Partial Purification and Characterization of a Factor for the Enhancement of Colony Formation *in Vitro* by Myeloid Progenitor Cells

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We have purified a factor, hematopoietic promoting factor (HPF), from porcine kidney extract (PKE), which exhibits a promoting activity on granulocyte/macrophage (GM) colony and burst-forming-unit-erythroid (BFU-E)-derived colony formation by progenitors from murine bone marrow cells *in vitro*. The addition of HPF resulted in an enhancement of the GM colonies as well as BFU-E-derived colonies, but did not enhance the colony-forming-unit-erythroid (CFU-E)-derived colony formation. HPF was added to the BFU-E cultures together with cytokines, such as recombinant murine interleukin-3 (IL-3), recombinant murine GM colony-stimulating-factor (GM-CSF) and recombinant human G-CSF, which have all been shown to enhance BFU-E growth. The combination of HPF plus these cytokines resulted in an enhancement of benzidine negative colony formation in comparison to the case of each cytokine alone; however, no increase was found on BFU-E colony formation.

HPF is able to enhance the granulopoiesis and erythropoiesis *in vitro*. And the synergistic activity of HPF is significantly affected by the presence of cytokines in the cultures.

Keywords kidney extract; colony-promoting activity (CPA); burst-promoting activity (BPA); purification; serum-free culture

Proliferation and differentiation of hematopoietic stem cells requires the presence of humoral regulators termed cytokines.[1,2] A number of cytokines have been isolated and the genes cloned.[2,3] A complex network between cytokines and hematopoietic progenitors on blood cell production is being clarified. Generally, cytokines have various biological effects, and act synergistically with other cytokines.[2,3]

In our previous paper, we reported that porcine kidney extract (PKE) has colony promoting activity (CPA) on granulocyte/macrophage (GM) colony formation.[4,5] One or more factors in PKE act synergistically with recombinant human granulocyte colony-stimulating factor (G-CSF), recombinant murine granulocyte/macrophage CSF (GM-CSF), recombinant murine interleukin-3 (IL-3), and recombinant human IL-6 on colony formation by progenitors from murine bone marrow cells (BMC) *in vitro*.[6] Additionally, PKE has burst-promoting activity (BPA) which is considered enhancement with erythropoietin (Epo).[7]

In this article, the purification of hematopoietic promoting factor(s) (HPF) from PKE, and the effect of HPF on colony formation by progenitors from murine BMC *in vitro* are described.

Experimental

Preparation of PKE PKE was prepared from porcine kidneys as previously described.[5]

Purification Procedure Hydrophobic Chromatography on a Butyl Toyopearl Column: PKE was applied to a TSKgel Butyl Toyopearl 650 (Tosoh, Japan) column (4.4 × 8.2 cm) previously equilibrated with 50% saturated ammonium sulfate. The column was washed with a 5 column volume of the same solution and then eluted with 450 ml of 50—0% ammonium sulfate gradient at a flow rate of 60 ml/h. Fractions of 5 ml were collected. And 500 μl aliquots of the fractions were dialyzed against phosphate-buffered saline (PBS), pH 7.4 and sterilized by filtration through a 0.45 μm filter; then CPA was measured.

Q-Sepharose Chromatography: A column of Q-Sepharose (2.5 × 11 cm, Pharmacia LKB Biotechnology AB, Sweden) was equilibrated with 20 mM Tris-HCl buffer, pH 7.40. Active fractions from Butyl Toyopearl were pooled, then applied to the column. The column was washed with a 5 column volume of equilibration buffer, and then eluted with 130 ml of a 0—0.6 M NaCl gradient at a flow rate of 60 ml/h. Fractions of 4 ml were collected. And 20 μl aliquots were diluted with 5 volumes of PBS containing 0.1% bovine serum albumin (BSA), then sterilized, and finally CPA was measured.

Gel-Filtration High Performance Liquid Chromatography (GF-HPLC): Active fractions from Q Sepharose were pooled, applied to a TSKgel G3000SW$_{XL}$ column (7.5 mm i.d. × 30 cm, Tosoh) previously equilibrated with 50 mM of a phosphate buffer, pH 6.7. Each absorbance peak at 280 nm was collected and CPA was measured. Active fractions were pooled and named HPF in this study.

