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PHYSIOLOGICAL CONCENTRATIONS OF ATRIAL NATRIURETIC FACTOR STIMULATE HUMAN ERYTHROID PROGENITORS IN VITRO

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Human α-ANF[1-28] potentiated erythroid colony formation up to four-fold in cultures containing erythropoietin. Both early and late erythroid precursor cells responded to α-ANF[1-28] [0.032 to 1 nM] in a dose dependent fashion. Removal of T lymphocytes and macrophages which have been shown to modulate erythropoiesis did not abolish the stimulatory effect. All major circulatory forms of ANF (α-ANF[1-28], α-ANF[4-28] and α-ANF[5-28]) had potent erythropoietic activity. These results indicate that concentrations of ANF reached during hypoxia stimulate erythroid progenitor cells in the presence of erythropoietin.

Stimulation of red cell production by hypoxia has been attributed to enhanced renal synthesis and release of a hormone called erythropoietin (Epo) (1). Epo is a glycoprotein acting on terminal stages of erythroid differentiation (2). Another hormone released in response to low oxygen pressure is atrial natriuretic factor (ANF) (3,4). This recently discovered peptide (5,6) is synthesized in the atria and has powerful diuretic, natriuretic and hypotensive actions (7-9). ANF also allows intravascular fluid to exit across the capillaries which leads to acute hematocrit rise (10). These observations and the increase in plasma ANF during hypoxia (up to 0.2 nM)(4) prompted us to assess whether ANF can control red cell mass by action on erythropoiesis.

The effect of ANF on human early and late erythroid progenitor cells called burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) cells, respectively, was studied in vitro. We report that in the presence of Epo, α-ANF[1-28]

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increases CFU-E and BFU-E numbers up to four-fold. This effect is dependent on the molecular form of ANF and concentration of ANF (0.032 to 0.96 nM), and is not affected by the presence of T cells or macrophages in the culture.

MATERIALS AND METHODS

Cell preparations: Bone marrow cells and peripheral blood cells were collected from normal human volunteers after obtaining informed consent. The cells were suspended in α-medium containing 10% fetal calf serum and separated by Ficoll-Hypaque gradient to obtain an enriched mononuclear cell population. Peripheral blood derived cells were further enriched by incubation in plastic dishes twice for 30 minutes to remove macrophages. Then the majority of T cells were removed by rosetting with sheep red blood cells (Lampire) and centrifugation. The remaining T cells were removed by incubating the cell suspension with Leu-1 antibody (Becton-Dickinson) and rabbit complement. Less than 0.3% of macrophages and T cells could be detected among the total cells in the suspension.

Erythroid progenitor BFU-E and CFU-E assay. Cells (5×10^4 per plate) were cultured in petri dishes using the methylcellulose technique (11). Human Epo (Toyobo) was added to the cultures on day 0. Human ANF analogs (Peninsula) diluted in α-medium were added to the cultures every 3 days starting on day 0. Control cultures were supplemented with α-medium alone. Triplicate plates were incubated at 37°C in a humidified atmosphere of 7% CO_2 in air. On day 8, cell aggregates which contained more than seven erythroid cells were counted as CFU-E. On day 14, aggregates of forty cells or more were counted as BFU-E. Hemoglobinization of the cells was confirmed by using a Soret band. Number of observations are indicated in the bottom part of the bar.

Data analysis: Statistical significances were obtained by using Kruskal-Wallis Analysis of Variance [one way by ranks].

RESULTS

Cell culture techniques have allowed detection of molecules that act on erythropoietic precursor cells. Demonstration of the stimulatory effect on BFU-E and CFU-E *in vitro* requires the presence of erythropoietin in the culture system (12-14). Addition of human α-ANF[1-28] alone at a concentration of 0.32 nM to cultures had no effect on erythroid precursors (Fig. 1). As expected, Epo alone stimulated both erythroid precursors ($p < 0.05$). The highest numbers of both BFU-E and CFU-E were observed in cultures containing Epo plus α-ANF[1-28] ($p < 0.005$). The stimulatory effect was dependent on the doses of α-ANF[1-28] and Epo (Fig. 2). For identical α-ANF[1-28] concentrations, BFU-E and CFU-E yield was higher in cultures containing 2 U of Epo than 0.25 U. A concentration-dependent potentiation of erythropoiesis was observed when α-ANF[1-28] levels were increased from 0.032 nM to

