Enhancement of Murine Erythropoiesis *in Vitro* by a Porcine Kidney Extract

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We have examined the effect of porcine kidney extract (PKE) on the growth of erythroid progenitor-derived colonies in a methylcellulose culture. The addition of PKE resulted in an enhancement of burst-forming-unit-erythroid (BFU-E)-derived colonies, and the enhancement of the colony was also observed in a low concentration of erythropoietin (Epo) and fetal calf serum (FCS).

The activity of PKE on BFU-E was compared with the erythroid growth factors, such as recombinant murine interleukin-3 (IL-3), recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF) and recombinant human granulocyte colony-stimulating factor (G-CSF) which are all well known to stimulate BFU-E growth. IL-3 showed a potent burst-promoting activity (BPA), but GM-CSF and G-CSF did not enhance BFU-E growth in our culture conditions.

In the cultures supplemented with Epo, the rapid loss of BFU-E was prevented with IL-3; however, PKE alone did not prevent the disappearance of BFU-E.

These results suggest that PKE possesses a BPA-like activity which is considered an enhancement of BFU-E.

Keywords kidney extract; burst-forming-unit-erythroid (BFU-E); burst-promoting activity (BPA); interleukin-3 (IL-3)

At least two major groups of erythroid progenitor cells, the burst-forming-unit-erythroid (BFU-E) and the colony-forming-unit-erythroid (CFU-E), have been recognized^{1,2} in *in vitro* culture systems. The growth of CFU-E, relatively mature erythroid progenitors, requires the presence of erythropoietin (Epo). Epo is able to stimulate the proliferation of CFU-E and promotes maturation leading to the hemoglobinization of proerythroblast. Epo and its recombinant protein have been purified as a glycoprotein with a molecular weight of 39000.^{3,4} On the other hand, the proliferation of BFU-E, an early erythroid progenitor, requires burst promoting activity (BPA) which is different from Epo.^{1,2} BPA stimulates the proliferation of BFU-E and may be responsible for the differentiation of BFU-E into CFU-E with a high responsiveness to Epo. BPA has been found in media conditioned by some human monocyte cell lines,⁵ various kinds of blood mononuclear cells,⁶ surface membrane vesicles,⁷ urine^{8,9} and serum.¹⁰

Interleukin-3 (IL-3) has been purified from a WEHI-3 conditioned medium^{11,12} and demonstrated to have all of the properties ascribed to BPA.¹³ In addition, granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and IL-4 have also been demonstrated to have "BPA-like" activity.^{1,14,15}

Previously, we have reported that porcine kidney extract (PKE) has colony promoting activity (CPA) on GM colony formation.¹⁶⁻¹⁸ In those papers, we showed some biological and biochemical properties of the CPA and suggested that the CPA of PKE appears to be a factor different from IL-3, G-CSF, GM-CSF and IL-4.

In this study, we examined the effects of PKE on colony formation by erythroid progenitors from murine bone marrow cells (BMC) *in vitro*.

Experimental

Preparation of PKE PKE was prepared as previously described.¹⁶ Briefly, porcine kidneys were homogenized with distilled water. The homogenate was centrifuged at $1 \times 10^5 g$ and the clear supernatants thus obtained were lyophilized. The lyophilized material was dissolved in distilled water, and saturated ammonium sulfate solution was added to the solution. The precipitate from 50 to 65% saturation of ammonium

sulfate was collected, dialyzed against distilled water, and lyophilized. The lyophilized material was dissolved in distilled water and insoluble material was removed by centrifugation. The clear supernatant obtained was dialyzed against phosphate-buffered saline (PBS), passed through a membrane filter (0.45 μm pore size, Fuji Photo Film Co., Ltd.), and stored at $-20^\circ C$ until use. The same preparation was used throughout the experiment. The results of repeated experiments showed good reproducibility on CPA of the preparations, and no detectable inactivation of CPA was observed during several months under the above storage conditions.

Mice Male mice of the ddY strain (Sankyo Labo Service Co., Sapporo) aged 6–10 weeks were used.

