Effect of a Hematopoietic Promoting Factor Derived from Porcine Kidney on the Proliferation of Mouse Hematopoietic Progenitor Cells in Liquid Culture

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We have partially purified a factor from porcine kidney, hematopoietic-promoting factor (HPF), which enhances granulocyte-macrophage colony-forming units (CFU-GM) and erythropoietic burst-forming unit (BFU-E) colony formation in the presence of various exogenous colony-stimulating factors (CSF) or erythropoietin (Epo) from mouse bone marrow cells. In this paper we examine the combined effects of HPF and/or stem cell factor (SCF) with interleukin-3 (IL-3) and interleukin-6 (IL-6) on the proliferation of primitive hematopoietic progenitor cells in liquid cultures for 7 or 14 d. The combination of IL-3 + IL-6 + HPF could not increase the number of CFU-GM, BFU-E, and day−8 colony forming units in spleen (CFU-S) in the cultures of unfractonated bone marrow cells, while this combination resulted in a marked increase of progenitors in cultures of c−kit+−enriched cells. In contrast, expansion of progenitors was observed by IL-3 + IL-6 + SCF or IL-3 + IL-6 + SCF + HPF in the culture of both unfractionated bone marrow cells and c−kit+−enriched cells after 7 d. The number of CFU-GM and BFU-E in the combination of IL-3 + IL-6 + SCF + HPF for c−kit+−cells showed the largest increase, 102-fold and 38-fold respectively after 14 d. These results show that HPF has promoting activity on hematopoietic stem cells and acts synergistically with SCF in the early stages of hematopoiesis.

Key words liquid culture; stem cell factor; kidney extract; CFU-GM; BFU-E; CFU-S

All mature blood cells are produced by the proliferation and differentiation of multipotential hematopoietic stem cells.10 It is well known that hematopoiesis is controlled by a highly complex network of hematopoietic micro-environments consisting of stromal cells, hematopoietic humoral regulators named cytokines and hematopoietic stem cells.2−8) Since stromal cells play an essential role in the survival and proliferation of primitive hematopoietic stem cells in long-term cultures in vitro, stem cell expansion was investigated in the presence of stromal layers.4−6) On the other hand, previous investigators have shown stem cell expansion in vitro using a combination of cytokines in the absence of stromal cells.7−9) These results indicate that the combination of interleukin-3 (IL-3) and interleukin-6 (IL-6) in liquid cultures could synergistically expand hematopoietic progenitors.10 In addition, a stem cell factor (SCF)/c−kit ligand plays an essential role in the early stage of hematopoiesis.11)

Previously, we reported a hematopoietic-promoting factor (HPF) obtained from porcine kidney, which enhances granulocyte-macrophage colony-forming (CFU-GM) and burst-forming-unit-erythroid (BFU-E) colony formation in the presence of various exogenous colony-stimulating factors (CSF) or erythropoietin (Epo) in vitro.10,11) However, HPF does not have CFU-GM and BFU-E colony-stimulating activity and does not enhance the colony formation of colony-forming-unit-erythroid, which are more mature than erythroid progenitors. In this experiment, we compared the effect of HPF and SCF on the growth of hematopoietic progenitors with unfractionated murine bone marrow cells and c−kit+−enriched cells.

MATERIALS AND METHODS

Growth Factors Recombinant human Epo (750 units/ml) was purchased from Sankyo Co. Ltd., (Tokyo, Japan). Recombinant mouse IL-3 (800 µg/ml), recombinant human IL-6 (580 µg/ml) and recombinant mouse SCF (300 µg/ml) were kindly provided by Kirin Brewery Co. Ltd., (Tokyo, Japan). Doses of these factors per culture dish were as follows: (1) Epo 2 units, (2) IL-3 1 ng, (3) IL-6 20 ng, (4) SCF 100 ng.

Preparation of HPF from Porcine Kidneys HPF was prepared from porcine kidneys as previously described.13) Briefly, crude extract prepared from porcine kidneys was subjected to hydrophobic chromatography on a Tskgel Butyl Toyopearl 650 (Toch, Tokyo, Japan) column equilibrated with 50% saturated ammonium sulfate. Colony-promoting activity (CPA) was estimated for the eluted fractions in the presence of recombinant human G-CSF. CPA was eluted with 42−35% ammonium sulfate. As the second purification step of an active fraction from Butyl Toyopearl was applied on a Q-Sepharose (Pharmacia LKB Biotechnology AB, Sweden) column equilibrated with 20 mm Tris-HCl buffer, pH 7.40. CPA was eluted with 0.2−0.3 M NaCl. As the third purification step, the active fraction obtained from Q-Sepharose was subjected to gel filtration-HPLC on a Tskgel G3000SWXL (7.5 mm i.d. × 30 cm, Tosoh) column equilibrated with 50 mm phosphate buffer, pH 6.7. Each absorbance peak at 280 nm was collected and the active fraction was pooled and used as HPF. The molecular weight of HPF was about 140 kDa following standard protein calibration. One unit represents the activity required to increase the number of colonies 2-fold in the CPA assay. In the above purification procedure, the specific activity of HPF was increased 100-fold compared with that of crude extract.