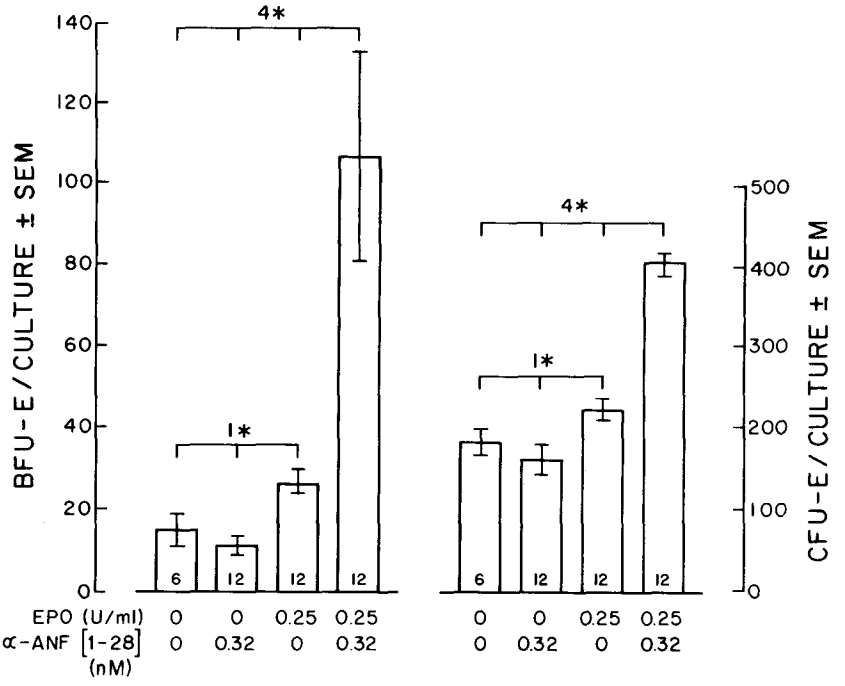

Fig. 1. Effects of α-ANF[1-28] on erythroid progenitors BFU-E and CFU-E in the presence and absence of Epo: 1* = $p < 0.05$; 2* = $p < 0.01$; 3* = $p < 0.005$; 4* = 0.001. Bars represent ± SEM.

0.96 nM. Potentiation usually peaked at 0.96 nM α-ANF[1-28]. Stimulation of erythroid colony formation by α-ANF[1-28] was not dependent on the presence of T lymphocytes or macrophages (Table 1). The number of peripheral blood derived BFU-E colonies increased three-fold in the presence of α-ANF[1-28] in cultures containing 0.25 U of Epo and less than 0.3% of T cells and macrophages. Potentiation of erythropoiesis depended on the molecular form of ANF (Peninsula) (Table 2). The stimulatory effect of Epo on both CFU-E and BFU-E colony formation was enhanced by α-ANF[1-28] as well as α-ANF[4-28] and α-ANF[5-28]. In fact, the number of BFU-E colonies, but not CFU-E colonies, was higher in cultures supplemented with α-ANF[4-28] ($p < 0.05$) and α-ANF [5-28] than in cultures containing α-ANF[1-28] (Table 2). The effect of α-ANF[1-11] and α-ANF-dimer on erythroid precursors was not significant.

DISCUSSION

These results indicate that all major circulatory forms (15) of ANF stimulate erythroid colony formation in vitro in the presence of erythropoietin. This newly described bioactivity of ANF does not depend on an intact N-terminal sequence but may

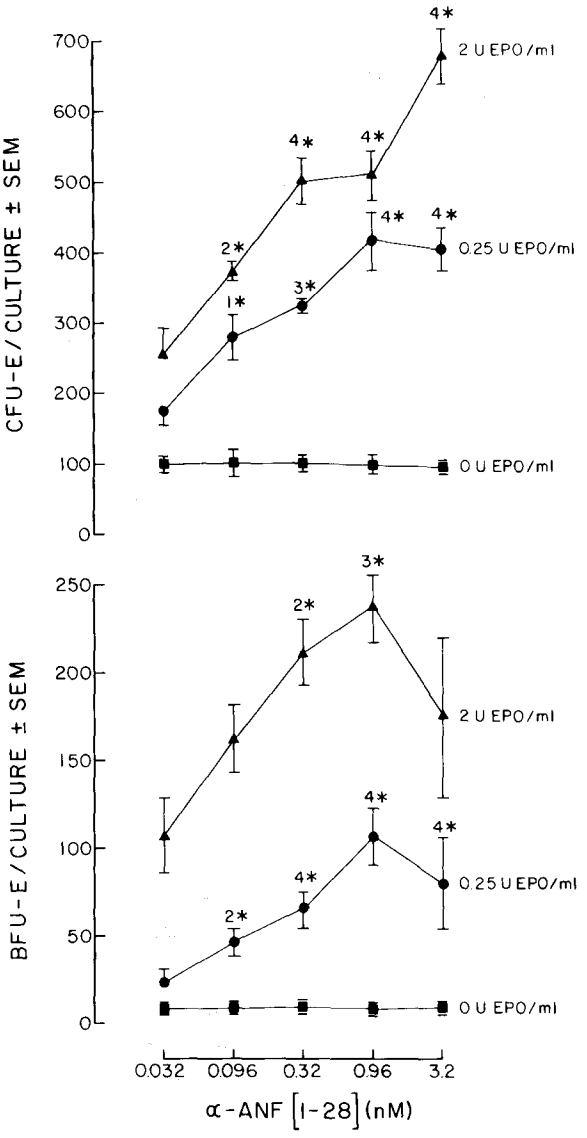

Fig. 2. Relationship between α-ANF[1-28] dose and erythroid progenitor BFU-E and CFU-E numbers in Epo containing cultures. Probabilities (Fig. 1) were determined using the lowest ANF concentration in each group as a reference point. N = 6 observations per group; bars represent ± SEM. Note log scale on abcissa.