Growth Factors Human recombinant Epo (ESPO Injection, Sankyo Co., Ltd.) was used in this study. Recombinant murine IL-3 (specific activity $>1 \times 10^5$ U/mg) and recombinant murine GM-CSF (specific activity 5×10^7 U/mg) were purchased from Genzyme, Inc. (Boston). Recombinant human G-CSF was kindly provided by Kirin Brewery Co. (Tokyo, Japan). Doses of these factors per culture dish were as follows: (1) IL-3, 100 units; (2) GM-CSF, 100 units (3) G-CSF, 1 μg .

CPA Assay The assay for CPA was carried out in methylcellulose cultures. 5×10^4 BMC from a mouse femur were cultured in 35 mm plastic Petri dishes (SH-S3512SW, Terumo, Tokyo) in 1 ml of Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 0.8% methylcellulose (Wako Chemicals, Tokyo), 20% horse serum, G-CSF, and test samples. Cultures were incubated at $37^\circ C$ in a humidified atmosphere of 5% O_2 , 10% CO_2 , and 85% N_2 for 7 d. Colonies consisting of 50 or more cells were scored using an inverted microscope. From a dose response study, 30 μl of PKE per dish was considered to be optimal for promoting activity in methylcellulose cultures. PKE alone did not support GM colony formation, even at 100 μl .

Erythroid-Burst (EB) Colony Assay Erythroid progenitors were assayed in a methylcellulose culture system as described by Iscove *et al.*¹⁹ In routine experiments, 2×10^5 BMC were cultured in 35 mm plastic Petri dishes in 1 ml of IMDM supplemented with 0.8% methylcellulose, 1% deionized bovine serum albumin (BSA, Sigma), 30% fetal calf serum (FCS, Mitsubishi Kasei Ltd.), $1 \times 10^{-4} M$ 2-mercaptoethanol (ME), 2 units of Epo, and test samples. Cultures were incubated at $37^\circ C$ in a humidified atmosphere of 5% O_2 , 10% CO_2 , and 85% N_2 for 8 d. EB colonies were stained with benzidine solution which was a freshly prepared mixture of 6 volumes of 0.3% diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6) and 1 volume of 0.5% H_2O_2 . The solution (0.5–1.0 ml) was gently overlaid on the cultures. After 20–60 min at room temperature, the colonies dyed brown-dark were scored using an inverted microscope. Benzidine positive colonies on 8 d cultures were defined as an EB colony. BFU-E-derived colonies contained 50 or more erythroblasts.

Trypsin Treatment 0.1 ml of PKE (13.3 mg protein/ml) was treated with 0.1 ml of trypsin solution (8 mg/ml, bovine pancreas, Sigma) at $37^\circ C$ for 2 h, then the treatment was stopped by the addition of 0.2 ml trypsin inhibitor (4 mg/ml, soybeans, Sigma) at $37^\circ C$ for 0.5 h. After

treatment, aliquots of 40 μ l were applied to the EB colony assay.

Delayed Addition The cultures of 2×10^5 BMC prepared in 0.9 ml of a methylcellulose medium containing PKE (10 μ l) or IL-3 (100 units) without Epo, or containing Epo (2 units) without PKE or IL-3, were incubated. The cultures on days 0, 2 and 4, were overlaid with 0.1 ml of Epo-solution (2 units), PKE or IL-3, and were incubated for another 8 d, then the EB colonies formed were scored.

Preincubation of BMC 1×10^7 BMC were incubated in a 25 cm² culture flask (MS-20050, Sumitomo, Tokyo) with 10 ml of IMDM containing 1% BSA, 30% FCS, 1×10^{-4} M ME, and 20 units of Epo and/or 0.5 ml of PKE at 37°C in a humidified atmosphere of 5% O₂, 10% CO₂, and 85% N₂ for 4 or 7 d beforehand. The nonadherent cells were harvested, washed three times with IMDM, and then assayed for EB colony formation.