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with a recovery of activity of 8.4%.

Preparation of Bone Marrow Cells Male mice of the ddY strain (Sankyo Labo Service Co. Ltd., Sapporo, Japan) aged 8—12 weeks were used throughout the experiments. Bone marrow cells were flushed out of the femur and tibia using phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA, Boehringer Mannheim GmbH, Germany) using a 25-gauge needle. Cells were treated with Gey’s solution to lyse erythrocyt, and recovered cells were allowed to adhere on a plastic petri-dish to remove the adherent populations in marrow cells. Cells obtained by the above treatment were used as unfractonated bone marrow cells (BM cells).

Preparation of c-kit Positive Cells C-kit\(^+\) cells were enriched from BM cells by one passage on a surface-activated T-25 cell culture flask (Applied Immune Sciences Inc., Santa Clara, CA), following the instructions of the manufacturer. This flask was prepared using a purified anti-mouse c-kit receptor (Rat IgG2b, Pharmingen, San Diego, CA). C-kit\(^+\)-enriched cells (c-kit\(^+\) cells) were analyzed with Epics\(^\text{®}\) Profile Analyzer (Coulter, Hialeah, FL) using an anti-rat IgG2b (Rabbit IgM, Pharmingen) labeled with fluorescein isothiocyanate as a secondary antibody. The results of the analysis indicated that the concentration of c-kit\(^+\) cells was about 10-fold higher than previous preparations. C-kit\(^+\) cells provided approximately only a 5-fold enrichment of in vitro colony-forming cells (CFC) and a 10-fold enrichment of day 8 colony-forming units in spleen (CFU-S).

Liquid Cultures BM cells (1.2 × 10\(^6\) cells) were suspended in 60 mm plastic petri-dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in 6 ml Iscove’s modified Dulbecco’s medium (IMDM, Gibco BRL, Grand Island, NY) containing 1% BSA, 5% fetal calf serum (FCS, Intergen, NY), 600 µg transferrin (Miles Inc., Kankakee, IL), 1% (v/v) lipoprotein-cholesterol solution (cholesterol 10.7 mg/ml, ICN ImmunoBiologicals, Inc., Lisle, IL) and cytokines. C-kit\(^+\) cells (2 × 10\(^4\) cells) were suspended in 2 ml of the above medium in 35 mm plastic petri-dishes (Falcon). Cultures were carried out at 37 °C in a humidified atmosphere of 5% O\(_2\), 10% CO\(_2\) and 85% N\(_2\). After 7 d of incubation, the nonadherent cells were harvested, washed three times with IMDM, and resuspended in fresh IMDM medium containing cytokines, then the cultures were further incubated for 7 d. After incubation, the nonadherent cells were assayed for their capacity for CFU-S, BFU-E and CFU-GM colony formation. The total number of cells at day-14 was obtained by the calculation:

\[
\text{the total number of cells at day-14} = \frac{x}{y} \times z
\]

where

- \(x\) = the number of harvested cells at day-7.
- \(y\) = the number of inoculated cells at day-7 (BM cells 1.2 × 10\(^6\) cells, c-kit\(^+\) cells 2 × 10\(^4\) cells).
- \(z\) = the number of harvested cells at day-14.

Total number of progenitors was calculated from the total number of cells and the number of progenitors per tested cells.

**In Vitro Colony Assays** The assay for in vitro colony formation was carried out in methylcellulose cultures as previously described with some modification.\(^{13}\) Briefly, BM cells (4 × 10\(^4\)) or c-kit\(^+\) cells (1 × 10\(^3\)) were seeded in 35 mm plastic petri-dishes in 1 ml IMDM supplemented with 0.8% methylcellulose (Wako Chemicals, Tokyo, Japan), 2% BSA, 1% FCS, 600 µg transferrin, 1% (v/v) lipoprotein-cholesterol solution, 2 units Epo and 1 ng IL-3. Each dish was incubated at 37°C in a humidified atmosphere of 5% O\(_2\), 10% CO\(_2\) and 85% N\(_2\) for 10 d. After incubation, benzidine-positive colonies were defined as BFU-E-derived colonies. Colonies consisting of 50 or more cells were scored using an inversion microscope.