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

Growth Factors Recombinant human Epo (Espo Injection®) was purchased from Sankyo Co., Ltd. (Tokyo, Japan). 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). The optimal concentration of each factor was determined from dose-response study of BPA in serum-supplemented cultures. However, because GM-CSF and G-CSF did not show BPA, the dose of these cytokines were used at an optimal concentration for CPA. 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.

Serum-Supplemented Cultures CPA Assay: The assay for CPA was carried out in methylcellulose (MC) cultures. BMCs from a mouse femur were cultured in 35 mm plastic Petri dishes (SH-S3512SW, Terumo, Tokyo) at 5×10^4 cells in 1 ml of Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 0.8% MC (Wako Chemicals, Tokyo), 20% horse serum, G-CSF, and test samples. Cultures were incubated at 37 °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. One unit is represented as an activity that increased the number of colonies twice.

Erythroid-Burst (EB) Colony Assay: Erythroid progenitors were assayed in a MC culture system as described by Iscove *et al.*[8] In routine experiments, 2×10^5 BMC were cultured in 35 mm plastic Petri dishes in 1 ml of IMDM supplemented with 0.8% MC, 1% deionized BSA (Sigma, U.S.A.), 30% fetal calf serum (FCS, Mitsubishi Kasei Ltd. Tokyo), 1×10^{-4} M 2-mercaptoethanol (ME), 4 units of Epo, and test samples. Deionized BSA was prepared according to the method of Tepperman *et al.*[9] with some modification. Cultures were incubated at 37 °C in a humidified atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 8 d. EB colonies were scored after benzidine staining. Benzidine positive colonies on 8 d cultures were defined as an EB colony. BFU-E-derived colonies contained 50 or more erythroblasts.

Serum-Free Cultures Erythroid Progenitors Colony Assay: BMCs were cultured in 35 mm plastic Petri dishes at 1—2×10^5 cells in 1 ml of IMDM supplemented with 0.8% MC, 1% deionized BSA, 600 μg transferrin (Miles Inc., Diagnostics Division, U.S.A.), 1% (v/v) lipoprotein-cholesterol solution (cholesterol 10.7 mg/ml, ICN Immuno Biologicals, Inc., U.S.A.), 4 units of Epo, and test samples. Cultures were incubated at 37 °C in a humidified atmosphere of 5% O_2, 10% CO_2, and 85%

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N_2. Benzidine positive colonies on 3 or 8 d cultures were defined as CFU-E-derived colonies or EB colonies, respectively. CFU-E-derived colonies contained 8 or more erythroblasts.

Benzidine Staining: Erythroid colonies were stained with a 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 dark brown were scored using an inverted microscope.

Results

Purification of HPF from PKE PKE was subjected to hydrophobic chromatography on a Butyl Toyopearl column. CPA was not detected in non-adherent fractions. Then, on decreasing the ammonium sulfate concentration, CPA was eluted with 35% (data not shown). As the second step of purification, an active fraction from Butyl Toyopearl was applied to a Q-Sepharose column (Fig. 1). CPA was absorbed, then after increasing the NaCl concentration, CPA was eluted with 0.3 M NaCl. As the third step of purification, the active fraction obtained from Q-Sepharose was applied to GF-HPLC on a G3000SW$_{XL}$ column. CPA was detected in a fraction (HPF) that was eluted at 8.66 min (Fig. 2). The molecular weight of HPF was about 140 kDa from the result of calibration by standard protein (data not shown). The dose–response curves of HPF for the growth of GM colonies are shown in Fig. 3. A three-fold increase of GM colonies was obtained with 6 μg of HPF, and the addition of 26 μg HPF resulted in an increase of 3.6-fold. One unit is represents the activity required to increase the number of colonies 2 times in CPA assay. PKE had a specific activity of 2.5 units per mg of protein. By the above purification procedure, about 3.8 mg of HPF was obtained from 4.65 g of PKE, which has a specific activity of 256 units per mg of protein. The specific activity of HPF was increased 100-fold compared to that of PKE, and the recovery of HPF activity from PKE was 8.4%.