Results

Effect of PKE Concentration on EB Colony Formation

The results are shown in Fig. 1. The addition of PKE into the cultures containing Epo resulted in an increase of EB colonies in comparison with the absence of PKE. A maximal increase of 2.4-fold was induced at 10 μ l of PKE. In contrast, no EB colony formation was observed at any PKE concentrations in the absence of Epo.

Effect of Epo and FCS Concentration on EB Colony Formation The influence of PKE on the requirements of Epo was tested. As shown in Fig. 2, no colonies were found in the presence of less than 0.2 units per dish of

Fig. 1. Effect of PKE Concentration on Erythroid-Burst Colony Formation

EB cultures were incubated in the absence (○) or the presence of 2 units of Epo (●). Numbers represent the mean $\pm$ S.E. of 9 dishes from three separate experiments. ○, Epo (-); ●, Epo (+).

Fig. 2. Effect of Epo Concentration on Erythroid-Burst Colony Formation

EB cultures were incubated in the absence (○) or the presence of PKE (●). Numbers represent the mean $\pm$ S.E. of 9 dishes from three separate experiments. ○, PKE (-); ●, PKE (+).

Epo alone, and a maximum stimulation was obtained at 2–4 units of Epo. The addition of PKE (10 μ l) to the cultures containing 0.5 units of Epo resulted in nearly the same colony formation in comparison with the cultures containing 2–4 units of Epo alone, and the plating efficiency was significantly increased corresponding to the concentration of Epo.

The influence of PKE on the requirements of FCS was tested. As shown in Fig. 3, the EB colony formation reached a plateau at a concentration 15–30% of FCS. The addition of PKE (10 μ l) to the cultures containing 5% of FCS resulted in nearly the same colony formation in comparison with the cultures containing 15–30% of FCS without PKE.

The Correlation between the Number of EB Colonies Formed and of BMC Plated We examined the plating efficiency of the EB colony with an inoculation of between 1×10^5 and 4×10^5 cells per dish. As shown in Fig. 4, a linear relationship between the cell number plated and the colony number formed was observed in the cases both Epo alone and in a combination of PKE and Epo, showing of correlation coefficients of 0.9969 and 0.9952, respectively.

Stability of BPA in PKE PKE was treated by heat and trypsin. As shown in Table I, the reduction of BPA was

Fig. 3. Effect of FCS Concentration on Erythroid-Burst Colony Formation

EB cultures were incubated in the absence (○) or the presence of PKE (●) with 2 units of Epo. Numbers represent the mean $\pm$ S.E. of 9 dishes from three separate experiments. ○, PKE (-); ●, PKE (+).

Fig. 4. Correlation between the Number of Erythroid-Burst Colony and BMC Plated

EB cultures were incubated in the absence (○) or the presence of PKE (●) with 2 units of Epo. Each value indicated a coefficient of correlation. Numbers represent the mean $\pm$ S.E. of 9 dishes from three separate experiments. ○, PKE (-), $r=0.9969$; ●, PKE (+), $r=0.9952$.

observed from 2.1 to 1.66 and 1.65 at 56 °C, 30 min and 70 °C, 30 min, respectively. And the BPA of PKE was completely inactivated by the trypsin treatment. Similar inactivation of the CPA of PKE on GM lineage CSFs has also been observed as shown in previous papers.¹⁶⁾ In addition, a mixture of trypsin and trypsin inhibitor did not affect the CPA or the BPA of PKE (data not shown).

Effect of IL-3, GM-CSF and G-CSF on EB Colony Formation The BPA of PKE was compared with that of IL-3, GM-CSF, and G-CSF. As shown Fig. 5, IL-3 enhanced EB colony formation, which was equivalent to