**CFU-S Assay** CFU-S was assayed using the method of Till and McCulloch\(^{14}\) with some modification. Mice were irradiated with X-rays (180 kVp, 20 mA) using 0.5 mm copper and 0.5 mm aluminum filters at a distance of 30 cm from the focus surface at a dose-rate of 1.45 Gy/min. BM cells or c-kit\(^+\) cells prepared from normal mice and recovered cells from liquid cultures were suspended in IMDM and injected intravenously into the recipient mice which were irradiated with 8 Gy of X-rays. After 8 d, the recipient mice were sacrificed and their spleens were removed and fixed in Tellysnikey’s solution. Colonies observed on the spleen surface were counted.

**RESULTS**

**Effects of HPF, SCF, IL-3, and IL-6 on Colony Formation** The combination of SCF and HPF with IL-3 and IL-6 on in vitro colony formation was investigated with BM cells or c-kit\(^+\) cells. In the case of BM cells, no colony formation was observed following the addition of Epo alone after 10 d culture (Fig. 1). Epo+HPF, Epo+SCF, and Epo+IL-6 also did not support any colony formation (data not shown). However, Epo + IL-3 stimulated colony formation, and Epo + IL-3 + IL-6 resulted in an enhancement of colonies compared with that of Epo + IL-3. The total number of colonies with the combination of SCF or HPF with Epo + IL-3 + IL-6 resulted in a synergistic increase over that seen with

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Fig. 1. Colony Formation of Unfractonated Bone Marrow Cells

Unfractonated bone marrow cells (4 × 10\(^6\) cells) were cultured in the presence of 2 units Epo and the following factors: IL-3 1 ng; IL-6 20 ng; SCF 100 ng; HPF 15 µg. Numbers represent means of 9 dishes from three separate experiments.

\* \(p < 0.01\) vs. IL-3; \** \(p < 0.01\) vs. IL-3 + IL-6.
10 days culture

\begin{align*}
\text{Epo} \\
+\text{IL-3} \\
+\text{IL-3+IL-6} \\
+\text{IL-3+IL-6+SCF} \\
+\text{IL-3+IL-6+HPF} \\
+\text{IL-3+IL-6+SCF+HPF}
\end{align*}

Colonies / 10,000 cells

20 days culture

\begin{align*}
\text{Epo} \\
+\text{IL-3} \\
+\text{IL-3+IL-6} \\
+\text{IL-3+IL-6+SCF} \\
+\text{IL-3+IL-6+HPF} \\
+\text{IL-3+IL-6+SCF+HPF}
\end{align*}

Colonies / 10,000 cells

Fig. 2. Colony Formation of c-kit⁺ Cells

C-kit⁺ cells (1 x 10⁶ cells) were cultured in the presence of 2 units Epo and the following factors: IL-3 100 ng; IL-6 20 ng; SCF 100 ng; HPF 13 μg. Numbers represent means of 9 dishes from three separate experiments. * p < 0.01 vs. IL-3. ** p < 0.05 vs. IL-3+IL-6.

Epo + IL-3 + IL-6. However, HPF and SCF did not act synergistically to stimulate colony formation by BM cells. In the culture of c-kit⁺ cells, SCF and HPF showed a weak promoting activity after 10 d culture (Fig. 2). When the culture period was extended to 20 d, the number of colonies of Epo + IL-3 considerably decreased, but the number of colonies of the other combinations did not. Macroscopic (> 1.0-mm in diameter) multilineage colonies comprising erythroid, granulocyte, macrophage and other cells appeared in the culture containing SCF and/or HPF (data not shown). These colonies were thought to be derived from primitive hematopoietic stem cells. Preparation of c-kit⁺ cells enriched the number of CFC and produced an increase in the ratio of committed progenitor cells to primitive stem cell in comparison with BM cells. These results suggest that the decreased colony formation at 20 d was due to Epo + IL-3 stimulating mainly committed progenitor cells and, furthermore, only the addition of HPF or SCF to an IL-3 + IL-6 combination was sufficient for maximum colony formation.