The Relationship between the HPF Concentration and the EB Colony Formation The effect of HPF concentration on EB colony formation was examined in serum-free cultures. The results are shown in Fig. 4. The addition of about 13 μg HPF resulted in a maximum increase of EB

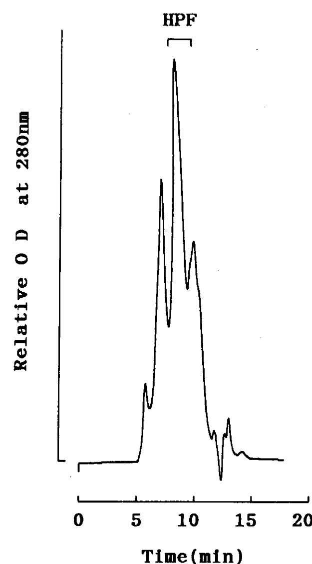

Fig. 2. Purification of HPF by TSKgel G3000SW$_{XL}$ Chromatography

The CPA preparation from the Q-Sepharose column was applied to a TSKgel G3000SW$_{XL}$ column previously equilibrated with 50 mM phosphate buffer, pH 6.7. Each absorbance peak at 280 nm was collected and CPA was measured.

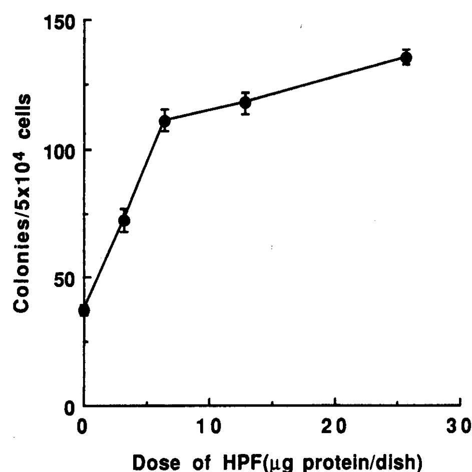

Fig. 3. Dose–Response Relationship between HPF Concentration and the Number of GM Colonies

Cultures were carried out in the presence of 1 μg of G-CSF. Numbers represent the mean ± S.E. of 6 dishes from two separate experiments.

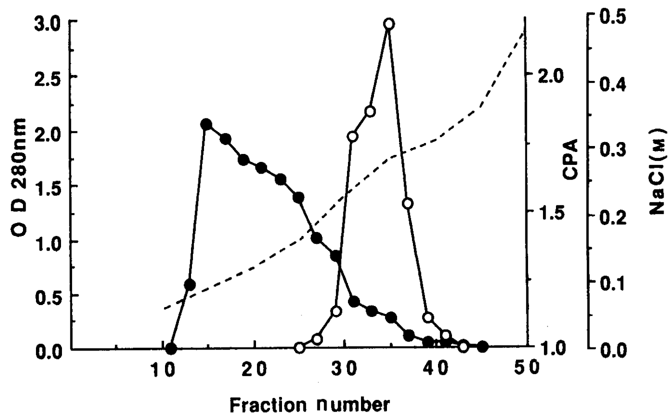

Fig. 1. Elution Profile of HPF on Q-Sepharose Column Chromatography

The CPA preparation eluted from the Butyl Toyopearl chromatography was applied to a Q-Sepharose column and eluted by 130 ml of a 0—0.6 M NaCl gradient as described in the text. Fractions of 4 ml were collected and their absorbance at 280 nm (●), conductivity (---), and CPA (○) was measured. CPA is represented as the ratio of the colonies in the presence of each fraction to the control colonies.