require an intact ring structure (Table 2) as do the renal actions of ANF (5-7). Both early (BFU-E) and late (CFU-E) erythroid progenitors respond to α-ANF[1-28] (Fig. 2) at concentrations detectable in hypoxemic rabbits(4). Removal of macrophages and T cells which have been shown to elaborate factors stimulating both CFU-E and BFU-E in vitro (16), did not abolish the enhanced colony formation due to α-ANF[1-28] (Table 1). This suggests that the stimulating effect of ANF is not mediated via these cells.

TABLE 1

THE EFFECT OF HUMAN α-ANF[1-28] AND ERYTHROPOIETIN ON PERIPHERAL BLOOD BFU-E IN THE ABSENCE OF T CELLS AND MACROPHAGES

Agent	BFU-E/culture ± SEM
Experiment 1	
Epo 0.25 U/ml	7.0 ± 0.9
Epo 0.25 U/ml + ANF 0.32 nM	22.7 ± 1.9**
Experiment 2	
Epo 0.25 U/ml	14.0 ± 2.0
Epo 0.25 U/ml + ANF 0.32 nM	42.3 ± 6.8*

* = $p < 0.025$;
** = $p < 0.005$; n = 3 observations per group.

It is well established that erythropoietin is important in the latter stages of erythropoietic differentiation by stimulating proliferation of CFU-E and morphologically recognizable erythroid cells (1). Humoral control of the early stages of erythropoiesis is not as well understood. In vitro stimulation of BFU-E has been observed in the presence of three species of molecules. Interleukin-3 (IL-3, also called multipotential growth factor, Multi-CSF) acts on many hemopoietic precursors and stimulates BFU-E burst formation in vitro (12). Granulocyte-macrophage colony

TABLE 2

POTENTIATION OF ERYTHROPOIETIN-STIMULATED ERYTHROPOIESIS FROM HUMAN BONE MARROW BY ANF-ANALOGS

ANF analog	Potentiation of BFU-E(%)	p^1	p^2	Potentiation of CFU-E (%)	p^1	p^2
ANF [1-28]	100 ± 24.9	0.001	-	100 ± 39.2	0.00003	-
ANF [4-28]	74.8 ± 15.3	0.003	NS	208 ± 41.0	0.00001	0.05
ANF [[5-28]	98.5 ± 38.6	0.003	NS	143 ± 26.7	0.00001	NS
ANF [1-11]	-1.5 ± 8.8	NS	0.001	14.8 ± 4.6	NS	0.003
ANF Dimer	40.2 ± 26.7	NS	0.04	5.4 ± 13.1	NS	0.025

The concentration of ANF analog was 0.32 nM. All cultures were supplemented with 0.25 U/ml of Epo. Potentiation is expressed relative to the effect of α-ANF[1-28] + Epo (100%). The actual numbers of BFU-E and CFU-E in the presence of α-ANF[1-28] and Epo were 103 ± 26 and 440 ± 176, respectively. In cultures containing Epo alone, the number of BFU-E and CFU-E was 54 ± 16 and 265 ± 160, respectively. p^1 = significance relative to Epo alone; p^2 = significance relative to potentiation by α-ANF[1-28]. Numbers are means ±SEM (n=6 observations/group).

stimulating factor (GM-CSF) is thought to act mainly on granulocyte and macrophage precursors and stimulates BFU-E in vitro if cultures are initiated in the presence of this factor and erythropoietin is added 3-5 days later (13). The third factor is erythroid potentiating activity (EPA). EPA stimulates in vitro growth of BFU-E and CFU-E from both human and murine marrow and colony formation by the K-562 human erythroleukemic leukemia line cells (14,17). In vivo enhancement of erythropoiesis was observed after administration of EPA to experimental animals (18). A significant increase in the number of reticulocytes is seen in the peripheral blood and erythroid precursors in the spleen in anemic ICR mice. The significance of EPA effects on erythropoiesis has been challenged since its molecular form is identical to the tissue inhibitor of metalloproteinases (TIMP) that is involved in collagen metabolism (19).

In this study, a fourth species of molecules with major short-term effects on the circulation was shown also to modulate erythroid precursor cell proliferation in vitro. In contrast to EPA, ANF release is controlled by a physiological mechanism that plays a pivotal role in regulation of erythropoiesis, namely, hypoxia (3,4). Production of erythropoietin is increased in response to low tissue oxygen pressure, leading to enhanced red cell production. Our results suggest that ANF may function similarly because ANF at concentrations induced in vivo by hypoxia augmented colony formation in cultures containing erythropoietin. This mechanism may be important in the long-term control of red cell mass and circulatory homeostasis.

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