with 

\[ \text{PGE, (10^{-8} M)} \] and low dosages of theophylline (10^{-5} M) potentiates the inhibitory action of the former agent on both BFU-E and CFU-C growth, but is ineffective on CFU-E (Fig. 5 shows 2 representative experiments).

**Effects of PGF_{\alpha} and db-cGMP on Erythroid and Myelomonocytic Precursors**

Addition of PGF_{\alpha} induces a significant inhibitory effect on the clonogenic capacity of CFU-C at 10^{-5} M (controls, 103.2 ± 5.0; PGF_{\alpha}, 77.8 ± 4.3, p < 0.02) but not at lower dosages. Similar results were reported by Kurland and Moore. On the other hand, this amount of PGF_{\alpha} (10^{-5} M) provokes either a mild (controls, 37.7 ± 2.2; PGF_{\alpha}, 27.3 ± 1.2, p < 0.02) or no decline of BFU-E expression, respectively, at plateau (3.0 IU/plate) or lower (0.5 IU/plate) levels of Ep. In either case, smaller dosages (10^{-5}, 10^{-6} M) of this agent do not cause significant modifications of the BFU-E number. Finally, PGF_{\alpha} does not modify the cloning efficiency of CFU-E at all tested levels. Combined treatment with PGF_{\alpha} (10^{-5} M) and indomethacin (10^{-4} M) is also ineffective (results not presented here).

In regard to db-cGMP (data not shown), this agent does not induce any significant fluctuation of the number of both BFU-E and CFU-E at concentrations of 10^{-4}, 10^{-5}, or 10^{-6} M, both at suboptimal and optimal levels of Ep (0.5 or 3.0 IU/plate, respectively). Furthermore, the same concentrations of db-cGMP do not significantly modify the in vitro expression of CFU-C.

**Effects of PGE_{\alpha} on BFU-E and CFU-E From Human Marrow, Either Unseparated or After Removal of Adherent Cells**

As shown in Fig. 6, addition of PGE_{\alpha} (from 10^{-7} up to 10^{-11} M) to unseparated human marrow results in a bell-type curve for BFU-E and CFU-E expression, with significant elevations at 10^{-7} and 10^{-9} M, respectively. Removal of adherent cells, however, fully effaces these effects. These observations were performed at plateau levels of Ep (2 IU/dish). Similar results were obtained at a dosage of 1 IU/plate (results not shown), which induces suboptimal growth of BFU-E.

**DISCUSSION**

The influence of PGEs on the in vitro expression of erythroid precursors in murine versus human marrow is species-dependent, i.e., these agents exert an inhibitory action on early progenitors in mouse and a stimulatory one on both early and late precursors in man.
In this regard, incubation of murine marrow with PGE₁-E₂ causes a dose-dependent inhibition of the cloning efficiency of both BFU-E and CFU-C, while not affecting CFU-E growth. As for CFU-C, a significant inhibitory action was observed down to a PGE₁ dosage of \(10^{-9} M\), versus \(10^{-10} M\) in the observations by Kurland and Moore. This difference may be accounted for by absence of adherent cells in the latter studies, as compared with their presence in those reported here. On the other hand, the decrease of BFU-E cloning efficiency in PGE₁ versus vehicle-treated marrow is significant down to the level of \(10^{-10} M\), i.e., within the range of in vivo concentrations of the hormonal mediators. The significance of these results is further strengthened by those obtained with PGE₁, as well as by the inhibitory effects of PGE₁ on BFU-E and CFU-C but not CFU-E expression in DBA/2 mice.

It is of interest that the PGE₁ inhibitory action on murine CFU-C is partially overcome by increasing amounts of CSF, as previously indicated by Kurland and Moore. A similar, although less well defined, phenomenon is observed for mouse BFU-E growth in the presence of increasing dosages of Ep. In this regard, it must be emphasized that BFU-E cloning efficiency is dependent on both Ep and factor(s) enhancing burst formation (BEF). In the present studies, BEF was present in the FCS and, therefore, its activity was constant in all plates: this may account for the less impressive competition exerted by Ep (as compared to that by CSF) on the PGE₁-induced inhibitory effect.