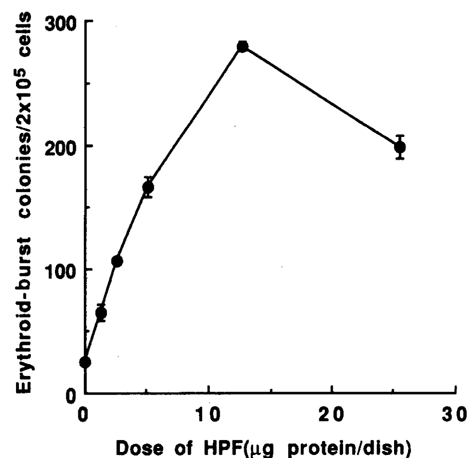

Fig. 4. Dose–Response Relationship between HPF Concentration and the Number of EB Colonies in Serum-Free Cultures

Cultures were carried out in the presence of 4 units of Epo. Numbers represent the mean ± S.E. of 6 dishes from two separate experiments.

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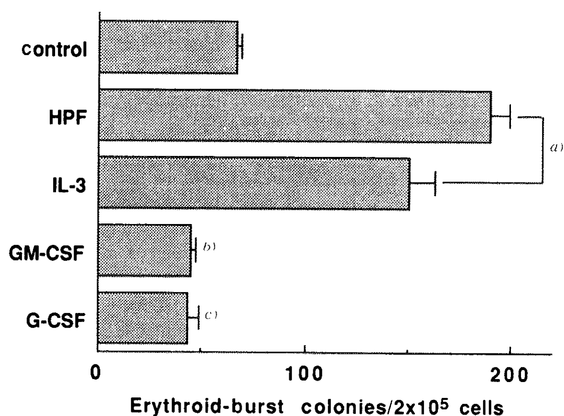

Fig. 5. Effect of Cytokines on EB Colony Formation in Serum-Supplemented Cultures

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)* $p<0.05$. *b)* $p<0.001$ *vs.* control. *c)* $p<0.01$. *vs.* control.

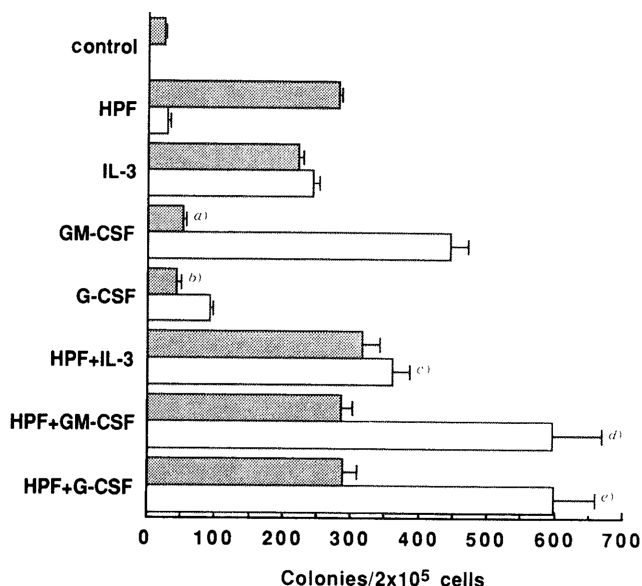

Fig. 6. Erythroid and Non-erythroid Colony Formation by Stimulation with Various Combinations of PKE and Cytokines in Serum-Free Cultures

Cultures were carried out in the presence of 4 units of Epo and each factor. Benzidine-positive or -negative colonies were scored after 8 d of incubation. Numbers represent the mean ± S.E. of 6 dishes from two separate experiments. ■, EB colonies; □, non-EB colonies. *a)* $p<0.001$ *vs.* control. *b)* $p<0.05$ *vs.* control. *c)* $p<0.01$ *vs.* IL-3. *d)* $p<0.05$ *vs.* GM-CSF. *e)* $p<0.001$ *vs.* G-CSF.

colonies. However, the increment of HPF dose to about 26 μg resulted in a decrease of colonies.

Effect of HPF and Some Cytokines on EB Colony Formation The BPA of HPF was compared with that of IL-3, GM-CSF, and G-CSF in the serum-supplemented cultures. As shown in Fig. 5, IL-3 enhanced EB colony formation, however, the BPA was less than that of HPF. GM-CSF and G-CSF did not enhance EB colony formation in our culture conditions.