TABLE I. Effect of Various Treatments of PKE

Treatments	CFU-GM ^{a)}	Ratio	BFU-E ^{b)}	Ratio
Control	120.4 ± 4.7	1.00	59.6 ± 1.6	1.00
Untreated PKE	216.1 ± 8.4	1.79	125.4 ± 6.3	2.10
56 °C, 30 min PKE	172.9 ± 3.2 ^{d)}	1.44	98.8 ± 5.4 ^{c)}	1.66
70 °C, 30 min PKE	166.8 ± 7.7 ^{c)}	1.39	98.1 ± 3.0 ^{c)}	1.65
Trypsinized PKE	106.1 ± 4.2	0.88	61.4 ± 4.4	1.03
37 °C, 2.5 h PKE	206.1 ± 10.1	1.71	117.0 ± 9.0	1.96

a) The number of CFU-GM colonies per 5×10^4 BMC stimulated by $1 \mu\text{g}$ of G-CSF. b) The number of erythroid-burst colonies per 2×10^5 BMC stimulated by 2 units of Epo. Data exhibits mean ± S.E. of 9 dishes from three separate experiments. c) $p < 0.01$ vs. untreated PKE. d) $p < 0.001$ vs. untreated PKE.

Fig. 5. Effect of IL-3, GM-CSF and G-CSF on Erythroid-Burst Colony Formation

Cultures were carried out in the presence of 2 units of Epo and each factor. Numbers represent the mean ± S.E. of 6 dishes from two separate experiments. a) $p < 0.001$, b) $p < 0.01$.

PKE. However, GM-CSF and G-CSF did not enhance EB colony formation in our culture conditions.

Effect of PKE on the Survival of BFU-E In order to investigate the supporting function of PKE on the survival of BFU-E, the delayed addition of Epo was examined. As shown in Fig. 6, in the control cultures (PBS + Epo), EB colonies decreased with a lag of Epo addition (PKE + Epo), showing a decrease in the survival of BFU-E. In the case of PKE, a rapid loss of BFU-E was also observed. In contrast with PKE, IL-3 could support the survival of BFU-E, since the number of EB colonies on day 4 of the addition of Epo were found to be 72% of day 0 of the addition, indicating the difference between PKE and IL-3 on their supporting activity.

The addition of PKE or IL-3 to Epo-containing cultures was also examined. In regard to the addition of PKE on day 4, the EB colonies were found to be 153% of the day 0 addition in the control cultures (PBS + Epo). When IL-3 was added on day 2, the number of EB colonies was nearly equal to that of the day 0 addition. However, the addition of IL-3 on day 4 resulted in a decrease in colony number. When the cultures were overlaid with PBS instead of PKE or IL-3 to Epo-containing cultures for 2 or 4 d, no difference in the number of EB colonies was observed. Furthermore, IL-3 alone did not support EB colony

TABLE II. Preincubation of BMC with Epo and/or PKE

Incubation period	Additive	Number of cells ($\times 10^{-4}$)	Number of BFU-E	BFU-E recovery rate (%)
0 time		1,000	2725 ± 141	100.0
4 d	PBS	257	381 ± 36	14.0
	Epo	208	1949 ± 258	71.7
	PKE	220	672 ± 52	24.6
7 d	PKE + Epo	363	3137 ± 162	115.2
	PBS	115	46 ± 20	1.7
	Epo	144	171 ± 34	6.3
	PKE	247	56 ± 13	2.1
	PKE + Epo	301	1660 ± 143	61.0

Mean ± S.E. of 6 dishes from two separate experiments.

Fig. 6. Effect of Delayed Addition of Epo, PKE or IL-3 on Erythroid-Burst Colony Formation

Cultures were started containing an "initial addition," to which was added Epo, PKE or IL-3 on different days. Each culture was incubated for 8 more days. Numbers represent the mean ± S.E. of 6 dishes from two separate experiments.

formation (data not shown).

Since PKE alone did not possess a supporting function for the survival of BFU-E, we studied the effect of the combination of Epo plus PKE on the ability to maintain BFU-E under liquid culture conditions. The results are shown in Table II. The number of BFU-E was reduced to 72 and 6% by day 4 or day 7 of preincubation with Epo, respectively. In the case of PKE, the recovery rates of BFU-E on days 4 and 7 were 25 and 2%. In the combination of Epo and PKE, 115 and 61% of BFU-E were recovered on those days, suggesting the synergistic action of Epo and PKE on maintenance of erythroid progenitors.