It is well established that PG of the E series induce an increase of intracellular levels of cAMP. Therefore, murine marrow cells were incubated with graded amounts of either db-cAMP, theophylline (an inhibitor of diphosphodiesterase), or theophylline + PGE₁. In all cases, treatment with these agents closely mimicked the inhibitory pattern induced by PGE₁ at the level of both BFU-E and CFU-C, whereas the CFU-E pool was once again unaffected. It is therefore suggested that intracellular levels of cAMP play a significant role in mediating the PGE₁ action. In this regard, however, it must be pointed out that PGE₁s apparently exert a stimulatory action on erythroid differentiation of untreated Friend leukemia cells independently of activation of the adenylate cyclase system.

It is emphasized that, whereas the inhibition by PGE₁-cAMP on CFU-C expression is confirmatory of previous evidence, the dampening action of these agents on the growth of murine BFU-E is novel.

In regard to the CFU-E pool, the present observations indicate that PGE₁ and their mediator(s) do not induce marked fluctuations in the proliferation of these late erythroid precursors in the mouse. Previous studies on murine marrow, focused, respectively, on CFU-E cloning efficiency or heme synthesis in erythroblasts, indicated that PGE₁ and/or db-cAMP exerted either a mild stimulatory or inhibitory action.

The slight decrease of BFU-E number induced by large amounts of PGF₂α₅ of uncertain significance. In this regard, this diminution was monitored when plating large amounts of both PGF₂α (\(10^{-4} M\)) and Ep (3 IU), but not in all other conditions (i.e., \(10^{-5} \) to \(10^{-9} M\) of PGF₂α and/or 0.5 IU of Ep). In addition, even comparatively large amounts (\(10^{-4}, 10^{-5} M\)) of the putative mediator of PGF₂α (i.e., cGMP) were ineffective on all tested precursors.

As previously mentioned, the action of the PGE₁-cAMP system on erythropoiesis is clearly species-dependent, in that stimulatory effects have been observed in humans, dogs, and sheep, inhibitory
actions in rats and guinea-pigs, variable influences in mice. In particular, Golde and Cline reported an enhanced CFU-E expression in PGE-treated human marrow. The present studies indicate a stimulatory effect by PGE, on the clonogenic capacity of both BFU-E and CFU-E in human marrow cultures. Interestingly, both phenomena are totally abolished by removal of adherent cells. It is generally conceded that human peripheral leukocytes and marrow-adherent cells release BEF. It is suggested therefore that PGE1 enhanced CFU-E and more particularly BFU-E expression, possibly via stimulation of BEF release by adherent cells. Similarly, enhanced CSF release by adherent cells is evoked by PGE addition. It should be mentioned that, at variance with results on human marrow, removal of adherent cells from mouse marrow does not dampen the inhibitory action exerted by the PGE-cAMP system on BFU-E expression (Peschle et al., preliminary observations).

In conclusion, the effect of the PGE-cAMP system on murine as well as human myelomonocytic precursor fall into a coherent, elaborate pattern. Accordingly, PGEs exert a direct, inhibitory action on CFU-C, but may also indirectly enhance proliferation and differentiation of these precursors, by triggering macrophage CSF production. At the level of erythroid precursors, instead, the stimulatory-inhibitory effects of the PGE-cAMP complex are dichotomized in different mammalian species. In this regard, an inhibitory influence is observed on murine BFU-E, independently from adherent cells. On the other hand, the stimulatory action of PGE on both BFU-E and CFU-E from human marrow is mediated by adherent cells, possibly via release of BEF.

REFERENCES

In vitro interactions of PGE and cAMP with murine and human erythroid precursors

GB Rossi, AR Migliaccio, G Migliaccio, F Lettieri, M Di Rosa, C Peschle and G Mastroberardino