Short-Term Liquid Culture

Short-term liquid culturing of BM cells or c-kit⁺ cells was performed with the combination of IL-3, IL-6, SCF, and/or HPF for 7 and 14 d, and these cultured cells were assayed. In the culture of BM cells, the combination of IL-3 + IL-6 + HPF showed a 2.6-fold increase of nucleated cells after 7 and 14 d (Fig. 3). The combination of IL-3 + IL-6 + SCF and IL-3 + IL-6 + SCF + HPF resulted in about a 24-fold expansion of nucleated cells after 14 d. In the culture of c-kit⁺ cells, a marked increase in the total number of cells was obtained after 7 d (85—130-fold) and 14 d (1100—2400-fold) with all combinations. On the other hand, in both cultures of BM cell and c-kit⁺ cell, the highest increase in the number of CFU-GM and BFU-E were obtained after 14 d with the combination of IL-3 + IL-6 + SCF + HPF (Figs. 4 and 5). In particular, this combination resulted in the highest increase of CFU-GM (109-fold) and BFU-E (38-fold) in

Fig. 3. Number of Cells in Liquid Culture

Unfractionated bone marrow cells (1 x 10⁶ cells) were suspended in 6 ml IMDM containing 1% BSA, 5% FCS, 600 μg transferrin, 160 μg cholesterol. C-kit⁺ enriched cells (2 x 10⁶ cells) were suspended in 2 ml of the above medium in 35 mm plastic petri-dishes. After 7 d of incubation, the nonadherent cells were harvested and assayed for colony formation. Recovered cells from each culture were re-suspended in the initial medium, then the cultures were further incubated for 7 d. The increase in number of cells after culturing was calculated from the number of harvested cells and inoculated cells. Data on the c-kit⁺ cells is a representative experiment of three separate experiments. (○) IL-3 + IL-6 + SCF; (△) IL-3 + IL-6 + HPF, (□) IL-3 + IL-6 + SCF + HPF, * p < 0.05.
the culture of c-kit⁺ cells. HPF acted synergistically with SCF in liquid cultures of either BM cells or c-kit⁺ cells. This suggests that this combination is the most efficient for the expansion of CFU-GM and BFU-E. However, the variation of CFU-S was different from CFU-GM and BFU-E; in the combination of IL-3 + IL-6 + HPF, and IL-3 + IL-6 + SCF + HPF there was a significant increase the number of CFU-S in comparison with the combination of IL-3 + IL-6 + SCF in c-kit⁺ cell cultures for 7 d, but none of the combinations could maintain CFU-S for 14 d (Fig. 6). In the cultures of BM cells with IL-3 + IL-6 + SCF + HPF, the numbers of CFU-S after 14 d of culture were maintained at the same value as the 7-d cultures. The difference between BM cells and c-kit⁺ cells in liquid cultures was thought to be due to the HPF and SCF acting on the expansion of progenitors in c-kit⁺ cells, while the effect of HPF + SCF on BM cells was negated due to the presence of accessory cells (macrophage, lymphocytes, neutrophil, or stromal cells) within the BM population. These results suggest that the action of HPF is inactivated by accessory cells, or HPF is bound to other cells rather than hematopoietic stem cells in BM cells.

DISCUSSION

We compared the effect of HPF and SCF on the growth of hematopoietic progenitors with BM cells and c-kit⁺ cells. Ogawa has reported that the hematopoietic growth factors may be divided into three categories: (1) late-acting lineage-specific factors, (2) intermediate-acting lineage nonspecific factors, and (3) factors acting on the kinetics of cell cycle dormant primitive progenitors. The
latter two categories include early-acting cytokines, IL-1, IL-3, IL-4, IL-6, IL-11, IL-12, LIF (leukemia inhibitory factor), G-CSF, GM-CSF and SCF. Early-acting cytokines may be loosely grouped together based on their functional similarities as follows: (a) IL-6, G-CSF, IL-11, IL-12 and LIF; (b) SCF; (c) IL-3, IL-4 and GM-CSF. Cytokines in each group may interact with those in other groups to stimulate the proliferation of primitive progenitors. Ogawa found that IL-6, G-CSF, IL-11, SCF and IL-12 act synergistically with IL-3 in support of colony formation from dormant murine hematopoietic progenitors. In addition, SCF could interact with IL-6, G-CSF, IL-11 and IL-12 to support the formation of multipotential blast cell and multilineage colonies. Okada et al. have reported that the more primitive stem cells require combinations of factors, such as SCF, IL-3 and IL-6. In the colony assays of BM cells or c-kit+ cells, HPF increased the number of colonies in combination with Epo + IL-3 + IL-6. Interestingly, macroscopic multilineage colonies appeared in the c-kit+ cell cultures containing HPF and/or SCF. However, HPF did not act synergistically with SCF on colony assays of either BM cells or c-kit+ cells. This indicates that HPF can also interact with IL-3 and IL-6 to support the formation of multilineage colonies, as does SCF. Thus, our results suggest that HPF appears to be a potent synergistic factor with other cytokines and one of the early acting factors. However, the function of HPF differs from SCF. In liquid cultures of BM cells, the combination of IL-3 + IL-6 + HPF cannot increase the number of cells and progenitors, while this combination results in a marked increase of cells and progenitors in liquid cultures of c-kit+ cells. Okada et al. have reported that the proto-oncogene c-kit encodes a transmembrane tyrosine kinase receptor for SCF, and that the c-kit molecule is expressed in primitive hematopoietic stem cells and plays an essential role in the early stage of hematopoiesis. The activity of HPF is affected by the presence of accessory cells in BM cells. Our results show that HPF acts on hematopoietic stem cells in the combination of IL-3 + IL-6 in a similar manner to SCF, and that the target cells of HPF are primitive and partially overlap those of SCF.