Discussion

The addition of PKE to the erythroid cultures containing Epo enhanced EB colony formation, and was able to decrease the requirement of both Epo and FCS concentrations for the growth of EB colony (Figs. 1–3). Epo was not detected in PKE, because PKE alone did not support the erythroid colony formation. A linear relationship between cell numbers plated and colony numbers was observed in the presence of PKE, and the enhancement ratios of each point by PKE were nearly constant (Fig. 4). These results suggested that the enhancing activity of PKE correlated directly with the number of BFU-E and the stimulation of Epo. From these results, it could be concluded that PKE contains the activity capable of potentiating the growth of murine BFU-E.

Ihle *et al.*^{11,12} purified IL-3 from a medium conditioned by a mouse myelomonocytic leukemia cell line WEHI-3. And Goldwasser *et al.*¹³ indicated that IL-3 had all of the properties ascribed to BPA, and presumed that IL-3 was identical to BPA. In addition, they reported that IL-3 alone does not support EB colony formation of murine BMC in the absence of Epo. It indicates that IL-3 is able to stimulate both the differentiation and the proliferation of the early stage of progenitors such as CFU-S and BFU-E, and then differentiation into a late stage of progenitors such as CFU-E. However, following terminal differentiation of CFU-E requires the presence of Epo. Additionally, some reports have demonstrated that IL-4, G-CSF and GM-CSF also have a potency of BPA-like activity.^{1,14,15}

BPA has the following biological characteristics¹³: (1) It greatly diminishes the requirement of Epo or FCS to stimulate EB growth. (2) It prevents the rapid loss of BFU-E *in vitro* in the absence of Epo.

In this study, IL-3 prevented the rapid loss of BFU-E, but, PKE alone could not prevent it (Fig. 6). Additionally, when PKE was combined with Epo in the preincubation of BMC, about 61% of BFU-E was recovered compared to that of the zero time control after 7 d of incubation (Table II). These results suggest that PKE acts synergistically with Epo in supporting the survival, proliferation and differentiation of erythroid progenitors. Thus, the factor(s) detected in PKE appear to have "BPA-like" activity.

We have already demonstrated that PKE stimulates the differentiation of immature hematopoietic stem cells.^{16,17} The CPA in PKE stimulates primitive hematopoietic stem cells in the presence of GM-CSF, but does not stimulate colony formation on murine BMC by itself.

The combination of PKE with IL-3, GM-CSF, or G-CSF was examined in the presence of one of these factors such as CSF in agar cultures.¹⁸ It was found that PKE acts synergistically with these factors. We have suggested that the CPA of PKE appears to be a factor different from IL-3, GM-CSF, and G-CSF because of its functional properties and chemical characteristics. Furthermore, since IL-4 does not enhance GM colony formation in the presence of GM-CSF or IL-3, the factor(s) in PKE would be different from IL-4.

Stability of CPA and the BPA-like activity of PKE were observed to have similar properties regarding heat stability and trypsin sensitivity (Table. I). Furthermore, partial purification of the factor(s) in PKE were carried out by hydrophobic column chromatography, ion exchange column chromatography, and gel filtration high-performance liquid chromatography. The partially purified factor(s) possessed a potent BPA-like activity as did CPA (data not shown). These results suggest that the key factor in PKE appears to be a multipotent regulator in hematopoiesis.

Most recently, Royet *et al.*²⁰ reported erythropoietic activity, termed mature-BPA (MBPA), in murine kidney cell extracts, that predominantly stimulates the proliferation of mature BFU-E progenitors giving rise to small bursts. They have also showed that MBPA is different from IL-3, IL-4, GM-CSF, and Epo. The activities detected in PKE are possibly different from MBPA, because PKE seems to act also on immature BFU-E and to stimulate large EB colony formation.

It has been well known that kidneys are one of the most important tissues in hematopoiesis by reason of the regulation of Epo and CSF production.^{21–24} It is suggested that the kidney possibly contains certain factors which regulate hematopoiesis, and the factor in PKE is one of those factors.

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