Similar investigation was carried out under serum-free culture conditions (Fig. 6). At this time, benzidine-positive colonies (EB colony) and benzidine-negative colonies (non-EB colony), which seem to closely resemble GM colonies, were counted. In the control (Epo alone), the number and size of EB colonies in the serum-free cultures were less than that of the serum-supplemented cultures. The addition of HPF resulted in an enhancement EB

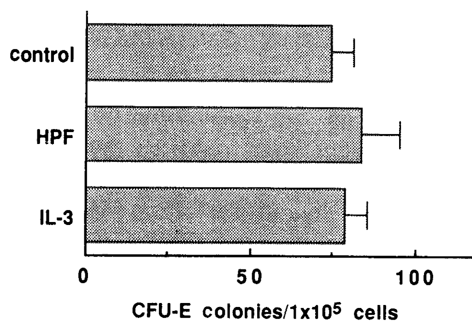

Fig. 7. Effect of HPF or IL-3 on CFU-E Growth in Serum-Free Cultures

Cultures were carried out in the presence of 4 units of Epo and each factor. Benzidine-positive colonies were scored after 3 d of incubation. Numbers represent the mean ± S.E. of 6 dishes from two separate experiments.

colony formation, but the number of colonies in the serum-free cultures was greater than in the case of the serum-supplemented cultures, and about 30 non-EB colonies were also found in the serum-free cultures. IL-3 induces both the numerous non-EB colonies as well as EB colonies. Furthermore, GM-CSF and G-CSF showed BPA in the serum-free culture conditions. The combination of HPF plus these cytokines resulted in an enhancement of non-EB colony formation in comparison to the case of each cytokine alone; however, no increases were found in EB colony formation.

On the other hand, we tested the effect of HPF or IL-3 on CFU-E-derived colony formation. Results are shown in Fig. 7. In both cases, no significant enhancement of colonies was observed.

Discussion

In this study, we have obtained partially purified HPF from PKE, and it was indicated that HPF possess both CPA and BPA. The addition of HPF to serum-free cultures for EB colony assay increased the number of colonies, as opposed to cultures containing serum. Furthermore, GM-CSF and G-CSF showed BPA under serum-free culture conditions in spite of being ineffective in the serum-supplemented cultures. The serum is known to contain several inhibitors for hematopoiesis, such as lipoprotein[10] and some glycoprotein.[11] These serum components may affect the BPA of HPF, GM-CSF and G-CSF. On the other hand, HPF did not enhance CFU-E-derived colony formation, even in the serum-free cultures. It has been known that CFU-E is a more mature erythroid progenitor than BFU-E.[12,13] This result is consistent with our previous observation that HPF acts on primitive hematopoietic stem cells.[4,5]

Previously, 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.[6] It was shown that HPF acts synergistically with these factors. We have suggested that the CPA of HPF appears to be a factor different from IL-3, GM-CSF, and G-CSF because of its functional properties and chemical characteristics.

When HPF and the cytokines were combined, the number of non-EB colonies increased significantly, compared to the results with each cytokine alone. However, no increase was found in EB colony formation. These results indicated that the synergistic activity of HPF is

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significantly affected by the presence of cytokines in the cultures.

Most recently, a new hematopoietic growth factor, which seems to act on most primitive hematopoietic stem cells at present, was reported by three different groups.[14-22] These factors seem to be an identical molecule and act as a ligand for the tyrosine kinase-type receptor, c-kit. Witte reviewed these factors, termed steel factor (SF),[23] though it was alternatively named mast cell growth factor,[14-16] stem cell factor,[17-19] or kit ligand[20-22] by each group. SF alone results in no significant colony number increase, but synergizes with IL-3, GM-CSF and G-CSF to stimulate progenitors in terms of the numbers of colonies.[24,25] The enhancing activity of SF is greater than that of IL-3. However, SF did not enhance M-CSF stimulated colony number or size.[25] There are some common properties we found to be in our previous investigation, though we did not examine the effect of HPF on the growth of mast cells or early B cell progenitors that has been supported by SF.

HPF is possibly one of the hematopoietic growth factors which regulate the proliferation and differentiation of early stage of progenitors.

In order to isolate the active molecule in HPF, we are examining the conditions of further purification.

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