Miura et al. have demonstrated that the combination of SCF + IL-6 result in a 21-fold increase of day-8 CFU-S after liquid culture of Lin− lineage marker-depleted c-kit+ cells for 7 d. They showed that c-kit+ cells provide approximately 20-fold enrichment of CFC. Neben et al. have demonstrated a 24000-fold expansion of progenitor cells, determined by in vitro limiting dilution analysis of cobblestone area-forming cells. In their findings, Neben used stem cell-enriched Lin− Sca1+ FUdR cells (2 d after 5-fluourouracil treatment, lineage marker-depleted, Sca-1-positive) in liquid cultures supplemented with SCF + IL-3 + IL-6 + IL-11 for 6 d. In our experiment, since c-kit+ cells provided approximately only a 5-fold enrichment of CFC and a 10-fold enrichment of day 8 CFU-S, the differences may be due to differences in the concentration of input stem cells and culture conditions. In liquid cultures of BM cells with the combination of IL-3 + IL-6 + SCF + HPF, the numbers of CFU-S were maintained at the same value as 7-d cultures after 14 d. On the other hand, in the cultures of c-kit+ cells, this combination led to a marked disappearance of CFU-S after 14 d. However, increases in CFU-GM and BFU-E were observed. These results suggest that the combination of IL-3 + IL-6 + SCF + HPF leads to excess cell proliferation and rapid maturation of hematopoietic stem cells, after which CFU-S numbers decrease markedly. Since stem cell proliferation is accompanied by differentiation at an early stage, the regulation of stem cell proliferation in our liquid culture will be the subject of further investigation.

Furthermore, in spite of the synergistic activity of HPF and SCF in liquid cultures of either BM cells or c-kit+ cells, its effect was not observed in colony assays. Iscove et al. in their investigation of the increase of pluripotential hematopoietic precursors in suspension cultures with IL-1 and IL-3, suggested that different kinds of cells are
detected in the methylcellulose colony assays and in the suspension culture assay. The cells detected in the suspension cultures are similar to those giving rise to the "blast" colonies described by Suda et al. In our cases, the combination of HPF or SCF with IL-3 + IL-6 are sufficient for maximum colony formation in the methylcellulose colony assay. However, the combination of HPF plus SCF with IL-3 + IL-6 additionally require the "self-renewal" division of stem cells in the suspension culture assay. Thus, we believe that the liquid culture requires the presence of more cytokine stimulation for synergistic activity of HPF and SCF to be expressed than in the colony assay. In order to investigate the properties of HPF function, further purification of hematopoietic stem cells are required. Moreover, further combination of HPF with other early-acting cytokines (G-CSF, IL-11 and LIF) should be tested for their effects on the proliferation of hematopoietic stem cells.

At present the N-terminal amino acid sequence in purified HPF shows no homology with any known molecules (unpublished data). It is possible that HPF is one of the hematopoietic growth factors which regulate the proliferation and differentiation of the early stage of progenitors. The expansion of hematopoietic stem cells in vitro is an important topic; some attempts are being made to use this in bone marrow transplantation. In this study, we showed that the combination of IL-3 + IL-6 + SCF + HPF was most efficient for the expansion of mouse hematopoietic progenitor cells. HPF will be a useful factor for manipulating primitive hematopoietic stem cells in vitro.

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